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THE OXIDATION AND UTILIZATION OF GLUCOSE AND ACETATE BY THE MAMMARY GLAND OF THE GOAT IN RELATION TO THEIR OVER-ALL METABOLISM AND TO MILK FORMATION

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It is well established that in ruminants dietary carbohydrate is largely fermented to short-chain fatty acids. Acetate, the major product of ruminal fermentation, is absorbed together with smaller amounts of propionate and butyrate to constitute a major source of energy. The paucity of alimentary glucose, when considered with the low concentrations of blood glucose and relative insensitivity to insulin which characterize ruminants, has tended to obscure the importance of glucose in ruminant metabolism. However, recent work based on the dilution of constantly infused isotope (Searle, Strisover & Chaikoff, 1954; Steele, Wall, de Bodo & Altszuler, 1956) has shown that rates of turnover and oxidation of glucose in sheep (Annison & White, 1961, 1962; Bergman, 1963; Ford, 1963) and cattle (Davis & Brown, 1962) are not markedly lower than in non-ruminants. Similar studies with acetate have confirmed the quantitative importance of this substrate in ruminant metabolism and shown that, relative to glucose, acetate makes a two- to three-fold greater contribution to total oxidative metabolism in the intact animal (Annison, Brown, Leng, Lindsay, West & White, 1963).

The dominant role of acetate in the over-all economy of ruminants is not reflected in the metabolism of all individual tissues. McClymont & Setchell (1956) demonstrated the uptake of glucose but not acetate by the sheep brain, and Annison, Scott & Waites (1963) showed that the testis and epididymis in the anaesthetized ram oxidize more glucose than acetate. Extensive studies on the metabolism of acetate and glucose by the isolated perfused mammary gland of the lactating goat have also shown that glucose is oxidized about twice as rapidly as acetate and is essential for milk secretion (Hardwick, Linzell & Price, 1961; Hardwick, Linzell & Mepham,

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1963). These findings were unexpected since Folley & French (1950) reported that slices of mammary glands of lactating sheep were inert towards glucose, and that glucose merely increased the utilization of acetate. During a study of mammary blood flow in conscious goats Linzell (1960*b*) found that the glucose uptake by the lactating gland was much greater than that required for milk lactose synthesis, suggesting that substantial amounts are oxidized under normal conditions, as in the isolated gland.

The present studies were undertaken to investigate the uptake and oxidation of glucose and acetate by the mammary gland of the normal animal in relation both to the over-all metabolism of these substrates, and to milk production. As in the experiments of Annison, Scott & Waites (1963) with the testicle of the ram, the isotope dilution technique has been combined with the measurement of mammary blood flow and measurements of the specific radioactivities of the substrate and the CO_2 in both arterial and mammary venous blood during a constant I.V. infusion of ^{14}C-labelled glucose or acetate. From these data values have been calculated for the amounts of acetate and glucose entering the circulation, the amounts taken up by the udder and for the contribution of the two substrates to CO_2 production by the whole body and by the udder. Knowledge of the uptake and extent of oxidation of glucose and acetate by the udder has allowed quantitative assessment of the role of these substrates as precursors of milk fat and lactose.

A preliminary account of the work has been given by Annison & Linzell (1964).

METHODS

Animals. Four adult Saanen goats were studied (Table 1). They were kept on a constant diet of hay, fed *ad libitum*, and a standard cereal ration fed twice daily according to the milk yield. The animals were prepared for the measurement of blood flow through one mammary gland (half the udder) and the collection of arterial and mammary venous blood by the exteriorization of a carotid artery, a 'milk' (caudal superficial epigastric) vein and the separation of the left and right halves of the udder to divide the blood vessels crossing between the two glands (Linzell, 1960*b*). The volumes of the empty glands were measured by displacement of water to compare the flows/g of tissue with those made previously (Linzell, 1960*b*) and to estimate the total udder blood flow assuming that the flow/g was the same in both glands.

Experimental procedure. The infusions of isotopes were carried out in the animals' own pens, using an apparatus similar to that of Jewell (1957). The peristaltic infusion pump of constant output was placed above the pen on a swivelling platform. The plastic outflow tubing passed to a leather harness worn by the animal through flexible metal tubing and a counterbalanced levelling device that followed the animal's movements. The plastic tube ended at a tap on the loose collar of the harness from where a catheter entered a jugular vein and another catheter in the carotid loop was similarly fixed to another tap on the collar. Thus blood samples could be collected without necessarily restraining the animal and I.V. infusions carried out whilst the animal was quite free to move in any way within its pen and to feed and drink as it customarily did on other days. The animals were accustomed to the apparatus before the experiment.

The catheters (Jugular: P.V.C. 'Portex' NT/3 Shore Scale 95, 1·4 mm bore, 2·0 mm o.d.; Carotid: Nylon 'Portex' Flexible Grade 3, 1 mm bore, 1·34 mm o.d.) were inserted into the carotid and jugular over flexible wires or nylon rod by Seldinger's (1953) technique under local anaesthesia after the morning milking on the day of the experiment.

TABLE 1. Details of Saanen goats used

Goat	Jill	Jill	Sally	Angel	Angel	Balham*
Age (yr)	7	7·5*	7	3·5	3·5	2·5
Lactation	7th	7th	6th	3rd	3rd	2nd
Time of experiments (weeks after parturition)	6, 12	36	12,15	17, 18	23	35
Weight (kg)	68, 63	62·5	74	69	66	60
Milk yield (l./day) (mean of 7 days)	4·93, 4·51	1·52	2·85, 2·61	3·16, 3·07	0	2·36
Mean milk composition (bulked from 3 days)						
Fat (g/100 ml.)	3·4	4·75	3·2	3·1	—	4·0
Lactose (g/100 ml.)	4·4	3·9	4·0	4·2	—	4·0
Volume of udder, (empty) (l.)	3·43	2·59	2·39	2·95	2·00	1·6
Mean udder blood flow (ml./min.)	1780	700	1170	1330	480	840

* At this time Jill and Balham were 6 weeks pregnant.

Uniformly labelled [^{14}C]-glucose in 0·9% (wt./vol.) NaCl (0·3 μc; 1 mg/ml.) was infused at 1 ml./min immediately after a priming dose (30 μc) of isotopic glucose (Annison & White, 1961). Sodium [1,2-^{14}C]-acetate in 0·9% (wt./vol.) NaCl was infused at 1 μc, 1 mg/min without a priming dose. Infusions of both isotopes were continued for 4 hr at constant rate. During the last hour of infusion three to six pairs of arterial and mammary venous blood samples (10–30 ml. each) were taken simultaneously at 10–15 min intervals. The venous samples were taken from the exteriorized 'milk' vein whilst the animal was standing and whilst another person manually compressed the external pudic vein at the base of the udder (Linzell, 1960*a*). In most experiments the milk formed during the hour of sampling was collected by milking out after the intravenous injection of 400 m.u. of oxytocin.

Estimates of mammary blood flow were made on other days at the same level of milk yield by the method previously described of measuring the flow in the exteriorized 'milk' vein by the thermodilution method and assessing the proportion of the total flow in this vein by manual clamping of the other main vein, the external pudic (Linzell, 1960*a*, *b*). This can only be done with the animal standing and the measurements were made on the milking stand with the goat feeding.

Radioactive materials. Sodium [1,2-^{14}C]-acetate, uniformly labelled [^{14}C]-glucose, sodium [^{14}C]-bicarbonate and calibrated *n*-hexadecane were obtained from the Radiochemical Centre, Amersham, Bucks.

CHEMICAL METHODS

Assay of radioactivity. All substrates were assayed by wet oxidation to $^{14}CO_2$ (Van Slyke & Folch, 1940), which was absorbed in 2 ml. of ethanolamine: methyl cellosolve (1:2 vol./vol.). Radioactivity was measured by liquid scintillation counting after adding 4 ml. of toluene containing 2,5-diphenyl oxazole (0·6%). Calibrated *n*-hexadecane (1 μc/g) was used as a reference standard.

Plasma glucose specific radioactivity. Glucose was isolated from plasma (5 ml.) as glucosazone after the addition of carrier glucose (20 mg) by the method described earlier (Leng & Annison, 1963) and assayed for radioactivity by oxidation to $^{14}CO_2$, as described above.

Plasma glucose concentrations were measured with glucose oxidase (Huggett & Nixon, 1957).

Blood acetate specific radioactivity. Acetate was isolated from blood by steam distillation as described earlier (Annison & White, 1962), and after titration the dried samples were oxidized to $^{14}CO_2$ and assayed for radioactivity as described above.

Specific radioactivity of blood carbon dioxide. Blood CO_2 was isolated as $BaCO_3$ as described by Annison & Lindsay (1961), and using the same gas transfer apparatus weighed amounts of $BaCO_3$ were heated gently with concn. H_2SO_4 (5 ml.) *in vacuo* and the liberated CO_2 absorbed in 2 ml. of ethanolamine:methyl cellosolve (1:2 vol./vol.). The solution was assayed for radioactivity as described above.

In the acetate experiment on the goat Angel during lactation the radioactivities of the titrated blood acetate and the $BaCO_3$ from the blood CO_2 were estimated on planchets and an end-window counter by Dr D. C. Hardwick as in the experiments of Hardwick *et al.* (1963).

Blood gases. Blood O_2 and CO_2 were measured on the same blood sample (1 ml.) by the procedures of Peters & Van Slyke (1932).

Analysis of milk. Milk total solids, ash, lactose and fat were determined by standard methods as described by Hardwick & Linzell (1960).

RESULTS

Experimental conditions. In experiments with conscious animals it is important to have the stable physiological conditions which were largely achieved in the present study. Experiments were usually started 1·5–2 hr after the morning milking when the animals were finishing their morning feed. They were not apparently upset by the harness or catheters. The worst disturbance seemed to be that they were made to stand for each pair of blood samples during the last hour of infusion, when they would otherwise have been lying down and ruminating. However they tolerated this patiently and frequently continued to ruminate during the taking of the blood samples. Their heart rates (95–110/min), mean blood pressure (90–95 mm Hg), plasma glucose and free fatty-acid concentrations were not significantly altered or different from other lactating animals in this herd. The arteriovenous differences of O_2, CO_2, glucose and acetate (Table 2) were within the range of values for conscious lactating goats obtained earlier (Linzell, 1960*b*).

Equilibration of bicarbonate pools. The specific radioactivities of CO_2 in arterial and mammary venous blood were followed during the continuous infusion of $NaH^{14}CO_3$ (Fig. 1). The time required to approach equilibration of body CO_2 pools (100–200 min) was appreciably less than with anaesthetized rams (Annison *et al.* 1963). Evidence of a CO_2 pool in the mammary gland was given by the much lower specific radioactivities of mammary venous CO_2 relative to arterial CO_2 during the early stages of the infusion. After 200 min the mean difference in the specific radioactivities of arterial and venous blood draining the mammary gland was roughly accounted for by the dilution of arterial blood with unlabelled CO_2 produced by the tissue.

The roughly constant specific radioactivities of $^{14}CO_2$ in arterial blood during the terminal stages of infusion of labelled glucose and labelled acetate (Table 2) indicated that body CO_2 pools were largely in equilibrium during the sampling periods.

The effect of oxytocin. In the first experiments the milk formed during the infusions was obtained by milking the animals either hourly or at the beginning and end of the blood sampling period. Milking releases oxytocin and in addition extra oxytocin (400 m-u.) was injected I.V. to ensure thorough emptying of the glands. However, oxytocin has been reported

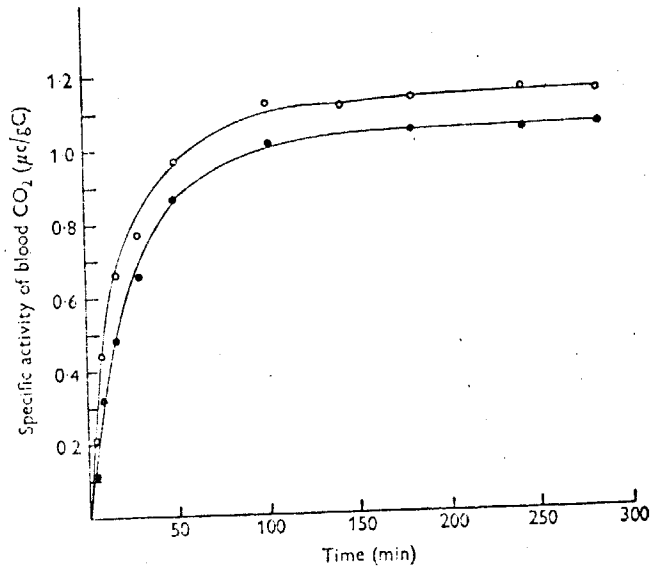

Fig. 1. Specific radioactivity–time curves of blood CO_2 in a lactating goat (Sally) receiving a constant I.V. infusion of $NaH^{14}CO_3$ (100 mg/100 ml. in 0·9 % (wt./vol.) NaCl; 0·5 μc/ml.) 1 ml./min. Open circles, arterial blood; closed circles, mammary venous blood.

in rats to have an insulin-like activity *in vitro* on mammary tissue (Goodfriend & Topper, 1961), in dogs to raise the plasma levels of free fatty acids (Mirsky, 1963) and glucose (Heidenreich, Kook & Reus, 1962) and in the human to increase the heart rate, cardiac output and hand blood flow and to decrease the blood pressure (Kitchin, Lloyd & Pickford, 1959). Although we calculated that the doses of oxytocin that we used were below the levels producing these metabolic responses and Assali, Holm & Parker (1961) reported that there were no cardiovascular effects in preg-

TABLE 2. Data from experiments on lactating goat Jill. Isotopes infused at constant rate into jugular vein and blood samples taken simultaneously from carotid artery and a mammary vein

(1) Infusion of (U^{14}C) glucose 0·3 μc/min in the 6th week of lactation

Sample	Time (min)	Blood glucose concentration (mg/100 ml.)	Blood glucose specific radioactivity (μc/gC)	Blood gases (vol. %) CO_2	Blood gases (vol. %) O_2	Blood CO_2 specific radioactivity (μc/gC)	Blood acetate (m-mole/l.)
A1	181	47	2·45	46·6	12·2	0·24	2·3
V1		34	—	54·2	6·6	0·39	0·9
A2	192	47	2·44	47·4	13·0	0·24	—
V2		30	—	56·4	6·6	0·39	—
A3	205	48	—	48·9	12·7	0·23	—
V3		34	—	58·5	6·3	0·40	—
A4	219	51	2·42	47·9	11·8	0·26	—
V4		33	—	56·5	5·9	0·41	—
A5	234	51	2·55	45·9	12·1	0·26	1·9
V5		36	—	54·9	6·8	0·38	0·6

(2) Infusion of (U^{14}C) acetate 1·0 μc/min in the 12th week of lactation

Sample	Time (min)	Blood acetate concentration (m-mole/l.)	Blood acetate specific radioactivity (μc/gC)	Blood gases (vol. %) CO_2	Blood gases (vol. %) O_2	Blood CO_2 specific radioactivity (μc/gC)	Blood glucose (mg/100 ml.)
A1	191	1·61	5·5	50·2	10·7	1·22	45
V1		0·37	3·3	57·0	5·0	1·21	28
A2	211	2·00	6·4	56·3	10·7	1·36	45
V2		0·57	3·2	64·6	4·6	1·15	18
A3	234	1·88	5·9	55·2	10·4	1·24	38
V3		0·69	3·0	64·3	4·4	1·25	24

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nant sheep, the influence of oxytocin in our experiments was examined and in some experiments no oxytocin was given and the animal was not milked during the infusions.

Single I.V. injections of oxytocin (synthetic or Pitocin, Parke, Davis & Co.) 400–1000 m-u. had no effect on the mean blood pressure, heart rate, or the levels of blood sugar or volatile fatty acids. Neither were there any significant arterio-venous differences between three pairs of samples taken over a period of half an hour before and after oxytocin, in respect of blood gases, substrates or radioactivities. On two occasions with different goats there was an increase in udder blood flow of 15–30% 7–11 min after oxytocin but on other days no effect in the same animals; random variations of this order were previously detected by Linzell (1960*b*).

In view of these findings data obtained in experiments with and without oxytocin have been pooled.

Metabolism of acetate. Acetate was an important source of energy in these goats as in other ruminants (Table 3). The mean entry rate (i.e. rate of entry into the total body pool) was 5·5 mg/kg/min during lactation but the mammary glands were taking up 11–41 mg/kg/min according to the rate of milk secretion. From the specific radioactivities of the arterial CO_2 it was calculated that 28% of the total circulating CO_2 came from this substrate. The specific radioactivity of the CO_2 in the mammary venous blood was not significantly different from that in arterial blood and thus it was concluded that the proportion of mammary CO_2 derived from acetate is the same as the average for the rest of the body (i.e. about 27%). One goat (Angel) was prematurely dried up when in full lactation and when studied 5 weeks later the acetate utilization was somewhat less (4·0 mg/kg/min for the whole animal and 9·5 mg/kg/min for the udder), but the proportion of total CO_2 derived from it was similar (23%). From these data and the mean mammary arterio-venous differences of acetate and CO_2 it was calculated that 46% of the acetate taken up by the udder was oxidized during lactation and 57% in the dry animal (Table 3).

The specific radioactivity of acetate in mammary venous blood was always measurably lower than that in arterial blood (Table 2), indicating some production of acetate in addition to the large acetate uptake.

Metabolism of glucose. The mean entry rate of glucose into the total body pool (3·6 mg/kg/min) was less than for acetate and the proportion of total CO_2 derived from it much less at 9% (Table 3). However the mammary glands took up 25–89·5 mg/kg/min according to the milk yield, which is 2–3 times the mammary acetate uptake and 8–25 times the body average. Moreover, in all experiments, the mammary venous CO_2-specific radioactivity was always higher than the arterial level, indicating a greater oxidation of glucose in the mammary glands than in the rest of the body.

TABLE 3. Metabolism of acetate and glucose by the whole animal and by the udder

Goat	Rate of milk secretion (ml./min)	Whole animal entry rate (mg/min/kg)		Udder: Uptake of substrate (mg/min/kg)		Udder: Percentage of substrate oxidized		Percentage of CO_2 derived from substrate: Whole animal		Percentage of CO_2 derived from substrate: Udder	
		Acetate	Glucose	Acetate	Glucose	Acetate	Glucose	Acetate	Glucose	Acetate	Glucose
Jill	3·3	5·3	5·3	41	89·5	29	34	21	10	21	49
Angel	2·2	6·5	3·6	24	49	69	20	37	9	33	33
Sally	1·9	4·9	3·0	24	75	51	26	27	11	27	49
Balham	1·6	5·3	3·1	32	76	38	16	27	8	27	29
Jill	1·0	—	3·2	11	25	—	31	—	7	—	34
Angel	0	4·0	3·6	9·5	9·5	57	76	23	14	23	20

Comparison of the specific radioactivities of circulating glucose and of mammary CO_2 (calculated from arterial and venous CO_2 concentrations and specific radioactivities) showed that 39% of the mammary CO_2 was derived from the oxidation of glucose in the lactating state and only 20% in the one dry animal. In lactation a smaller proportion of the total glucose taken up by the udder was oxidized (25%) than in the case of acetate, whereas in the dry animal 76% of the glucose was oxidized (Table 3).

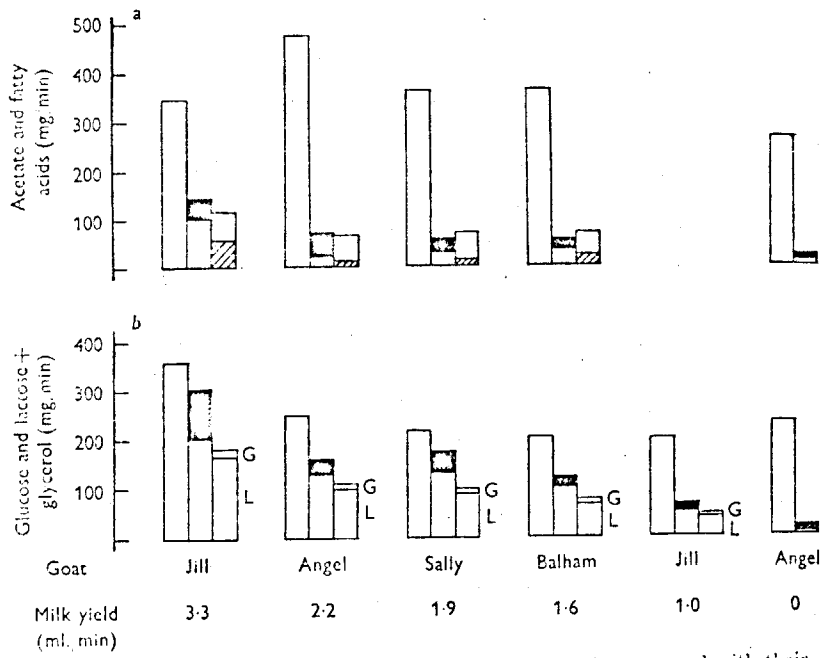

Fig. 2. The mammary uptake of acetate (*a*) and glucose (*b*) compared with their total utilization and the output of their derivatives in the milk. Left-hand column, total entry rate into circulation; middle column, uptake by the udder; right column, output in the milk. ▨, Proportion oxidized, in *a*, acetate, in *b*, glucose; ▨, Fatty acids that could be formed from acetate; G, Glycerol; L, Lactose.

Mammary metabolism of acetate and glucose in relation to milk formation. Measurements were made of the net mammary uptake (blood flow times A-V difference) of acetate and glucose for comparison with the output of their major derivatives in the milk, fatty acids and lactose plus glycerol respectively. It has been assumed that over each period of study when the mean daily milk yield did not vary more than 10%, the metabolic activity [illegible] did not vary significantly, so that experiments done on

goats showed that minute-to-minute, hour-to-hour and day-to-day variations in blood flow and A-V differences were random so long as the milk yield was steady, results which are confirmed in the present work (e.g. Table 2). It is well known that the rate of milk secretion readily falls in response to mild stress (e.g. fasting, a change in environment, slight illness) but in none of the experiments reported here were the milk yields on the days of the experiments significantly different from the previous few days or from each other. The results are shown in Fig. 2. It will be seen that during lactation the mammary glands use a large proportion of the total acetate and glucose available to the whole body. This is particularly true of glucose where in the fully lactating animals 60–85% of the total glucose entering the circulation was used by the udder. In the case of acetate 14–15% was taken up by the udder in three animals and in the highest yielding one (Jill) 41%. These unexpectedly high uptake figures gain credence when compared with output of fat and lactose in the milk. After subtracting the proportion of glucose oxidized the milk lactose and glycerol accounted for 79–95% of the glucose uptake. In the case of acetate the amount oxidized left only sufficient to form 17–29% by weight of the fatty acids in three animals and 45% in the high yielding one.

DISCUSSION

There are no published data on the rates of entry and oxidation of acetate and glucose for the whole body in goats and none for lactating dairy animals so that we can only compare our figures with those made in non-lactating ruminants. The goat is similar to the sheep and cow in that more of the total CO_2 is derived from acetate than from glucose. Acetate entry rates were similar to the figures of Annison & Lindsay (1961) in sheep but about half those of Essig, Norton & Johnson (1961). Entry rates of glucose were almost the same as those reported for sheep by Annison & White (1961) and Kronfeld & Simesen (1961) but about 1½–2 times the values of Bergman (1963) and Ford (1963) in sheep and Davis & Brown (1962) in steers. It seems highly likely that such measurements will be affected by the time after feeding and the diet and plane of nutrition. Our goats were studied after feeding and were on a standard diet similar to those fed to lactating dairy cows.

The results show clearly that during lactation the udder uses both substrates at many times the average rate of the other organs and in addition that over 4 times as much CO_2 originates from glucose in secreting mammary tissues as in the rest of the body so that in most cases more CO_2 is derived [illegible] as was found in isolated perfused goat

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mammary glands (Hardwick *et al.* 1963). The proportion of mammary CO_2 derived from acetate is the same as in the rest of the body, but because the net uptake of acetate is less than for glucose a greater proportion of the acetate is oxidized than is glucose. Another difference is that during lactation acetate and glucose together account for about two-thirds of the total mammary CO_2, whereas they account for just over one-third in the whole animal. A major part of the remainder probably arises from propionate and butyrate in the whole animal but the contribution of these substrates to oxidative metabolism has not yet been measured in the goat. Plasma-free fatty acids (FFA), which may account for as much as 25% of the CO_2 production in starved (24 hr) sheep (West & Annison, 1964), is not a major source of CO_2 in the fed goat. Isotope dilution experiments with ^{14}C-labelled stearic, oleic and palmitic acids have shown that under these conditions the contribution of plasma FFA to total CO_2 production is only about 2–3% (Annison, Fazakerley, Linzell & Nicholls, unpublished).

A most unexpected finding was that in these goats the fully lactating udder was apparently using 60–85% of the total glucose produced in the body. At first this figure seemed improbably high but when the amount of glucose that was being oxidized by the udder was calculated it was seen that the remainder was in reasonable agreement with the amounts of lactose and glycerol being put out in the milk (Fig. 2); a great deal of isotopic evidence from several laboratories has shown that glucose is the main precursor of these substances. Even if no glucose were oxidized by the mammary tissue milk lactose production would demand nearly half of the circulating glucose available in full lactation. It should be mentioned that the Saanen goats used in this work are a dairy breed selected for high milk yields and that the udder in these animals was 2·5–5% of the body weight, as large or larger than the liver, and could well be taking 15–20% of the cardiac output. Further evidence in favour of the magnitude of the udder uptake of substrates is that after single injections of labelled substrates Kleiber, Black, Brown, Baxter, Luick & Stadtman (1955) recovered 50% of glucose in milk lactose in a cow, Kleiber, Smith, Black, Brown & Tolbert (1952) recovered 9–12% of acetate in the milk fat of cows and Lascelles, Hardwick, Linzell & Mepham (1964) recovered 38 and 67% of tritiated chylomicra in the milk fat of two goats.

The proportion of the total circulating acetate that is used by the secreting udder (14–41%) is less than for glucose but a greater proportion of that taken up is oxidized by the mammary glands (29–69%). It has been known since the experiment of Popjak, French, Hunter & Martin (1951), who injected ^{14}C-labelled acetate into a lactating goat, that in ruminants acetate is the main source of milk fatty acids up to and including C16. It has been known also for a long time that secreting mammary

tissue produces more CO_2 than the O_2 used because an oxygen-poor molecule (fat) is being produced from an oxygen-rich one (acetate in ruminants). In lactating goats Linzell (1960*b*) found the R.Q. to be 1·24 ± 0·23 (S.D.). The oxidation of glucose and acetate would give an R.Q. of 1 and from the present data of the amount of acetate available for forming fatty acid, it can be calculated that sufficient O_2 would be 'spared' to raise the R.Q. to 1·1–1·28, which is in reasonable agreement with the observed values (1·21–1·51).

Knowledge of the amount of acetate that is oxidized is also interesting in determining how much of the milk fatty acids is formed from acetate and how much from blood triglycerides. The fatty acids that can be formed from acetate constitute the major part of the total ruminant milk fatty acids. However, Lascelles *et al.* (1964) showed that tritiated chylomicra from intestinal lymph are avidly taken up by the lactating goat's udder and incorporated into milk fat and Barry, Bartley, Linzell & Robinson (1963) found in the same goats used in this work, that sufficient chylomicra are taken up to account for most of the milk fat formed. These findings now appear more reasonable when allowance is made for the amount of acetate oxidized by the udder because sufficient remains to form only 17–45% of the milk fatty acids so that a considerable proportion of milk fat must be derived from triglycerides.

In our experiments we have been unable to demonstrate any effects of 'oxytocin' (endogenous or exogenous) on mammary metabolism that could not have occurred by chance, but we were chiefly interested in deciding whether the doses we injected were influencing our results. The hypothesis that, in addition to squeezing the milk from the alveoli, oxytocin released during milking and suckling also has a metabolic effect on the udder (e.g. increasing the uptake and oxidation of glucose) is plausible. Our results suggest that the small amounts released (40–400 m-u. in goats according to various authors) at the morning and evening milking would have a negligible effect, but the idea merits more detailed study.

SUMMARY

1. The isotope dilution method of measuring substrate turnover and oxidation has been used on four trained conscious female goats under normal animal-house conditions and combined with measurements of substrate uptake and oxidation by the mammary gland.

2. The total entry rate of acetate in the whole animal was 4·9–6·5 mg/kg/min in lactation and 4·0 mg/kg/min in a dry goat; 21–37% of total CO_2 was produced from acetate. In both lactating and dry goats the glucose entry rate was 3·1–5·3 mg/kg/min and glucose accounted for 7–14% of total CO_2 production.

3. During full lactation the udder took up 24–41 mg/kg/min of acetate and 49–89·5 mg/kg/min of glucose; 21–33 % of the mammary CO_2 came from acetate and 29–49 % from glucose; 29–69 % of the acetate taken up and 16–34 % of the glucose were oxidized.

4. During lactation the udder accounted for 60–85 % of the glucose used by the animal and 15–41 % of the acetate.

5. After allowing for the amount oxidized sufficient acetate remained to form only 17–45 % of the milk fatty acids, but there was enough glucose to form all the lactose and milk fat glycerol.

6. The injection of oxytocin (400 m-u.) did not significantly alter the rates of entry or oxidation of glucose or acetate under our conditions.

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The Oxidation and Utilization of Palmitate, Stearate, Oleate and Acetate by the Mammary Gland of the Fed Goat in Relation to their Overall Metabolism, and the Role of Plasma Phospholipids and Neutral Lipids in Milk-Fat Synthesis

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1. Measurements were made of milk yield, mammary blood flow and arteriovenous differences of each plasma lipid fraction, and their specific radioactivities, during the infusion of [U-^{14}C]stearate, [U-^{14}C]oleate, [U-^{14}C]palmitate and [1-^{14}C]acetate into fed lactating goats. 2. Entry rates of fatty acids into the circulation were 4·2mg./min./kg. body wt. for acetate, and 0·18, 0·28 and 0·42mg./min./kg. for stearate, oleate and palmitate respectively. Acetate accounted for 23% of the total carbon dioxide produced by the whole animal, and contributed to the oxidative metabolism of the mammary gland to about the same extent. Corresponding values for each of the long-chain acids were less than 1%. 3. There were no significant arteriovenous differences of phospholipids, sterols or sterol esters, and their fatty acid composition showed no net changes during passage through the mammary gland. 4. There were large arteriovenous differences of plasma triglycerides, and their fatty acid composition showed marked changes across the gland. The proportions of palmitate and stearate fell, and that of oleate increased. 5. Arteriovenous differences of plasma free fatty acids (FFA) were small and variable, but a large fall in the specific radioactivity of each of the long-chain acids examined indicated substantial uptake of plasma FFA, accompanied by roughly equivalent FFA release from mammary tissue. The uptake of FFA was confirmed by the extensive transfer of radioactivity into milk. The FFA of milk were similar in composition and radioactivity to the milk triglyceride fatty acids, and quite unlike plasma FFA. 6. The formation of large amounts of oleic acid (18–21mg./min.) from stearic acid was demonstrated. 7. During the terminal stages of the [^{14}C]acetate infusion, milk triglyceride fatty acids of chain length C_4–C_{14} showed specific radioactivities that were 75–90% of that of blood acetate, and that of palmitate was roughly one-quarter of this value. Oleate and stearate were unlabelled. 8. The results confirmed that milk fatty acids of chain length C_4–C_{14} arise largely from blood acetate, and palmitate is derived partly from acetate and partly from plasma triglyceride, the latter fraction being almost the sole precursor of oleate and stearate.

The precursors of the fatty acids of ruminant milk fat are known to be largely acetate, which gives rise to acids of chain length C_4–C_{16}, and plasma triglycerides, which supply most of the C_{18} acids and a proportion of the other long-chain acids. Plasma FFA,* a lipid fraction of considerable metabolic significance (Steinberg, 1963; Annison, 1964), have attracted attention as possible precursors of milk fat, but recent studies have shown very small and variable arteriovenous differences of FFA across the lactating mammary glands of fed goats and cows (Barry, Bartley, Linzell & Robinson, 1963; Kronfeld, 1965; Hartmann & Lascelles, 1964). However, mammary blood flow is high during lactation so that small arteriovenous differences could represent substantial uptake of substrate. In the present studies the metabolism of FFA by the lactating mammary gland of the normal conscious fed goat has been examined in conjunction with measurements of FFA entry rate into the circulation in the whole animal. Isotope-dilution techniques were combined with measurements of arteriovenous differences and mammary blood flow (Annison & Linzell, 1964) to assess the uptake, oxidation and transfer into milk of plasma

* Abbreviation: FFA, free fatty acids.

FFA. Changes in the concentration, fatty acid composition and specific radioactivity of the major plasma lipids across the mammary gland, and the transfer of radioactivity into milk, were also followed. Similar measurements were made during the infusion of [^{14}C]acetate.

This work was carried out with trained lactating goats surgically prepared to allow the venous drainage from a single mammary gland to be sampled (Linzell, 1960*a,b*) for the assay of blood constituents, and for blood flow measurement. The use of fully trained undisturbed animals was essential to avoid the changes in FFA concentration that accompany even mild excitement. The plasma FFA concentrations observed in this work were uniformly low.

A preliminary report of this work was given to The Biochemical Society (Annison, Fazakerley, Linzell & Nicholls, 1965).

MATERIALS AND METHODS

Experimental animals. The surgical and infusion procedures were as described by Annison & Linzell (1961). The ^{14}C-labelled fatty acids were infused intravenously for 4–8hr. at 2μc/min. by an infusion arrangement that allowed the goats to move freely in their own pens during the experiment. Trained lactating goats were used that had been surgically prepared some years earlier to allow the collection of arterial and mammary venous blood, and the measurement of blood flow through one mammary gland, by exteriorizing one carotid artery, one main mammary vein and dividing blood vessels crossing between the two glands.

Catheters were inserted under local anaesthesia into one jugular vein and an exteriorized carotid artery on the morning of the experiment. Four pairs of simultaneous samples of arterial and mammary venous blood were taken at 15min. intervals during the last hour of infusion. Mammary venous samples were taken from the vein loop with the precaution of manually clamping the other main vein to obtain a representative mammary venous sample (Linzell, 1960*a,b*, 1966). Since the two glands forming the udder were of equal size and giving an equal volume of milk, total uptake by the udder could be calculated. The animals were given hay *ad lib.*, and about 1kg./day of a concentrate was fed in roughly two equal parts at the times of milking (09.30 and 16.00hr.). The infusions were carried out on fed animals during the period 11.00–16.00hr. They were milked hourly until 5hr. after the end of the infusion, an intravenous injection of oxytocin (200milli-units) being used to aid milk ejection.

In the experiments now reported labelled stearate was infused into goat Jill (body weight 63kg.) when she was giving 2·92l. of milk daily (59% of peak yield) of 4·4% fat, mean rate of blood flow through the udder 1140ml./min., haematocrit 23%. Goat Balham was used for three experiments with labelled oleate, palmitate and acetate. She weighed 68·5–73kg., the haematocrit was 23–27% and milk fat 4%. On the days of the experiments the corresponding milk yields (l./day) and rates of blood flow through the udder (ml./min.) were: 2·67, 950; 2·07, 790; 2·24, 790 (55–69% of peak yield).

^{14}C-labelled fatty acids. [U-^{14}C]Stearic acid, [U-^{14}C]oleic acid, [U-^{14}C]palmitic acid and sodium [1-^{14}C]acetate were obtained from The Radiochemical Centre, Amersham, Bucks. The long-chain acids were bound to goat albumin (prepared by the method of Kekwick & Mackay, 1954) by the procedure of Laurell (1957) and the resultant clear solutions diluted with 0·9% NaCl (without addition of carrier fatty acid) to adjust the solution to 2μc/ml. Sodium [1-^{14}C]acetate was dissolved in 0·9% NaCl and carrier sodium acetate added to adjust the solution to 0·5mg. and 2μc/ml.

Extraction and fractionation of blood lipids. Blood samples (30ml.) were immediately centrifuged (3000rpm) at 2° and the concentration of FFA in 2ml. of the separated plasma was determined by the method of Dole (1956). Lipid was extracted from the remainder of the plasma by the method of Folch, Lees & Sloane-Stanley (1957). Plasma FFA was isolated from a portion of the isolated lipid by the procedure of McCarthy & Duthie (1962) and stored in solution in light petroleum (b.p. 40–60°) at −10° until examined by gas radiochromatography. The major part of the plasma lipid was separated into phospholipid and a neutral lipid fraction (containing triglycerides, free sterol and sterol esters) by silicic acid chromatography (Hirsch & Ahrens, 1958).

The component lipids of these major fractions were separated by thin-layer chromatography on silicic acid (silica gel G; E. Merck A.G., Darmstadt, Germany). Phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and lysolecithin were isolated from the phospholipid fraction by the solvent system chloroform–methanol–water (65:25:4, by vol.), and the neutral lipid fraction yielded triglyceride, free sterol and sterol esters when fractionated with the solvent system hexane–diethyl ether (17:3, v/v). Separated lipids were recovered from thin-layer plates by scraping off the silicic acid containing each fraction and eluting with chloroform–methanol (1:1, v/v) and then pure methanol. Yields of isolated lipids were determined by analysis with the following methods: phospholipid fraction (as total phosphorus after digestion, Allen, 1940); triglyceride (Carlson & Wadstrom, 1959); free and esterified sterol (with the modified Liebermann–Burchard reagent of Huang, Chen, Weller & Raftery, 1961). Isolated lipid fractions were stored in chloroform–methanol (2:1, v/v) at −10° until assayed for radioactivity and fatty acid composition.

Extraction and fractionation of milk lipids. Extracted fat was subjected to column chromatography (Hirsch & Ahrens, 1958) to yield phospholipid and neutral lipids. The neutral lipid was separated into triglyceride and sterol (free plus esterified) fractions by thin-layer chromatography. Milk fat FFA was isolated from a portion of the extracted lipid by the method of McCarthy & Duthie (1962).

Analysis of fatty acids. Methyl esters of plasma FFA were prepared by treatment with an excess of freshly prepared diazomethane in ether and examined by gas–liquid chromatography. The analytical data supplied by gas radiochromatography (see below) were supplemented by additional analyses with systems in which β-ionization detectors were used, but with column stationary phases and operating conditions similar to those used in the radiochromatographs. The fatty acid composition of lipid fractions was determined after preparation of the methyl esters of the component fatty acids by transesterification

lipids were refluxed with 5ml. of methanol–benzene–conc. H_2SO_4 (20:5:1, by vol.) for 120min. and the methyl esters extracted with light petroleum (b.p. 40–60°). Milk triglyceride was saponified with KOH, and the fatty acids were separated by partition chromatography on A.R. silicic acid (100 mesh; Mallinckrodt Chemical Works, New York, N.Y., U.S.A.) with methanol–water (10:1, v/v) as stationary phase. Fatty acids were eluted in two stages by using first *n*-hexane (100vol.) saturated with stationary phase (10vol.), followed by a mixture of *n*-hexane (50vol.) and toluene (50vol.) saturated with stationary phase. A mixture of higher fatty acids (including the C_{12} acid) emerged first, followed by well-separated fractions containing acids of chain length C_{10}, C_8, C_6 and C_4. Strict temperature control (25°) was necessary for good fatty acid separations. The solvent system allowed fractions collected from the column to be assayed for radioactivity by liquid scintillation after adding a suitable amount of phosphor in toluene. Quenching corrections were made by using internal standards. Alternate fractions were titrated with 0·01N-NaOH under CO_2-free conditions with 1ml. of Nile Blue (0·001% in ethanol) as indicator. The remaining fractions were assayed for radioactivity. The higher acid fraction eluted from the column was methylated with diazomethane and examined by gas radiochromatography.

Assay of radioactivity. Lipids were dissolved in toluene containing 2,5-diphenyloxazole (4g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0·01g./l.) and assayed for ^{14}C content in a Tritomat liquid-scintillation counter (Isotope Developments Ltd., Bucklow, Berks.). Corrections for quenching were made by adding known amounts of radioactivity (*n*-[^{14}C]hexadecane) as internal standards.

The ^{14}C-labelled long-chain fatty acids (chain length greater than C_{16}) were assayed for specific radioactivity by gas radiochromatography (James & Piper, 1963). Methyl esters of fatty acids were transferred to the gas radiochromatograph as described by Hitchcock & James (1964). The esters were separated on 130cm. straight or coiled columns (internal diam. 4mm.) containing 100–120-mesh Celite supporting 20% Apiezon L grease at 200° or 80–100-mesh Celite supporting 20% ethylene glycol adipate polymer at 170°.

The radiochromatographs used in these investigations were standardized daily by running methyl palmitate of known specific radioactivity.

Other methods of analysis. Concentrations of acetate, O_2 and CO_2 in blood, the specific radioactivities of CO_2 and acetate and the concentrations of total fat in milk were measured as described by Annison & Linzell (1964).

RESULTS

Mammary uptake of plasma triglyceride. As reported previously (Barry *et al.* 1963) there was a consistent and significant fall in the concentration of plasma triglyceride across the mammary gland. Uptakes of plasma triglycerides by the mammary gland were calculated from flow rates of plasma through the mammary gland and arteriovenous-difference data, and in the four experiments were equivalent to 63–82% of the output of milk triglycerides (Table 1). When [U-^{14}C]stearate was infused there was no detectable labelling of plasma triglyceride, but when [U-^{14}C]oleate and [U-^{14}C]-palmitate were infused appreciable amounts of these labelled acids were incorporated into plasma triglycerides (Table 2). In each pair of samples the oleic acid content of the venous plasma triglyceride increased and the stearic acid content decreased relative to arterial plasma triglyceride (Table 2). The plasma triglyceride-palmitate content fell during passage across the gland in two of the three experiments, but the specific radioactivity was largely unchanged, indicating that triglyceride arising in the gland, which would contain unlabelled palmitate synthesized in the gland, is not in equilibrium with plasma triglyceride. This conclusion is strongly supported by the absence of short-chain acids (which occur in milk triglycerides) in venous plasma triglyceride.

Mammary uptake of sterol and sterol esters. There was no significant uptake of sterol, free or esterified (Table 1). Slight labelling of the sterol ester fraction occurred during the infusion of [U-^{14}C]-palmitate, but the specific radioactivity of palmitate was similar in the arterial and mammary venous samples. The fatty acid composition of plasma sterol ester showed only slight changes during passage through the mammary gland (Table 3), but the possibility of fatty acid exchange in the gland cannot be ruled out. The sterol ester fraction, which accounts for the major portion of plasma lipid, was rich in linoleic acid, as observed by previous workers (Garton, 1963).

Mammary uptake of plasma phospholipid. In agreement with the observations of Blackwood (1934) no evidence was obtained for the uptake of significant amounts of phospholipid by the mammary glands (Table 1). Examination of individual phospholipid fractions (phospholipid choline, sphingomyelin, phosphatidylethanolamine and lysolecithin) revealed only small and variable arteriovenous differences. The extent of incorporation of infused labelled fatty acids into plasma phospholipids was too low for assay by gas radiochromatography. Fatty acid analyses were carried out on all isolated phospholipid fractions. No evidence was obtained of significant changes in the fatty acid content of any phospholipid fraction during passage through the mammary gland, but since the fractions were not labelled the possibility of fatty acid exchange cannot be excluded.

Uptake and oxidation of fatty acids by mammary tissue. (*a*) Long-chain acids. Although only small positive or negative arteriovenous differences in FFA concentrations were observed, in each instance the specific radioactivity of the labelled fatty acid declined markedly across the gland (Table 4). Oxidation accounted for only a small fraction of the radioactive fatty acid removed by the gland since the specific radioactivity of carbon dioxide

Table 1. *Mean values for the concentration of plasma lipids in four pairs of arterial and venous blood samples taken during ^{14}C-labelled fatty acid infusions*

The uptakes of triglyceride and acetate by the mammary gland are compared with the output of milk triglyceride.

Expt.	Sample	Sterol (mg./100ml.) Total	Sterol (mg./100ml.) Free	Phospholipid (mg. of P/100ml.)	FFA (m-equiv./l.)	Volatile fatty acid (m-equiv./l.)	Triglyceride (mg./100ml.)	Plasma flow (ml./min.)	Milk yield (l./day)	Mammary uptake: Triglycerides (mg./min.)	Mammary uptake: Acetate (mg./min.)	Output of triglycerides in milk (mg./min.)
[U-^{14}C]Stearate	Arterial	139	22	6·2	0·15	2·0	21·2	877	2·92	74	82	97
	Venous	123	28	5·6	0·14	0·7	12·7					
[U-^{14}C]Oleate	Arterial	172	34	9·4	0·18	1·4	27·1	693	2·67	63	48	81
	Venous	173	33	8·9	0·18	0·6	18·0					
[U-^{14}C]Palmitate	Arterial	121	43	7·5	0·24	1·2	30·8	608	2·07	63	18	76
	Venous	135	42	8·5	0·23	0·8	20·5					
[1-^{14}C]Acetate	Arterial	97	37	5·7	—	1·2	24·2	608	2·24	51	48	81
	Venous	104	38	5·7	—	0·3	15·8					

Table 2. *Composition and specific radioactivities of the fatty acids of arterial and mammary venous plasma triglycerides during the infusion of [U-^{14}C]palmitate, [U-^{14}C]stearate and [U-^{14}C]oleate*

Mean values for three pairs of samples taken during the last hour of infusion are shown. The shorthand nomenclature of Dole, James, Webb, Rizack & Sturman (1959) was used in this and subsequent Tables to indicate chain length, number of double bonds and branched-chain acids (br).

Substrate infused.....	[U-^{14}C]Palmitate				[U-^{14}C]Stearate				[U-^{14}C]Oleate			
	Arterial		Venous		Arterial		Venous		Arterial		Venous	
Fatty acid	(%)	Sp. act. (μc/g.)	(%)	Sp. act. (μc/g.)	(%)	Sp. act. (μc/g.)	(%)	Sp. act. (μc/g.)	(%)	Sp. act. (μc/g.)	(%)	Sp. act. (μc/g.)
10:0	0·4	—	0·6	—	0·2	—	0·4	—	2·7	—	4·1	—
12:0	1·4	—	1·6	—	0·3	—	0·7	—	2·6	—	3·9	—
12:br?	0·4	—	—	—	0·1	—	0·3	—	0·4	—	0·3	—
14:0	1·2	—	2·0	—	1·8	—	1·7	—	1·8	—	1·1	—
14:1	—	—	0·1	—	0·4	—	0·1	—	1·8	—	1·1	—
14:br?	0·7	—	0·1	—	1·4	—	0·2	—	0·7	—	0·7	—
15:0	0·6	—	0·1	—	1·5	—	0·5	—	0·4	—	0·3	—
16:0	27·9	14·1	21·1	12·7	23·4	—	14·8	—	21·7	—	21·3	—
16:1	3·4	—	5·1	—	5·2	—	3·6	—	6·6	—	5·0	—
16:br?	—	—	—	—	3·5	—	5·1	—	0·6	—	0·9	—
17:0	—	—	—	—	2·9	—	1·4	—	3·0	—	2·1	—
18:0	36·5	—	31·4	—	34·6	—	31·6	—	28·8	—	21·8	—
18:1	23·3	—	31·7	—	21·8	—	35·5	—	24·5	20·6	32·1	14·6
18:2	3·2	—	6·2	—	2·9	—	4·3	—	4·4	—	3·8	—

Table 3. *Fatty acid composition of the sterol esters of arterial and mammary venous plasma samples withdrawn simultaneously during the infusion of labelled long-chain fatty acids*

The results for a representative pair of samples from each experiment are shown.

Substrate infused.....	[U-^{14}C]Stearate		[U-^{14}C]Oleate		[U-^{14}C]Palmitate	
Fatty acid	Arterial (%)	Venous (%)	Arterial (%)	Venous (%)	Arterial (%)	Venous (%)
10:0	0·4	0·4	0·3	0·5	0·5	0·6
12:0	0·6	0·9	0·5	0·4	0·7	0·9
12:br?	2·0	4·3	—	—	—	—
14:0	0·7	0·9	0·5	0·9	0·4	0·4
14:1	0·6	0·9	0·4	0·5	0·3	0·2
14:br?	0·3	0·5	0·3	0·7	0·4	0·2
15:0	0·7	0·9	0·2	0·3	0·2	0·2
16:0	8·9	10·2	10·4	12·1	7·7	6·9
16:1	3·8	4·5	2·1	1·9	1·1	0·9
16:br?	0·1	0·1	0·1	—	0·6	0·6
17:0	2·2	3·3	0·7	0·5	0·4	0·4
18:0	2·4	4·2	3·2	4·3	5·5	2·6
18:1	28·1	26·6	27·9	30·0	24·3	24·9
18:2	48·0	41·0	50·1	46·9	54·6	56·9
18:3	1·2	1·3	3·0	1·0	3·3	4·3

in mammary venous blood was not significantly different from that in arterial blood in any of the experiments with long-chain fatty acids (Table 5).

Changes in the composition of plasma FFA across the mammary gland were evident when [^{14}C]stearic acid was infused, but only minor differences were observed in the experiments with labelled oleic acid and palmitic acid (Table 4).

The uptake of radioactivity by the mammary gland and the amount of radioactivity secreted in milk at the end of the period of the infusion are shown in Fig. 1. The ^{14}C-labelled milk fat of declining specific radioactivity continued to be secreted for some hours after the end of the ^{14}C-labelled fatty acid infusion (Fig. 2).

The extensive conversion of stearic acid into oleic acid in the mammary gland was clearly shown when the uptake and production of long-chain fatty acids by the mammary gland was calculated from the arteriovenous differences, and the rates of blood flow and milk production (Table 6). This was confirmed in the stearate experiment, where labelled oleic acid appeared both in the milk and in the mammary venous plasma FFA (Tables 4 and 9). Values for the production of milk C_{18} fatty acids were closely similar to the amounts taken up by the mammary gland, confirming that blood triglycerides are almost the sole precursors of the C_{18} acids in milk (Table 6).

(*b*) Acetate. Mean concentrations of acetate in arterial and mammary venous blood during the infusion of [1-^{14}C]acetate (Table 5) indicated an acetate uptake of 0·95m-mole (57mg. of free acid)/min. The contribution of acetate to carbon dioxide produced by the mammary gland was 23%, and 33% of the acetate taken up by the gland was directly oxidized. These values are very similar to those reported previously (Linzell, 1960*b*; Annison & Linzell, 1964).

Rates of entry and oxidation of fatty acids. (*a*) Long-chain acids. Total plasma FFA concentrations and FFA specific radioactivities were sufficiently constant during the terminal stages of the labelled fatty acid infusions to allow calculation of rate of entry of fatty acids into the plasma (Table 5). The low concentrations of plasma FFA reflected the use of fed undisturbed animals, where FFA mobilization from adipose tissue is minimal (Steinberg, 1963). The specific activity of arterial blood carbon dioxide increased only slowly during the final hour of infusion, and comparison of this specific activity with that of plasma fatty acid showed that the contribution of long-chain fatty acid to total oxidative metabolism was low (Table 5).

(*b*) Acetate. The mean concentration and specific radioactivity of acetate in arterial blood was 1·5m-moles/l. and 17·8μc/g. of C respectively. The acetate entry rate was 4·2mg./min./kg. body wt. and the contribution of acetate to carbon dioxide production in the whole animal was 23% (Table 5), both values being within the range reported by Annison & Linzell (1964).

Incorporation of plasma FFA into milk. When ^{14}C-labelled fatty acids were infused there was a lag period of about 2hr. before milk fat became

Table 4. *Fatty acid composition and specific radioactivities of plasma FFA in arterial and mammary venous blood obtained during ^{14}C-labelled fatty acid infusions*

Mean values for three pairs of samples taken during the last hour of infusion are shown.

Substrate infused.....	[U-^{14}C]Stearate				[U-^{14}C]Oleate				[U-^{14}C]Palmitate			
	Arterial		Venous		Arterial		Venous		Arterial		Venous	
Fatty acid	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)
10:0	—	—	—	—	0·5	—	0·2	—	0·1	—	0·2	—
12:0	0·9	—	0·3	—	1·6	—	0·7	—	0·2	—	0·1	—
12:br	0·2	—	0·2	—	—	—	0·5	—	1·8	—	0·9	—
14:0	2·5	—	2·9	—	3·4	—	2·0	—	2·2	—	2·0	—
14:1	0·4	—	—	—	0·3	—	1·0	—	0·3	—	0·3	—
14:br?	—	—	—	—	0·4	—	0·7	—	0·8	—	0·9	—
15:0	3·2	—	3·4	—	3·6	—	0·2	—	0·9	—	1·2	—
16:0	25·1	—	28·0	—	15·4	—	19·7	—	17·6	90	17·5	45
16:1	4·0	—	2·4	—	3·8	—	3·4	—	3·5	—	4·2	—
16:br?	—	—	—	—	1·8	—	1·4	—	1·7	—	1·5	—
17:0	1·0	—	1·0	—	2·3	—	0·7	—	0·7	—	0·5	—
18:0	33·7	252	40·4	84	35·2	—	37·6	—	31·4	—	30·1	—
18:1	25·7	4	19·5	49	25·6	166	27·3	100	36·1	—	37·3	—
18:2	3·0	—	3·1	—	2·9	—	7·5	—	2·7	—	3·3	—
18:3	—	—	—	—	4·4	—	—	—	—	—	—	—

Table 5. *Mean values for the concentrations and specific radioactivities of plasma fatty acids in four pairs of arterial and venous blood samples obtained during ^{14}C-labelled fatty acid infusions*

Entry rates of stearic acid, oleic acid, palmitic acid and acetic acid are shown with estimates of their contribution to CO_2 production in the whole animal.

Expt.	Sample	Plasma concn. of infused substrate (m-equiv./l.)	Sp. act. of infused substrate (μc/g. of C)	Sp. act. of blood CO_2 (μc/g. of C)	Entry rate (mg./min./kg.)	Contribution to total CO_2 (%)
[^{14}C]Stearate	Arterial	0·047	252	0·25	0·18	0·1
	Venous	0·050	84	0·24		
[^{14}C]Oleate	Arterial	0·051	166	0·40	0·28	0·2
	Venous	0·063	100	0·41		
[^{14}C]Palmitate	Arterial	0·042	90	0·58	0·42	0·6
	Venous	0·042	45	0·59		
[^{14}C]Acetate	Arterial	1·5	17·8	4·2	4·2	23
	Venous	0·3	14·9	4·3		

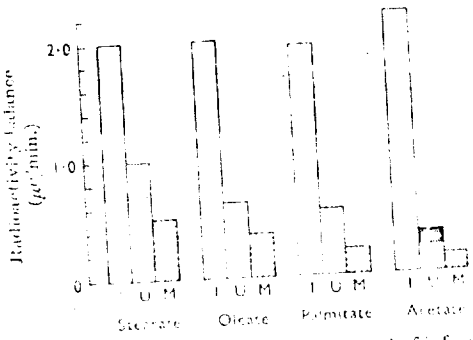

Fig. 1. Radioactivity balance (μc/min.) at end of infusion: I, rate of infusion into animal; U, uptake of whole udder; M, radioactivity in milk. The black bar in the acetate experiment represents the amount of acetate oxidized by the mammary glands.

appreciably radioactive (Fig. 2). In the experiment with long-chain acids maximum specific radioactivity occurred 1–2hr. after the end of the infusion (Fig. 2).

(c) Acetate. Sodium [1-^{14}C]acetate was infused at 2·2μc/min. for 450min. Maximum specific radioactivity of milk fat was observed after 7hr. Although 210min. after the end of the infusion the milk fat showed 80% of this maximum value (Fig. 2). The mean specific radioactivity of acetate in arterial blood during the infusion was 17·8μc/g. of C. There was little change in fatty acid composition during the experiment, and the specific radioactivities of the even-numbered acids C_{12}–C_{16} reached maximum values after 7hr. (Table 7). Examination of the short-chain acids of the triglyceride isolated from this sample showed that the specific radioactivities of the even-numbered acids C_4–C_{10}, like those of the C_{12} and C_{14} acids, were 75–95% of that of circulating acetate, but that of palmitate was approx. 25% of this value (Table 7). These results confirm that acetate is the major precursor of the C_4–C_{14} acids, but that palmitate arises both from acetate (and ketone bodies) and by transfer from plasma triglyceride.

(*b*) Long-chain acids. The fatty acid composition of the milk showed only slight changes during the infusion, but the specific radioactivity of the fatty acid being studied increased steadily until 1–2hr. after the end of the infusion (Tables 8, 9 and 10).

The milk-fat sample of highest specific radioactivity obtained during each infusion was fractionated into triglyceride and free fatty acids and the component fatty acids were examined by gas radiochromatography (Tables 8, 9 and 10). The specific radioactivities of each fraction were of the same order in each experiment. The milk FFA fraction was similar in composition to that of the corresponding milk triglyceride, and obviously dissimilar in composition and specific radioactivity to plasma FFA.

DISCUSSION

The combination of arteriovenous-difference studies and isotope-dilution techniques allowed measurement of the rates of entry and oxidation of individual plasma free fatty acids in the whole animal, and in the mammary gland, since mammary blood flow was known. The low specific radioactivity of $^{14}CO_2$ in arterial and mammary venous blood relative to that of circulating ^{14}C-labelled fatty acid showed the limited extent of FFA oxidation in both the mammary gland and in the whole animal. Low overall oxidation of plasma FFA and low entry rates were consistent with the low plasma FFA concentration, which is characteristic of fed, unexcited animals (Steinberg, 1963).

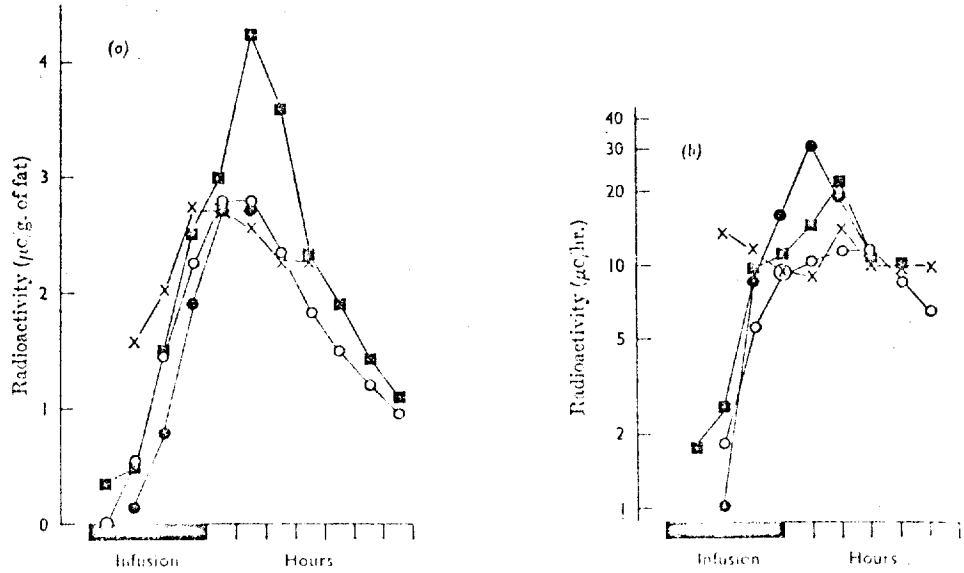

Fig. 2. Time-course of the appearance of radioactivity in milk fat, after the infusion of long-chain fatty acids for 4hr. and acetate for 7·5hr. The end of infusion is synchronized. ×, Acetate; ■, oleate; ●, stearate; ○, palmitate.

Table 6. *Mammary uptake (from plasma triglyceride) and secretion (as milk triglyceride) of stearate and oleate*

Substrate infused	Uptake across mammary gland (mg./min.)		Secreted in milk (mg./min.)		Net production of oleic acid (mg./min.)
Fatty acid.....	18:0	18:1	18:0	18:1	
[U-^{14}C]Stearate	24·5	4·2	9·1	21·4	21·4
[U-^{14}C]Oleate	23·6	2·6	8·2	16·3	17·4
[U-^{14}C]Palmitate	26·1	3·6	7·8	17·7	17·7

The small positive or negative arteriovenous differences of plasma FFA suggested the absence of significant FFA removal by mammary tissue, but the marked decline in the specific radioactivity of labelled FFA across the gland indicated substantial uptake of plasma FFA. This was confirmed by the considerable transfer of radioactivity into milk fatty acids, since the specific radioactivity of other possible precursors of milk fat was far too low to account for the radioactivity appearing in milk. The extent of the reduction of the specific radioactivity of ^{14}C-labelled FFA across the gland and the absence of net uptake indicated release of FFA from mammary tissue. The origin of FFA produced by mammary tissue is uncertain, but, if plasma triglycerides are hydrolysed before transfer across the surface membrane of capillary cells, liberated fatty acids would equilibrate with plasma FFA, and the fraction escaping absorption would appear in mammary venous plasma. It is known that mammary venous plasma in the goat contains more lipoprotein lipase than arterial plasma (Barry *et al.* 1963). The composition of mammary venous FFA resembled that of the fatty acids of mammary venous-plasma triglyceride (Tables 2 and 5), but was unlike that of mammary tissue, which, like milk fat, contains short-chain and medium-chain fatty acids. When [U-^{14}C]stearate was infused, labelled oleate appeared in mammary venous-plasma FFA (Table 5). This is difficult to explain since the dehydrogenation of stearate is almost certainly an intracellular process, and there was no evidence that fatty acids synthesized in the gland from acetate (chain length C_4–C_{16}) escaped from the mammary gland. A consistent increase in the oleic acid content of the mammary venous

Table 7. *Composition and specific radioactivities of the fatty acids of milk fat secreted during and after the infusion of* [1-^{14}C]*acetate* (3·3μc/*min. for* 7·5*hr.*)

Only the long-chain acid fraction was examined in the total milk fat analyses. Sample numbers refer to hours after the start of infusion. Specific radioactivity of blood acetate, 17·8μc/g. of C.

	Total milk fat						Milk triglyceride	
	Sample 5		Sample 8		Sample 11		Sample 7	
Fatty acid	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)
4:0							1·3	13·3
6:0							2·8	13·8
8:0							8·3	15·3
10:0							12·9	13·9
12:0	5·1	10·3	4·5	14·0	4·3	6·5	3·6	16·8
12:1	0·6	—	—	—	0·5	—	0·4	—
14:0	0·6	—	—	—	—	—	—	—
14:1	12·5	9·1	11·8	11·3	13·4	4·9	10·2	12·6
16:0	1·0	—	0·5	—	0·7	—	0·4	—
16:1	—	—	—	—	—	—	—	—
18:0	29·2	3·1	30·1	4·0	32·1	1·7	24·5	4·3
18:1	1·0	—	1·0	—	1·0	—	0·9	—
18:2	—	—	—	—	—	—	—	—
20:0	10·0	—	12·2	—	12·3	—	9·8	—
20:1	30·7	—	29·1	—	28·5	—	23·3	—
20:2	2·7	—	2·2	—	1·5	—	1·5	—

Table 8. *Incorporation of radioactivity into the long-chain fatty acids of milk triglycerides and free fatty acids during the infusion of* [U-^{14}C]*palmitic acid* (1·95μc/*min. for* 4*hr.*)

Time after start of infusion (hr.)	1		3	5				10	20
	Triglyceride		Triglyceride	Triglyceride		Free fatty acid		Triglyceride	Triglyceride
Fatty acid	(%)	Sp. act. (μc/g. of C)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)
4:0	1·4			6·3		—	—	—	4·8	—
6:0	3·9			5·2		5·1	—	—	5·2	—
8:0	12·4			14·1		9·2	—	—	11·7	—
10:0	0·2			—		—	—	—	—	—
12:0	0·9			—		0·9	—	—	—	—
14:0	28·2	0·8	7·5	30·7	13·4	42·8	15·0	8·3	27·2	5·8
16:0	1·5			—		—	—	—	—	—
16:1	0·7			—		—	—	—	—	—
17:0	10·7			18·0		13·4	—	—	16·7	—
18:0	36·7			23·6		26·4	—	—	32·4	—
18:1	3·0			1·9		2·7	—	—	1·9	—
18:2										

the total triglyceride fraction suggested either the preferential removal of triglycerides not containing oleic acid, or the resynthesis and release from mammary tissue of triglycerides containing oleic acid arising from the dehydrogenation of stearic acid. In the present experiments the extent of incorporation of labelled stearic acid into plasma triglyceride was too low for changes in triglyceride specific radioactivity across the gland to be assessed, and further work with labelled triglycerides is necessary to resolve these problems.

Comparison of the rates of plasma triglyceride uptake (calculated from arteriovenous differences and mammary blood flow) with those of milk triglyceride production has clearly shown that the C_{18} acids of milk could be derived almost entirely

Table 9. *Incorporation of radioactivity into the long-chain fatty acids of milk triglycerides and free fatty acids during the infusion of [U-^{14}C]stearic acid (2·02 μc/min. for 4 hr.)*

Time after start of infusion (hr.)	0		1·5		2·5		4		5		6			
	Triglyceride		Triglyceride		Triglyceride		Triglyceride		Triglyceride		Triglyceride		Free fatty acid	
Fatty acid	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)
10:0	5·3	—	—	—	—	—	3·5	—	—	—	6·3	—	5·9	—
12:0	6·6	—	—	—	—	—	7·0	—	—	—	7·2	—	6·7	—
14:0	15·6	—	—	—	—	—	15·6	—	—	—	16·4	—	9·6	
14:br?	2·2	—	—	—	—	—	3·4	—	—	—	2·5	—	0·8	—
15:0	1·1	—	—	—	—	—	1·8	—	—	—	—	—	—	—
16:0	31·0	—	—	—	—	—	34·1	—	—	—	37·3	—	35·8	
16:1	1·2	—	—	—	—	—	3·3	—	—	—	1·9	—	—	—
18:0	7·3	—	—	1·9	—	20·5	5·9	39·9	—	34·0	5·0	28·7	9·9	8·6
18:1	29·3	—	—	0·6	—	4·8	24·7	10·4	—	16·1	22·1	16·7	28·5	9·9
18:2	1·8	—	—	—	—	—	0·8	—	—	—	1·3	—	2·8	—

Table 10. *Incorporation of radioactivity into the long chain fatty acids of milk triglycerides and free fatty acids during the infusion of [U-^{14}C]oleic acid (2·06 μc/min. for 4 hr.)*

Time after start of infusion (hr.)	1		2		3		4		5		6				8	
	Triglyceride		Triglyceride		Triglyceride		Triglyceride		Triglyceride		Triglyceride		Free fatty acid		Triglyceride	
Fatty acid	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)	(%)	Sp. act. (μc/g. of C)
10:0	7·2	—	3·4	—	4·7	—	7·6	—	7·6	—	5·0	—	2·3	—	9·6	—
12:0	4·9	—	3·8	—	4·9	—	5·8	—	4·4	—	5·7	—	4·8	—	5·6	—
14:0	12·3	—	12·7	—	13·2	—	13·7	—	13·8	—	13·8	—	9·8	—	13·2	—
14:br?	1·9	—	—	—	2·0	—	—	—	1·4	—	—	—	1·0	—	—	—
16:0	16·6	—	28·8	—	28·3	—	29·4	—	30·5	—	30·0	—	29·7	—	30·4	—
16:1	1·6	—	2·7	—	1·3	—	2·5	—	2·7	—	2·4	—	7·7	—	2·3	—
17:0	0·9	—	—	—	0·9	—	—	—	2·2	—	—	—	—	—	—	—
18:0	13·0	—	13·1	—	13·9	—	11·9	—	8·0		13·8	—	12·7	—	13·0	—
18:1	28·6	—	32·7	3·1	28·0	7·9	26·5	20·1	26·7	30·8	24·8	30·6	26·8	15·1	23·1	22·2
18:2	2·7	—	2·8	—	2·9		2·5		2·6		2·7	—	5·1	—	2·6	—

in plasma triglyceride (Table 5). The extensive conversion of stearic acid into oleic acid, first reported in udder fat of the perfused cow's udder by Lauryssens *et al.* (1969), was convincingly demonstrated *in vivo* (Table 5). Glascock & Reinius (1956) reported the occurrence of tritium-labelled oleic acid in the milk fat of cows given tritium-labelled stearic acid.

There was no detectable uptake of free or esterified cholesterol by the mammary gland, confirming the early observations of Lintzel (1934), as the amounts secreted in milk are so small that removal of an equivalent amount from arterial plasma would not be detectable. Patton & McCarthy (1963) demonstrated rapid uptake of oleate and linoleate by the cholesterol ester fraction of milk when the acids were infused through the teat canal, suggesting that the cholesterol esters of milk originate in the gland. The present studies have not implicated the plasma cholesterol fraction in any aspect of milk lipid synthesis, but only experiments with isotopically labelled esters would provide unequivocal evidence on this point. Clarenburg & Chaikoff (1966) have suggested that in the rat 80% of milk cholesterol is synthesized in the mammary gland.

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

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

MATERIALS AND METHODS

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

The incorporation of ^{14}C from [1-^{14}C]- or [2-^{14}C]-propionate into glucose was consistent with the conversion of propionate into a symmetrical C_4 compound before conversion into phosphoenolpyruvate, and was in agreement with the established pathway, namely propionate → methylmalonate → succinate → phosphoenolpyruvate → glucose. The equal distribution of ^{14}C between C-2 and C-3 of lactate arising from [2-^{14}C]propionate was evidence against metabolic pathways involving the direct conversion of propionate into lactate (or pyruvate), e.g. propionate → acrylate → lactate → pyruvate (Mahler & Huennekens, 1953). The slight incorporation of ^{14}C into acetate and β-hydroxybutyrate from [1-^{14}C]propionate implies the production of acetyl-CoA from propionate by a pathway other than the well-established route of propionate → succinate → pyruvate → acetyl-CoA, which would result in loss of ^{14}C as $^{14}CO_2$. Comparison of the specific radioactivities of blood propionate, acetate and β-hydroxybutyrate (Table 1) indicated that this pathway was not quantitatively significant. The high specific radioactivity of β-hydroxybutyrate relative to that of acetate probably reflected the greater rate of acetate entry into body pools. James, Peeters & Lauryssens (1958) reported the production of ^{14}C-labelled acetate from [1-^{14}C]propionate by the perfused goat udder.

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

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

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

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

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

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

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

SUMMARY

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

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

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

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

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

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

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

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

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

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The heat increment of steam-volatile fatty acids in fasting sheep

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The heat increment of feeding represents the additional heat produced by an animal consequent upon the ingestion of food. In man and dog it is usually called the specific dynamic effect (S.D.E.), or less correctly the specific dynamic action (S.D.A.), and is measured by giving the fasting subject an amount of food containing sufficient energy to meet the basal requirement and by determining the increase in heat production which results. This procedure entails the continuation of measurements until such time when metabolism is again basal. It was shown by Glickman, Mitchell, Lambert & Keeton (1948) that often insufficient time was allowed in such studies to permit the complete metabolism of the food, hence many estimates of the S.D.E. of food by this technique are too low. In farm animals the technique employed by Kellner & Köhler (1900) and by Fingerling (1914) to estimate heat increments was to give individual foods or nutrients as additions to a ration which already permitted small positive retentions of energy and to measure the increase in the 24 h heat production which resulted. The two methods do not give identical results since heat increment is smaller when fasting and not maintenance is the base-line, as has been shown in many experiments with a variety of species. Between-species comparisons of the heat increment of feeding must clearly be made at similar nutritional planes.

In Table 1, the results of published experiments are presented which allow an estimate to be made of species differences in the heat increments of feeding. It will be noted that data are not available for man at maintenance levels of feeding.

Table 1. *Heat increment* (*Cal./100 Cal. metabolizable energy*) *of feeding in different species evaluated with fasting as the base-line* (S.D.E.) *and with maintenance as the base-line*

	Starvation base-line			Maintenance base-line				
Nutrient	Man[1]	Rat[2]	Sheep	Man	Rat[2]	Pig[3]	Steer[4]	Sheep[5]
Fat	4	6	—	—	17	9	35	29
Carbohydrate	6	9	7[6]	—	23	17	37	32
Protein	30	25	—	—	31	26	52	54
Mixed rations	10–17[7]	~17[8]	~22[9]	—	~31[8]	10–40[10]	35–70[10]	35–70[11]

(1) Lusk (1931).
(2) Kriss, Forbes & Miller (1932).
(3) Fingerling (1914).
(4) Kellner & Köhler (1900).
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The data of Table 1 show that above maintenance the ruminant animal loses approximately twice as much heat in metabolizing the carbohydrate of its diet as does the non-ruminant rat and pig. With mixed diets the increments of heat range from 35 to 70% in the ruminant but from only 10 to 40% in the pig. Comparisons of the S.D.E. in the fasting animal are difficult to make, but it appears that here also the ruminant animal loses more energy as heat compared with the non-ruminant rat and man. The large effect of nutritional plane is very evident.

There have been several attempts to explain the high heat increment of the ruminant. Kellner (1920), following the work of Zuntz, ascribed it to the work of digestion (*Verdauungsarbeit*). He took it to include not only intestinal work in the sense originally used by von Mering & Zuntz (1877), but the mechanical work of prehension and mastication of food. The extent of this energy expenditure has not been measured directly. Earlier, the high heat increment had been ascribed to a stimulating effect of the steam-volatile fatty acids on metabolism (Grouven, 1864). This theory was revived by Ritzman & Benedict (1938) only to be rejected since they could not reconcile the high heat increments found in the non-ruminant but herbivorous horse with a stimulation of metabolism by the lower steam-volatile fatty acids, not realizing, perhaps, the massive nature of microbial fermentation in the large intestine of that species.

More recently, attempts have been made to explain the high heat increments of ruminants in terms of the thermodynamics of the intermediary metabolism of the end-products of ruminant digestion. Marston (1948*a*) suggested that the transfer of at least some of the absorbed fatty acids into the cycles of intermediary metabolism involved considerable thermodynamic losses. He showed also (Marston, 1948*b*) that the heat of fermentation, that is the free energy of the microbial fermentation, could not account for more than 15% of the total heat increment in the ruminant. He later stated that heat loss occurring in the dissimilation of acetic acid 'is probably responsible for a major part of the relatively high rate of heat production of the fed ruminant' (Marston, 1951).

McClymont (1952) expanded Marston's contention that inefficiency of free-energy capture in the dissimilation of acetic acid was the main reason for the high heat increment of the ruminant, and suggested that a high heat increment of acetic acid and of butyric acid might be inferred from known facts about their intermediary metabolism. He suggested that very rapid oxidation in non-lipogenic tissue was the main reason for the high heat increment, and that two-carbon units arising from acetic acid or butyric acid undergo a continuous partition between oxidation and lipogenesis, the former being, thermodynamically, a wasteful process.

To arrive at this hypothesis, McClymont had perforce to make certain assumptions. The first one was that the metabolism of the steam-volatile fatty acids in the ruminant, particularly of acetic and butyric acids, is indeed associated with very large losses of heat. He quoted unpublished results of Marston's which suggested that the heat increment of acetic acid was 70%, and of propionic acid 30%, and adduced the rather unsatisfactory experiments of Lusk (1921) in which an increase in the heat production of a dog was noted on the giving of 3 g acetic acid. He quoted also the results of the experiments of Dye & Marsters (1943) with nephrectomized dogs, who had found that

when sodium acetate was injected intravenously the excess oxygen consumption was equivalent to 49% of the theoretical amount needed to oxidize the acetate completely. A lower value of 39% was obtained in the nephrectomized eviscerated animal. With butyric acid the value was 26% in both types of animal preparation. McClymont (1952) concluded from this work that the S.D.E. of acetic and butyric acids could be placed at 50–70% and that the resulting heat increment due to their metabolism would account for a major proportion of the total heat increment, reported as 30–60% of the metabolizable energy. This conclusion, however, is difficult to reconcile with the results of calorimetric experiments with rats by McManus, Bender & Garrett (1943) in which acetic acid fed in the form of triacetin constituting 20% of the diet was used as efficiently as a source of energy as a mixture of glycerol and glucose.

With the possible exception of Marston's work, details of which are not available, none of the above experiments appeared to us to provide an unequivocal proof that the metabolism of the end-products of rumen fermentation is in fact associated with large losses of heat under normal conditions, whereas the experiments of McManus *et al.* (1943) suggest the contrary. For this reason the contribution of the steam-volatile acids to the heat increment of sheep has been studied in some detail by indirect methods of calorimetry. This paper primarily deals with the heat increments found in starved sheep when acetic, propionic and *n*-butyric acids were given singly. Subsequent papers will deal with the more physiological experiments in which a range of steam-volatile acid mixtures was given to fasted and fed sheep and in which marked associative effects were found.

EXPERIMENTAL

Animals. Three adult castrated male sheep (Halfbred × Down) were used as experimental animals. A fistula into the rumen was made by the technique of Phillipson & Innes (1939) and of Stoddard, Allen, Hale, Pope, Sorenson & Winchester (1951), and the fistula closed with a cannula of Perspex. These animals were kept in pens and fed *ad lib.* until 10 days before an experiment began. They were then placed in cages and given a ration sufficient to maintain their body-weight.

Plan of experiments. Each of the three sheep was given acetic, propionic or butyric acid to supply approximately 700 Cal. combustible energy daily. Each was also given a mixture of acetic, propionic and butyric acids in the molar proportions of 5:3:2 to supply 1100 Cal. Four further experiments were made. Two were with acetic acid, one with a mixture of the acids to supply 600 Cal. and one with a glucose solution supplying 600 Cal. combustible energy. With the exception of the last two experiments with acetic acid the experiments all conformed to one plan. The sheep was starved for 4 days during which dilute saline was dripped into the rumen usually at the rate of 6·5 l./day. During the next 2 days a fatty-acid solution containing the same amount of saline was given under identical conditions. The sheep then reverted to the dilute-saline regimen for 2 days. A second fatty-acid solution was then given for 2 days and the experiment ended with a 2-day period in which the dilute saline was again given. In one experiment the second fatty-acid solution was replaced by a glucose solution containing penicillin. The antibiotic was added to reduce possible rumen bacterial

activity. The total period without solid food was thus 12 days, the whole period being spent within a respiration chamber under controlled ambient conditions. In the last two acetic-acid runs the animals received dilute saline for the first 4 and last 3 days of a 10-day experiment and acetic acid during the intervening 3 days. At the end of each experiment the rumen of the sheep was inoculated with ingesta from a normal sheep's rumen and the animal allowed limited food for a few days before being given unlimited access to food. Recovery was usually uneventful. At least 4 weeks were allowed between experiments with any one individual. Under this system of management of the animals there was no cumulative loss of condition in the series of experiments. The average loss of body-weight during a 12-day experiment was 6·6 kg with individual losses ranging from 5·3 to 10·0 kg. Part of this loss must be accounted for as loss of ingesta from the gut.

Steam-volatile fatty-acid mixtures. In much of the previous work on the metabolism of the steam-volatile fatty-acids, the acids have been given in buffered solutions as the sodium salts. If continued, such administration gives rise to a marked systemic alkalosis (cf. Johnson, 1955) and is clearly not a suitable method to use if CO_2 production is to be measured over long periods. Elsden & Phillipson (1948) have mentioned that acetic acid could be dripped into the rumen at the rate of 4–5 g/h (up to 2 g-equiv./24 h) with no change in the alkali reserve of the blood. Preliminary experiments with other fistulated sheep showed that the mixed acids themselves could be infused into the rumen without producing any gross acidosis, in amounts which supplied up to 1100 Cal. gross energy daily, and that there was no necessity to buffer the solutions provided they were dilute. Accordingly, all steam-volatile acid mixtures were given in dilute solution, to which was added an equal volume of a neutral physiological saline solution, containing per l., 6 g NaCl, 0·4 g KCl, 0·2 g $MgCl_2$, 0·2 g $CaCl_2$ and 0·45 g NaH_2PO_4.

Table 2 summarizes the data relating to the steam-volatile fatty-acid solutions used.

Continuous-drip feed pump. For such relatively long-term experiments it was thought necessary to administer the solution to the animals at a steady rate throughout rather

Table 2. *Characteristics of solutions of steam-volatile fatty acids and glucose used in the experiments**

Substance	Approx. volume of solution supplied/24 h (l.)	Normality	Moles supplied/24 h	Energy supplied/24 h (Cal.)
Acids:				
Acetic	6·5	0·515	3·35	700
Propionic	6·5	0·293	1·91	700
Butyric	6·5	0·206	1·34	700
Mixture of the three in the molar proportions 5:3:2	6·5	0·529	3·44	1100
Mixture (same)	4·2	0·448	1·88	600
Glucose	4·2	38·2 g/l.	0·89	600

* The values for the number of moles and the energy supplied varied slightly from experiment to experiment, depending upon the output of the pumps used to introduce the solutions into the rumen.

than in large amounts at intervals. To introduce the acids a pump was found necessary since gravity-flow systems were not found to be satisfactory. It consisted of a slow-speed motor (Drayton Regulator and Instrument Co. Ltd), geared to operate the plungers of two Record syringes as pistons. The liquid flow from the reservoir to the chamber via the syringes was controlled by two sets of Bunsen valves made from plastic tubing (Vinyl 'Portex', laboratory grade 6c, Portland Plastics Ltd). Rubber tubing was unsatisfactory since it lost its elasticity in the presence of the acids. By alteration of the size of the syringes, the stroke of the pistons or the reduction gearing, a very wide range (1–30 l./24 h) of fluid output could be obtained. The outflow from the pump was connected to a tube leading into the rumen by drainage tubing (Vinyl 'Portex' drainage tubing 6HD, Portland Plastics Ltd) which first passed through a seal in the side of the respiration chamber and then over a series of pulleys and counterpoise weights that allowed the animal to lie and stand at will. The tube into the rumen was protected by an outer sheath of Polythene tubing and was so constructed that the acid outlet was unlikely to be restricted and the acid leaving it unlikely to come into direct contact with the rumen wall without prior dilution by rumen contents.

The output of the pump was very constant from day to day but in practice the 24 h input was always measured gravimetrically. Two such pumps have been in operation for nearly 2 years without any mishap. The valves are renewed periodically and the pistons greased but otherwise maintenance is negligible. Pl. 1, 1, shows a fistulated sheep in the respiration chamber, Pl. 1, 2, a photograph of the pump and Pl. 1, 3, the internal lead into the sheep's rumen.

Calorimetric methods. Two respiration apparatuses were used to determine the CO_2 production, O_2 consumption and CH_4 production of the sheep. Measurements were made at 12 h intervals throughout except in the final two experiments with acetic acid, when the interval was 24 h. Urine was collected quantitatively at the same intervals. The analytical and other methods employed in the respiration experiments were those previously described (Blaxter, Graham & Rook, 1954). The computations involved are discussed in the text.

Sampling of blood and rumen liquor. Blood samples from the jugular vein were withdrawn daily, heparin being used as anticoagulant. Rumen-liquor samples were also taken daily via the fistula every 24 h.

Analysis of rumen liquor. After filtration through glass wool the total steam-volatile acid content was determined by the method of Elsden, Hitchcock, Marshall & Phillipson (1946). A further sample collected under liquid paraffin was used for pH estimation with the glass electrode. In some experiments samples were examined qualitatively by direct microscopy by Dr Constance Higginbottom.

Analysis of blood. Blood sugar was determined by the method of Nelson (1944) with the copper reagent of Somogyi (1945). The CO_2-combining capacity of plasma was determined after equilibration with a 5% CO_2-air mixture by the method given by Peters & Van Slyke (1932). Ketone bodies were determined by a modification of the method of Thin & Robertson (1952). Proteins were first precipitated with trichloroacetic acid as suggested by El Hawary & Thompson (1954). The colour reagent

comprised 0·1 ml. salicylic aldehyde in 8 ml. 4N-KOH. After incubation at room temperature overnight, 0·5 ml. of the coloured solution was added to 4 ml. water and the resulting solution read in a Unicam Spectrophotometer at a wavelength of 540 mμ, in 1 cm cells. The total steam-volatile acid content was determined by steam distillation of the whole blood by the method of Scarisbrick (1952); 0·1 ml. silicone (M.S. Antifoam A, Hopkin & Williams Ltd, Chadwell Heath, Essex) in carbon tetrachloride, 4% (w/v) was used as an antifrothing agent, 4 ml. blood were used in each estimation and three 40 ml. volumes of distillate collected.

Analysis of urine. N was determined by Kjeldahl's method with $CuSO_4$ and Se as catalysts. Ketones were determined initially by the method of Greenberg & Lester (1944) as modified by Pugh (1954) and later by that used for the determination of ketone bodies in blood.

RESULTS

The results are presented first for the twelve experiments with the three acids given separately and as a mixture. Results for the remaining four experiments are given in less detail.

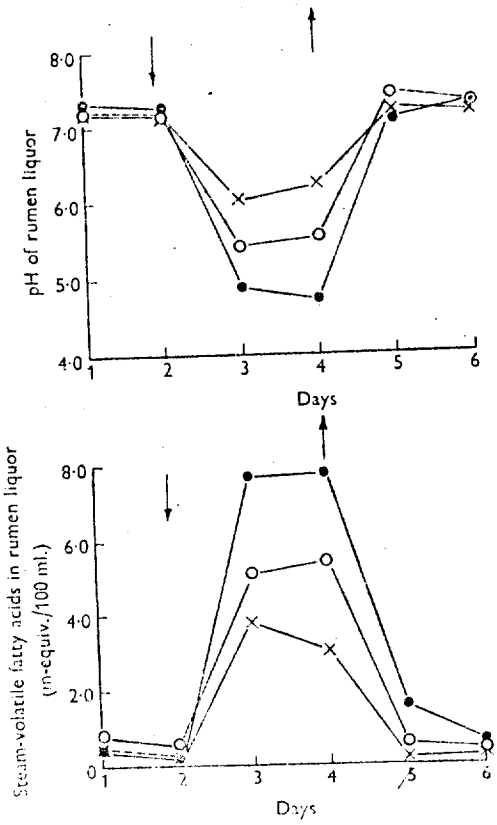

Fig. 1. Mean pH and mean concentration of steam-volatile fatty acids in rumen liquor of fasting sheep as affected by the infusion of 700 Cal. energy as acetic, propionic or *n*-butyric acid. ●—●, acetic acid; ○—○, propionic acid; ×—×, butyric acid; ↓, beginning of infusion; ↑, end of infusion.

Conditions within the rumen. As was to be expected from the large fluid intake used in these experiments the character of the rumen contents changed markedly as starvation proceeded. By the end of the 4th day the contents, although still green, contained relatively little vegetable debris and samples drawn at later stages were colourless and contained many epithelial cells. The bacterial count showed a steady decline throughout and the flora was a much simplified one, being largely composed of short rods and cocci. The reduced activity of the rumen flora by the end of the 4th day of starvation was indicated by the fact that during the previous 24 h the mean total methane production per animal amounted to 2·03 l. as compared with the 26–28 l. produced by a fed animal. The mean production of methane during the 12th day of starvation was 0·58 l.

In Fig. 1, the mean results for the pH and steam-volatile acid concentration in rumen liquor are given. It will be noted that for equal calorie intakes as steam-volatile acid the depression of the rumen pH and the elevation of its steam-volatile acid content was greatest for acetic acid and smallest for butyric acid, that is, in proportion to the number of hydrogen ions supplied. The mean values obtained at the end of 24 and 48 h for the rumen concentration of steam-volatile acid and the pH at 48 h are shown in Table 3.

Table 3. *Concentration of steam-volatile fatty acids in rumen liquor of fasting sheep, receiving infusions of steam-volatile fatty acids into the rumen, 24 and 48 h after the beginning of infusion, and rumen pH at 48 h*

(Mean values for three sheep)

Acid infused	Actual amount supplied (moles/24 h)*	Concentration of steam-volatile fatty acids in rumen (m-equiv./100 ml.) After 24 h	After 48 h	Rumen pH after 48 h
Acetic	3·32	7·67	7·73	4·73
Propionic	1·94	5·41	5·44	5·54
Butyric	1·32	3·85	3·03	6·24
Mixture of the three in the molar proportions 5:3:2	3·49	6·80	6·72	5·37
None	None	—	—	6·9–7·4
Standard error of means†	—	±0·39	0·01–0·04 ±0·39	±0·12

* Values in this column are actual intakes. They differ slightly from those given in Table 2 owing to the variable output of the infusion pumps.

† The standard error of differences between the concentration of the acids at the 24th and 48th h obtained from the acids × days component in the analysis of variance was ±0·36.

There were no significant differences between values for steam-volatile acid concentration obtained at the 24th and 48th hour. The acids must, therefore, have been absorbed, and the concentration noted at the 48th hour represented a steady-state relationship in which the amount leaving the rumen was equal to the amount infused. The differences in concentration of the acids and the pH ascribable to the effects of the acid infused were highly significant statistically. Even the highest values observed for concentration of steam-volatile fatty acids are not abnormal and are in fact lower

than many values recorded in the literature for sheep on natural foods (Phillipson, 1942; El-Shazly, 1952; Johns, 1955).

Prestarvation pH values in these sheep were in the region of 6·0–6·5. The increase in rumen pH to 6·9–7·4 on starvation is in agreement with the results of Phillipson (1942). A rumen pH of 4·7 is low but values as low have been recorded in sheep in which fermentations giving rise to a fair proportion of lactic acid occurred (Phillipson, 1952; Hungate, Dougherty, Bryant & Cello, 1952). It is remarkable, however, that in the absence of the natural buffers of food and in the absence of the stimulus of solid food on the secretion of bicarbonate into the rumen via the saliva, the pH was not more seriously depressed by the acid infusion. It may be concluded that the steam-volatile acids may be given to supply 700–1100 Cal./day without any serious accumulation in the rumen.

Fermentation of acids. Despite the fact that the sheep were starved, the rumen still contained micro-organisms and some methane was produced. Methane production is a good indication of the extent to which bacterial fermentation is proceeding within the rumen and the experiments thus provide information on whether or not the acids are further degraded with the production of methane. In Fig. 2, the mean production of methane by the sheep receiving the individual acids is shown. It is clear that in none was there an increase in the production of methane, which provides support for the finding of Beijer (1952) that solutions of the sodium salts of these acids are not fermented in vitro by rumen bacteria.

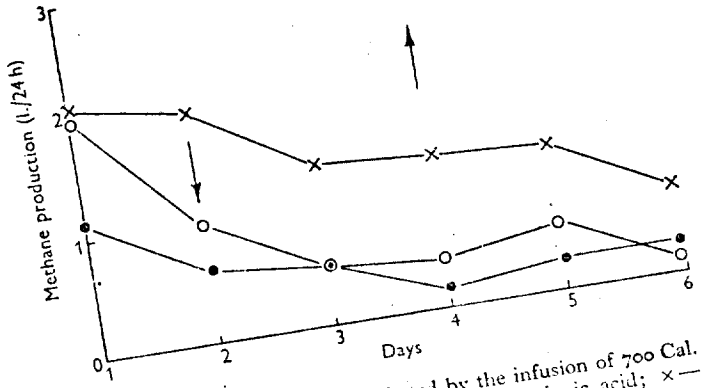

Fig. 2. Methane production of fasting sheep as affected by the infusion of 700 Cal. energy as acetic, propionic, or *n*-butyric acid. ○—●, acetic acid; ○—○, propionic acid; ×—×, butyric acid; ↓, beginning of infusion; ↑, end of infusion.

Composition of the blood. Fig. 3 summarizes the observations made on the concentration of glucose, steam-volatile fatty acids and ketone bodies in the whole blood, and the CO_2-combining capacity of the plasma. The mean values obtained 48 h after the beginning of the infusion are shown in Table 4, together with their standard errors.

From the concentration of steam-volatile acids given in Table 4 it is clear that the infusion of acetic acid resulted in a very considerable accumulation in peripheral blood. There were small, but nevertheless statistically significant, increases with butyric acid and with the mixture but no change at all with propionic acid. Annison (1954) found the steam-volatile acid content of venous (jugular) blood from fed sheep to range from 0·056 to 0·143 m-equiv./100 ml. After the sheep had fasted for 48 h

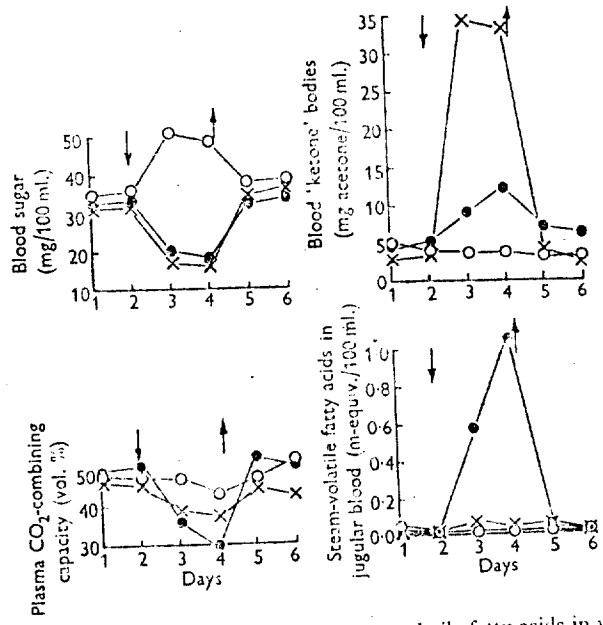

Fig. 3. Concentration of sugar, 'ketone' bodies and steam-volatile fatty acids in whole blood, and the CO_2-combining capacity of the blood plasma, of fasting sheep as affected by the infusion of 700 Cal. energy as acetic, propionic or *n*-butyric acid. ●—●, acetic acid; ○—○, propionic acid; ×—×, butyric acid; ↓, beginning of infusion; ↑, end of infusion.

Table 4. *Concentration of sugar, ketone bodies and steam-volatile acids in the jugular blood, and the CO_2-combining capacity of the blood plasma of fasting sheep receiving infusions of steam-volatile fatty acids into the rumen*

(Mean values for three sheep 48 h after the beginning of infusion)

Acid	Steam-volatile fatty acids* (m-equiv./100 ml.)	Sugar (mg/100 ml.)	Ketone bodies (expressed as acetone) (mg/100 ml.)	CO_2-combining capacity of plasma (vol. %)
None (starvation values before infusion)	0·014 ± 0·002	33·5	4·1	47·4
Acetic	1·043 ± 0·031	17·7	12·1	29·6
Propionic	0·013 ± 0·002	48·1	3·6	42·3
n-Butyric	0·049 ± 0·004	16·1	33·2	37·0
Mixture of the three in the molar proportions 5:3:2	0·056 ± 0·003	45·1	3·6	41·0
Standard error of means	—	± 2·12	± 1·51	± 1·18

* Separate standard errors are given for the steam-volatile fatty acids since the mean concentration when acetic acid was given was almost 100-fold that observed when the others were supplied.

the values had fallen to below 0·03 m-equiv./100 ml. Reid (1950*b*) reported arterial (carotid) blood values from fed sheep ranging from 0·102 to 0·195 m-equiv./100 ml., and when allowance is made for the considerable arterio-venous differences that exist (these may be of the order of 50% in individual animals (Reid, 1950*b*; Annison, 1954), it can be seen that the venous concentrations are similar to those obtained by Annison. There is little doubt that when acetic acid was given alone the very high level of steam-volatile acid in jugular blood represented a considerable departure from normality and was hardly compatible with McClymont's (1952) contention that acetic acid is very rapidly oxidized. On the assumption that the acid is distributed throughout the whole of the body water and that the venous concentration of steam-volatile acid represents the concentration of acetic acid in this water, some 18 g acetic acid are present in the tissues at any one time. This is clearly a minimal estimate. No comparable accumulation of propionic acid was found and with *n*-butyric acid the amount in the tissues, on a similar assumption, was approximately 150 mg.

These results suggest that when acetic acid is given alone it is not metabolized particularly rapidly, whereas *n*-butyric acid and propionic acids are. When the mixture containing 1·72 g-equiv. acetic acid was given, no accumulation of volatile fatty acid in the blood occurred, which suggests that the presence of either propionic or butyric acids or both, acting synergistically, facilitates the metabolism of acetic acid. From Table 3 it can be seen that administration of acetic or *n*-butyric acid resulted in a highly significant depression of the blood glucose, whereas propionic acid and the mixture produced a rise in sugar levels to normal and above. The implications of these results are dealt with in the discussion.

From the blood-ketone values reported in Table 3, it is apparent that *n*-butyric acid and, to a lesser extent, acetic acid, caused a highly significant increase in ketone bodies. Jarrett, Potter & Filsell (1952), Johnson (1955) and Clark & Malan (1956), among others, have demonstrated in vivo the ketogenic nature of the C_4 acid for ruminants. The experiments of Pennington (1952) and of Seto, Tsuda & Umezu (1955) have shown that the rumen epithelium is a major site for the conversion of butyric acid to ketone bodies. The finding of a smaller, but nevertheless significant, rise in blood ketones with acetic acid is in agreement with the results of Jarrett & Potter (1950), Jarrett *et al.* (1952) and Clark & Malan (1956). All the above-mentioned workers have demonstrated the antiketogenic nature of propionic acid. The absence of an increase in blood ketones after the infusion of the mixed steam-volatile acids is further confirmation of the antiketogenic nature of the C_3 acid.

As might be expected, the CO_2-combining capacity of the venous plasma was reduced when acetic acid was given. The reduction was from 50 vol. % CO_2 to 29·6. It has already been noted that the rise in concentration of steam-volatile fatty acids in the blood was 1 mg-equiv./100 ml. and, since 1 mg-equiv. CO_2 = 22·4 ml. CO_2 (the carbon dioxide is held as the bicarbonate), it would appear that the major cause of the acidosis was the peripheral accumulation of acetic acid. There was, however, concomitantly an increase in the ketones of the blood, and clearly these would account in part for the depression of the CO_2-combining capacity. With butyric acid a slightly less severe acidosis developed. Analogous reasoning suggests that the major cause

was accumulation of ketones, presumably β-hydroxybutyric acid and acetoacetic acid (Clark & Malan, 1956), in the blood stream rather than the accumulation of the unoxidized acid. The considerable production of acetoacetic acid from *n*-butyric acid in rumen epithelium was shown by Pennington (1952). With propionic acid there was no ketosis and no accumulation of the acid in the blood. The small drop in the CO_2-combining capacity of the plasma that occurred during the 2nd day of acid infusion may have been due to the accumulation of lactic or pyruvic acids which were not estimated in these experiments. A rapid rise in the blood levels of these two acids consequent upon the injection of propionate into the blood stream of sheep was noted by Jarrett & Potter (1950), and Pennington & Sutherland (1954) have found that when propionate is metabolized alone by sections of sheep-rumen epithelium, lactic acid is

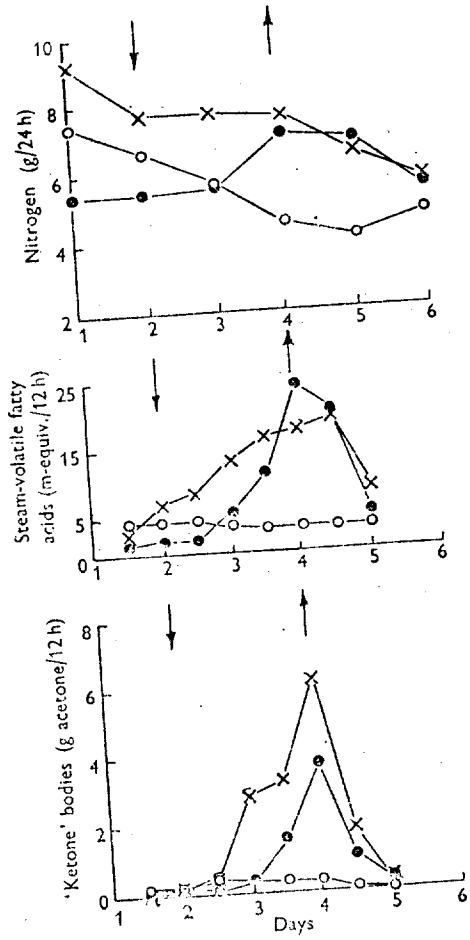

Fig. 4. Quantities of nitrogen, 'ketone' bodies and steam-volatile fatty acids excreted in the urine of fasting sheep as affected by the infusion of 700 Cal. energy as acetic, propionic or *n*-butyric acid. ●—●, acetic acid; ○—○, propionic acid; ×—×, butyric acid; ↓, beginning of infusion; ↑, end of infusion.

produced in amounts which account for between 30 and 50% of the propionate disappearing.

Composition of the urine. In Fig. 4 the mean values are shown for the nitrogen and steam-volatile acid content of urine for the three sheep, and of urine 'ketone' bodies for sheep P, when the single acids were infused into the rumen.

The excretion of nitrogen in the urine rose markedly when acetic acid was given and fell when the C_3 acid was supplied. The summarized data for N excretion given in Table 5 show that the difference between the two acids was statistically significant. With *n*-butyric acid no significant change in nitrogen excretion occurred. These results indicate a marked protein-sparing effect of propionic acid but a considerable increase in protein katabolism with acetic acid. The administration of the C_2 and C_4 acids resulted in a significant increase in urine content of the steam-volatile acids. The loss of these acids from the body via the kidney represented a very small proportion of the acid intake. Thus with acetic acid some 1670 m-equiv. of acid were introduced into the rumen each 12 h and yet the maximal excretion did not exceed 25 m-equiv., or 1·5%.

Table 5. *Changes in excretion of nitrogen (g/24 h) by fasting sheep receiving infusions of steam-volatile fatty acids into the rumen*

(Mean values for three sheep during the last 24 h of infusion)

Acid	Mean increase (+) or decrease (−) in N excretion (g/24 h)	Odds that change significantly different from zero
Acetic	+1·74	13:1
Propionic	−1·09	5:1
n-Butyric	+0·08	—
Mixture of the three in the molar proportions 5:3:2	−1·02	3:1
Standard error of means	±0·50	—
Significant difference, $P=0·05$	±1·97	—

The amounts of ketones contained in the urine reflect the changes that occurred in the ketone content of the blood as a result of the acid infusions. Thus the ketonuria was more pronounced with the C_4 than the C_2 acid and with the C_3 acid there was no change.

Respiratory metabolism. In Fig. 5 the O_2 consumption and CO_2 production of the three sheep given the three steam-volatile fatty acids are given, together with the mean respiratory quotients. It is clear that ingestion of acetic acid to supply 700 Cal./day resulted in a greater increase in O_2 consumption and in CO_2 production than did the ingestion of either propionic acid or *n*-butyric acid.

In order to determine the heat increments of food by indirect calorimetry the usual procedure is to use the non-protein respiratory quotient to arrive at that proportion of the O_2 consumed needed for the complete dissimilation of fat and that needed for dissimilation of carbohydrate. In the present experiments, when the infusions of acids were begun after 4 days of starvation following feeding at a maintenance level, the non-protein respiratory quotients were all close to 0·7, indicating that metabolism

of glycogen reserves and any carbohydrate reaching the tissues from the gut was negligible, and that the sheep were using body fat and protein as the sole sources of energy. The calorific value of O_2 under these conditions was taken to be 4·69 Cal./l. An increase in the R.Q. during an experiment, in which a steam-volatile acid is given,

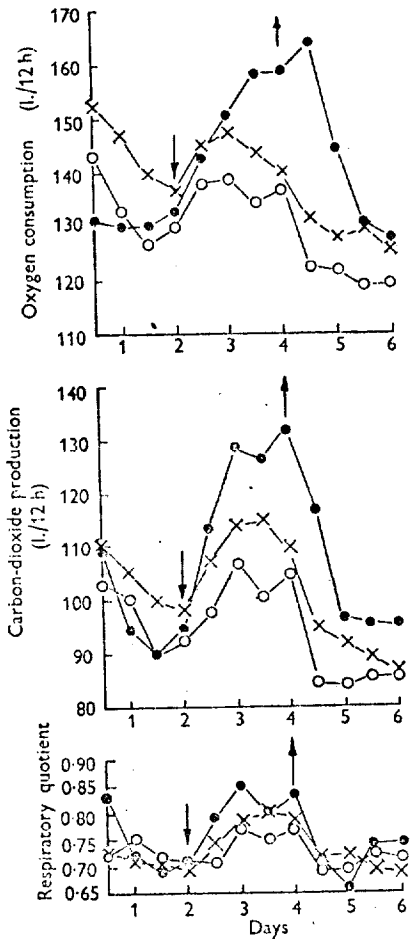

Fig. 5. O_2 consumption, CO_2 production and uncorrected respiratory quotients of fasting sheep as affected by the infusion of 700 Cal. energy as acetic, propionic or *n*-butyric acid. ●—●, acetic acid; ○—○, propionic acid; ×—×, butyric acid; ↓, beginning of infusion; ↑, end of infusion.

to a value approaching 0·8 does not, however, for several reasons necessarily mean that the calorific value of O_2 increases by 2·5% to a value of 4·80 as might be inferred from the Zuntz-Schumberg tables as modified by Lusk (1931). Firstly, as shown in Table 6, for complete dissimilation of acetic acid, although the R.Q. is 1·00, the same as for carbohydrate, the calorific value of O_2 is not 5·047 but 4·674 Cal./l., that is 7% less. Secondly, it has been shown that acetic acid by accumulating in the tissues leads to acidosis and a loss of bicarbonate in the plasma. This additional CO_2 is not

to be considered in computing a non-protein respiratory quotient. The same arguments apply to the C_3 and C_1 acids. Finally, when butyric acid is administered any urinary excretion of ketone bodies, implying only a partial oxidation of the C_1 acid means that oxygen has been consumed without a concomitant loss of CO_2 and the calorific value of this oxygen is not known though it is obviously small. A calculation of the calorific value of the oxygen so used can be made by first assuming complete combustion of butyric acid and then deducting the heat of combustion of the acetoacetic acid formed from it.

Table 6. *Calorific value of O_2 in the dissimilation of fat, carbohydrate and the steam-volatile fatty acids, and overall O_2 consumption and CO_2 production associated with other reactions in which the steam-volatile acids may participate*

Variable	Heat of combustion or of reaction (Cal./mole)	O_2 consumed (l./mole)	CO_2 produced (l./mole)	R.Q.	Calorific value of O_2 (Cal./l.)	Reference
			Complete dissimilation			
Tripalmitin	7657	1623·5	1143·1	0·703	4·716	Lusk (1931)
Mixed fat	—	—	—	0·707	4·686	Lusk (1931)
Starch	680·4	134·4	134·4	1·000	5·063	Swift & French (1951)
Mixed carbohydrate	—	—	—	1·000	5·047	Lusk (1931)
Acetic acid	209·4	44·8	44·8	1·000	4·674	Calculated from heat of combustion and chemical composition
Propionic acid	367·2	78·4	67·2	0·857	4·683	
n-Butyric acid	524·3	112·0	89·6	0·800	4·681	
			Other reactions			
Release of CO_2 from bicarbonate due to systemic acidosis	None	None	22·4	—	—	—
Oxidation of *n*-butyric acid to acetoacetic acid	?	22·4	None	—	?	—
Glucogenesis from propionic acid	?	11·2	None	—	?	—

The method finally adopted to calculate heat productions during the acid runs was to first compute the O_2 consumption, CO_2 production and urinary-N excretion to be expected in the experimental periods. This computation was done by fitting quadratic regressions to the results obtained on the 3rd, 4th, 8th and 12th days of starvation, when no acids were given (eight points) and interpolating to obtain values for the 5th, 6th, 9th and 10th days when the acids were supplied. From these interpolated values heat production was computed by the use of the non-protein R.Q. and the tables of Zuntz & Schumberg (Lusk, 1931).

The actual O_2 consumption and CO_2 production during the periods when the acids were infused were corrected for protein metabolism in the usual way. The non-protein R.Q.'s were not, however, calculated but, instead, each l. of O_2 consumed was assumed to be associated with the production of 4·7 Cal. This method is justified since the non-protein R.Q.'s obtained during the periods of acid infusion result from the oxidation of mixtures of fat and fatty acids, and the calorific values l. of O_2 for fat and the three

fatty acids differ by less than 0·5% (see Table 6). The errors resulting from a neglect of the respiratory quotients are negligible.

This method of calculation of heat production when the acids were given, by ignoring the CO_2 production, effectively minimizes errors that might arise owing to loss of bicarbonate from the tissues during the acidosis. Results for sheep P with this method are given in Table 7.

Table 7. *Heat production in experiments with sheep P when acetic, propionic or butyric acids were given separately, or their mixture in the molar proportions* 5:3:2, *by infusion into the rumen*

Acid given	Twelve-hour period of experiment	Base-line of heat production* (Cal./24 h)	Heat production in experimental period (Cal./24 h)	Increase in heat production (Cal./24 h)
Acetic	1	1269	1345	76
	2	1271	1572	301
	3	1276	1514	238
	4	1282	1514	232
Propionic	1	1160	1214	54
	2	1151	1263	112
	3	1143	1245	102
	4	1136	1263	127
Butyric	1	1276	1444	168
	2	1256	1437	181
	3	1238	1316	78
	4	1221	1331	110
Mixture	1	1354	1527	173
	2	1326	1518	192
	3	1310	1410	100
	4	1297	1411	114

* Calculated by interpolation of CO_2 production, O_2 consumption and urinary N excretion in starvation periods when no acids were given.

In Table 8, the mean increments of heat in Cal./24 h from each of the three sheep are given. The values obtained for the first 12 h period of acid infusion were not used in computing the mean increments. With butyric acid allowance was made for the oxygen used in oxidizing the acid to acetoacetic (see footnote 1, Table 8). In order to compute the calorie equivalent of the acids completely oxidized within the body, the calorie equivalent of the weight of acid infused was corrected for the amount of acid accumulated in the tissues (see footnote 2, Table 8), the amount of acid excreted in the urine (see footnote 3, Table 8) and the calorie equivalent of the quantity of ketone bodies excreted (see footnote 4, Table 8).

The results given in Table 8 show that acetic acid had the highest heat increment and that propionic acid and *n*-butyric acid had significantly lower ones. The last line of Table 8 shows that the increment of heat as a percentage of the calories metabolized was 41% for acetic acid, 13% for propionic acid and 16% for *n*-butyric acid. The value obtained for acetic acid is considerably lower than the value reported by McClymont (1952) from the unpublished results of Marston in which the introduction of 70 g

acetic acid into a sheep resulted in a rise in heat production approximately equivalent two-thirds of the energy in the acetic acid. It is, however, similar to that obtained y Dye & Marsters (1943) from the increase in O_2 consumption when sodium acetate was injected into nephrectomized eviscerated dogs. The value for butyric acid is considerably lower than that obtained by the last-mentioned workers.

A mixture of acetic, propionic and butyric acids in the molar proportions of 5:3:2 would be expected to give a heat increment of 27%. The observed value was 17%, which is considerably lower. Furthermore, the level of intake of the steam-

Table 8. *Mean heat increments (Cal./24 h) of each of three sheep receiving infusions of steam-volatile fatty acids into the rumen, and calculation of heat increment in terms of metabolized fatty acids*

	Acetic acid (Cal./24 h)	Propionic acid (Cal./24 h)	Butyric acid (Cal./24 h)	Mixture of the three in the molar proportions 5:3:2 (Cal./24 h)	Standard error of means
Sheep P	257	114	123	135	±27·6
Sheep T	220	83	95	222	±33·3*
Sheep S	288	90	112	209	—
Mean	255·1	95·7	110·4	188·7	—
Heat attributed to O_2 used in oxidizing butyrate to acetoacetate†	0	0	11·8	0	—
Corrected heat increment	255·1	95·7	98·6	188·7	—
Calorific value of acid infused	694	711	691	1115	—
Correction:					
Tissue accumulation‡	35	0	0	0	—
Urinary excretion of fatty acids§	7	0	18	0	—
Unoxidized 'ketone' bodies in urine‖	27	0	52	0	—
Calorific value of acid metabolized	625	711	621	1115	—
Heat increment as percentage of acid metabolized	40·8 ± 5·3	13·5 ± 4·7	15·9 ± 5·4	16·9 ± 3·0	

* The standard error for the mean values in line 4 (±33·3) was obtained from the sheep × acid interaction term in the analysis of variance. The error applying to results for individual sheep was obtained from the 12 h period × acids × sheep interaction term.

† Calculated from the observed increase in ketone excretion by sheep given butyric acid. Excretion of 0·125 mole acetoacetic acid would be associated with the consumption of 2·8 l. O_2. This quantity of O_2 if full calorific value for dissimilation of butyrate is assumed would give rise to 13·2 Cal. If the heat associated with the oxidation of *n*-butyric acid to acetoacetic acid is taken as 11 Cal./mole, the difference, 13·2 − 1·4 = 11·8 Cal., represents the overestimation of the heat production due to neglect of the ketosis.

‡ Calculated from the increase in the steam-volatile acids in the tissues from the 24th h to the 48th h. It was assumed that the jugular-blood concentration represented the concentration of acid in the whole body water of the sheep which was taken to be 60% of the body-weight. The mean body-weight was 59·0 kg. No correction was necessary for ketone concentration in the tissues as there was no change during the 24 h concerned.

§ The mean increase in the excretion of steam-volatile acid in the urine of sheep given acetic and *n*-butyric acids was 54·0 m-equiv./24 h in both instances.

‖ The mean increases in excretion of ketone bodies were for acetic acid 3·7 g and for butyric acid 7·0 g acetone/24 h.

volatile acid mixture was higher and, as previously pointed out, higher intakes might be expected to result in higher heat increments.

Table 9 shows that there were no significant differences between sheep, though it was thought, in the course of the experiments, that the smallest sheep (S) showed the most pronounced effects. There was no significant variation in the results from 12 h period to 12 h period during the final 36 h of experiment for any of the acids, though here it was suspected that metabolism did not reach a steady state when acetic acid

Table 9. *Analysis of variance of the individual heat increments (Cal./24 h) of each of three sheep receiving infusions of steam-volatile fatty acids into the rumen*

Component	Degrees of freedom	Mean square	Variance ratio	Significance
Total	35	—	—	—
Sheep	2	1465·5	—	NS
Acids	6	49313·9	14·81	**
Sheep × acids	11	3328·2	—	—
Total experiments	24	—	—	—
Within individual experiments	2	—	—	—
12 h periods	6	649·7	—	NS
12 h periods × acids	4	3012·4	1·31	NS
12 h periods × sheep	12	2857·3	1·25	NS
12 h periods × acids × sheep		2283·8	—	—

NS = not significant statistically, $P > 0·05$.
** = significant statistically, $0·01 > P > 0·001$.

was given. The continued accumulation of acetic acid in peripheral blood suggests that the metabolism was not, in fact, constant, and it was invariably found that in the first 12 h after the infusion of acetic acid the heat production and oxygen consumption remained elevated. This was not so apparent with propionic and butyric acids, as may be seen in Fig. 5. Presumably, this continued metabolism reflected the gradual reduction of the accumulated acetic acid in the tissues.

Effect of sugar. In a previous paper (Armstrong & Blaxter, 1956) the metabolic effects were presented of a solution of sugar dripped into the rumen of sheep P starved for 8 days and given antibiotics. These results showed that the heat increment due to glucose was 6·4%, a value similar to that found in man and in the rat. Some fermentation of the sugar took place despite precautions to avoid it, but was of no great magnitude.

Further experiments with acetic acid. Two further experiments with sheep S and T were carried out in which acetic acid was given to supply about 700 Cal. (approx. 3·3 moles) 24 h, and, in these, the period of infusion was prolonged to 72 h. Metabolism measurements were made at 24 h intervals instead of 12 h intervals. The results showing the composition of the blood and rumen contents are given in Table 10. The values were similar to those obtained in the earlier series, but the degree of acidosis judged by the alkali reserve was considerably greater in sheep T than it was in the previous experiment with this sheep. With sheep S a metabolic equilibrium of acid accumulation in the blood appeared to have been reached by 24 h when the CO_2-combining capacity of the plasma was 20–25 vol. %. Sheep T had reached an equilibrium of 12–14 vol. % at 48 h.

Table 10. *pH and concentration of steam-volatile fatty acids in rumen liquor, CO_2-combining capacity of plasma, concentration of steam-volatile fatty acids, ketones and sugar in whole blood of two sheep given 3·3 moles acetic acid/24 h for 72 h by infusion into the rumen*

Variable	Sheep	Preliminary period (days before infusion)		Period of infusion. End of day			Recovery period. End of day		
		2	1	1	2	3	1	2	3
pH of rumen liquor	S	7·13	7·44	4·78	4·66	4·44	7·54	7·54	7·45
	T	7·01	7·05	5·00	4·86	4·52	7·41	7·57	7·41
Acids in rumen liquor (m-equiv./100 ml.)	S	0·73	0·94	7·10	8·76	9·98	0·82	0·76	0·65
	T	0·44	0·50	7·54	7·29	8·74	1·13	0·84	0·69
Plasma CO_2-combining capacity (vol. %)	S	41·5	39·0	23·0	20·0	25·0	37·0	36·0	41·0
	T	43·0	43·0	25·0	14·0	12·5	31·0	—	34·0
Blood sugar (mg/100 ml.)	S	27·3	28·6	13·1	25·1	29·7	46·2	36·9	36·4
	T	31·5	28·4	22·2	18·6	26·4	55·9	—	39·5
Total ketones in blood (mg acetone/100 ml.)	S	6·73	5·89	14·80	9·75	31·95	10·76	3·70	9·57
	T	6·22	5·55	14·46	14·63	12·11	11·77	—	8·41
Acids in blood (m-equiv./100 ml.)	S	0·010	0·013	1·237	1·110	1·210	0·010	0·003	0·010
	T	0·003	0·003	0·963	1·340	1·710	0·010	—	0·003

The O_2 consumption and urinary N excretion were used to compute the increase in heat production by the method previously outlined, and the calorie equivalent of O_2 was taken to be 4·70 Cal./l. irrespective of the non-protein R.Q. The results are given in Table 11.

Table 11. *Heat production in experiments with sheep S and T when about 700 Cal. acetic acid were given by infusion into the rumen*

Sheep and calorific value of acid given	Period of measurement (h)	Base-line of heat production (Cal./24 h)	Heat production in experimental period (Cal./24 h)	Increase in heat production (Cal./24 h)
Sheep S (705 Cal.)	0–24	1296	1322	26
	24–48	1284	1497	213
	48–72	1255	1544	289
Sheep T (732 Cal.)	0–24	1330	1364	34
	24–48	1330	1586	256
	48–72	1336	1659	323

Table 12 shows that the mean heat increment expressed as a percentage of the acid metabolized in these experiments was 41·1% in excellent agreement with the previous result of 40·8%. Inclusion of the two values obtained in these experiments with the previous two narrows the margin of error attached to the mean estimate of heat increment to about ±4%.

Carbon retention. The estimate of the heat increment for acetic acid may be checked independently from the carbon retention of the sheep. In the calculation of the heat increments, given in Tables 8 and 12, O_2 consumption only was used since the calorific value of O_2 consumed in the complete dissimilation of acetic acid is very close to the

Table 12. *Mean heat increments (Cal./24 h) of sheep S and T when receiving infusions of acetic acid into the rumen, and calculation of heat increment in terms of acetic acid metabolized*

(Values for final 48 h of infusion)*

	Sheep S	Sheep T
Mean increase in heat production	251	289
Calorific value of acetic acid	705	732
Correction:		
Tissue accumulation of fatty-acid and 'ketone' bodies†	18·4	27·3
Urinary excretion of fatty acid‡	8·0	13·0
Unoxidized urine 'ketone' bodies§	42·7	16·6
Calorific value of acetic acid metabolized	635·9	675·1
Therefore, heat increment as percentage of acid metabolized	39·5	42·8

* For methods of computation see footnotes ‡, § and ‖, Table 8.
† Mean body-weight of sheep S 51·0 kg, of sheep T 65·6 kg.
‡ Mean increase in urinary excretion of fatty acid (m-equiv./24 h): sheep S 39·7, sheep T 61·9.
§ Mean increase in 'ketone' bodies in urine (g acetone/24 h): sheep S 5·93, sheep T 2·27.

Table 13. *Computation of heat increment of acetic acid given by infusion into the rumen in sheep S and T from their metabolism of carbon and nitrogen**

Component	Sheep S	Sheep T
Carbon balance (g/24 h)		
C supplied as acetic acid	+80·79	+83·91
C excreted by lungs as CO_2	−42·96	−39·89
C excreted in urine as acetone	−3·68	−3·96
C excreted in urine as acetic acid	−0·95	−1·49
C released from bicarbonate in tissues owing to acidosis†	−0·17	+1·39
C of acetic acid accumulated in tissues	+0·10	−3·68
C of 'ketone' bodies accumulated in tissues	−1·62	+0·28
C of body spared from oxidation	+31·51	+36·56
Carbon and nitrogen balance (g/24 h)		
N of body spared from oxidation	−1·61	−0·33
C spared as protein	−5·15	−1·05
C spared as fat‡	+36·66	+37·61
Energy balance (Cal./24 h)		
Energy of depot fat spared from oxidation	+460	+472
Energy of body protein spared from oxidation	−56	−11
Energy retained as acetic acid in tissues§	−1	+30
Energy retained as 'ketone' bodies in tissues§	+19	−3
Total energy balance	+422	+488
Energy intake as acetic acid	+705	+732
Therefore heat increment	+283	+244
Heat increment from oxygen consumption (see Table 12)	+251	+289

* To avoid confusion the signs attached to the values represent the algebraic operation of the terms. Thus C excreted in respiratory CO_2 is a loss of C. It, however, contains some CO_2 arising from the fall in alkali reserve, so for sheep T in which the alkali reserve fell markedly the sign becomes positive. The C of acetic acid accumulated in the tissues does not contribute to the retention of fat or protein and therefore has a negative sign. Since sheep T showed a decrease in blood ketone content during the experiment the C of the ketones is given a positive sign.

† Calculated from the body-weight of sheep, on the assumption that the total body water is 60% of the weight and that the reduction in the CO_2 content and increase in the acetic-acid content of the plasma and whole blood applies to the whole body water.

‡ The total carbon retained as fat and protein less the carbon retained as protein. The factors of Blaxter & Rook (1953) were used in making the computation.

§ The accumulation of acetic acid and of ketones in the body represents a gain of energy to the animal and is not therefore included in the heat increment.

calorie value of O_2 consumed in the combustion of body fat. The respiratory CO_2 was ignored. In Table 13, the carbon retention of sheep S and T in the second series of experiments has been computed. The components to be subtracted from the intake of C are the respiratory C in the CO_2 exhaled and the C of the urine. For simplicity of presentation the values are expressed as the mean changes in C excretion over the base values, in the 24th–72nd h of experiment.

The carbon retained in the body when acetic acid is given represents the carbon from body tissue spared by oxidation of the acid. From this carbon balance and that for nitrogen it is possible to compute the amounts of body fat and protein that have been spared when 700 Cal. energy as acetic acid were given. Details of the computation are given in Table 13, and footnotes explain the logic of the steps taken.

The results given in Table 13 show that heat increments computed from a knowledge of the increase in O_2 consumption are in agreement with those calculated independently from the carbon and nitrogen retention of the animal within the range of analytical error to be expected in this type of work.

DISCUSSION

Blood sugar of the ruminant. The findings about the effect of the steam-volatile fatty acids on the blood sugar of the fasting sheep are of some interest. The mean value for the blood sugar in the fed sheep in Britain has been given by Reid (1950*a*) as 39·1 mg/100 ml., and in fasting it falls to values ranging from 26 to 35 mg/100 ml. The values we have obtained in fasting sheep are similar to these, and the few for sheep fed at the maintenance level range from 40 to 43 mg/100 ml.

The rise in blood sugar when propionic acid was given is in agreement with the observations of others with ruminants (Johnson, 1955; Clark & Malan, 1956) and is in agreement with the fact that propionic acid is glucogenic (Ringer, 1912; Deuel, Butts, Hallman & Cutler, 1935–6).

With acetic acid the fall in blood sugar is in agreement with the classical work of Ringer & Lusk (1910), which showed that it is not converted into glucose. The critical experiments of Reid (1951) showed that acetic acid will not relieve insulin coma in the sheep, whereas propionic acid is as effective as glucose. There are metabolic pathways whereby acetate carbon could be incorporated into the glucose molecule (Lorber, Lifson & Wood, 1945; Potter & Heidelberger, 1950) notably through entry of acetyl coenzyme A into the tricarboxylic-acid cycle but such pathways do not result in any net increase in carbohydrate.

Considerable uncertainty exists as to the effect of *n*-butyric acid on the blood sugar of ruminants. Jarrett *et al.* (1952) found a marked rise in the blood glucose of sheep after intravenous injection of the sodium salt, but pointed out that, since the injections were followed by marked respiratory distress and partial collapse, the response in blood sugar may have been due to glycogenolysis stimulated by the sympatho-adrenal system. Potter (1952) reported that intravenously injected sodium butyrate relieved insulin coma in sheep and caused a rise in blood glucose. On the other hand, in experiments in which butyric acid was orally administered to goats, Schultz & Smith (1951) found that after a brief initial rise in blood sugar there was a marked decline

to one-half the initial value. Clark & Malan (1956) found a significant drop in blood sugar in most of the sheep they dosed with sodium butyrate. They point out that the results obtained by injecting the acid into the blood stream may be misleading since the normal route of absorption of butyric acid is via the epithelium of the rumen, where a large-scale conversion to ketones takes place (Pennington, 1952). The results of our experiments would suggest that in the fasting sheep, and over considerable periods of time, butyric acid is not glucogenic. It will be noted from Fig. 3 that the levels of sugar were depressed after 24 h and remained so until the acid infusion was stopped.

The fact that the blood-sugar levels rose when the three acids were administered together is in agreement with the finding by Clark & Malan (1956) of a glucogenic effect for mixtures of acetic–propionic and propionic–butyric acids in sheep.

Metabolism of acetic acid. The results obtained in the five experiments with acetic acid show that when given as the sole source of energy it has a high heat increment. The four experiments with mixtures of steam-volatile acids, however, suggest that when it is given with propionic acid and *n*-butyric acid in the molar proportions 5:3:2 the increment of heat is very considerably less. The magnitude of the heat increment of mixtures of steam-volatile acids of different composition, particularly those more closely resembling the products of rumenal fermentation will be discussed in a subsequent paper. The present results suggest very strongly, however, that it is not correct to consider the metabolism of each acid separately in order to find the cause of the high heat increment of the ruminant animal.

It is of considerable interest, to see to what extent the metabolic changes and high heat increment of acetic acid given as the sole source of energy can be explained in terms of the biochemistry and thermodynamics of intermediary metabolism, as elucidated in studies on tissues and isolated enzyme systems in vitro. The fact that the blood sugar falls when acetic acid is given suggests that an excessive demand is placed on glycolysis. The increase in urinary N excretion further suggests gluconeogenesis from protein or an excessive demand for the intermediaries of carbohydrate metabolism. This, in turn, suggests that very little glucose is being formed from acetic acid.

The known main pathways of acetic-acid metabolism, apart from special systems such as cholesterol synthesis, are entry into the tricarboxylic-acid cycle, and entry into the cyclic process of lipogenesis. The first step in the entry of acetic acid in both instances is the formation of acetyl coenzyme A, which takes place at the expense of the pyrophosphate bond of adenosine triphosphate, generated in the tricarboxylic-acid cycle or in glycolysis. Condensation of acetyl coenzyme A with oxaloacetic acid would allow entry of acetic acid into the tricarboxylic-acid cycle, where it would be oxidized or, since the steps of glycolysis are reversible, could form glucose. The factor limiting its entry would be the supply of oxaloacetate, a product of carbohydrate metabolism.

For lipogenesis from acetyl coenzyme A, the first stage would be the condensation of two molecules of acetyl coenzyme A, to form acetoacetyl coenzyme A. For the synthesis of the higher homologues, hydrogen equivalents in the form of reduced diphosphopyridine nucleotide and reduced flavin adenine dinucleotide must be

available. The supply of both can come only from the oxidation of sugar. Their absence would undoubtedly lead to hydrolysis of acetoacetyl coenzyme A and hence to the ketosis observed.

The biochemical findings in the in vivo experiments suggest that in the sheep given acetic acid as the sole source of energy, metabolic blocks occurred at three points, the supply of oxaloacetic acid, the supply of the reduced coenzymes for lipogenesis, and possibly the supply of adenosine triphosphate for the initial acetylation of coenzyme A. Understandably, acetic acid accumulated as shown by the rise in the blood steam-volatile fatty-acid content, the fall in the alkali reserve and the rise in the urinary excretion of steam-volatile acid. These three blocks probably resulted from the low supply of glucose in the fasting animal. The provision of carbohydrate or a substance that would enter with ease the tricarboxylic-acid cycle would clearly facilitate the metabolism of acetic acid. Undoubtedly, the low heat increment of the mixture of steam-volatile fatty acids, in which propionic acid was present, can be explained in this way. Furthermore, the low heat increment of diets containing triacetin as a source of part of the energy can be explained by the concomitant presence of carbohydrate in the diet together with glycerol (McManus *et al.* 1943).

Obviously, since a considerable portion of the carbon of the acetic acid was retained in the body, the lack of the products of glycolysis was not absolute. In fact, it was noticeable that the increase in urinary N excretion was greater when acetic acid was given on the 9th and 10th days of starvation than when it was given on the 5th and 6th, when probably glycogen reserves were less exhausted.

The reason for the observed high increment of heat is not clear. Once a two-carbon unit has entered the tricarboxylic-acid cycle or the lipogenetic cycle through gluconeogenesis from protein it is difficult to see why there should be excessive losses of heat apart from those attributable to hydrolysis of peptide bonds of tissue proteins and deamination of amino-acids. There are two possible explanations. The first is that there is an alternative metabolic pathway for the complete dissimilation of acetic acid to carbon dioxide and water in which the efficiency of free-energy capture is considerably less than in oxidation in the tricarboxylic-acid cycle. The second is that the accumulation of acetic acid and the metabolic acidosis which occurs produce spontaneous hydrolysis of the pyrophosphate bond of adenosine triphosphate or of the thioester bond of acyl derivatives of coenzyme A.

That severe acidosis due to mineral acid may increase the heat increment of food in the ruminant and that neutralization of the mineral acid can abolish it has been shown experimentally by Mollgaard & Thorbek (1941). Working with milking cows in the respiration apparatus at Copenhagen, they found that the heat increment of A.I.V. silage before neutralization of the mineral acid was 84% of the metabolizable energy. After neutralization with $NaHCO_3$, the heat increment fell to 24%. It is of interest that in our experiments the heat increments of the acids and of glucose were inversely related to the CO_2-combining capacity of the plasma at the end of each experiment, as shown in Fig. 6. The regression was significant statistically. The hypothesis that the high heat increment of acetic acid may be due to hydrolysis of high-energy linkages under conditions of acidosis is clearly an extension of the acid-stimulus theory of the

specific dynamic effect advanced by Benedict & Ritzman (1927). Further work in vivo and in vitro is needed to establish or refute it.

Metabolism of propionic and butyric acids. The low heat increment of propionic acid is not surprising in view of its glucogenic nature. The oxidation of glucose, besides yielding adenosine triphosphate and acetyl coenzyme A and the hydrogen equivalents in the form of diphosphopyridine and flavin adenine dinucleotide necessary for fatty-acid synthesis provides a source of glycerol in the free or phosphorylated form necessary for the synthesis of fat or of phosphatides.

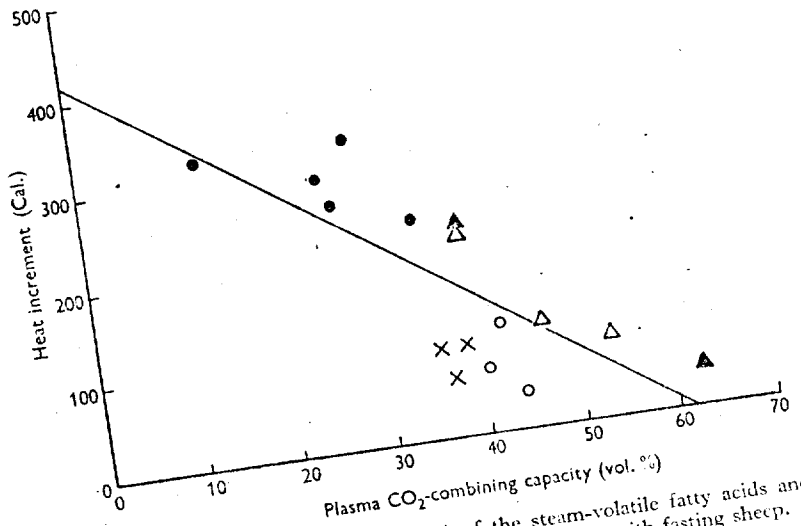

Fig. 6. Relation between the heat increment (H.I.) of the steam-volatile fatty acids and the CO_2-combining capacity of the plasma (P) in individual experiments with fasting sheep. H.I. = 420 − 6·85 P. ●, acetic acid; ○, propionic acid; ×, butyric acid; △, steam-volatile fatty-acid mixture; ▲, glucose.

The low heat increment for butyric acid is by no means easy to explain. Butyric acid like acetic acid finally undergoes oxidation in the form of two-carbon fragments. Hence one would expect it to have a similar effect on tissue metabolism. The fact that considerable amounts of butyrate absorbed from the rumen are metabolized in the epithelial wall lining the rumen, primarily to acetoacetate (Pennington, 1952) and that the oxidation of acetoacetate by extrahepatic tissues is considered by many (see Stadie, 1945) to be the pathway for the utilization of a considerable portion of fat metabolized in normal, fed animals may have some bearing on the point. In our experiments two factors are noteworthy. Firstly, there was no peripheral accumulation of acid such as occurred with acetic acid, and consequently a considerably less severe acidosis. Secondly, there appeared to be no increase in N katabolism. The need for further study of the metabolism of butyric acid has been pointed out by Phillipson & Cuthbertson (1956).

Heat increment of the ruminant. The single experiment with sugar giving a value for

the heat increment of 6·6%, together with the values obtained for the heat increment of steam-volatile acid mixtures, might be taken to suggest that the heat increment associated with the metabolism of the end-products of ruminant carbohydrate digestion is possibly about double that of carbohydrate digestion in simple-stomached animals. This estimate is based on a single experiment with glucose and, if errors in this experiment are similar to those observed in the series reported in detail, the true value for the heat increment of glucose may lie in the region of 10%. Indeed, the value for the S.D.E. of glucose in man is often quoted as 10%, and the calculations of Soskin & Levine (1946) from in vitro studies suggest that since the oxidation of 1 mole of glucose provides the energy for the phosphorylative synthesis of 6–12 moles of glycogen, the S.D.E. might be predicted to lie within a range of 6–16%. Further experiments with ruminants are clearly necessary.

These considerations suggest that the heat increments of absorbed mixtures of steam-volatile acids in the fasting ruminant may not greatly exceed those of absorbed glucose.

SUMMARY

1. Experiments in which three fistulated sheep were starved for periods of 12 days were carried out to study the heat production associated with the dissimilation of acetic, propionic and *n*-butyric acids when given to supply about 700 Cal. The metabolism of a mixture of these acids in the molar proportions 5:3:2 was also studied as well as the metabolism of glucose.

2. Infusion of acetic acid into the rumen for 48 or 72 h caused in five experiments a rise in the concentration of steam-volatile acids in the rumen, a fall in the pH of the rumen to 4·6, a considerable rise in the concentration of steam-volatile acids in the blood, acidosis, ketosis and a fall in the blood sugar. There was a rise in the urinary excretion of nitrogen, ketones and steam-volatile acids.

3. The increment of heat was computed from the oxygen consumption and N metabolism, and the value found to be 40·8% in three experiments of 48 h duration, measurements being made in the final 36 h. In two experiments of 72 h duration, the heat increment during the 24th–72nd h was 41·1%.

4. In the latter two experiments, the increment of heat was also computed independently from the carbon and nitrogen retention and almost identical results were obtained.

5. Administration of propionic acid in three experiments of 48 h duration resulted in a marked increase in blood sugar and a heat increment of 13%. There was no ketosis or acidosis and there was a reduction in the urinary N excretion, that is an N-sparing effect.

6. Administration of butyric acid in three experiments of 48 h duration resulted in marked ketosis, very slight accumulation of the acid in peripheral blood with a consequent slight decline in the plasma carbon dioxide-combining capacity. The increment of heat was 15·9%.

7. A mixture of the acids gave a heat increment of 16·9%, which was considerably lower than what would be expected from summation of the heat increments of the separate components of the mixture. Glucose given under conditions in which minimal fermentation took place in the rumen had a heat increment of 6·6%.

8. The results are discussed in relation to knowledge of the metabolic pathways whereby the acids might be dissimilated, and it is suggested that the reason for the metabolic findings with acetic acid is linked with the shortage of intermediaries and reduced coenzymes which arise in carbohydrate dissimilation. The high heat increment is not to be explained in this way and the hypothesis is advanced that an increase in the rate of non-enzymic hydrolysis of the pyrophosphate bond of adenosine triphosphate and thioester linkage of coenzyme A occurs because of the resulting metabolic acidosis.

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EXPLANATION OF PLATE

1. View from above of the respiration chamber showing the infusion pump (A), the acid reservoir (B) and the lead into the rumen of the sheep passing through the counterpoise system (C).
2. Infusion pump showing the Record syringes and plungers (D), the Bunsen valves (E) and the slow-speed motor (F).
3. Internal cannula used to introduce the acids. The acid lead (G) has a piece of stainless steel wire inside, which together with an outer sheath of Polythene tubing prevents blockage. The screw cap (H) holds the cannula in place.

Note added in proof. In subsequent experiments in which *n*-butyric acid was given to fasting sheep for periods longer than 48 h considerable day-to-day variation in the heat increment was found. Under these conditions the mean value was 23·6 Cal./100 Cal. acid metabolized, which is higher than the values given in this paper.

Intravenous Prednisolone and Acetic Acid on the Antral Phase of Gastric Secretion

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In dogs with Heidenhain pouches, the outputs of H^+ elicited by endogenous gastrin were increased by administration of prednisolone intravenously; the outputs of Na^+ were unchanged. An instillment of acetic acid reduced the outputs of H^+ and increased the outputs of Na^+, without provoking mucosal hemorrhage. When prednisolone was administered intravenously and acetic acid was administered topically, simultaneously, the outputs of H^+ were higher than control values but lower than when prednisolone was given alone; the outputs of Na^+ were higher than those noted after exposure of the pouches to acetic acid alone and hemorrhage occurred consistently. The results indicated that the degree of mucosal damage caused by acetic acid alone was increased by simultaneous administration of prednisolone. Nevertheless, the damaged mucosa still secreted increased outputs of H^+ in response to the prednisolone.

Although it is generally accepted that adrenal steroids can be implicated in the pathogenesis of certain types of peptic ulceration,[1-6] the literature relative to their effects on gastric acid secretion is inconsistent. Some workers have reported that these steroids increase the output of gastric acid and have attributed their ulcerogenic properties to this action.[7-14] However, others have claimed that gastric acid secretion is unaffected by these hormones.[15-20] It is now established that exposure of the gastric mucosa to organic acids results in increased insorption of H^+—in exchange for Na^+ exsorbed into the gastric juice—and that this increased insorption of H^+ damages the gastric mucosa.[21-26]

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In the present investigation, in dogs with vagally denervated fundic pouches, we have studied the effect of prednisolone, given intravenously, on gastric secretion stimulated by endogenous gastrin. In addition, we have defined the changes in the gastric secretory response to endogenous gastrin following exposure of the pouches to acetic acid. Furthermore, we have compared these changes with those induced by exposure of the pouches to the same solution of acetic acid when the animals also received prednisolone intravenously.

Materials and Methods

Experimental Animals.—Three adult mongrel dogs (two male, one female), average weight 18 kg, were used in the study. Under general anesthesia, a standard vagally denervated fundic pouch was constructed in each animal. Each pouch was equipped with a light, nonirritant vitallium cannula which drained into an external collecting tube. At least one month was allowed to elapse between operation and commencement of tests. The animals were accustomed to the laboratory and only minimal restraint was required during experiments. The dogs were maintained on a standard diet and their weights remained constant.

Secretory Tests.—Prior to an experiment, the animal was deprived of food but not water for at least 18 hours. At least three days intervened between tests on the same animal. In each test, the stimulus for gastric secretion was a standard meat meal consisting of 14.5 oz of canned beef which was generally consumed in less than one minute.

Four groups of experiments were performed in all dogs:

Group 1. Control tests, meat meal only; eight experiments on each dog. Basal (unstimulated) secretions were collected for one hour. The standard meat meal was then administered and, thereafter, gastric juice was continuously collected in 15-minute samples for four hours.

Group 2. Meat meals after 125 mg of intravenously administered prednisolone; three experiments on each dog. After a 30-minute basal period, 125 mg of prednisolone in 2 ml of fluid were injected into a vein in the forelimb. After a further 30 minutes, the animal was fed, and thereafter gastric juice was collected, as before, for four hours.

Group 3. Meat meal after acetic acid instilled into the pouches and 125 mg prednisolone given intravenously; three experiments on each dog. After a 30-minute basal period, 125 mg of prednisolone in 2 ml of fluid were injected into a vein in the forelimb. Simultaneously, 40 ml of an acetic acid solution (100 millimol), at pH 2, were instilled into the pouch where they remained for 30 minutes. During this period, small amounts of the instillment were frequently withdrawn into a syringe and immediately reinjected into the pouch, thus ensuring that the mucosa was continuously bathed by the instillment. After this time, the pouch was emptied and the animal fed. The pouch was allowed to drain freely until 15 minutes after the removal of the solution of acetic acid. Thereafter, 15-minute collections of gastric juice were commenced and continued until four hours had elapsed after feeding.

Group 4. Meat meal after acetic acid instilled into the pouches, but no prednisolone given; three experiments on each dog. The protocol of these tests was identical to that in Group 3, except that the animals were not given prednisolone. The capacities of the pouches were considerably more than 40 ml and instillation of this volume of fluid into the pouches did not appear to disturb the animals in any way.

Analyses Carried Out on Each Sample of Gastric Juice.—The volume of each sample of gastric juice was recorded after first centrifuging to remove cellular debris. The concentrations of the electrolytes were determined as follows: H^+, by titration

against decinormal NaOH to pH 3.5 using Topfer's reagent; Na^+ and K^+, using flame photometer; and Cl^-, using an automatic chloride titrator.

Analysis of Data

Secretory Outputs.—In each secretory test, calculations were made of the secretory volumes and of the outputs of the H^+, Na^+, K^+, and Cl^- in the gastric juice, during each hourly period after feeding. In groups 3 and 4, outputs for only the last 45 minutes of the first hour were available, and hence these were compared with outputs similarly calculated in groups 1 and 2. In each experimental group, the mean hourly outputs of each constituent for all the tests in the group were determined. Each group, in addition, determined the means of the concentrations and of the outputs of the electrolytes, each 15 minutes, as well as the sums of the mean outputs during the last three and three-quarter hours of the tests.

The secretory outputs during the various hours between the different groups were compared by *t*-tests for unpaired data; in comparisons pertaining to the first hourly periods, the outputs during the last 45 minutes of these hours were used.

Electrolytic Interrelationships in the Gastric Juice.—In each experimental group, the regression relationships relating the concentrations in the gastric juice of (1) H^+ and Na^+, and (2) (Cl^- - H^+) and (Na^+ + K^+) were calculated; these relationships were calculated for the pooled data from tests on individual animals and for the pooled data from all the tests on all the animals. The regressions were calculated using all the available 15-minute observations after feeding; only those observations relating to basal (unstimulated) periods were excluded. The slopes of the regression relationships were compared between the various groups by analyses of covariance.[7]

Results

Secretory Outputs.—The detailed patterns of secretion in all the groups can be seen by examining the mean 15-minute values.

The mean secretory volumes, the mean outputs of the electrolytes during the various hours in the different groups, and the results of comparisons of these outputs between the groups are shown in Table 1.

In groups 1 and 2, the secretory volumes and the outputs of H^+, Cl^-, and K^+ were highest in the third hour; the outputs of Na^+ were highest in the second hour, thereafter decreasing in successive hours. In groups 3 and 4, the secretory volumes and the outputs of H^+, Cl^-, and K^+ were highest in the fourth hour (except for the output of K^+ in group 3). The output of Na^+ was highest in the second hour in group 3 and in the third hour in group 4.

In group 2, the mean secretory volumes and the outputs of H^+, Cl^-, and K^+ were significantly higher than those in group 1 during all the hourly periods. However, the outputs of Na^+ during corresponding hours were not significantly different in these two groups.

In group 3, blood was constantly present in the gastric juice during the second poststimulatory hour and sometimes even during the first. Once this hemorrhage had started, it usually persisted throughout the test. Bleeding into the gastric juice did not occur in groups 1, 2, and 4.

In group 4, the secretory volumes and the outputs of H^+, Cl^-, and K^+ were significantly lower than those in group 1 during each of the first three poststimulatory hours. The outputs of Na^+ in group 4 were significantly lower than those in group 1 during the first hour, not significantly different during the second hour, and significantly higher during the third and fourth hours.

In group 3, the outputs of H^+ were significantly lower than those in group 2 during each of the first three hours. The secretory volumes and the outputs of Cl^- and K^+ were significantly lower in group 3 than in group 2 during the first two hours; they were also appreciably lower in the third hour, although the differences were not significant ($.1 < P < .05$). The outputs of Na^+ in group 3 were consistently higher than those in group 2—the differences being significant during the second and third, but not during the first and fourth hours.

Hours after feeding	Volume (ml) 1	2	3
Group 1:M*	4.76 (0.69)	12.35 (1.22)	18.38 (1.35)
2:M, P†	15.13 (2.13) §	35.50 (4.03) §	46.91 (4.27) §
3:M, P, A‡	4.82 (1.16) ‖¶	22.37 (2.30) ‖¶	36.29 (3.72) ¶
4:M, A	1.06 (0.34) §	5.51 (1.49) §	11.86 (0.85) §

* M = meat meal.
† P = intravenously administered prednisolone.
‡ A = instillate of acetic acid.

The secretory volumes and the outputs of H^+, Cl^-, and K^+ in group 3 were significantly higher than those in group 4 during all the hourly periods. The outputs of Na^+ were also consistently higher in group 3 than in group 4, although the differences were significant only during the first and second hours.

The differences between the mean secretory outputs during the various hours in groups 1 and 4, expressed as percentages of the mean outputs in group 1, are shown in Table 2. This table contains comparable data in relation to groups 2 and 3. Clearly, in group 4 relative to group 1, and in group 3 relative to group 2, the percentage reductions in the mean secretory volumes and in the mean outputs of Cl^- and K^+ were closely similar during the various hours. These percentage reductions were less than those in the mean outputs of H^+ during corresponding hours. In terms of the mean outputs of Na^+, in contrast, there were percentage increments in group 4 relative to group 1 and group 3 relative to group 2—except during the first hour in group 4 when the output of Na^+ was reduced, but to a lesser degree than the outputs of the other electrolytes.

In general, the percentage reductions in the mean secretory volumes and in the mean outputs of H^+, Cl^- and K^+ were marginally greater in group 4 relative to group 1 than in

Table 1.—Mean Secretory Volumes and Outputs of Electrolytes, per hour

	Outputs of Electrolytes (mEq)															
	H^+				Cl^-				K^+				Na^+			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0 20 (0 9)		1.526 (0.16)	2.530 (0.20)	2.098 (0.30)	0.860 (0.14)	2.249 (0.21)	3.175 (0.24)	2.573 (0.37)	0.041 (0)	0.111 (0)	0.162 (0)	0.131 (0)	0.196 (0)	0.282 (0)	0.210 (0)	0.132 (0.03)
2 03 (0 2)		5.391 (0.67) §	7.376 (0.75) §	6.636 (0.79) §	2.518 (0.38) §	5.985 (0.72) §	7.970 (0.78) §	7.203 (0.83) §	0.094 (0.17) §	0.283 (0.04) §	0.374 (0.03) §	0.297 (0.03) §	0.212 (0.05)	0.227 (0.05)	0.193 (0.06)	0.162 (0.05)
0 66 (0 7) ¶		1.954 (0.41) ‖¶	4.434 (0.68) ‖¶	4.972 (0.80) ¶	0.716 (0.19) ‖¶	3.431 (0.36) ‖¶	5.866 (0.63) ¶	6.191 (0.73) ¶	0.025 (0) ‖¶	0.149 (0) ‖¶	0.277 (0.03) ¶	0.266 (0) ¶	0.267 (0.04) ¶	1.012 (0.26) ¶	0.716 (0.23)	0.487 (0.24)
0 032 (0)		0.285 (0.07) §	1.178 (0.12) §	1.720 (0.20)	0.126 (0.03) §	0.822 (0.21) §	2.048 (0.12) §	2.407 (0.21)	0.006 (0) §	0.037 (0) §	0.091 (0) §	0.108 (0)	0.077 (0) §	0 368 (0.10)	0.415 (0.03) §	0.284 (0.03) §

§ Significantly different from group 1; standard error of mean in parentheses.
‖ Significantly different from group 2.
¶ Significantly different from group 4.

group 3 relative to group 2. However, the percentage increments in the mean outputs of Na^+ were considerably higher in group 3 relative to group 2 than in group 4 relative to group 1. Furthermore, it was noteworthy that the sums of the mean hourly outputs of Na^+ were virtually the same in groups 1 and 2, while they were markedly higher in group 3 than in group 4.

Electrolytic Interrelationships in the Gastric Juice.—In each group—in individual animals and in the group as a whole—there were highly significant linear correlations relating the concentrations in the gastric juice of (1) H^+ and Na^+, and (2) (Cl^- − H^+) and (Na^+ + K^+) (Table 3). Although there were minor differences in the slopes of these regressions between the groups (Table 4), it is clear from the Figure that these interrelationships were not appreciably different in any of the groups.

Comment

Our results have shown clearly that a single injection of 125 mg of prednisolone, administered intravenously 30 minutes before feeding, results in increased outputs of gastric acid from vagally denervated canine fundic pouches. Since the steroid did not have a significant effect on the outputs of Na^+, these findings would be consistent with a selective increase in parietal secretion. We have noted similar changes in the secretory response to endogenous gastrin when vagally denervated canine fundic pouches were exposed to instillments of prednisolone in a range of doses, and when dogs with vagally denervated fundic pouches were given prednisolone, intramuscularly, for periods up to three months.

The effects of instillments of acetic acid on the secretory response to endogenous gastrin were clearly similar whether or not the animals received prednisolone intravenously. Thus, when the secretory outputs were compared in groups 1 and 4, and in groups 2 and 3, the secretory volumes and the outputs of H^+, Cl^-, and K^+ were reduced during the first three poststimulatory hours in groups 3 and 4,

Table 2.—Percentage Differences Between Groups 1 and 4 and Groups 2 and 3

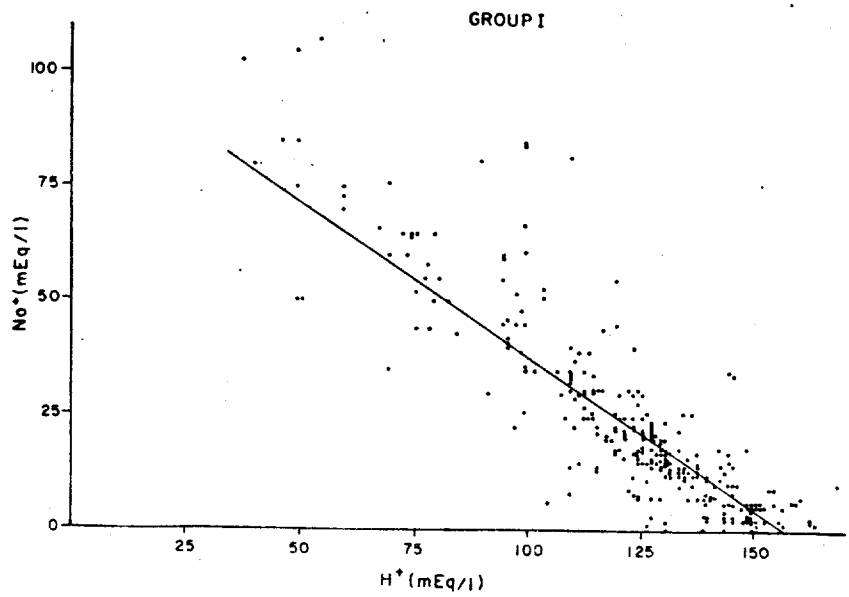
Hours after feeding	% Change (Difference Between 1 and 4 × 100)				% Change (Difference Between 2 and 3 × 100)			
	1	2	3	4	1	2	3	4
Volume	−77.7	55.4	35.5	5.9	68.1	37.0	−22.6	−11.6
H^+	−93.8	81.3	53.4	−18.0	−82.5	−63.8	−39.9	−25.0
Cl^-	−85.3	63.4	35.5	−6.5	71.5	−42.7	−26.4	−14.0
K^+	85.3	66.6	43.8	−17.6	73.4	−47.3	−25.9	−10.4
Na^+	−60.7	30.5	97.6	−115.2	25.9	−345.8	271.0	−200.6

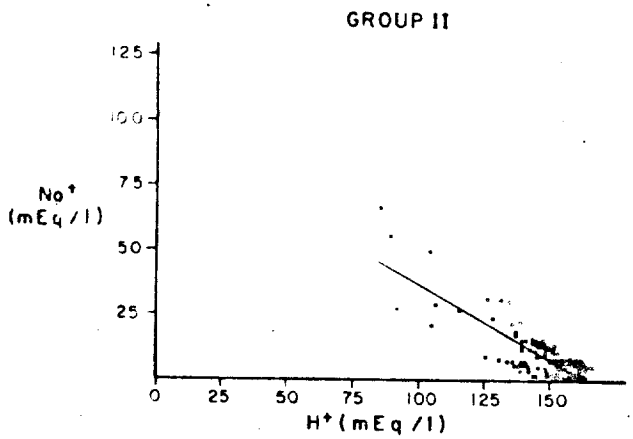
Table 3.—Electrolytic Interrelationships in the Gastric Juice

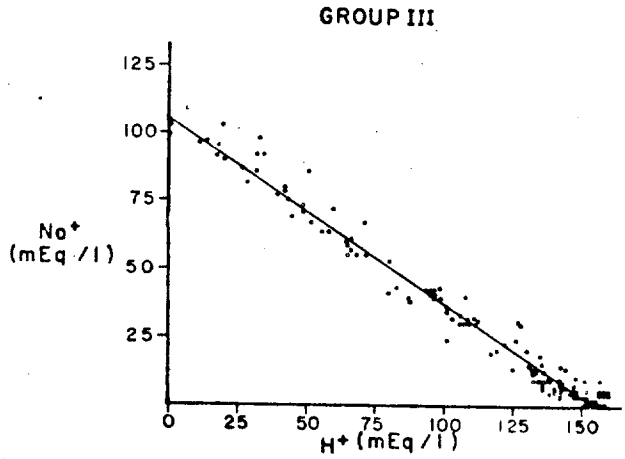
	Coefficients of Linear Correlation				Linear Regression Equation for Group
	Individual Animals*			Group*	
H^+ vs Na^+ in group:					
1:M	−0.88	−0.90	−0.92	−0.80	Y = 105.8 − 0.67X
2:M, P	−0.66	−0.73	−0.87	−0.80	Y = 95.3 − 0.59X
3:M, P, A	−0.98	−0.99	−0.99	−0.99	Y = 104.7 − 0.68X
4:M, A	−0.84	−0.92	−0.93	−0.87	Y = 109.7 − 0.72X
(Cl^- − H^+) vs (Na^+ − K^+) in group:					
1:M	0.88	0.88	0.88	0.87	Y = −2.7 − 0.75X
2:M, P	0.53	0.69	0.89	0.77	Y = 3.5 − 0.63X
3:M, P, A	0.98	0.98	0.98	0.98	Y = −5.9 − 0.86X
4:M, A	0.72	0.87	0.88	0.83	Y = −2.6 − 0.70X

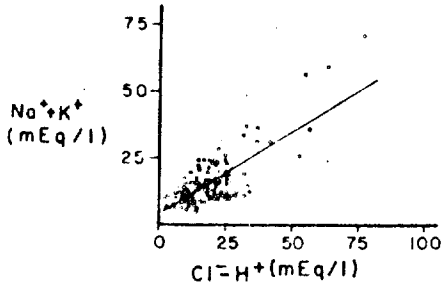
* $P < .001$.

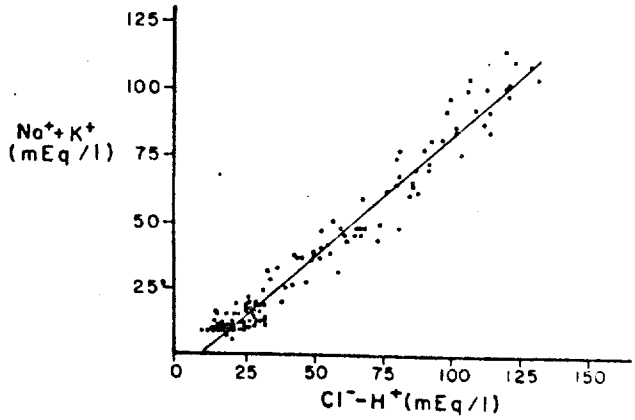
Regression relationships between concentrations in gastric juice of H^+ vs Na^+ and of ($Cl^- - H^+$) vs ($Na^+ + K^+$).

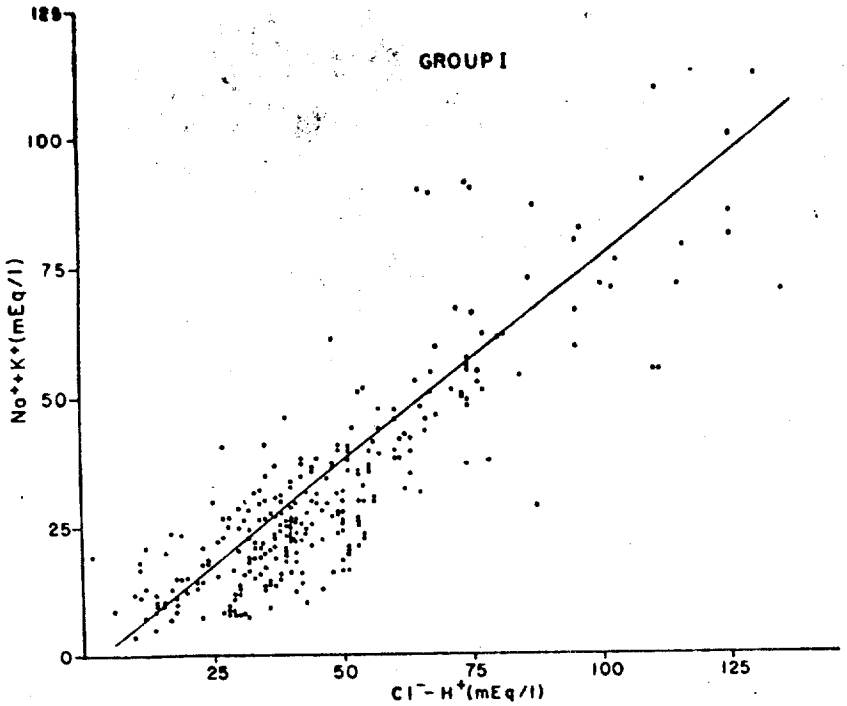


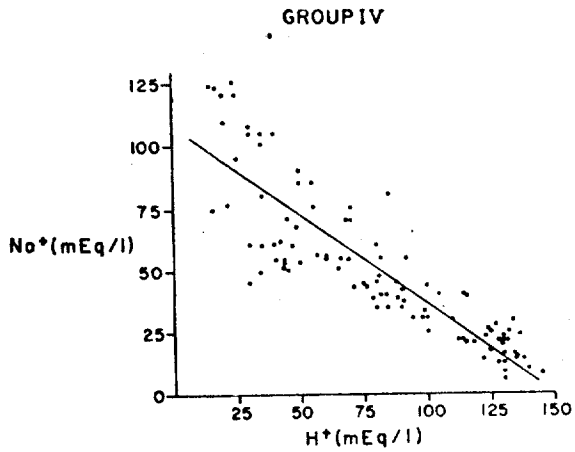


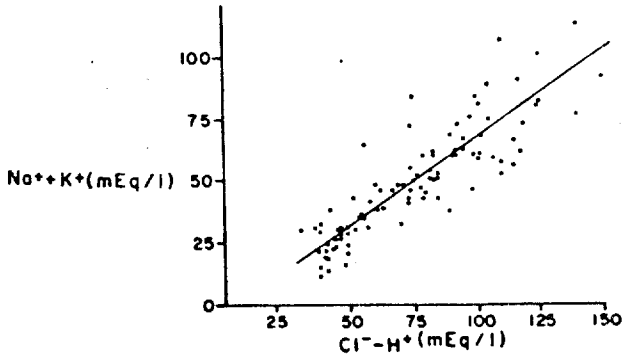












whereas the outputs of Na^+ were generally increased. The reduced secretory volumes and outputs of Cl^- clearly indicated inhibition of gastric secretion, whereas the increased outputs of Na^+ were the result of enhanced exchange-diffusion, demonstrated in a previous study (unpublished data). The overall similarities between the changes evoked by exposure of the pouches to acetic acid, whether or not the animals received prednisolone intravenously, were clearly emphasized by the similar percentage changes in the mean secretory outputs in group 3 compared with group 2, and in group 4 compared with group 1. Our results thus suggested that mucosal permeability was equally affected by exposure to acetic acid, whether or not the animals received prednisolone intravenously. The administration of prednisolone did not prevent the changes in the secretory response induced by exposure of the pouches to acetic acid, for the organic acid still inhibited gastric secretion and still induced increased exchange-diffusion.

It is important to note that the outputs of Na^+ were consistently higher in group 3 rather than in group 4, the differences being significant during the first two hours. Since prednisolone itself did not increase the output of Na^+ in the gastric juice (group 2), the higher outputs of this ion in group 3 rather than in group 4 would suggest that acetic acid caused more exchange-diffusion when prednisolone was also given intravenously. As mucosal permeability appeared to be equally affected by exposure to acetic acid in groups 3 and 4, the greater exsorption of Na^+ during the first two hours in group 3 rather than in group 4 was probably a reflection of greater availability of H^+. Thus, during the first two hours in group 4, there were clearly few H^+ secreted, even assuming that all the Na^+ were derived by exchange-diffusion and that the true secretion of H^+ was the sum of the outputs of the H^+ and Na^+. This low secretion of H^+ resulted in few of these ions being available for insorption across the damaged mucosa, thus limiting the exsorption of Na^+. In contrast, secretion of H^+ in group 3

Table 4.—Analyses of Covariance

Groups Compared	F (Slope) Values: H^+ vs Na^+	F (Slope) Values: $(Cl^- - H^+)$ vs $(Na^+ + K^+)$
1: M vs		
2: M, P.	7.343 SD*	3.050 NS†
3: M, P, A.	5.888 SD	12.311 SD
4: M, A.	0.476 NS	0.887 NS
4: vs		
2	3.179 NS	0.884 NS
3	1.057 NS	12.988 SD

* Significantly different.
† Not significantly different.

was more profuse during these hours, and hence there were more H^+ available for insorption. As a result, the highest output of Na^+ occurred in the second hour in group 3 but not until the third hour in group 4. Thus, by increasing the secretion of H^+, the administration of prednisolone increased the availability of this ion and so resulted in increased insorption of H^+ and increased exsorption of Na^+. This was a particularly significant finding, since it is well established that the degree of mucosal damage caused by exposure of the gastric mucosa to organic acids is proportional to the actual number of H^+ insorbed.[24,25] The observation thus indicated that the degree of mucosal injury caused by exposure of the mucosa to acetic acid was worse when the animals were given prednisolone intravenously than when the steroid was not given. This was substantiated by our observation that hemorrhage from the gastric mucosa was almost constantly observed in group 3 but not in group 4.

Even ignoring the fact that the outputs of H^+ were falsely low estimates of the actual secretion of H^+ in group 3 (and in group 4), it was clear that the mucosa damaged by exposure to acetic acid was still capable of responding to stimulation by intravenously administered prednisolone, for the outputs of H^+ were generally higher in group 3 than in either group 1 or group 4. Evidently enhancement of gastric mucosal permeability and mucosal damage from increased insorption of H^+ did not impair the ability of the mucosa to secrete increased outputs of acid in response to stimulation by prednisolone.

Our results have shown clearly that in secretions elicited from the vagally denervated gastric mucosa, the electrolytic interrelationships in the gastric juice are not altered by the administration of prednisolone intravenously, or by exposure of acetic acid, or by both in combination.

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GASTRIC MUCOSAL BLOOD FLOW FOLLOWING DAMAGE BY ETHANOL, ACETIC ACID, OR ASPIRIN

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The gastric mucosal blood flow in vagally denervated oxyntic gland area (Heidenhain) pouches was estimated by aminopyrine clearance in four 30-min periods after irrigations with acid solutions of ethanol, acetic acid, and aspirin. Mucosal blood flow increased initially when either ethanol or acetic acid was used and subsequently declined with appearance of evidence of gastric mucosal barrier damage. In spite of similar evidence for gastric mucosal barrier damage the mucosal blood flow with the aspirin solutions was, by the fourth period, significantly greater than the mucosal blood flow with the acetic acid solutions. Studies with acetic acid and aspirin solutions were repeated in vagally innervated oxyntic gland area (Pavlov) pouches. The pattern of mucosal blood flow was similar and in those experiments with homogeneous base lines the mucosal blood flow of the aspirin studies in the fourth period was again significantly greater than that of the acetic acid study. Although a greater mucosal blood flow might be considered a beneficial and protective influence, invariable bleeding characterized the fourth period of the aspirin experiments while only inconstant and minimal bleeding was noted in the fourth period of the acetic acid experiments.

The normal gastric mucosa forms a nearly impermeable barrier to the diffusion of sodium and hydrogen ions. This barrier, by preventing the diffusion of H^+ from the gastric lumen through the mucosa, maintains the concentration of secreted acid and protects the stomach from injuring itself by its own secretions. If this barrier is disrupted by noxious substances, back diffusion of H^+ and a large outpouring of Na^+ occur. Bleeding frequently follows. In man as well as in dogs numerous chemicals and drugs have been proven or are strongly suspected to be causes of the induction of this acute gastric injury.[1-6]

Davenport has proposed[7] that the increased permeability of the mucosal barrier after injury permits rapid diffusion of

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acid into the mucosa. There the acid liberates histamine, which stimulates acid secretion and increases the permeability of mucosal capillaries. The acid diffusing into the mucosa also ruptures capillaries, producing interstitial hemorrhage and bleeding into the lumen. The central features of this concept are rapid back diffusion of acid and release of histamine. The combined effect of these two influences on the gastric mucosal blood flow is unknown.

This paper reports an attempt to measure the response of the gastric mucosal blood flow after damaging the gastric mucosal barrier. The method used to measure gastric mucosal blood flow was the gastric mucosal clearance of aminopyrine.[8] The technique of this measurement avoids the difficulties of surgical trauma, shock, and anesthesia and permits repeated study of the same animals. The gastric mucosal clearance of aminopyrine was measured after damaging the gastric mucosal barrier with acid solutions of ethanol, acetic acid, and aspirin.

Materials and Methods

Part I

Three dogs with vagally denervated oxyntic gland area (Heidenhain) pouches were used. The animals were healthy and accustomed to the procedure and were fasted for at least 20 hr before each experiment. Hematocrit (Wintrobe tube) determinations were performed frequently. Initially the hematocrit of each dog was about 50%, but it subsequently fell and fluctuated from 38 to 48%. Neither profound anemia nor manifestations of agranulocytosis developed. Experiments were carried out at intervals of no less than 1 week. The details of an experiment in which aminopyrine was infused have been described,[7] as has the method of irrigating the pouches.[8,9,10] Briefly, an experiment consisted of placing a dog in a supporting sling, filling the pouch with a solution of 100 mN HCl and 54 mN NaCl, and beginning an infusion of aminopyrine in saline through a hind leg vein. A loading dose of 20 mg. per kg was given in 10 min and was followed by a constant maintenance infusion of 5 mg per kg per hr. Twenty minutes after the start of this maintenance dose the first of four 30-min periods was begun. The pouch was rinsed twice, gently aspirated, and filled with 40 ml of a solution of 100 mN HCl and 54 mN NaCl. Approximately 9.5 ml were removed, measured, and saved and the time was noted (zero time). At both the beginning and the end of a period, aspiration did not completely empty the pouch. The small residual or dead space volume has been estimated in each dog by a phenolsulfonphthalein dilution technique[8,9] and this volume is considered in the calculation (vide infra). At 15 min, 6 to 8 ml of venous blood were drawn from a hind leg vein and centrifuged and the plasma was saved. At 30 min (the completion of the first or base line period), the fluid in the pouch was aspirated, measured, and saved. This procedure was repeated in identical manner for three subsequent periods with the exception that the pouch was filled with a test solution in the second period. The solution of 100 mN HCl and 54 mN NaCl again was used in the third and fourth periods as in the first. The three test solutions were (1) 9% (w/v) ethanol in 100 mN HCl and 54 mN NaCl, (2) 100 mM acetic acid and 54 mN NaCl, and (3) 20 mM aspirin (acetylsalicylic acid) in 100 mN HCl and 54 mN NaCl. Each solution was tested in each dog three or four times. To compare our technique with that of Jacobson et al.,[7] at least one experiment was done on each dog in which, after the completion of the first period, histamine (50 μg per kg-hr) was infused with aminopyrine. The infusion was continued for three subsequent 30-min periods, during each of which the pouch was filled with the solution consisting of 100 mN HCl and 54 mN NaCl.

To establish whether the gastric mucosa damaged by the test solutions was permeable to aminopyrine, two controls were carried out with each of the test solutions on each dog. The solution with which the pouch was filled in all control periods contained aminopyrine (150 to 200 μg per ml), a concentration nearly equal to the greatest concentration found at the end of any 30-min period during an experiment. For the control animals, the first and two subsequent periods were preceded by two base line periods.

Total acidity of the initial and final solutions from each period was measured by titration of a 1-ml sample with 0.1 N NaOH to the phenolphthalein end point. The net flux of H^+ across the mucosa per 30-min period were determined by subtracting the initial from the final H^+ mass. A positive net flux of H^+ means a gain of H^+ in the pouch lumen and, although evidence for active acid secretion, indicates only,

the minimal amount that may have been secreted. A large negative net H^+ flux means a loss of H^+ from the pouch lumen and indicates that the gastric mucosal barrier is damaged. One cannot infer, however, from the negative flux that there was no H^+ secreted. Acid secretion may have occurred and have been masked by the loss of H^+ diffusing across the damaged gastric mucosal barrier.

The hemoglobin concentration of the final solution from each period was estimated by comparison with samples of hemoglobin substrate powder of known concentration. These standards were made by adding hemoglobin substrate powder to 0.1 N HCl. The pouch aspirate was compared with these standards against a white background and the concentration was recorded to the nearest multiple of 5 mg per 100 ml. When the hemoglobin concentration was less than 40 mg per ml, one easily distinguished concentration ranges of less than 5 mg per 100 ml. The results are expressed in milligrams of hemoglobin per 100 milliliters. (If all of the red blood cells in a solution with a hematocrit of 1% were lysed completely, the solution would then have a hemoglobin concentration of 333 mg per 100 ml.)

The concentration of aminopyrine in each pouch and plasma sample was determined by the method of Brodie and Axelrod.[19] A 1- to 2-ml sample was alkalinized, extracted into ethylene dichloride, washed twice with sodium borate, re-extracted into an acid solution, and measured at 260 mμ on a Beckman double beam spectrophotometer. An aminopyrine solution standard with a concentration of 100 μg per ml gave an absorbance reading of 0.43. Neither aspirin, acetic acid, ethanol, nor hemoglobin interfered with this determination.

The plasma aminopyrine clearance (PAC) was calculated for each period from the equation:

$$Cl = \frac{(G_{30} \times V_{30}) - (G_0 \times V_0)}{P \times 30}$$

where Cl is the PAC in milliliters per minute, G_0 the intial concentration of aminopyrine in the pouch, G_{30} the concentration at the end of the period, V_0 the initial volume of the gastric pouch (40 ml plus the residual volume and minus the amount aspirated, usually about 9.5 ml), V_{30} the final volume (the amount aspirated plus the residual volume), and P the plasma aminopyrine concentration.

The sample mean and sample standard deviation of the PAC's were calculated for each period. For each dog the mean PAC of each period of one test solution was compared with the mean PAC of the same period for the other two test solutions. Comparisons between periods were made by the Student's t-test.

Part II

Two dogs with vagally innervated oxyntic gland area (Pavlov) pouches were used. The intact vagal innervation was demonstrated by a 2-fold or greater increase in the acid secreted per 15-min period following an intravenous injection of regular insulin (1.2 U per kg). Dog G's pouch was small and was filled with only 30 ml of the appropriate solution rather than the 40 ml as used with the other dogs. The details of the experimental procedure otherwise have been described in part I. Before all experiments had been completed, dog G began refusing food and lost weight; she was not used again. The two test solutions were (1) 100 mM acetic acid and 54 mM NaCl and (2) 20 mM aspirin (acetylsalicylic acid) in 100 mM HCl and 54 mM NaCl. Two controls were carried out with each test solution on each dog. Aminopyrine, H^+, and hemoglobin determinations were performed as indicated previously. In addition, Na^+ concentration in the initial and final solutions from each period was measured by an internal standard flame photometer. The net Na^+ fluxes per 30-min period were calculated by subtracting the initial from the final Na^+ mass. The net flux of Na^+ was almost invariably positive, indicating a net movement of Na^+ into the pouch lumen. After introduction of the test solutions positive net fluxes of Na^+ became larger, indicating that the gastric mucosal barrier had been broken.[7]

Results

Part I

Comparison of technique. During maximal histamine stimulation, Jacobson et al.[8] observed a minimal range of the ratio of gastric aminopyrine concentration to plasma aminopyrine concentration of 25 to 35. A maximal plasma aminopyrine clearance of 18 ml per min was recorded. In our dogs the minimal range of the ratio was 25 to 38, and the range of maximal plasma aminopyrine clearance was 21.2 to 30.3 ml per min. This comparison indicates satisfactory reproduction of their technique.

TABLE 1. *Percentage of aminopyrine recovered from oxyntic gland area pouches after five 30-min periods of irrigation*[a]

Test solution	Vagally denervated pouches (3 dogs)			Vagally innervated pouches (2 dogs)		
	N[b]	Period	Percentage aminopyrine recovered	N[b]	Period	Percentage aminopyrine recovered
100 mM HCl and 54 mM NaCl	36	1 and 2	99.7 ± 5.9	24	1 and 2	102.6 ± 3.4
9% (w/v) ethanol in 100 mM HCl and 54 mM NaCl used in period 3	6	3	97.9 ± 3.7			
100 mM HCl and 54 mM NaCl	6	4	98.1 ± 3.5			
100 mM HCl and 54 mM NaCl	6	5	95.9 ± 2.5			
100 mM acetic acid and 54 mM NaCl used in period 3	6	3	97.2 ± 5.9	4	3	103.4 ± 7.6
100 mM HCl and 54 mM NaCl	6	4	97.5 ± 2.7	4	4	99.6 ± 1.9
100 mM HCl and 54 mM NaCl	6	5	99.9 ± 1.9	4	5	103.9 ± 4.0
20 mM aspirin in 100 mM HCl and 54 mM NaCl used in period 3	6	3	100.6 ± 4.2	4	3	101.6 ± 3.5
100 mM HCl and 54 mM NaCl	6	4	98.0 ± 2.6	4	4	100.9 ± 2.4
100 mM HCl and 54 mM NaCl	6	5	100.8 ± 4.7	4	5	101.8 ± 4.5

[a] Values are means ± SD of two experiments done with each test solution. The aminopyrine concentration was either 150 or 200 μg per ml in all solutions.
[b] N, number of observations.

Control animals. The percentage of aminopyrine recovered in five consecutive 30-min periods is presented in table 1. Two experiments were done with each of the three test solutions in each of the 3 dogs. The data of base line periods from all tests were pooled and were expressed as one result. The figures show an over-all aminopyrine recovery of 96 to 101% and were interpreted as indicating that the loss of aminopyrine through the damaged gastric mucosa of the vagally denervated oxyntic gland area pouch was negligible.

Experiments. The mean net volume and H^+ changes, hemoglobin concentration, and plasma aminopyrine clearances for dogs C, F, and H are presented in table 2. The data of the base line periods for each dog were pooled and were expressed as one result.

Second period (test solution). In each dog the highest mean PAC was found in the presence of the ethanol solution. In all 3 dogs this mean PAC was significantly greater than the mean PAC found with the aspirin solution, but not greater than the mean PAC of the acetic acid solution. The mean PAC of the acetic acid solution was significantly greater than the mean PAC of the aspirin solution in 2 of 3 dogs. The expected stimulation of acid secretion by ethanol with a net gain of H^+ in the pouch[11] and a similarly expected net loss of H^+ with both acetic acid and aspirin solutions[2] were observed.

Third period (follow-up 1). (To avoid awkwardness, those experiments in which ethanol was used henceforth are called the ethanol test or experiment, those experiments with acetic acid, the acetic acid tests, and so forth. The specific test solution, however, was used only in the second period.) The mean PAC of the ethanol tests was significantly greater than the mean PAC of the acetic acid tests in 2 of 3 dogs. There were no significant differences between the mean PAC of the aspirin and the mean PAC of the acetic acid tests. A positive net flux of H^+ in the ethanol tests and negative net fluxes of H^+ in the acetic acid and aspirin experiments again oc

TABLE 2. *Plasma aminopyrine clearance and net H^+ and volume changes in vagally denervated oxyntic gland area pouches of 3 dogs after four 30-min periods of irrigation*[a]

Solution	N[b]	Period	Net volume	Net H^+	Hb[c]	PAC[d]	Significant differences[e]
Dog C							
100 mN HCl and 54 mN NaCl	9	1	0.8	−47	0	5.25 ± 1.10	
9% (w/v) ethanol in 100 mN HCl and 54 mN NaCl	3	2	2.9	184	0	10.55 ± 1.24	$P < 0.05$*
100 mN HCl and 54 mN NaCl	3	3	1.4	59	0	8.61 ± 0.28	$P < 0.05$*
							$P < 0.05$†
100 mN HCl and 54 mN NaCl	3	4	0.6	−136	0	6.28 ± 0.80	$P < 0.01$†
100 mM acetic acid and 54 mN NaCl	3	2	2.4	−677	0	9.18 ± 2.39	
100 mN HCl and 54 mN NaCl	3	3	0.6	−589	0	5.38 ± 1.58	
100 mN HCl and 54 mN NaCl	3	4	0.5	−345	0	4.04 ± 0.60	
20 mM aspirin in 100 mN HCl and 54 mN NaCl	3	2	1.7	−587	0	6.76 ± 2.00	
100 mN HCl and 54 mN NaCl	3	3	1.3	−631	0-5	5.59 ± 1.50	
100 mN HCl and 54 mN NaCl	3	4	1.5	−380	5-10	5.69 ± 0.31	$P < 0.025$†
Dog F							
100 mN HCl and 54 mN NaCl	15	1	1.1	34	0	9.79 ± 1.94	
9% (w/v) ethanol in 100 mN HCl and 54 mN NaCl	3	2	5.9	551	0	14.95 ± 1.29	$P < 0.05$*
100 mN HCl and 54 mN NaCl	3	3	1.9	45	0	11.22 ± 0.91	$P < 0.025$†
100 mN HCl and 54 mN NaCl	3	4	1.0	−67	0-trace	10.84 ± 2.51	$P < 0.10$†
100 mM acetic acid and 54 mN NaCl	3	2	4.3	−875	0	13.72 ± 0.57	$P < 0.05$*
100 mN HCl and 54 mN NaCl	3	3	2.4	−766	0-trace	7.71 ± 1.17	
100 mN HCl and 54 mN NaCl	3	4	2.3	−592	0-30	6.79 ± 0.95	
20 mM aspirin in 100 mN HCl and 54 mN NaCl	3	2	1.4	−1132	5-10	9.99 ± 2.25	
100 mN HCl and 54 mN NaCl	3	3	2.0	−783	40-1000	9.14 ± 1.74	
100 mN HCl and 54 mN NaCl	2	4	2.7	−271	100-1000	9.91 ± 0.60	$P < 0.05$†
Dog H							
100 mN HCl and 54 mN NaCl	14	1	1.2	−49	0	4.92 ± 1.02	
9% (w/v) ethanol in 100 mN HCl and 54 mN NaCl	3	2	3.7	231	0	10.98 ± 1.64	$P < 0.005$*
100 mN HCl and 54 mN NaCl	3	3	1.4	0	0-trace	8.08 ± 0.87	
100 mN HCl and 54 mN NaCl	2	4	0.6	−132	0-10	6.79 ± 0.75	
100 mM acetic acid and 54 mN NaCl	4	2	2.9	−917	0	9.37 ± 2.19	$P < 0.05$*
100 mN HCl and 54 mN NaCl	4	3	3.1	−867	0	5.65 ± 2.38	
100 mN HCl and 54 mN NaCl	4	4	4.6	−653	0	5.01 ± 2.28	
20 mM aspirin in 100 mN HCl and 54 mN NaCl	3	2	3.5	−510	0	5.26 ± 0.73	
100 mN HCl and 54 mN NaCl	3	3	6.8	−357	10-20	7.64 ± 2.08	
100 mN HCl and 54 mN NaCl	3	4	7.3	−233	20-80	8.31 ± 1.01	$P < 0.10$†

[a] Mean net changes of volume in milliliters and of H^+ in microequivalents per 30 min. A negative sign denotes loss of contents from the pouch.

[b] Number of observations.

[c] Range of hemoglobin concentration at end of 30-min period in milligrams per 100 milliliters.

[d] PAC, plasma aminopyrine clearance in milliliters per minute, mean ± SD.

[e] PAC of each period of a test solution compared with PAC of same period of other two test solutions. *, mean PAC of this period is significantly greater than the mean PAC of same period of aspirin study. †, mean PAC of this period is significantly greater than mean PAC of same period of acetic acid study.

curred. Some bleeding was noted in the dogs tested with aspirin.

Fourth period (follow-up 2). The characteristic feature of this period was that the mean PAC of the aspirin experiments was greater than the mean PAC of the acetic acid test in all 3 dogs. This difference was significant at probability levels of $P < 0.025$ (dog C), $P < 0.05$ (dog F), and $P < 0.10$ (dog H). Continued net loss of H^+ from the pouch occurred in the acetic acid and aspirin experiments. For the first time a negative net H^+ flux was observed in the ethanol experiments. This negative net flux and the bleeding noted in the pouch fluid of 2 of 3 dogs indicate that this 9% (w/v) ethanol solution can break the mucosal barrier in dogs.

Although the amount of bleeding was variable in the dogs, each dog's response

TABLE 3. *Plasma aminopyrine clearances and net electrolyte changes in vagally innervated oxyntic gland area pouches of 2 dogs after four 30-min periods of irrigation*[a]

Solution	Period	Experiment 1			Experiment 2			Experiment 3			Experiment 4			Experiment 5		
		H^+	Na^+	PAC[b]	H^+	Na^+	PAC[b]	H^+	Na^+	PAC[b]	H^+	Na^+	PAC[b]	H^+	Na^+	PAC[b]
Dog D																
100 mN HCl and 54 mN NaCl	1	112	24	4.12	−9	32	4.89	122	−3	8.87	53	173	6.08			
100 mM acetic acid and 54 mN NaCl	2	−1114	730	9.67	−1376	636	6.63	−1180	694	14.61	−1635	902	6.58			
100 mN HCl and 54 mN NaCl	3	−959	344	8.37	−869	503	5.56	−484	486	11.16	−989	858	5.67			
100 mN HCl and 54 mN NaCl	4	−408	293	5.96	−496	343	4.26	35	43	10.47	−711	645	4.00			
100 mN HCl and 54 mN NaCl	1	−14	79	5.61	146	22	8.92	319	76	9.96	−36	156	5.01	81	81	5.76
20 mM aspirin in 100 mN HCl and 54 mN NaCl	2	−920	146	6.96	104	222	10.36	−226	271	12.06	−476	350	7.84	−272	296	7.98
100 mN HCl and 54 mN NaCl	3	−519	572	7.48	5	107	9.90	−21	174	11.76	−519	432	7.99	−286	464	8.04
100 mN HCl and 54 mN NaCl	4	−411	162	7.33	88	98	7.46	255	135	10.86	−388	373	7.85	−169	394	8.39
Dog G																
100 mN HCl and 54 mN NaCl	1	168	175	4.78	28	217	6.28	−35	140	5.48						
100 mM acetic acid and 54 mN NaCl	2	159	344	4.42	−462	611	6.26	−463	761	6.58						
100 mN HCl and 54 mN NaCl	3	−137	267	3.44	−355	438	4.56	−291	456	3.07						
100 mN HCl and 54 mN NaCl	4	52	277	2.56	−157	347	3.78	−214	498	4.81						
100 mN HCl and 54 mN NaCl	1	96	114	6.46	269	75	9.57	233	248	8.66	302	178	7.65			
20 mM aspirin in 100 mN HCl and 54 mN NaCl	2	66	192	9.92	−184	109	10.48	−291	321	7.55	133	275	7.21			
100 mN HCl and 54 mN NaCl	3	118	227	10.67	209	192	9.31	−20	521	9.14	−25	255	7.94			
100 mN HCl and 54 mN NaCl	4	268	229	13.03	310	167	8.61	84	450	10.14	141	308	8.07			

[a] Electrolyte changes in microequivalents per 30 min. A negative sign denotes loss of contents from the pouch.

[b] PAC, plasma aminopyrine clearance in milliliters per minute.

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was remarkably consistent. Dog C bled in the fourth period in each of three aspirin experiments. She did not bleed in any period of the acetic acid or ethanol tests. By contrast, dog F's mucosa appeared more fragile as there was mild bleeding in the fouth period of two acetic acid experiments and severe bleeding in all three aspirin experiments. Bleeding was so severe in her pouch by the end of the third period in the first aspirin experiment that the procedure was discontinued. Dog H bled minimally in one ethanol test and regularly in the third and fourth periods of all the aspirin experiments. The characteristic features of these aspirin experi-

TABLE 4. *Plasma aminopyrine clearance and net electrolyte and volume changes of experiments with satisfactory base lines in vagally innervated oxyntic gland area pouches of 2 dogs*[a]

Solution	N[b]	Period	Net volume	Net H^+	Net Na^+	Hb[c]	PAC[d]	Significant differences[e]
Dog D								
100 mN HCl and 54 mN NaCl	6	1	1.0	31	91	0	5.25 ± 1.02	
100 mM acetic acid and 54 mN NaCl	3	2	3.1	−1375	756	0	7.63 ± 1.77	
100 mN HCl and 54 mN NaCl	3	3	0.9	−939	568	0–5	6.53 ± 1.59	
100 mN HCl and 54 mN NaCl	3	4	0.9	−538	427	0–2.5	4.74 ± 1.06	
20 mM aspirin in 100 mN HCl and 54 mN NaCl	3	2	2.9	−556	264	trace–5	7.59 ± 0.55	
100 mN HCl and 54 mN NaCl	3	3	1.9	−441	489	10–20	7.84 ± 0.31	
100 mN HCl and 54 mN NaCl	3	4	1.8	−323	310	15–25	7.86 ± 0.53	$P < 0.025$†
Dog G								
100 mN HCl and 54 mN NaCl	4	1	1.7	64	162	0	5.75 ± 0.77	
100 mM acetic acid and 54 mN NaCl	3	2	4.9	−255	572	0	5.75 ± 1.17	
100 mN HCl and 54 mN NaCl	3	3	2.1	−261	387	0	3.69 ± 0.77	
100 mN HCl and 54 mN NaCl	3	4	2.2	−106	374	0–5	3.72 ± 1.14	
20 mM aspirin in 100 mN HCl and 54 mN NaCl	1	2	3.1	66	192	0	9.92	
100 mN HCl and 54 mN NaCl	1	3	2.6	118	227	trace	10.67	
100 mN HCl and 54 mN NaCl	1	4	3.7	268	229	20	13.03	

[a] The experimental data from which this group are drawn has been presented in table 3. The selected experiments were for dog D, acetic acid tests 1, 2, and 4 and aspirin tests 1, 4, and 5 and for dog G, acetic acid tests 1, 2, and 3 and aspirin test 1. Mean net changes of volume in milliliters and of electrolytes in microequivalents per 30 min. A negative sign denotes loss of contents from the pouch.

[b] N, number of observations.

[c] Range of hemoglobin concentration at end of 30-min period in milligrams per 100 milliliters.

[d] PAC, plasma aminopyrine clearance in milliliters per minute, mean ± SD.

[e] PAC of each period of acetic acid study compared with PAC of same period of aspirin study. †, mean PAC of this period was significantly greater than mean PAC of same period in acetic acid study.

ments were that the dogs invariably bled, that this bleeding increased during the experiment, and that the bleeding was always greater than any that occurred with acetic acid or ethanol.

Part II

Controls. Table 1 shows an over-all aminopyrine recovery of 99.6 to 104.8%. These results were interpreted as indicating that the loss of aminopyrine through the damaged gastric mucosa of the vagally innervated oxyntic gland area pouch was negligible.

Experiments. All experiments for the 2 dogs are presented in table 3. It was hoped that in the first or base line period of each experiment the dog's pouch would be in the resting state with a low PAC and minimal H^+ secretion. Marked variation in these base line measurements, however, was observed. Dog D, for instance, had in the first period a PAC range from 4.12 to 9.96 ml per min and a net H^+ flux range from −36 to 319 μEq per 30-min period. Comparisons of the results of these experiments with heterogeneous base lines were without significant differences. As the physiological state of the stomach determines in part its response to drugs, it seemed that more meaningful comparisons could be made between groups of experiments with more homogeneous base line periods as had been planned originally. Experiments where the dog's pouch was in the resting state (low PAC and minimal H^+ secretion) during the first period were identified. Three experiments with satisfactory base lines for each test solution were obtained for dog D (acetic acid tests 1, 2, and 4 and aspirin tests 1, 4, and 5) and for the acetic acid solution for dog G (acetic acid tests 1, 2, and 3). Unfortunately only one acceptable experiment with the aspirin solution was obtained in dog G (aspirin test 1) before she stopped eating.

Comparison of selected groups. The data from the selected experiments with satisfactory base lines are presented in table 4. For dog D the comparison indicates that the mean PAC of the fourth period of the aspirin test was significantly greater than the mean PAC of the fourth period of the acetic acid test. There were no other significant differences between mean PAC's. For dog G the acetic acid results are complete and mimic those of dog D. The one satisfactory aspirin test did show an increase in mean PAC from the first through the fourth period. As the data are incomplete for dog G, tests of significance were not applied. Bleeding invariably occurred in the aspirin tests and progressively increased through the fourth period. By contrast, bleeding was seen infrequently with the acetic acid tests, was minimal, and when present was not necessarily worst in the final period. The positive net Na^+ flux and the negative net H^+ were both larger in the acetic acid tests than in the aspirin tests.

Discussion

The aminopyrine clearance technique is based on the work of Shore et al.[12] and Jacobson et al.[5] This clearance would reflect gastric mucosal blood flow if the following assumptions were made: (1) that aminopyrine is equally distributed between plasma and red blood cells, (2) that there is rapid equilibration of aminopyrine between plasma and red blood cells, (3) that aminopyrine is cleared completely from the blood by the gastric mucosa in one passage, and (4) that aminopyrine is not secreted actively by the stomach. With regard to the first assumption, plasma aminopyrine concentrations have been shown to equal whole blood concentrations and to be independent of drug binding to hemoglobin.[13] Determination of plasma aminopyrine concentration is more accurate than measurement of aminopyrine in whole blood and therefore is more representative of whole blood aminopyrine concentration. Assumptions 2, 3, and 4 have not been proven conclusively to be true, but there are sound reasons to accept them as valid.[13, 14] Although conceivable, it seems unlikely that ethanol, acetic acid, or aspirin interferes with diffusion of aminopyrine from plasma to gastric pouch lumen. The results reported here as plasma

aminopyrine clearance are assumed for all practical purposes to reflect accurately the gastric mucosal blood flow.

Alterations of gastric mucosal blood flow in response to injury induced by solutions of ethanol, acetic acid, and aspirin were determined in vagally denervated pouches. The three test solutions provoked large net negative H^+ fluxes, large net positive Na^+ fluxes, or bleeding, indicating that the gastric mucosal barrier had been damaged. These results are similar to extensive previous studies with ethanol, acetic acid, and aspirin.[2, 11] The degree and direction of change of the mucosal blood flow varied for these solutions. In the ethanol experiments the second or test period was characterized by an increase in acid secretion and in mucosal blood flow. A change in the gastric acid secretory rate obligates a directionally corresponding change in mucosal blood flow.[8] As the ethanol had stimulated acid secretion in this test period, the observed increase in the mucosal blood flow is appropriate. A small additional contribution may have been the influence of absorbed ethanol.[11] The circulating ethanol, by depressing the central nervous system vasomotor center, might have vasodilated the mucosal vessels, reduced peripheral resistance, and thereby contributed to the augmented flow. The mucosal blood flow of the second period of the acetic acid study also increased, although in this period there was evidence of gastric mucosal barrier damage. As experiments in which secreted H^+ is trapped by glycine have shown only minimal acid secretion after irrigation with acetic acid,[6] this increase in mucosal blood flow cannot be explained reasonably as a requirement of increased acid secretion as with ethanol. There is, however, a rapid release of histamine with peak concentrations in the gastric venous blood 5 min after exposure of the entire gastric mucosa to acetic acid.[15] Thus, histamine is present and could be exerting an influence that increases the gastric mucosal blood flow.

As the ethanol and acetic acid experiments proceeded, evidence of gastric mucosal barrier damage appeared and the mucosal blood flow declined. This decline was more precipitous in the acetic acid study where the net negative loss of H^+ from the lumen was larger than that in the ethanol experiments. This suggests that the decreasing mucosal blood flow is related causally to the back diffusion of H^+. This is consistent with the observation that diffusion of H^+ into guinea pig mucosa induces vasoconstriction and stasis.[16] One may envisage in the healthy stomach a gastric mucosal blood flow delivering adequate amounts of buffers to neutralize the few H^+ diffusing back across the gastric mucosal barrier. After damage to this barrier the marked increase in the back diffusion of H^+ overwhelms these buffers and the effects of the unbuffered H^+ on mucosal vessels decrease the mucosal blood flow.

An unexpected discovery of this work was that in the fourth period of the aspirin studies the mucosal blood flow was significantly greater than that in the fourth period of the acetic acid experiments. As this discrepant response was unexplained and as a vagally stimulated cholinergic vasodilator mechanism has been recognized in the gastric vascular bed,[17] the experiments with the acetic acid and aspirin solutions were repeated in dogs with vagally innervated pouches. In these studies (part II) the response of the mucosal blood flow with either solution was similar to the response for that same solution in dogs with vagally denervated pouches. Furthermore, in a group of experiments with homogeneous base lines the gastric mucosal blood flow in the fourth period of the aspirin study was again significantly greater than that of the acetic acid study. It is concluded that the changes of the mucosal blood flow after injury induced with acetic acid and aspirin are essentially independent of intact vagal innervation.

Similar magnitudes of negative net H^+ flux occurred in both the acetic acid and aspirin experiments. If back diffusion of H^+ after injury is related causally to decreasing mucosal blood flow as it seems to have been in the acetic acid and ethanol experiments, then some mechanism is pre-

venting the expected decrease of mucosal blood flow in the aspirin experiments. Part II of this work indicates that this effect is not mediated by the vagus nerve. After gastric mucosal injury with concentrations of acetic acid and salicylic acid identical with those used in these experiments, histamine release into an isolated gastric vein, was similar in timing and peak concentrations.[15] This suggests that the mechanism preventing the expected decrease of gastric mucosal blood flow in the aspirin experiment is not operating through a change in release of histamine. Other vasoactive substances may be liberated from the damaged gastric mucosa. In particular, the kinins are intriguing as they act in the pathogenesis of inflammation and aspirin may inhibit their formation or action.[18] Finally, one might expect greater mucosal blood flow to be a beneficial and protective influence by preventing hypoxia and by diluting, neutralizing, and rapidly removing noxious agents, e.g., H^+ and aspirin, diffusing across the damaged gastric mucosal barrier. Although the final period of the aspirin studies had a significantly greater mucosal blood flow than the final period of the acetic acid study, the former was characterized, paradoxically, by invariable bleeding and the latter by inconstant and minimal bleeding. What this peculiar toxic property of aspirin is and whether it is also responsible for the response of the mucosal blood flow remain unknown.

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038

Quantitative Chemical Analysis

SECOND EDITION

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THE UNIVERSITY OF TEXAS AT AUSTIN

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Titration of a Weak Acid with a Strong Base

This type of titration may be illustrated by the general equation

$$HA + OH^- \rightarrow H_2O + A^-$$

in which HA represents a weak monoprotic acid, and A^- is its anion (salt). This case differs from the strong acid–strong base titration in four important respects: (1) The hydrogen ion concentration at the start is considerably smaller

(pH is larger) than for a strong acid. (2) In the early stages of titration the pH of the solution increases rather rapidly, because the ionization of the weak acid is suppressed by the common ion effect of the anion (highly ionized salt) formed by neutralization; see pp. 60–62. (3) For a considerable distance on either side of half-neutralization (i.e., from about 10 to 90% or about 20 to 80% titration, depending on K_a of the acid) the titration curve is approximately linear because this is a *buffer region* (see pp. 62 and 75). (4) The stoichiometric point does not occur at pH 7.00, because of the reverse reaction in which the anion A^- (the conjugate base of HA) acquires some protons from water, leaving the solution somewhat alkaline.

EXAMPLE 5

50.00 ml of 0.1000 N acetic acid, HAc, are titrated with 0.1000 N NaOH solution; K_a of HAc $= 1.8 \times 10^{-5}$. Calculate the pH values for construction of the titration curve.

a. In the acetic acid solution, before any alkali is added, $[H^+] = [Ac^-]$, and $[HAc] = 0.1000$ (approximately); actually, [HAc] is slightly less than 0.1000, but the slight amount of HAc that is ionized can be ignored without introducing appreciable error (see p. 58).

$$K_a = \frac{[H^+][Ac^-]}{[HAc]}$$

hence,

$$[H^+] = \sqrt{K_a[HAc]} = \sqrt{(1.8 \times 10^{-5})(0.100)}$$
$$= \sqrt{1.8 \times 10^{-6}} = 1.34 \times 10^{-3}$$

and pH = 2.87. For comparison, note that 0.100 N solution of HCl has a pH of 1.00.

b. As soon as some sodium hydroxide has been added, neutralizing an equivalent amount of acetic acid and forming an equivalent amount of acetate ion, the common ion effect comes into play, and the calculation of the hydrogen ion concentration must take this effect into account.

$$K_a = \frac{[H^+][Ac^-]}{[HAc]} \quad \text{and} \quad [H^+] = K_a \frac{[HAc]}{[Ac^-]}$$

Addition of 1.00 ml of 0.1000 N NaOH (i.e., 0.100 gme of NaOH) neutralizes 0.100 gme of HAc and leaves $5.000 - 0.100 = 4.900$ gme of HAc, and forms 0.100 gme of Ac^-, in a total volume of 51.00 ml.

$$[HAc] = 4.900/51.00 = 0.0961 \quad \text{and} \quad [Ac^-] = 0.100/51.00 = 0.00196$$

$$[H^+] = 1.8 \times 10^{-5} \times 0.096/0.00196 = 8.8 \times 10^{-4}$$

and pH = 3.05.

The above calculation using the Henderson equation (5-45) is an approximation that is valid in the buffer region of the titration. Near the start of the titration, where $[Ac^-] \ll [HAc]$, and near the stoichiometric point, where $[Ac^-] \gg [HAc]$, the exact

method of equation (5-44) should be used. For the above example,

$$[H^+] = (1.8 \times 10^{-5})(0.096 - [H^+])/(0.00196 + [H^+])$$

Solving the quadratic equation, $[H^+] = 6.5 \times 10^{-4}$ and $pH = 3.19$, a value somewhat different from that calculated above. The closer the calculated point is to the initital point or to the stoichiometric point, and the smaller the value of K_a, the greater the difference between the approximate and the exact calculations.

Because the reacting solutions in this example are of the same concentration, and the acetic acid unneutralized and the acetate ion formed by neutralization are in the same solution, it is not necessary, in the approximate calculations, to use the actual molar concentrations of the substances. It is more convenient to use the "equivalent volumes" of HAc and Ac^-, which are, of course, in the same ratio as their molar concentrations; that is, the original concentration and the final volume would cancel in the calculation of $[H^+]$ from the K_a expression. For example, addition of 1.00 ml of 0.1000 *N* NaOH can be considered as neutralizing 1.00 ml of 0.1000 *N* HAc, leaving 49.00 ml of acid unneutralized, and forming the equivalent of 1.00 ml of 0.1000 *N* Ac^-. Hence,

$$[H^+] = 1.8 \times 10^{-5} \times 49.00/1.00 = 8.8 \times 10^{-4} \quad \text{and} \quad pH = 3.05$$

the same value that was calculated previously from the actual molar concentrations of HAc and Ac^-.

It will be noted in this example that addition of only 1 ml of NaOH solution changed the *p*H from 2.87 to 3.05, a change of 0.18 *p*H unit (or from 2.87 to 3.19, through 0.32 *p*H unit if the exact calculation is used). This is a greater change in pH than was produced by addition of 10 ml of alkali in the titration of 0.1000 *N* HCl (Example 1), and illustrates in a striking way the influence of even a small amount of acetate ion in suppressing the ionization of acetic acid. These calculations also illustrate the fact that the solution of acetic acid has relatively small buffer capacity.

When 10.00 ml of NaOH have been added,

$$[H^+] = 1.8 \times 10^{-5} \times 40.00/10.00 = 7.2 \times 10^{-5} \quad \text{and} \quad pH = 4.14$$

When 25.00 ml of alkali have been added,

$$[H^+] = 1.8 \times 10^{-5} \times 25.00/25.00 = 1.8 \times 10^{-5} \quad \text{and} \quad pH = 4.74$$

In all cases of weak monoprotic acids, at the half-neutralization point $[HA] = [A^-]$, hence $[H^+] = K_a$, and $pH = pK_a$.

Additional points up to the stoichiometric point are calculated in exactly the same way as illustrated above.

c. At the stoichiometric point the system is an aqueous solution of sodium acetate. The acetate ion is the conjugate base of acetic acid:

$$Ac^- + H_2O \rightleftharpoons HAc + OH^-$$

and the solution at this point is not neutral, but slightly alkaline. The hydroxyl ion concentration of the solution depends upon the K_a of acetic acid, the concentration of acetate ion, and K_w, and is calculated by equation (5-21) or (5-39) as derived in Chap. 5:

$$[OH^-] = \sqrt{\frac{K_w[Ac^-]}{K_a}}$$

By using the known numerical values for the three terms under the radical, $[OH^-]$ can be calculated, then converted to pH in the usual way through K_w or pK_w. Because the relations developed above for acetic acid apply generally to the conjugate bases of weak acids, and the pH of the solution, rather than $[OH^-]$, is usually the item desired for plotting and/or for the selection of an indicator, the conversion can be done once and for all, for the various cases, as follows.

$$[H^+] = \frac{K_w}{[OH^-]} = \frac{K_w}{\sqrt{K_w[A^-]/K_a}} = K_w\sqrt{\frac{K_a}{K_w[A^-]}} = \sqrt{\frac{K_w^2 K_a}{K_w[A^-]}} = \sqrt{\frac{K_w K_a}{[A^-]}}$$

Now take the logarithm of the expression, remembering that multiplication (division) of numbers is performed by adding (subtracting) their logarithms, and that the square root is taken by dividing the logarithm by 2.

$$\log [H^+] = \tfrac{1}{2}(\log K_w + \log K_a - \log [A^-])$$

Changing signs (i.e., multiplying the equation by -1):

$$-\log [H^+] = \tfrac{1}{2}(-\log K_w - \log K_a + \log [A^-])$$

Table 23-2 Titration of 50.00 ml of 0.1000 *N* Acetic Acid with 0.1000 *N* Sodium Hydroxide

NaOH Added, ml	Equiv. ml of HAc Unneutralized	Equiv. ml of Ac^- Formed	Total Volume, ml	$[H^+]$	pH
0.00	50.00	0.00	50.00	1.34×10^{-3}	2.87
1.00	49.00	1.00	51.00	8.8×10^{-4}	3.05
5.00	45.00	5.00	55.00	1.62×10^{-4}	3.79
10.00	40.00	10.00	60.00	7.2×10^{-5}	4.14
20.00	30.00	20.00	70.00	2.7×10^{-5}	4.57
25.00	25.00	25.00	75.00	1.8×10^{-5}	4.74
30.00	20.00	30.00	80.00	1.20×10^{-5}	4.92
40.00	10.00	40.00	90.00	4.5×10^{-6}	5.35
45.00	5.00	45.00	95.00	2.0×10^{-6}	5.70
49.00	1.00	49.00	99.00	3.7×10^{-7}	6.43
49.90	0.10	49.90	99.90	3.6×10^{-8}	7.44
49.99	0.01	49.99	99.99	3.6×10^{-9}	8.44
50.00	0.00	50.00	100.00	1.9×10^{-9}	8.72
	Excess NaOH, ml			$[OH^-]$	
50.01	0.01		100.01	1.0×10^{-5}	9.00
50.10	0.10		100.10	1.0×10^{-4}	10.00
51.00	1.00		101.00	1.0×10^{-3}	11.00
55.00	5.00		105.00	4.8×10^{-3}	11.68
60.00	10.00		110.00	9.1×10^{-3}	11.96

But $-\log [H^+] = pH$, $-\log K_w = pK_w$, and $-\log K_a = pK_a$. Therefore,

$$pH = \tfrac{1}{2}(pK_w + pK_a + \log [A^-])$$

$[A^-]$ is the anion (salt) concentration, in moles per liter, at the stoichiometric point; at 25°C, $pK_w = 14.00$.

In this example, $50.00 \times 0.1000 = 5.000$ gme of HAc are neutralized, and the volume at the stoichiometric point is 100.00 ml. Therefore, $[Ac^-] = 0.05000$, and $\log 0.050 = 0.70 - 2 = -1.30$. $K_a = 1.8 \times 10^{-5}$, and $pK_a = 4.74$. Then

$$pH = \tfrac{1}{2}(14.00 + 4.74 - 1.30) = 8.72$$

and the stoichiometric point of the titration occurs in slightly alkaline solution.

d. Beyond the stoichiometric point the pH is calculated from the amount of excess alkali and the total volume of the solution, exactly as in the case of the strong acid–strong base titration. The acetate ion present in the solution has no direct influence on the ionization of the strong base.

Table 23-2 shows the calculated pH values for several points in this titration, and the titration curve is shown in Fig. 23-2. The differences between the weak acid and the strong acid cases, given on pp. 287–288, are now obvious. The solution becomes neutral (pH 7.00) at about 49.7 ml of added NaOH, or about 0.6% before the stoichiometric point. Phenolphthalein has the midpoint of its color change interval at pH 8.8,

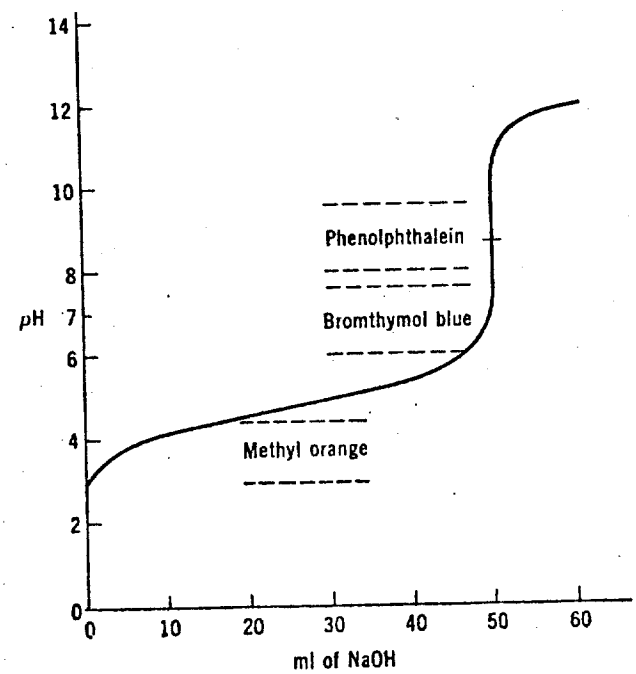

Fig. 23-2 Titration curve. 50.0 ml of 0.100 N acetic acid titrated with 0.100 N sodium hyrdoxide.

and is therefore a suitable indicator. Methyl orange, although suitable for use in the titration of 0.1 *N* HCl, is entirely unsuitable for the titration of acetic acid; methyl orange, with midpoint of its color change interval at *p*H 3.8, would change color very gradually, and would be mostly in its alkaline form when only about 10% of the acetic acid had been neutralized.

All the variations (dilution before titration, use of solutions of different normalities, or of weighed amounts of solid weak acids dissolved in a known volume of water for titration) previously illustrated for the titration of a strong acid, can be applied to the titration of weak acids by expressing the concentrations of HA and A^- in terms of millimoles (derived from milliequivalents) at each desired calculation point, and calculating $[H^+]$ and/or *p*H by the use of K_a of the acid being titrated.

EXAMPLE 6

50.0 ml of 0.100 *N* HAc are diluted to 75.0 ml and titrated with 0.125 *N* NaOH.

a. At the start, $50.0 \times 0.100 = 5.00$ gme (also millimoles) of HAc are contained in 75.0 ml of solution.

$$[HAc] = 5.00/75.0 = 0.0667$$

$$[H^+] = \sqrt{(1.8 \times 10^{-5})(0.067)} = 1.10 \times 10^{-3}$$

and $pH = 2.96$.

b. After addition of 1.0 ml of 0.125 *N* NaOH ($= 0.125$ gme), an equivalent amount of HAc is neutralized and an equivalent amount of Ac^- is formed, in a total volume of 76.0 ml.

$$[HAc] = (5.00 - 0.125)/76.0 = 0.0642$$

$$[Ac^-] = 0.125/76.0 = 0.00164$$

$$[H^+] = \frac{(1.8 \times 10^{-5})(6.4 \times 10^{-2})}{1.64 \times 10^{-3}} = 7.0 \times 10^{-4}$$

and $pH = 3.15$. Other points in the titration are calculated in the same way.

c. The volume of NaOH required to reach the stoichiometric point is $50.0 \times 0.100/0.125 = 40.0$ ml, and the total volume of solution is then 115.0 ml. This volume of solution contains 5.00 gme of Ac^-, and $[Ac^-] = 5.00/115.0 = 0.0435$; $\log 0.0435 = -1.36$. $pH = \frac{1}{2}(14.00 + 4.74 - 1.36) = 8.69$.

d. Beyond the stoichiometric point, the *p*H is calculated from the amount of excess alkali added and the volume of the solution, exactly as in previous examples.

From the ionization constant of the weak acid being titrated, it is possible to calculate the error that would be produced if the titration were stopped at a *p*H other than the *p*H of the stoichiometric point.

EXAMPLE 7

Calculate the percent error that would result from the use of bromcresol purple indicator (*p*H interval 5.2–6.8) in the titration of 50.0 ml of 0.100 *N* HAc with 0.100 *N* NaOH. The midpoint of the indicator color change is at *p*H 6.0. Assume that titration

is stopped at this pH, and calculate the ratio [HAc]/[Ac$^-$] at this pH, which corresponds to $[H^+] = 1.0 \times 10^{-6}$.

$$\frac{[HAc]}{[Ac^-]} = \frac{[H^+]}{K_2} = \frac{1.0 \times 10^{-6}}{1.8 \times 10^{-5}} = 5.6 \times 10^{-2}$$

That is, there is 0.056 mole of unneutralized HAc for every 1.00 mole of Ac$^-$ that has been formed or every mole of HAc that has been neutralized; stated in another way, if 1.056 moles of HAc were taken, then at pH 6.0, 1.00 mole has been titrated and 0.056 mole is not yet titrated. Hence,

$$\% \text{ error} = \frac{0.056}{1.056} \times 100 = 5.3$$

an error so large as to be intolerable.

Effect of Concentration of Weak Acid. If acetic acid of lower concentration, e.g., 0.010 N, is titrated with ~~0.010~~ N NaOH, the following conditions will prevail, compared to titration of more concentrated solutions: (1) the pH at the start and early in the titration will be *slightly* higher. (2) The pH at intermediate stages will be approximately the same, because [H$^+$] is determined by the *ratio* [HAc]/[Ac$^-$]. (3) The pH at the stoichiometric point will be slightly lower, owing to the lower concentration of the anion. (4) The pH after the stoichiometric point will be significantly lower because of the lower concentration of excess alkali added. Because of conditions (3) and (4), the rate of change of pH with addition of titrant in the vicinity of the stoichiometric point is more gradual, and the end point, shown by color change of the indicator, will not be as sharp as when more concentrated solutions are used. The practical limit of feasibility for acetic acid titrations is about 0.010 N solution, unless errors of the order of one percent can be tolerated.

Effect of Acid Strength. For a given normality of acid being titrated, the following effects will be noted as the acid becomes progressively weaker (smaller K_a): (1) The initial pH is higher, and the rate of change in early stages of titration is greater; indeed, when K_a is about 10^{-10} (e.g., boric acid) or less, the greatest rate of change of pH is at the start of the titration. (2) The rate of change of pH near the stoichiometric point is more gradual. (3) The reverse reaction is more extensive, hence the stoichiometric point pH is higher and the rate of change of pH with excess alkali is smaller. These factors, illustrated in Fig. 23-3, result in an indistinct end point (gradual change of color of the indicator), and the titration will not give reliable results. The situation just described is true for titration of 0.10 N solutions of acids for which K_a is less than about 10^{-7}.

Combining the effect of the concentration of solution and the K_a of the weak acid, it may be stated that if the product K_a[HA] is less than about 10^{-8}, the titration is not feasible.

CHANGES IN THE SECRETORY ACTIVITY OF THE GASTRIC GLANDS RESULTING FROM THE APPLICATION OF ACETIC ACID SOLUTIONS TO THE GASTRIC MUCOSA.

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Several theories have been advanced to explain the mechanism of the regulation of the acidity of the gastric juice. They range from the purely physiological, such as that the concentration of acid in the juice depends on the strength of the stimulation applied to the glands, to the purely physico-chemical, such as that the variations in the gastric acidity are due to a simple exchange of ions between the gastric contents and the blood. Nevertheless the problem of the regulation of the gastric acidity is still far from solution. An interesting study by the late Professor V. V. Savich and co-workers stimulated us to return to the investigation of this problem.

Working on dogs with a Pavlov or a Brestkin-Savich (innervated) gastric pouch, Gorbunova, Lebedinskaïa and Savich [1933] demonstrated that, after the introduction into the main stomach of 300 c.c. of 1 per cent. acetic acid, there was a marked change in the secretory response of the pouch to ingestion of milk or of a 5 per cent. solution of Liebig's Extract, or to a rectal injection of 6 per cent. alcohol. On the day after the administration of acetic acid, when the pouch secretion was tested in any of the above-mentioned ways, it was found that the secretion was diminished in volume and that it contained much mucus and had a lower acidity. On the third day the mucus content of the secretion was reduced, but the acidity of the juice still remained below normal. These experiments showed that the acetic acid acted not only on the mucous membrane with which it came in direct contact but also on the mucosa of an isolated pouch *par distance*: that is, the important fact established by Gorbunova, Lebedinskaïa and Savich was that as a result of the introduction of acetic acid into the main stomach the juice secreted by the pouch was diminished in volume and altered in composition. That was what interested us most in this work. For a number of years it has been held in our laboratory that the variations so often occurring in the composition of

the gastric juice are due chiefly to changes in the secretory activity of the different cellular groups which form part of the gastric glands [*cf.* Babkin, 1931, 1934, 1938 *a* and *b*]. A good means of testing this theory was offered by acetic acid, a stimulant which is capable of altering the functional activity of the gastric mucosa. Such an investigation was the more desirable since Gorbunova, Lebedinskaïa and Savich did not determine systematically the total chloride, neutral chloride and pepsin of the gastric juice, all three of which are extremely important components of the secretion. For this reason, it may be remarked, their theory that the secretion of water and the secretion of acid by the gastric glands are two distinct functions would require further investigation.

Methods.

The experiments were all of the chronic type and were performed on five dogs, K, T, B, C, and H. Dogs K, T, and B were œsophagotomised dogs provided with a metal gastric fistula. Dogs K and B were otherwise perfectly normal animals. In Dog T (for special reasons unconnected with this work) all the gastric arteries were tied and cut on Aug. 3. [This procedure has been carried out on several dogs in our laboratory by Drs. J. C. Armour and D. R. Webster (results not yet published), who showed that it does not interfere with the secretory function of the gastric glands when they are stimulated by sham-feeding or histamine. Even a week or two after the ligature of the arteries the secretory response to either stimulus was almost normal.] The experiments with acetic acid on Dog T were started on Sept. 24, 52 days after the operation.

The secretion of gastric juice was stimulated in the dogs with œsophagotomy and a gastric fistula by sham-feeding with meat for 5 to 15 minutes or by subcutaneous injection of histamine, and the direct effect of previous administration of acetic acid on the reflex phase of gastric secretion was studied. In Dog C, which was equipped with a Pavlov pouch and a metal fistula in the main stomach, acetic acid solution was introduced into the main stomach and the secretory response of the pouch to a test-meal of meat or to rectal injection of 200 c.c. of 5 per cent. ethyl alcohol was studied. Dog H was provided with a Bickel pouch (*i.e.* one deprived of all extrinsic innervation) and a metal fistula in the main stomach. In this animal the effect of the introduction of acetic acid into the main stomach was reflected on the secretory function of the denervated pouch during the second (or pyloric) and third (or intestinal) phases only.

In all these animals from 100 to 300 c.c. (according to body-weight) of a 1 per cent. solution of acetic acid were introduced through the metal fistula into the stomach and kept there for from two to three hours, after which the stomach was emptied and the sham-feeding or other gastric stimulus applied. Usually about one-fifth of the acetic acid solution was recovered from the stomach; the rest passed into the duodenum and was presumably absorbed. In a few experiments the dogs vomited a small amount of the acetic acid solution. In such cases the experiment was discontinued, or the quantity of

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solution lost was replaced and the experiment continued. In one of the dogs with œsophagotomy and a gastric fistula the effect of introduction into the stomach of 2·2 per cent. lactic acid was also tested. Control experiments were performed before and for several days after administration of the acid solutions.

The total and free acidities of the gastric juice were determined by titration with ·02 N solution of NaOH, phenolphthalein and Töpfer's reagent being used as indicators. The total chloride was estimated by Wilson and Ball's [1928] modification of Van Slyke's method and the values were expressed in milli-equivalents per litre. For the determination of the peptic power Nirenstein and Schiff's modification of Mett's method [Hawk and Bergeim, 1937] was employed. To determine the amount of mucus in the gastric juice, samples were centrifuged for 5 minutes at constant speed and the volume of the sediment measured.

Results.

The results of experiments on two of the dogs with œsophagotomy and a gastric fistula (Dogs K and T) are shown in Tables I. and II.

Table I.

Dog K (*dog with œsophagotomy and a gastric fistula*).—*H.* = 0·6 mg. of histamine phosphate injected subcutaneously. *Sh.F.* = 5 minutes' sham-feeding with meat. In the experiments in which acetic acid solution was previously introduced into the stomach, histamine was injected or sham-feeding performed 20 minutes after the stomach had been emptied of the acetic acid. In the experiments of Dec. 18 and Jan. 15 the dog vomited part of the acetic acid solution, which was collected and re-introduced into the stomach.

Date of expt.	Stimulus.	Volume of gastric secretion, c.c.	Mucus content, c.c.	Free acidity, m.eq./l.	Total acidity, m.eq./l.	Total Cl content, m.eq./l.	Peptic power, Mett units.	Remarks.
Dec. 11	*H.*	66·5	0·6	131	143	164	33	
„ 13	*H.*	80·7	1·1	133	145	169	33	
„ 18	*H.*	*58·2*	*13·1*	*45*	*69*	*137*	*15*	Before histamine administration, 250 c.c. 1 per cent. *acetic acid* introduced into stomach for 2 hrs. 25 min.
„ 20	*H.*	60·9	1·3	133	142	167	20	
Jan. 8	*Sh.F.*	65·8	4·2	124	142	166	200	
„ 10	*Sh.F.*	39·4	2·8	120	137	166	208	
„ 15	*Sh.F.*	*91·2*	*11·0*	*54*	*76*	*137*	*50*	Before sham-feeding, 200 c.c. 1 per cent. *acetic acid* introduced into stomach for 2 hrs. 25 min.
„ 17	*Sh.F.*	55·1	3·5	122	137	167	200	
„ 23	*Sh.F.*	*11·3*	*11·9*	*71*	*92*	*150*	*96*	Before sham-feeding, 100 c.c. 1 per cent. *acetic acid* introduced into stomach for 1 hr.

Dog K (Table I.).—In all the experiments where variable amounts of 1 per cent. acetic acid solution were previously introduced into the

stomach and allowed to remain there for different periods of time, the subsequent response of the gastric glands to histamine administration or to sham-feeding with meat was uniformly altered. The gastric juice now differed from the normal "histamine" or "sham-feeding" juice in the following respects:—

(1) There was a great increase in visible mucus. (*N.B.*—The mucus content of the normal sham-feeding juice is usually greater than that of histamine juice.)
(2) The free and total acidities were much reduced.
(3) The total chloride concentration was reduced.
(4) The peptic power of both the sham-feeding and the histamine juice was diminished.

The volume of the secretion provoked by sham-feeding or by histamine was diminished after the application of acetic acid in most of the experiments, as we shall see later. However, in some cases, as in the experiment of Jan. 15 (Table I.), it was increased. Since this was an experiment involving sham-feeding, there was one factor—the appetite of the animal—which was difficult, if not impossible, to control. Nevertheless, in spite of the greater volume of secretion in this experiment, the free and total acidities, total chloride concentration, and concentration and total output of pepsin were lower than in the control experiments (*e.g.* experiments of Jan. 10 and 17), where the volume of secretion was only about half as large. A very instructive comparison may be drawn between this and the histamine experiments of Dec. 18 and 20, where the volumes of secretion in the control and the test experiments were practically equal (viz. 60·9 c.c and 58·2 c.c.).

Dog T (Table II.).—The experiments on this animal fully confirm the results obtained on the previous dog (K). Various doses of histamine were tried in order to produce a volume of gastric secretion equal to that of the control experiment, but the volume of the secretion in all the histamine experiments after the application of acetic acid was less than in the corresponding control experiments. Although in one of the sham-feeding experiments (Oct. 2) the acetic acid markedly diminished the volume of secretion, in another (Oct. 10) the inhibition was doubtful. This cannot be ascribed to the occasional vomiting of a small amount of acetic acid solution, because vomiting occurred in other experiments (Oct. 2 and Nov. 1) and yet there was no increase in the volume of secretion. In the same experiment (Oct. 10) there was a less marked reduction of the peptic power of the juice obtained after the preliminary introduction of acetic acid into the stomach than in the experiment of Oct. 2. We did not try to analyse this fact because our interest was centred on changes in the acidities and the concentration of chloride in the gastric juice and in the secretion of mucus by the gastric mucosa under the influence of acetic acid. In that respect in all eight of the acetic acid experiments on the two dogs, K and T, we

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obtained uniform results. Undoubtedly acetic acid, on being applied locally to the gastric mucosa, caused a great secretion of mucus, whereas in control experiments, if there was no secretion of gastric juice before histamine administration or sham-feeding, a scanty amount of mucus mixed with acid was discharged from the gastric fistula. The volume of this secretion of mucus was 0·2 to 0·6 c.c. per 15 minutes. After the administration of acetic acid the volume of mucoid secretion (in the interval between the emptying of the stomach of acid solution and

TABLE II.

Dog T (dog with œsophagotomy and a gastric fistula).—*H.*—subcutaneous injection of histamine phosphate. *Sh.F.*—5 minutes' sham-feeding with meat. In the experiments in which acetic acid solution was previously introduced into the stomach, histamine was injected or sham-feeding performed 15 minutes after the stomach had been emptied of the acetic acid. In the experiments of Oct. 2, Oct. 10, and Nov. 1 the dog vomited about 50 c.c. of the acetic acid solution, which was re-introduced into the stomach.

Date of expt.	Stimulus.	Volume of gastric secretion, c.c.	Mucus content, c.c.	Free acidity, m.eq./l.	Total acidity, m.eq./l.	Total Cl concent., m.eq./l.	Peptic power, Mett units.	Remarks.
Sept. 21	*Sh.F.*	88·2	3·2	125	136	158	138	Before sham-feeding, 250 c.c. 1 per cent. *acetic acid* introduced into stomach for 2 hrs. 25 mins.
Oct. 2	*Sh.F.*	*47·5*	*4·1*	*59*	*75*	*146*	*41*	
„ 4	*Sh.F.*	62·2	2·8	120	135	161	187	
„ 8	*Sh.F.*	53·1	1·8	115	129	160	188	Before sham-feeding, 250 c.c. 1 per cent. *acetic acid* introduced into stomach for 2 hrs. 25 mins.
„ 10	*Sh.F.*	*63·6*	*3·1*	*80*	*97*	*148*	*117*	
„ 11	*Sh.F.*	88·9	1·2	112	127	162	112	
„ 16	*H.*	84·7	1·3	132	143	162	78	1 mg. histamine phosphate injected.
„ 18	*H.*	*34·7*	*4·3*	*72*	*89*	*153*	*35*	Before injection of 1 mg. histamine phosphate, 250 c.c. 1 per cent. *acetic acid* introduced into stomach for 2 hrs. 25 mins.
„ 30	*H.*	119·4	1·3	137	147	168	31	0·75 mg. histamine phosphate injected.
Nov. 1	*H.*	*15·3*	*6·8*	*71*	*90*	*151*	*70*	Before injection of 0·75 mg. histamine, 250 c.c. 1 per cent. *acetic acid* introduced into stomach for 2 hrs. 25 mins.
„ 13	*H.*	39·7	0·9	124	138	171	71	0·5 mg. histamine phosphate injected.
„ 15	*H.*	*69·4*	*6·1*	*90*	*108*	*150*	*27*	Before injection of 0·75 mg. histamine phosphate, 250 c.c. 1 per cent. *acetic acid* introduced into stomach for 2 hrs. 25 mins.
„ 20	*H.*	37·1	1·3	123	142	166	87	0·6 mg. histamine phosphate injected.

the beginning of sham-feeding or injection of histamine) was many times greater than that of the control for the same length of time. The following are the average figures for this mucoid secretion in all the experiments in which acetic acid was introduced in the two dogs:—

	Volume per 15 min., c.c.	Free acidity, m.eq./l.	Total acidity, m.eq./l.	Total Cl. concent., m.eq./l.	Peptic power, Mett units.
Dog K . .	5·9	23·7	56·9	125	38
Dog T . .	2·9	14·7	29·5	122	83

It will be noted that in Dog T the acidities and the concentration of the total chloride were lower and the peptic power somewhat higher than in Dog K.

During the course of the secretion induced by sham-feeding or histamine, the secretion of mucus, large at the beginning, gradually diminished (the first samples of gastric juice always contain more mucus). Nevertheless the total and free acidities and the total chloride even in the last samples of gastric juice obtained by sham-feeding or histamine after administration of acetic acid were much lower than in the control experiments. Thus the lowering of the acidity and of the total chloride content did not depend only on the admixture of mucus with the gastric juice. Some other factors presumably also played an important part in this phenomenon.

The reaction of the gastric glands to the irritating effect of acetic acid is illustrated in Table III., which shows the results of two experi-

TABLE III.

Dog K: Expt. Dec. 11.—Gastric secretion in response to subcutaneous injection of 0·6 mg. histamine phosphate.

Dog K: Expt. Dec. 18.—Gastric secretion in response to subcutaneous injection of 0·6 mg. histamine phosphate after preliminary introduction of acetic acid into stomach.

Volume, per 15 min., c.c.	Mucus content, per 15 min., c.c.	Free acidity, m.eq./l.	Total acidity, m.eq./l.	Total Cl concent., m.eq./l.	Peptic power, Mett units.	Volume, per 15 min., c.c.	Mucus content, per 15 min., c.c.	Free acidity, m.eq./l.	Total acidity, m.eq./l.	Total Cl concent., m.eq./l.	Peptic power, Mett units.
10·0	0·42	107	122	161	125	14·0	5·50	30	58	130	19
23·3	0·06	134	146	168	16	27·7	7·30	42	66	136	16
13·7	0·06	136	147	165	10	10·5	0·45	66	91	142	10
12·5	0·08	138	150	163	23	4·3	0·13	64	89	145	10
7·0	0·03	136	146	158	19	1·7	0·05	52	76	142	13
0·7	0·00	89	119	..	..	..	..	..	..	..	..
67·2	0·65	131	143	164	33	58·2	13·43	45	69	137	20

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ments on Dog K, in which the gastric secretion was stimulated by the same dose of histamine injected subcutaneously with and without preliminary treatment with acetic acid. In all other experiments the same relations were observed, the difference between the volume of mucus secreted in control experiments and that secreted in experiments in which acetic acid was previously introduced into the stomach being

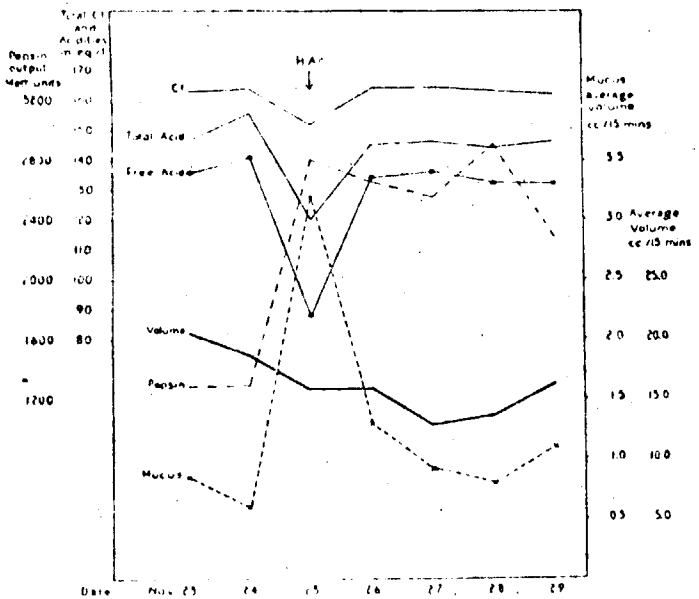

FIG. 1. The effect of previous introduction of acetic acid solution into the stomach on the gastric secretory response to sham-feeding with meat in a dog with œsophagotomy and a gastric fistula.

less marked when the stimulus was sham-feeding than when it was histamine.

Dog B.—Prolonged experiments were performed on Dog B, also an œsophagotomised dog with a gastric fistula. The after effects of a single introduction into the stomach of a 1 per cent. solution of acetic acid or of a 2·2 per cent. solution of lactic acid were studied in the case of each acid for several days. Fig. 1 illustrates a typical experiment which lasted 7 days. The gastric secretion obtained by 15 minutes' sham-feeding with meat was collected every day. Control observations had been made on the first two days and then on the third day 300 c.c. of 1 per cent. acetic acid solution were introduced into the empty stomach. After 2 hours and 25 minutes the gastric contents (17 c.c.) were removed through the opened fistula. Half an hour later sham feeding with meat was performed in the usual manner. The effect of the acetic acid was striking. The volume of secretion diminished on the day

that the acetic acid was administered (Nov. 25) and continued reduced until the fourth day, after which it began to rise. The volume of visible mucus was five times greater after acetic acid on the day of its administration. In some of the samples of juice collected on that day it formed half of the fluid. The volume of mucus fell sharply the next day and the following day returned to normal. The total chloride concentration and the free and total acidities were markedly reduced on the day that acid was administered; they all returned practically to normal within the next two days (Nov. 26 and 27). A peculiar effect of acetic acid administration on the peptic power of the juice was observed in this dog. Both the concentration and the output of pepsin rose sharply, remained high for four days, and then began to fall. The results of this experiment leave no doubt that the changes in the composition of the gastric juice were due to the increased activity of the mucus-producing and perhaps of some other glandular cells caused by the acetic acid, and not to the diminished volume of the gastric secretion.

The introduction into the main stomach of 300 c.c. of 2·2 per cent. lactic acid in the same dog had a somewhat different effect on the gastric secretion elicited by a subsequent sham-feeding of meat. The volume of secretion was not diminished, but on the first day tended to rise slightly. The volume of mucus secreted was tripled or quadrupled on the day of introduction of the lactic acid, but diminished thereafter. Correspondingly the concentration of total chloride and the total and free acidities fell slightly on the first day, but next day had already returned to normal or almost normal. No definite effect was produced by the lactic acid on the concentration and output of pepsin in the sham-feeding secretion.

Dog C (Pavlov-pouch Dog).—Gorbunova, Lebedinskaïa and Savich (*l.c.*) observed that the introduction of 300 c.c. of 1 per cent. acetic acid into the main stomach of dogs with an innervated gastric pouch elicited from the pouch a scanty secretion containing a great amount of mucus. On the second day, 100 c.c. of 6 per cent. ethyl alcohol were administered per rectum, which produced from the pouch a secretion of gastric juice having a low acidity and a high mucus content. On the third day alcohol was again injected and produced from the pouch a secretion in which the mucus content was practically normal but the acidity still low.

We repeated these experiments of Gorbunova, Lebedinskaïa and Savich on a dog with a Pavlov pouch. Five series of experiments, each series lasting several days, were performed on this animal. In four series 300 gm. of minced beef, alone or mixed with 200 c.c. of water, were used as a test-meal. About 2 to 2½ hours before the meat was given, 200 or 300 c.c. of 1 per cent. solution of acetic acid were introduced into the main stomach. By the end of this period most of the

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acetic acid had left the stomach and the gastric secretion of the pouch had ceased. Acetic acid itself elicited from the Pavlov pouch a secretion of 5 to 8 c.c. of gastric juice, having a somewhat lower concentration of total chloride (152 to 158 m.eq. per litre), and usually a lower free acidity (86 to 94 m.eq. per litre) and total acidity (105 to 110 m.eq. per litre) than the secretion in control experiments with meat. The concentration, and often the volume of mucus in the pouch secretion, was increased, while the peptic power of the juice was moderate. In the

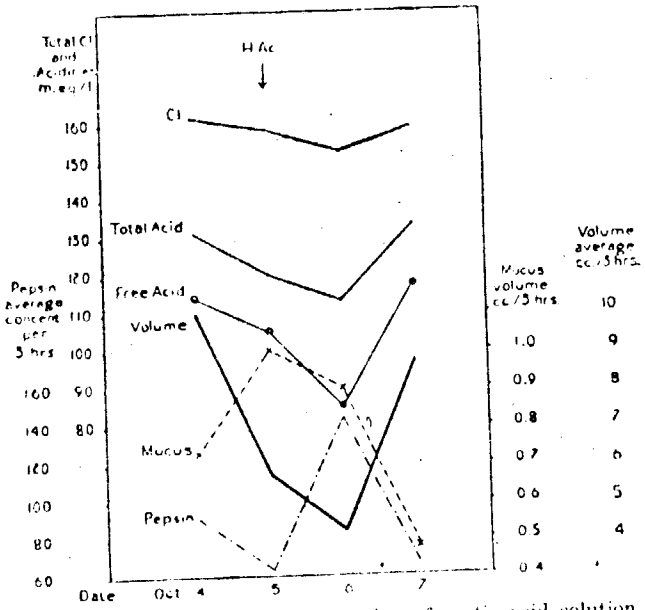

Fig. 2.—The effect of previous introduction of acetic acid solution into the main stomach on the secretory response of a Pavlov pouch to a test-meal of meat in a dog.

fifth series, after the introduction of acetic acid solution into the stomach, 200 c.c. of 5 per cent. alcohol were injected into the rectum and the gastric secretion was collected from the pouch.

The facts observed by Gorbunova, Lebedinskaïa and Savich were confirmed, viz. that after the introduction of acetic acid into the main stomach the Pavlov pouch responded to a test-meal of meat or to a rectal injection of alcohol with a smaller gastric secretion, containing a greater amount of mucus and possessing a lower acidity and a lower total chloride concentration. No marked changes were noted in the peptic activity of the juice.

Fig. 2 represents one of a series of typical experiments. The greatest depression of the curves for the total chloride concentration, for both free and total acidity and for volume of the secretion, occurred on Oct. 6, the day after administration of acetic acid. This coincided

with the highest concentration of mucus in the juice (Oct. 4, 7·4 per cent.; Oct. 5, 18·5 per cent.; Oct. 6, 20·0 per cent.; and Oct. 7, 5·5 per cent.), although the total volume of mucus on Oct. 6 was somewhat less than on the day that acetic acid was administered.

These effects of acetic acid were also quite marked during the late afternoon gastric secretion, provoked by a second feeding of the animal after the cessation of the secretion on meat. The second meal consisted of oatmeal porridge (100 g. of oatmeal cooked in 200 c.c. of milk and 100 c.c. of water, with the addition of 1 g. of sodium chloride) and the gastric juice from the pouch was collected over a period of 6 hours. The following data were obtained on the same days that the main experiment (shown in fig. 2) was performed:—

Date.	Volume, c.c.	Free acidity, m.eq./l.	Total acidity, m.eq./l.	Mucus content, per cent.	Remarks.
Oct. 4	35·0	121	137	8·1	Acetic acid solution introduced into main stomach in the morning.
„ 5	*18·0*	*94*	*115*	*23·0*	
„ 6	5·4	44	64	54·2	
„ 7	12·0	83	104	31·0	

It is evident from both series of experiments that the greatest depression of the pouch secretion and the proportionally greater content of mucus were observed not on the day of acetic acid administration but on the following day.

These experiments, in confirmation of the data of Gorbunova, Lebedinskaïa and Savich, show that, when a solution of acid is introduced into the main stomach, it acts not only on the gastric mucosa with which it comes in direct contact but also in some unknown way on the mucosa of the Pavlov pouch, which is completely separated from the main stomach. However, the effect of preliminary administration of acetic acid on the volume and the composition of the histamine or sham-feeding gastric secretion *par distance* in the Pavlov-pouch dog was not so immediately noticeable as had been its local effect in the œsophagotomy dogs, although in the former it seemed to last somewhat longer.

It might be objected, in regard to the experiments involving sham-feeding or ingestion of meat, that the animals perhaps lost their appetite after administration of the acetic acid solution and that this may have affected the volume and the composition of the gastric secretion. This supposition is invalidated by the experiments on the Pavlov-pouch dog in which gastric secretion was provoked by the rectal injection of

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200 c.c. of 5 per cent. ethyl alcohol before and after introduction into the main stomach of 300 c.c. of 1 per cent. acetic acid (Table IV.).

TABLE IV.

Dog C (Parlow-pouch Dog).—Illustrating the effect of previous introduction into the main stomach of 300 c.c. of 1 per cent. acetic acid solution on the pouch secretion elicited by rectal injection of 200 c.c. of 5 per cent. ethyl alcohol. In the experiment of Feb. 3 acetic acid was kept in the stomach for 3 hours; alcohol was injected 30 minutes after the stomach had been emptied of acid. With the exception of those for total volume of secretion and percentage of mucus, the figures represent the average per hour of secretion.

Date of expt.	Stimulus.	Total volume, c.c.	Average volume, c.c./hr.	Mucus content, per cent.	Free acidity, m.eq./l.	Total acidity, m.eq./l.	Cl concent., m.eq./l.	Peptic power, Mett units.
Feb. 2	Alcohol	9·5	3·2	2·1	109	132	154	74
Feb. 3	Acetic acid	8·2	2·7	2·4	104	122	157	80
	Alcohol	8·5	2·8	5·3	93	108	153	52
Feb. 4	Alcohol	10·3	3·4	3·5	94	110	154	144

Dog H (Dog with Bickel Pouch).—The secretory response of the Bickel (denervated) pouch to a meal consisting of 100 g. of meat mixed with 200 c.c. of water was much less affected by previous introduction of acetic acid into the main stomach than was that of a normally innervated stomach or pouch. The variations in the volume of fluid and of mucus and in the different constituents of the juice (fig. 3)

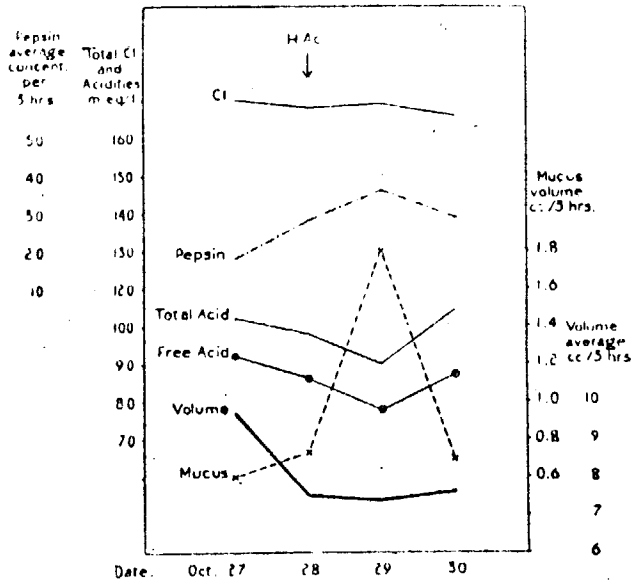

FIG. 3.—The effect of previous introduction of acetic acid solution into the main stomach on the secretory response of a Bickel pouch to a test-meal in a dog.

seem to indicate that the acetic acid after it had left the stomach still affected somewhat the secretion of the isolated pouch. However, it is to be noted (1) that these variations were slight; (2) that in many control experiments the volume of secretion and especially the volume of mucus, which is abundantly produced by this kind of pouch, underwent analogous variations; and (3) that the figures for pepsin concentration in the Bickel-pouch juice are in general so small that the observed variations signify very little. Nevertheless one fact is clear, namely, that in this type of experiment the lowest free and total acidities coincided with the highest content of mucus in the juice.

Unfortunately, as there was no other dog in the laboratory with a Bickel pouch and a gastric fistula, this type of experiment could not be repeated on another animal.

DISCUSSION.

The experiments we have reported confirmed the observation of Gorbunova, Lebedinskaïa and Savich [1933] that the introduction of acetic acid into the main stomach affects the character of the gastric secretion of a Pavlov pouch. They also indicated clearly that the secretory activity of different histological components of the gastric mucosa must receive due consideration in any discussion of the regulation of the acidity of the gastric juice. The introduction of acetic acid solution into the stomach was used as a means of stimulating chiefly one group of epithelial cells of the gastric mucosa, namely, those producing mucus or a mucoid secretion. Acetic acid, whether acting directly on the mucosa or at a distance, produced a secretion of mucus greater than normal, which was accompanied by a diminution in the acidity of the gastric juice and in most cases by a slight diminution in the total chloride concentration. The lowering of the acidity was due not to the diminished rate of secretion but to the increased mucus content of the juice, as may be seen from fig. 1 (Nov. 25 and 26—sham-feeding with meat) and also from Table IV. (Feb. 2 and 4—rectal administration of alcohol). By "mucus" we do not mean only visible mucus in the form of lumps and threads, which could be centrifuged, and the amount of which merely serves as an indication of the changes in the production of the "mucoid secretion." It is very probable that not only is a certain amount of neutral or alkaline fluid secreted by the mucous cells of the gastric glands along with the gastric "mucus," as was shown by Florey [1931] to occur in the case of the colonic mucus, but there is another large group of cells in the gastric mucosa, namely, the mucous (or chief) cells of the neck, which also are able to produce a neutral or alkaline mucoid secretion. Integrity of the vagal innervation of the stomach seems to be an important factor in the increased production of

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mucus by the gastric mucous membrane in response to the application of acetic acid solution.

Already one of us [Babkin, 1929] has emphasized the importance of the part played by the mucous cells of the neck in the regulation of the acidity of the gastric juice. Hollander [1934] called the secretion of all the cells of the gastric glands other than the acid-producing cells a "diluting secretion," or "alkaline component," and supposed it to have a chloride concentration of about 100 m.eq. per litre—that is, lower than that of the acid secretion of the parietal cells. Some clinicians—Kalk [1932], Katsch [1933], Maclagan [1934], and others—consider that the level of the acidity of the gastric juice depends on the activity of the "mucous cells of the neck," which produce a neutral, watery secretion. To what extent the diminution of the acidity after the administration of acetic acid was due to actual neutralization of the hydrochloric acid of the gastric juice; and to what extent to dilution by excessive secretion of the surface epithelium cells and mucous cells of the neck, we are unable to say. It is true that in many of our experiments the activity of the parietal cells was diminished, as evidenced by the diminished volume of the gastric secretion, and the activity of the mucous and mucoid cells increased. In other experiments, in spite of a practically normal secretion of visible mucus, both the free and the total acidity were low. However, this fact cannot be explained by Rosemann's [1907] theory that the parietal cells are capable of producing hydrochloric acid in varying concentrations, because in our experiments with diminution of the acidity of the juice the concentration of total chloride likewise diminished (*cf.*, for example, the data in Table III.). This fact refutes one of the fundamental postulates of Rosemann's theory, namely, that the concentration of total chloride in the gastric juice remains constant, but that the relative amount of chloride combined with *H* ion or with *Na* ion varies. It seems to point to the admixture of the parietal secretion with a secretion possessing a lower chloride concentration.

One other point has to be mentioned. The data here presented are not in agreement with the "diffusion theory" of Teorell [1933, 1935, 1939, 1940] concerning the regulation of the gastric acidity. According to Teorell the hydrochloric acid of the gastric juice is diffused into the gastric mucosa or the blood and is exchanged against sodium chloride *i.e.* *Na* ions replace the *H* ions and neutral chloride is formed. The theory was based on experiments on cats, in which a very small amount (usually 3 to 5 c.c.—maximum 10 c.c.) of an acid of known concentration was introduced into the stomach, and samples of gastric juice were taken for chemical analysis every 15 minutes for 1½ hours. We do not dispute Teorell's facts. We consider, however, that the conditions necessary for the exchange of the ions of an acid solution which is present in the stomach occur rarely under normal

circumstances. They may occur perhaps when an exceedingly small amount of gastric juice remains for some reason in a resting or very feebly secreting stomach for a considerable time. However, Teorell's explanation, if accepted *in toto*, implies that gastric mucus possesses no acid-binding capacity, which does not agree with the data obtained in this investigation. In our experiments the gastric juice never stagnated either in the whole stomach or in a pouch, but was able to flow freely through the fistula. Thus, for instance, in the sham-feeding experiments, both on the day of administration of acetic acid and on the day after (fig. 1—Nov. 25 and 26) the rate of secretion was the same, being more than 60 c.c. per hour, yet the total and the free acidity and the chloride concentration of the juice differed greatly on the two days. Tables I. and II. show that in some of the experiments in which acetic acid had been administered the volume of the gastric secretion was greater than in the control experiments, and yet the acidity of the juice was lower in the former than in the latter. To explain this phenomenon according to the "diffusion theory," the fact of the greater secretion of mucoid fluid must be completely ignored and an additional hypothesis must be introduced, namely, that the acetic acid increased enormously the ability of the gastric mucosa to exchange the ions of the juice. Even if this assumption were correct, it would not provide an adequate explanation for many of our experiments. Thus, for example, in the Pavlov-pouch dog, in which the acetic acid did not come in direct contact with the pouch mucosa, the greatest depression of the activity occurred not on the actual day of administration of the acid but on the following day, when the mucus content was high and the total chloride concentration was particularly low (see fig. 2).

The problem of the regulation of the gastric acidity is a complicated one, as the present experiments plainly show. For the study of this problem it is essential to have a thorough understanding of the secretory activity of the various cellular groups which form the gastric glands and mucosa, including a knowledge of the composition of the secretion from each group of glandular cells, and of the mode or modes of regulation of the secretory process in each group.

Summary.

1. In dogs with œsophagotomy and a gastric fistula, 1 per cent. acetic acid solution, introduced into the stomach and kept there for 2 to 2½ hours, greatly affected the secretory response to subsequent sham-feeding with meat or subcutaneous injection of histamine, diminishing the volume of the gastric secretion, lowering the total chloride concentration and the free and total acidities, and increasing the content of mucus.

2. Analogous results were obtained in a Pavlov-pouch dog and in

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a much lesser degree in a Bickel pouch dog, the introduction of acetic acid into the main stomach in these cases influencing the secretory activity of the pouch *par distance*.

3. Evidence is advanced that the lowering of the acidity and of the total chloride concentration of the gastric juice in these experiments was due to the neutralizing and diluting effect of the mucoid secretion, an excessive production of which was caused by the introduction of acetic acid into the stomach.

We are greatly indebted to Mrs. Olga Komarov for her help in a number of these experiments.

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674. THE LACTIC ACID AND VOLATILE FATTY ACID CONTENTS OF FIVE LOCALLY MADE TYPE CHEESES

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(With 3 Figures)

A study has been made of the lactic acid and lower volatile fatty acid contents of five locally made type cheeses, Coulommier, Gervais, Pont l'Évêque, Dutch and Smallholder. The Smallholder cheese is of the cheddar variety. All the cheeses were made from the same batch of milk on the same day, and the conditions of ripening of each type have been as closely akin as possible to those normally encountered in the areas peculiar to the individual cheeses. The changes which have been studied relate to those which occur between the time of manufacture and the normal time of placing the cheeses on the market. No attention has been paid to later maturing changes.

It might be reasonably expected that lactic acid, as a strong acid, would have a dominant effect on pH and titratable acidity. This is true in *in vitro* studies on the activity of hetero-fermentative strains of lactobacilli, but in milk, as noted by Pederson & Bagg (1), the buffering effect of milk constituents results in higher degrees of acidity than might be expected from the pH. These effects will be exaggerated in cheese. It may be noted here, in passing, that estimations of lactic acid are in fact estimations of lactate and that therefore titratable acidity and pH will not be directly related to the figures obtained for lactic acid. Troupe & Kobe (2) have in fact shown that the reaction which readily occurs between lactic acid, in the monomeric form, and methanol results in a fall of titratable acidity.

In general, the higher volatile fatty acids, caproic, caprylic and capric, seem to be characteristic of cheeses in which moulds play an integral part, such as Roquefort (Currie) (6). They seem to be characteristic also of the later stages of ripening of harder cheeses such as Cheddar (Suzuki, Hastings & Hart (4); Peterson & Johnson (5)). Acetic acid appears to be the most commonly occurring acid in the early stages of cheese ripening, but Suzuki *et al.* (4) noted the presence of formic acid although later references to its presence are not frequent. In the present work this latter acid has been encountered in all the fatty acid analyses performed. Formic acid occurs as a result of bacterial metabolism, for example in silage (Barnett) (6), but its occurrence in combined form in lipids has been noted only in recent years (Barnett & Duncan (7); McInnes, Hansen & Jessop (8)). It is not clear whether the formic acid in cheese results from bacterial metabolism or from lipid breakdown. Butyric acid, in the early stages of ripening, may result from the breakdown of lactose via lactic acid as Hillig & Montgomery (9) suggest may happen in cream. Again, Wolf (10, 11) states that lactic streptococci and lactobacilli contain butyrinases and that it is possible that increases in butyric acid may be caused by butyrinase liberated from the dead cells of lactobacilli.

EXPERIMENTAL

Cheese. The cheese samples used in this work were obtained from the North of Scotland College of Agriculture (Craibstone Farm). The different cheeses were made at the same time from the same batch of milk and the starter used was a mixed strain type including acid producing strains (three strains of *Streptococcus cremoris* and one strain of *Str. lactis*). In addition, the starter included two V.P. positive strains—one, an active acid producer (*Str. diacetylactis* type) and the other a non-acid producer.

As far as possible the cheeses were each made and stored under conditions suitable to their type, and except for Smallholder sufficient individual cheeses were made to enable one cheese to be used as a complete representative sample. Thus, four Coulommiers were made, four Gervais, five Pont l'Évêques, three Dutch cheeses and two large Smallholders. The Smallholders were sampled by means of a scoop at the necessary intervals.

The aim of the work was to study the changes that occur up to the point at which the cheeses would normally be marketed. Thus, the Coulommier was sampled for 3 days, the Gervais for 3 days, the Pont l'Évêque for 4 weeks, the Dutch for 2 weeks and the Smallholder for 7 weeks, the initial samples being removed and stored at the time of manufacture.

Each sample when removed from the maturing room was transported to the laboratory and kept in a deep-freeze at −20° C. in an air-tight plastic bag till all the samples had been collected. During the different analyses the samples were never kept out of the deep freeze for longer periods than were absolutely necessary. All cheese analyses were performed on rind-free samples.

ANALYTICAL

Volatile fatty acids (V.F.A.). The acids were extracted from the cheese samples by the method of Barnett & Tawab (12). An amount of distillate from the Markham, equivalent to between 0·5 ml. and 1·0 ml. of N/50NaOH, was used for each V.F.A. estimation by gas chromatography, using the technique of James & Martin (13). While the procedure adopted was similar in all essentials to that of these latter authors, the recoveries of V.F.A. were not as good as had been hoped for. After considerable experimentation, it was found that excellent recoveries could be obtained by incorporating the following three slight modifications, viz.:

(1) Before evaporating the above neutralized distillate to dryness, 0·1 ml. 2N-NaOH should be added to prevent loss of formic and acetic acids.

(2) The micro-column used had a total length of 17 cm. and was made from tubing 4 mm. in diameter. The column was slightly flared at one end and sealed at the other. The aperture was situated on the side of the tube about 3 mm. from the closed end and the tube was packed first with a 5 mm. thick wad of fibreglass, followed by 2 cm. of anhydrous Na_2SO_4 and lastly by 2 cm. of Celite.

(3) The free V.F.A. were transferred to the column and washed through it with a total of 1·8 ml. of ether. The variation in results obtainable by the use of the normal micro-column and the slightly modified one are indicated by the following trials.

Trial A. (1) A standard mixture of volatile fatty acids from C_1 to C_{10} was prepared in ethereal solution. An aliquot of this solution was shaken with 2N-NaOH solution, the ether removed by evaporation and the extract distilled, after acidification, from the Markham to obtain the titration value.

(2) An aliquot similar to that used in (1), was loaded directly on to the micro-column, and 1·8 ml. anhydrous ether were used for washing through the column. The acid filtrate was collected in 2 N-NaOH and distilled from the Markham in the usual fashion, after removal of the ether by evaporation. The titration value was compared with that obtained in (1) above. The acid recoveries were 98–100% in both cases.

Trial B. Step (1) in Trial A was repeated, but the neutralized distillate was treated according to the procedure described above as being used for V.F.A. determinations on cheese. The acid filtrate from the micro-column was again collected in 2 N-NaOH, the ether evaporated and the acids removed by distillation from the Markham.

Trial C. Trial B was repeated, but a cheese extract of known titration value was used instead of the standard ethereal solution of V.F.A.

Each of these trials was prepared in duplicate. One of the duplicates was put through a standard James and Martin micro-column, while the other was put through the modified column. The results are shown in Table 1. It is clear that the modified column gave a greatly improved recovery with B and C. It was found that the improvement was attained in part by the modified column itself, but maximum recovery was obtained by combining the modifications. It is considered that, since equivalent results were obtained with repetitions of the different trials, the identical recoveries in Trial A were due to the absence of small amounts of phosphoric acid which were present in Trials B and C. It is conceivable that the phosphoric acid has a retarding effect on the passage of the ether solution, under air pressure, down the column.

Table 1. *The recoveries of total volatile fatty acids directly from a standard ethereal solution (A_2), from a standard ethereal solution subjected to normal pre-treatment before being loaded on to the micro-column (B) and from a cheese extract (C) treated similarly to B (see text)*

Trial no.	Recovery of volatile fatty acids (%)			
	Standard micro-column		Suggested micro-column	
	(1)	(2)	(1)	(2)
A_2	100	100	100	100
B	80	82	98	98
C	80	80	97	98

Good separations have been obtained, where both lower and higher V.F.A. were involved, by using two different vapour sources, one steam, the other ethylene glycol mono-ethyl ether, for the vapour jacket. This is in accord with the findings of Van der Kamer, Gerritsma & Wansink (14) who used a special thermostatically controlled heating arrangement for the outer jacket. It may be noted that the quantities of formic acid with which we have been involved in cheese have been such that they have not been lost during the transfer of the free V.F.A. to the micro-column by suction.

Lactic acid. Lactic acid has been determined in cheese in various ways, but in the present work it was decided to use a method incorporating the colorimetric technique of Barker & Summerson (15), chiefly because the method has been used in these laboratories, in modified form, in studies on the lactic acid content of silage (Barnett (16)). Kleinert (17) used the method, adapted from that described by Davidson (18), for milk. The following procedure, based on the work of these authors, has been used here and found to give satisfactory

results, and its efficiency has been confirmed by recovery tests with mixtures of fresh cheese and standard lactate.

Of the cheese sample, 1 g. is minced, in the case of hard cheese, and placed in a Pyrex test-tube (200 mm. × 25 mm.) with 30 ml. N/4 NaOH. If the cheese is a soft one, the sample is placed in the tube and blended, using a spatula, with the NaOH. The tube and its contents are kept in a water-bath at 70° C. for 1 hr. with occasional shaking. While still hot the contents of the tube are transferred quantitatively to a 100 ml. standard flask and the volume made up to the mark after cooling. A 1 ml. portion is taken in a 50 ml. standard flask and diluted to about 35 ml. with water. The procedure thereafter is similar to that described by Davidson (18).

After development of the colour with the *p*-hydroxy-diphenyl reagent, the reading is made on an EEL colorimeter using a green filter. In calculating the lactic acid content of the cheese samples, volume correction factors, as used by Davidson for milk, were used for cheese because the protein and fat content of 10 ml. of milk approximates to the amounts of these components present in 1 g. of cheese while the amounts of $Ca(OH)_2$ suspension and $CuSO_4$ solution were similar in both. In practice, six samples were examined at the same time along with a standard (lithium lactate) and a blank.

RESULTS

The pH changes during the ripening periods of the different cheeses are shown in Fig. 1, while the total titratable acidity changes in the fresh cheese are indicated in Fig. 2. It is clear that, considering the results for all the samples analysed, there was no close correlation between pH and total titratable acidity, but such an overall correlation could hardly

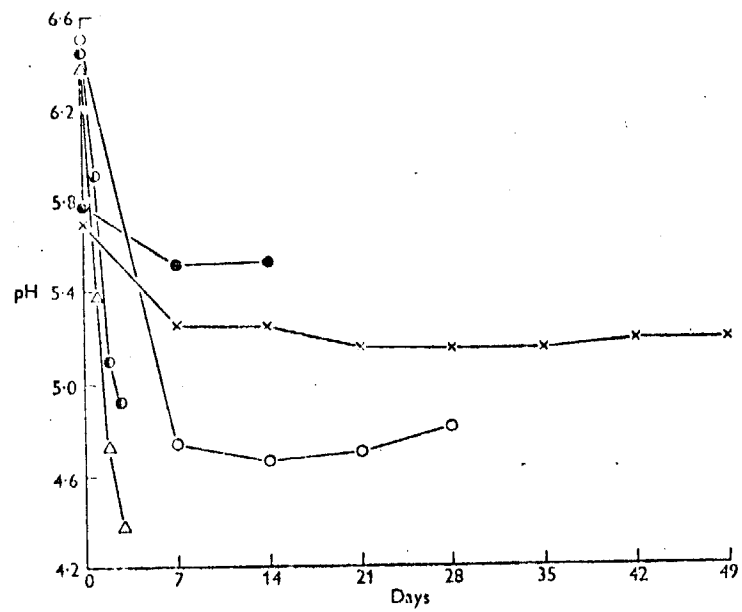

Fig. 1. The change in pH of the different type cheeses over the ripening period peculiar to each cheese. ×—×, Smallholder; ○—○, Pont l'Évêque; ●—●, Dutch; △—△, Coulommier; ◐—◐, Gervais.

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be expected since so many factors govern titratable acidity in cheese. For each particular cheese, however, the relationship between pH and titratable acidity is obvious (Fig. 3).

Changes in the total V.F.A. content of the cheese are indicated in Table 2. These results have to be considered in the light of the fact that the terms 'volatility in steam', 'water solubility' and 'fat solubility' as applied to fatty acids are only relative. Thus in the present work the quantities of cheese extract used in the Markham were such that any

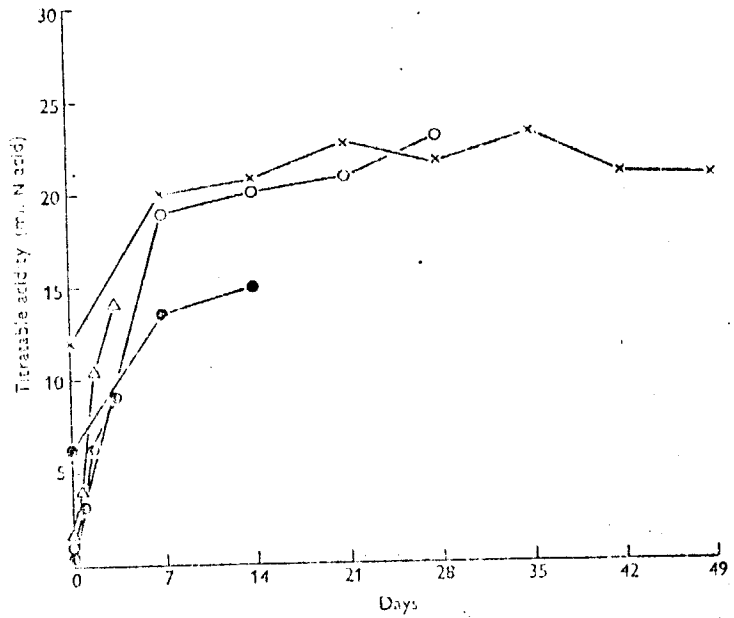

Fig. 2. The titratable acidity (in ml. N acid/100 g. fresh cheese) of the different cheese samples throughout the ripening period peculiar to each cheese. The symbols used correspond to those in Fig. 1.

acids from C_1 to C_9, in amount likely to be encountered, would have been soluble in the volume of aqueous distillate collected. Again, it is not always realized that even palmitic acid is slightly volatile in steam while myristic acid will distil from a Markham at the rate of about 1 mg./50 ml. of distillate under normal conditions of operation. These remarks bring into prominence the amounts of water insoluble fatty acids which were obtained from the different samples examined. The amounts varied from 16·3 to 50·0% of the total acids distilled as measured by titration values. A preliminary suspicion would be attached to the extraction of the other solution of fatty acids with sodium hydroxide employing the mild extraction method used by Smiley, Kosikowsky & Dahlberg(19) and by Barnett & Tawab(12), but, as one of the tests of the method, various triglycerides of higher and lower fatty acids were subjected, in ethereal solution, to the complete extraction technique and gave rise to no free fatty acid production. Again, it will be seen from Table 2 that for each cheese analysed, under identical conditions of extraction, there was a steady increase or decrease in the amounts of this insoluble fraction. Runs with different samples of the water-insoluble fraction on the gas chromatographic column indicated minute traces of caproic acid (c. 0·0006% of the dry matter of the sample of cheese involved), but no other acid, although the runs were continued under high nitrogen

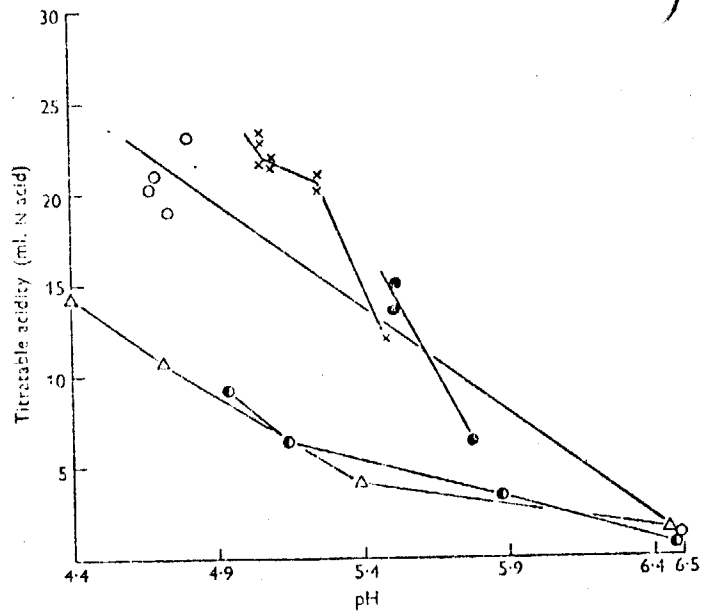

Fig. 3. The relation between titratable acidity and pH for each type of cheese over the ripening periods considered. The symbols used correspond to those in Fig. 1.

Table 2. *The volatile fatty acids, expressed as ml. N/10 alkali, obtained by distillation of extracts of the various cheese types at different stages of maturity*

Type	Age	Volatile acidity (≡ml. N/10 alkali) in 100 g. fresh cheese: Water soluble	Water insoluble	Total	Water insoluble volatile acids (% of total volatile acids)
		Soft cheese			
Coulommier	Start	3·8	3·8	7·6	50·0
	1 day	3·5	1·3	4·8	27·1
	2 days	11·1	2·6	13·7	19·0
	3 days	13·2	2·6	15·8	16·5
Gervais	Start	3·1	1·6	4·7	34·0
	1 day	4·2	2·6	6·8	38·2
	2 days	9·5	3·2	12·7	24·4
	3 days	11·1	4·7	15·8	29·8
Pont l'Évêque	Start	5·2	2·1	7·3	28·8
	1 week	9·5	4·2	13·7	30·6
	2 weeks	9·5	4·2	13·7	30·6
	3 weeks	9·0	5·8	14·8	39·2
	4 weeks	9·5	9·5	19·0	50·0
		Hard cheese			
Dutch	Start	11·6	2·6	14·2	18·3
	1 week	13·7	4·2	17·9	23·5
	2 weeks	17·0	4·2	21·2	19·8
Smallholder	Start	11·0	2·6	13·6	19·1
	1 week	15·8	3·7	19·5	19·0
	2 weeks	17·0	3·7	20·7	17·9
	3 weeks	19·0	3·7	22·7	16·3
	4 weeks	20·0	5·2	25·2	20·7
	5 weeks	20·0	5·3	25·3	20·9
	6 weeks	21·1	6·3	27·4	23·0
	7 weeks	21·1	6·3	27·4	23·0

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pressure for 60 hr. which would have been sufficient to bring out lauric and perhaps myristic acids. Moreover, the fractions recrystallized from aqueous alcohol melted over the range 47°–51°. Presumably, then, higher acids are involved, but it is significant that oleic acid, which is present in greatest abundance in the triglycerides of milk fat, was not present. The acid is somewhat volatile in steam and a trace of it would have resolved the solid obtained into an oil. It must be assumed, therefore, that these acids are produced by selective lipolysis of the triglycerides. The problem is being further investigated.

Table 3. *The individual volatile fatty acids, expressed as mg./100 g. fresh cheese, obtained by gas chromatography of the distillates from the various cheese types at different stages of maturity*

Type	Age	Volatile acids (mg./100 g. fresh cheese) Formic	Acetic	Butyric	Total
		Soft cheese			
Coulommier	Start	4·7	13·0	5·6	23·3
	1 day	5·0	19·1	0·0	24·1
	2 days	6·5	57·3	0·0	63·8
	3 days	7·7	65·7	0·0	73·4
Gervais	Start	3·7	13·1	0·0	16·8
	1 day	2·9	16·4	1·9*	21·2
	2 days	9·0	35·4	0·0	44·4
	3 days	7·7	53·1	0·0	60·8
Pont l'Évêque	Start	4·8	18·7	7·3	30·8
	1 week	5·8	37·9	0·0	43·7
	2 weeks	7·8	33·9	2·5	44·2
	3 weeks	13·6	25·4	7·5	46·5
	4 weeks	12·9	27·0	9·9	49·8
		Hard cheese			
Dutch	Start	5·8	50·6	0·0	56·4
	1 week	15·5	50·6	0·0	66·1
	2 weeks	18·2	57·6	10·0	85·8
Smallholder	Start	11·6	52·7	0·0	64·3
	1 week	5·8	73·3	3·7	82·8
	2 weeks	7·8	73·8	7·5	89·4
	3 weeks	9·7	75·8	11·2	96·7
	4 weeks	15·4	75·4	14·8	105·6
	5 weeks	19·4	80·9	17·4	117·7
	6 weeks	15·5	85·9	22·3	123·7
	7 weeks	15·5	85·9	22·3	123·7

* Iso-butyric.

The results for the individual water-soluble V.F.A. contents of the different cheese samples are indicated in Table 3. It is immediately clear that acetic acid was the most abundant, while formic acid was a much commoner constituent than butyric acid. Indeed, except at the start of the ripening process with Coulommier, butyric acid did not appear at all in this cheese while only a trace of iso-butyric acid was found on the second day of ripening of Gervais. Pont l'Évêque and Smallholder cheeses, however, contained considerable amounts of butyric acid in the later stages of the ripening period. Despite the tailing of the chromatograms to exhaustion no other V.F.A. were detected. When it is remembered that these results are calculated on the fresh cheese it is very obvious that the production of the acids was very much greater in the soft than in the hard cheese; this of course would be expected owing to the accelerating effect of moisture on ripening. The presence and subsequent disappearance of butyric acid at the start of the ripening period

Coulommier is quite characteristic of fermentations in which fatty [illegible] are involved. The same remarks apply to the iso-butyric acid present on the first day [illegible] the ripening of the Gervais and to the butyric acid at the start of the ripening of the Pont l'Évêque. These acids may form metabolic substrates for different organisms at different pH levels. The

Table 4. *The lactic acid content of each cheese at different stages of ripening, expressed as a percentage of the fresh cheese*

(Only the samples relevant to each ripening period were analysed, see text.)

Type	Cheese	Lactic acid content (% of fresh cheese) Start	Days 1	2	3	Weeks 1	2	3	4	5	6	7
Soft	Coulommier	0·883	1·325	1·457	1·811	—	—	—	—	—	—	—
	Gervais	0·883	1·060	1·264	1·345	—	—	—	—	—	—	—
	Pont l'Évêque	0·856	—	—	—	1·916	1·590	1·101	1·060	—	—	—
Hard	Dutch	0·937	—	—	—	1·508	1·345	—	—	—	—	—
	Smallholder	0·954	—	—	—	1·590	1·291	1·073	1·590	1·073	1·550	1·351

results for the lactic acid content of the different samples are given in Table 4. With the soft cheese over the short ripening period studied the lactic acid increased from about 0·9% (fresh material) to about 1·8% in the Coulommier and to about 1·3% in the Gervais. The figures for the hard cheeses were in the main higher than those for the soft cheeses as might be expected because of the longer ripening period. All of them were much higher after one week of ripening than they were at the start of the process, but some lower values were subsequently obtained, presumably due to metabolism of the lactic acid by the cheese microflora.

DISCUSSION

The work reported here has, with particular regard to lactic acid, served to confirm much of what has been done at other centres. The formation, in the early stages of ripening, with a subsequent complete or partial disappearance of this constituent has been noted by other workers (e.g. Hurel & Mocquot(20)), while the later fluctuations in the lactic acid content of Smallholder cheese are somewhat similar to those noted by Suzuki *et al.*(9) in Cheddar cheese. It might appear permissible to suggest that some of the starter organisms are hetero-fermentative in nature and that variations in lactic acid content should be paralleled by corresponding variations in acetic acid content. This suggestion is not apparently tenable, however, because the acetic acid figures for Smallholder cheese (Table 3) showed no such fluctuation. One can but assume that, in the ripening process, the lactic acid initially formed acts as a substrate for some organism or organisms and that its later formation and disappearance are due to the breakdown and utilization of components other than lactose such as amino acids and protein. For example, the hydrolytic deamination of alanine would give rise to lactic acid.

The lack of variety of fatty acids noted in these early stages of cheese ripening suggests that lipolytic and deamination effects were not greatly in evidence. Nevertheless, the high percentage of higher volatile fatty acids, that is acids higher than caproic, is of interest and not readily explicable. Although the synthesis of higher fatty acids by microorganisms has been studied (e.g. by Bornstein & Barker)(21), the synthesis of acids higher than caproic from lower V.F.A. has not been noted, as far as the authors are

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aware. Tha[illegible]ng so, one is led to the conclusion that very small amounts of higher f. acids are readily broken off from some lipid constituent in cheese. It would appear that there is here a field for further investigation.

The occurrence of formic acid in such quantity as has been noted makes it surprising that more attention has not been paid in the past to its possible significance. Doubtless this is due to the difficulty of identifying and determining this substance except by the chromatographic methods that are now available.

SUMMARY

1. A study has been made of the development of titratable acidity, lactic acid and volatile fatty acids in five locally made type cheeses. The study of the changes involved has been made over the period of ripening up to the stage at which each type would normally be marketed. All the cheeses were made from the same batch of milk at the same time.

2. Slight modifications to the micro-column devised by James & Martin(13), and its use, have been suggested which have resulted in improved recovery of volatile fatty acids.

3. The volatile fatty acids have been determined *in toto* and also as separate constituents. Formic acid has been noticed as a common constituent of the acid mixture in all the cheeses, while, over the ripening period involved and apart from unidentified acids higher than caproic, the only other volatile acids encountered have been acetic and butyric, and on one occasion iso-butyric.

4. Over the short ripening period studied, the lactic acid content of the soft cheeses rose, but after the initial rise in Pont l'Évêque and in Dutch cheese the results showed a downward trend. Fluctuations in the lactic acid content of the Smallholder cheese have been noted which compare with the corresponding findings of other workers.

5. All the results presented have been calculated on the basis of fresh cheese because this is the customary practice and comparisons with other work would be difficult if this had not been adhered to.

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Glucose and Acetate Utilization by Hyperplastic, Alveolar Nodule Outgrowths and Adenocarcinomas of Mouse Mammary Gland[1]

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SUMMARY

The utilization of glucose-1-^{14}C, -6-^{14}C, -6-^{3}H, and acetate-1-^{14}C was studied in slices of normal gland, both suckled and nonsuckled; outgrowths of hyperplastic alveolar nodules (HAN's); and mammary adenocarcinomas excised from pregnant and lactating C3H mice. The major metabolic products (CO_2, lipids, fatty acids, and lactate) of these substrates were isolated and assayed for radioactivity.

With the exception of lactate production, normal lactating glands showed the highest metabolic activity in all the parameters studied, uptake of substrates, production of CO_2, lipogenesis, and pentose phosphate cycle activity. Slices of HAN outgrowth and of nonsuckled, normal tissue from lactating mice exhibited very similar metabolic activity, which was lower than that of the normal, suckled gland. Tumor slices exhibited the lowest metabolic activity of the tissues studied from lactating mice. Lactate accumulation from glucose was observed in normal, nonsuckled tissue and in HAN outgrowth, as well as in adenocarcinoma.

While the addition of glucose to neoplastic slices stimulated incorporation of acetate into fatty acids, the extent of stimulation was much less than that observed in normal, lactating tissue from the same animal. Slices of the HAN outgrowth and of the nonsuckled, normal tissue from lactating mice also responded to the addition of glucose by increasing acetate conversion to fatty acids; the extent was intermediate between that exhibited by the normal tissue and by the adenocarcinoma.

The metabolic activity of all tissues excised from pregnant mice was lower than that of tissues from lactating mice. Therefore, the metabolism of the HAN outgrowth and of the adenocarcinoma is influenced by the physiological state of the host. The manner and means of glucose and acetate utilization by normal glandular tissue from pregnant mice was very similar to that by the HAN outgrowth from the same animals. Again, the tumor was the least active tissue studied.

The type of lipid synthesized from acetate-1-^{14}C by normal glandular tissue and by HAN outgrowth from pregnant mice was similar, 40 to 50% of the labeled lipid being triglyceride. The onset of lactation increased the percentage of incorporation into triglyceride almost two-fold in both the normal and preneoplastic tissues.

The types of fatty acids synthesized from acetate-1-^{14}C by slices of normal glands, of HAN outgrowths, and of adenocarcinomas prepared from tissues taken from pregnant mice were similar, predominantly 16 carbons long. Slices of normal gland, both suckled and nonsuckled, from lactating mice produced mainly ^{14}C fatty acids 10 to 12 carbons long. In contrast, the type of fatty acids produced by the HAN outgrowth and by the tumor was less responsive to the onset of lactation. The fact that the HAN outgrowth cannot produce the pattern of fatty acids typical of the lactational state is the first indication that this preneoplastic tissue is metabolically distinct from comparable normal mammary gland tissue.

INTRODUCTION

Local hyperplasias that arise in the mammary glands of C3H mice infected with mammary tumor virus have been shown by Blair *et al.* (7) and DeOme *et al.* (10, 12, 13) to be morphologically recognizable precursors of mouse mammary tumors. These HAN's[3] when transplanted into gland-free mammary fat pads of isologous mice, proliferate to produce cell population outgrowths that are each derived from a single preneoplastic hyperplasia (7, 11). Serially transplanted samples of mammary tissue (either normal or HAN's) into gland-free mammary fat pads of young female C3H mice result in the development of outgrowths with a histological pattern characteristic of the type of tissue transplanted (12).

In previous reports from this laboratory (23, 24), the transplantation technique was used to provide sufficient tissue to study the levels of activity of a number of enzymes in HAN's outgrowth from virgin mice. These studies revealed that the preneoplastic and neoplastic tissues of the mouse mammary gland are metabolically responsive to changes in the physiological state of the animal and, hence, are under some neurohormonal influence. Furthermore, the levels of enzy-

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[3] The abbreviations used are: HAN, hyperplastic alveolar nodule; PPC, pentose phosphate cycle.

matic activity in the outgrowth of the HAN's were closer to that of normal lactating gland than to that of the adenocarcinoma and were indistinguishable from that of normal gland from a pregnant mouse (23, 24).

In this report, the metabolic activity of the outgrowth of the HAN's was compared to that of normal outgrowths, adenocarcinomas, and normal glands, both suckled and nonsuckled. Incorporation of specific substrates into CO_2, fatty acids, and lactate has been determined in slices of the following tissues taken from lactating[4] or pregnant mice: both HAN and normal mammary gland outgrowths in gland-free fat pads; mammary adenocarcinomas; normal lactating mammary glands; and normal mammary glands with nipples that had been seared, making milk removal from those glands impossible. For this purpose, tissue slices were incubated with glucose labeled with ^{14}C in position 1 or with ^{14}C or ^{3}H in position 6 or with acetate-1-^{14}C. The use of these specific labels allows evaluation of various major metabolic pathways in each tissue.

The stimulatory effect of glucose on fatty acid synthesis from acetate is well established in normal lactating mammary glands (1, 4, 20). Such a stimulatory effect must rely on the operation of sophisticated metabolic controls within the cell. Therefore, it is of special interest to examine the stimulatory effect in the outgrowths of the preneoplastic HAN's and their tumors. Hence, the studies with labeled acetate were carried out both in the presence and absence of glucose.

Both pregnant and lactating mice were used to compare the response of the preneoplastic and neoplastic tissues to the onset of lactation with that of normal mammary gland. The normal outgrowths and the tissues from the glands with seared nipples provide control tissues for comparison with the hyperplastic outgrowths. In all of these tissues, the hormonal environment was the same and the biosynthetic products produced within the tissues were not being removed.

MATERIALS AND METHODS

Animals and Their Treatment

All mice were females of the C3H/Crgl strain and were maintained on Diablo Labration *ad libitum*. At 3 weeks of age, they received tissue implants, as described elsewhere (12, 24), and when 4 months old they were placed with males. Pregnant mice were put into separate cages and, after parturition, were allowed to lactate by feeding their young for 9 to 14 days. All litters were adjusted to 8 pups shortly after parturition. The dams were sacrificed, and their tissues were excised for study only if the stomachs of their pups were filled with milk. Tissues were collected from pregnant mice between the 16th and 18th day of pregnancy.

Origin and Description of Outgrowth Tissue

HAN Outgrowth. The HAN outgrowth used in these experiments was a subline (designated here as 1-P) of HAN outgrowth line F, which was originally described by Blair *et al.* (7). HAN outgrowth subline 1-P, derived from line F, transplant generation 15, was used in the studies reported here when in its 18th to 22nd transplant generation in relation to the hyperplastic nodule of origin. At the time of study, HAN outgrowth subline 1-P (Fig. 1) still closely resembled outgrowth line F (7) by morphological and histological criteria, but the alveoli were no longer distended with a milk-like secretion in virgin mice 14 weeks after transplantation. When the outgrowth was obtained from mice that had lactated for 9 to 14 days, however, its alveoli were distended and filled with a secretion resembling milk (Fig. 2).

Although outgrowth subline 1-P, like outgrowth line F did not give rise to tumors during the first 14 weeks after transplantation into gland-free fat pads of 3-week-old virgin mice, small tumors were occasionally found as early as 16 weeks. When 20 of these subline outgrowths were allowed to proliferate for 24 weeks, about half of them gave rise to 1 or more tumors. No tumors were found when 20 mice were given implants of normal tissue that were allowed to proliferate for the same length of time.

Normal Outgrowth. Implants of normal mammary gland in gland-free fat pads of 3-week-old virgin mice yielded outgrowths of normal mammary tissues, which almost completely filled the pads within a period of 9 to 12 weeks. DeOme *et al.* (12) have shown that the responsiveness of normal mammary gland outgrowths to endogenous hormones is such that their development parallels that of the host's own mammary glands.

Comparison between HAN Outgrowth and Normal Outgrowth. Both the HAN outgrowth and the normal outgrowth completely filled the Number 4 mammary fat pad of lactating mice 9 to 14 days postpartum. Under these conditions, the parenchymal cells in the 2 types of tissues were similar by morphological and histological criteria. There were usually more adipose cells present in the normal outgrowth (Fig. 3) than in the HAN outgrowth (Fig. 2).

Spontaneous Adenocarcinomas

Small spontaneous adenocarcinomas were occasionally palpated in the HAN outgrowths in virgin mice 4 to 6 months after transplantation. These mice were placed with males and isolated when pregnant. Tissues were collected from pregnant mice on the 16th to 18th days of gestation. Some of these mice were allowed to bear their young and lactate for 9 to 14 days, after which time the tumors were excised for study. The morphology of the tumors (Fig. 4) was typical of the mouse mammary adenocarcinomas described by Dunn (14).

Preparation of Normal Lactating Mammary Glands with Seared Nipples

The nipples of both No. 4 mammary glands of mice 13 to 14 days pregnant were completely removed by means of electric cautery. These animals delivered their young normally and maintained them with milk from other glands. Between 9 and 14 days postpartum, both the suckled and nonsuckled tissues were harvested for the experiments.

[4] The term "lactating" as used here was defined by Cowie *et al.* (9) to include both the production of milk by and the removal from the mammary gland.

Incubation Procedures

Mice were killed by cervical dislocation, and normal mammary glands, HAN outgrowths, and normal outgrowths were excised under a dissecting microscope. After removal of lymph nodes, the tissues were sliced to a thickness of 0.4 mm with the McIlwain-Buddle tissue chopper (30). The slices were washed free of milk (24) with ice-cold Krebs-Henseleit bicarbonate buffer, pH 7.3 (25), and then were gently blotted on filter paper.

Tumors were excised when approximately 1 cm in diameter. Only firm, nonnecrotic tissue was selected and sliced. Tumor slices were not washed but were immediately incubated as described below.

A precisely weighed portion of the slices from each tissue was separately incubated with isotopically labeled and unlabeled substrates according to the conditions described in the appropriate tables. The incubation vessel was a small culture tube (100 x 16 mm) with a removable center well suspended from the rubber stopper. The incubation vessel was closed with a gas-tight, self-sealing rubber serum cap. At the end of the incubation period, 0.1 ml of a freshly prepared 1:1 mixture of ethanolamine and 2-ethoxyethanol was injected through the serum cap into the center well of each incubation vessel. This was immediately followed by 0.2 ml of 0.05 N HCl injected into the compartment containing the incubation medium and slices. Incubation vessels were mechanically shaken for 1 hr to ensure complete absorption of the CO_2 by the ethanolamine mixture.

Analytical Procedures

Assay of $^{14}CO_2$. The contents of the center wells were quantitively rinsed into counting vials with 10 ml of a 0.5% solution of PPO dissolved in the mixture of toluene: 2-ethoxyethanol (2:1) (5). Radioactivity was assayed in a liquid scintillation spectrometer.

Assay of Labeled Fatty Acids and Total Lipids. The aqueous phase of the incubation mixture was decanted, and the slices were washed with water. The fatty acids in the washed tissues were saponified and extracted with hexane as reported previously (3). Aliquots of the hexane extracts were counted in the scintillation spectrometer in the same PPO solution described above for $^{14}CO_2$ assay.

In experiments for the analysis of the type of lipid synthesized, the total lipid of the washed slices was extracted several times into hot chloroform:methanol (2:1). The extract was washed with water evaporated to a small volume, and the lipids were extracted into petroleum ether (b.p. 30–60°). An aliquot of the total lipid extract was assayed for radioactivity, as described above.

Separation and Assay of Fatty Acids. A portion of the hexane extract containing the fatty acids was concentrated to a small volume in a cold water bath with the use of a stream of nitrogen gas. The methyl esters of the fatty acids were formed by the addition of an excess of diazomethane. The methyl esters were separated by gas-liquid chromatography. Each fatty acid fraction was collected in scintillation medium and assayed for isotopic content as described previously (3).

Separation and Assay of the Major Classes of Lipids. A portion of the petroleum ether extract containing the total lipid fraction was concentrated to a small volume under a stream of nitrogen gas. This concentrate was applied to thin layers (0.25 mm) of silica gel on glass plates (28). The constituent lipids were separated chromatographically on the gel with the use of a mixture of diethyl ether:petroleum ether:acetic acid (15:85:1.5). The areas of the gels containing the various lipid classes, identified by suitable standards applied to the same plate, were removed from the plate, and the adsorbed lipids were eluted with appropriate solvents (16). The eluates were evaporated under nitrogen gas and assayed for radioactivity in the liquid scintillation spectrometer in the same solvent mixture used for the total lipid assay.

Assay of the ^{14}C Content of the Carboxyl Carbon of Fatty Acids. Fatty acids in a portion of some of the hexane extracts were converted to their potassium salts by extraction into an excess of aqueous KOH. The hexane was evaporated under a stream of nitrogen gas. One aliquot of each aqueous extract was assayed in the scintillation spectrometer in the same solvent used for CO_2 assay. Another portion of this solution was decarboxylated by a modification of the Schmidt reaction (27, 32). The $^{14}CO_2$ released during this reaction was trapped in 1 N KOH, a portion of which was also assayed in the liquid scintillation spectrometer under conditions identical with those for the potassium salts.

Assay of ^{14}C-Labeled Lactate. ^{14}C-labeled lactic acid was extracted from the aqueous phase of the incubation mixture by continuous extraction with anhydrous diethyl ether for 24 hr, during which time it was collected in 1.5 ml of 0.02 N KOH. The lactate-containing solution was made up to 2.0 ml with water, and 0.1-ml aliquots were assayed on the liquid scintillation spectrometer in the same medium used for $^{14}CO_2$ assay.

Glucose and Acetate Uptake. Separate aliquots of the aqueous phase of the incubation mixture were assayed for unutilized substrates as follows. (*a*) Glucose was determined enzymatically by the glucose oxidase method (Worthington Biochemical Corp., Freehold, N. J.). (*b*) Labeled acetate was isolated by continuous steam distillation (29), and the radioactivity of an aliquot was determined in the same manner as was used with lactate. Uptake was calculated as the difference between added and unutilized substrate.

Tissue Composition. Sliced and washed tissues from several lactating mice were pooled as needed, and exactly 1-g portions of each tissue type were extracted 5 times with boiling Bloor's mixture (ethanol:ether, 3:1) followed by 2 additional extractions with hot ether. The defatted tissue residues were reserved for the determination of dry weights. The combined lipid extracts were evaporated with a minimum of heating under a gentle stream of nitrogen to a volume of approximately 2 ml in a sidearm flask (15). The lipids were then removed with 5 successive extractions with petroleum ether (b.p. 30–60°), which were pooled in tared vials. After removal of the solvent under nitrogen, the lipid samples were dried to constant weight in a vacuum at 40°. The total lipid content was then determined gravimetrically.

The defatted tissue residues were further treated as described in Table 1. If one assumes that the final tissue residue represents the total protein content of the tissue,[5] the difference between the net weight and the sum of the lipid and protein contents must be equal to the amount of water in the tissue.

Radioactive Substrates

Glucose-1-^{14}C, -6-^{14}C, and -6-^{3}H were purchased from New England Nuclear, Boston, Mass. Acetate-1-^{14}C was prepared from $Ba^{14}CO_3$ as described by Calvin *et al.* (8).

RESULTS

In order to compare the metabolic activities between the various mammary tissues studied, one must consider the differences in the relative amounts of protein in each tissue type. Thus, for facilitation of comparisons between tissues, the uptake and conversion of isotopically labeled glucoses and acetate to metabolic products have been expressed as the amount of substrate taken up or product formed per mg of protein. Since the utilization of glucose per mg of protein by each tissue type did not differ to any large degree (Table 2), the conclusions based on the mμmoles of substrate converted to product per mg of protein and those derived from calculations based on the uptake of glucose would be similar.

Tissue Composition. Normal mammary outgrowth had the highest and mammary adenocarcinoma had the lowest lipid content of all tissues studied (Table 1). Whereas the protein content of the normal outgrowths was somewhat lower than that of either the tumors or the normal lactating glands, the values obtained with the normal lactating glands and the outgrowth of HAN's were similar. The relatively high lipid content and low protein content of the normal outgrowth may be a reflection of the presence of a greater number of adipose cells in this tissue, relative to that found in the HAN outgrowth (compare Figs. 2 and 3).

Glucose and Acetate Uptake by Tissues from Lactating Mice. The disappearance of glucose from the medium containing slices prepared from the adenocarcinomas was significantly lower than from that containing slices from any of the other tissues (Table 2). The glucose uptake by slices of HAN and normal outgrowths and by the normal lactating glands was similar. The acetate uptake in the absence of glucose was similar in all tissues. Only in slices from normal glands did the addition of glucose increase the uptake of acetate.

Conversion of Glucose to CO_2, Fatty Acids, and Lactate by Tissues from Lactating Mice. Comparing the conversion of the isotopes of glucose-1-^{14}C, glucose-6-^{14}C, and glucose-6-^{3}H to fatty acids reveals that the activity of the HAN outgrowths resembled that of the normal outgrowths, both of which were 15 to 25 times as active as the adenocarcinomas. The normal lactating mammary gland was 2 to 3 times more active in this regard than was either kind of outgrowth and was, thus, more than 30 times more active than the mammary tumor (Table 3).

[5] Close agreement between the value for the final tissue residue and that from protein determinations of homogenates (17) of these tissues established the validity of this assumption.

Table 1

Composition of normal, preneoplastic, and neoplastic mammary gland slices obtained from lactating C3H mice

One-g samples of washed tissue slices were extracted for their total lipid contents as described in text. The defatted tissue residues were then treated with a 10% NaCl solution at 90° for 1 hr to remove nucleic acids. After the tissue residues were washed successively with distilled water, ethanol, and ether, they were dried to constant weight in a vacuum at 40°. Each value recorded below is given as the percentage of tissue slices, wet weight, and is the mean ± S.E. of from 3 to 5 separate determinations.

Kind of tissue	% of wet weight in: Lipid	Protein[a]	Wa[illegible]
Normal gland	10.6 ± 0.1	12.6 ± 0.8	76.7 [illegible]
Normal outgrowth	24.7 ± 0.7	9.3 ± 1.3	66.0 [illegible]
HAN outgrowth	7.2 ± 1.6	11.8 ± 1.8	81.0 [illegible]
Adenocarcinoma	3.2 ± 0.7	15.0 ± 0.7	81.8 [illegible]

[a] Defatted dry weight.

The incorporation into fatty acids of ^{3}H from C-6 of glucose relative to that of ^{14}C from the same carbon was similar in all 4 tissues.

Carbon dioxide production from glucose-1-^{14}C by the HAN outgrowths and the normal outgrowths was also nearly the same (Table 3). On the other hand, the $^{14}CO_2$ yields from the glucose carbon in the experiments with normal lactating slices were about twice as high as those with the outgrowth slices and about 5 times as high as those with slices of the adenocarcinoma.

The conversion of the 6th carbon of glucose to CO_2 by these tissues is also shown in Table 3. Slices prepared from normal lactating gland oxidized almost 3 times as much of the carbon of glucose as did the slices from the tumor, whereas the conversion of glucose-6-^{14}C to $^{14}CO_2$ by slices from the normal outgrowths and from the HAN outgrowths was not significantly different from that by the normal tissue.

With mammary tumor slices, the yields of lactate-^{14}C from glucose-1-^{14}C were 5 times higher than with normal lactating mammary gland slices (Table 4). The yields of lactate-^{14}C from glucose-6-^{14}C were more than 2 times higher from normal lactating glands than from tumors. In contrast to the normal lactating glands, both outgrowths, the normal as well as the HAN, converted both glucose-1-^{14}C and glucose-6-^{14}C to lactate-^{14}C slightly more actively than did the tumors.

Conversion of Acetate to Fatty Acids and CO_2 by Tissues from Lactating Mice. The recovery of the isotope of acetate-1-^{14}C in fatty acids was studied both in the absence and presence of glucose. As in the experiments with labeled glucose, the activity of the HAN outgrowth closely resembled that of the normal outgrowth (Table 3). When glucose was added to the incubation medium, the conversion of the isotope of acetate-1-^{14}C to fatty acids by slices from normal lactating mammary gland was about 2 times more active than by either outgrowth. Such conversion by both outgrowths exceeded that observed with tumors by about 10-fold.

Omission of glucose from the incubation medium decreased the incorporation of acetate into fatty acids by all tissues but to varying extents (Table 3). Again, slices of the normal and the preneoplastic outgrowths exhibited similar incorporation

Table 2

Uptake of glucose and acetate by slices of normal, preneoplastic, and neoplastic mammary tissues from lactating mice

Twenty mg of tissue slices were incubated at 37° for 3 hr in 0.2 ml of Krebs-Henseleit bicarbonate buffer (pH 7.3) containing 2.0 μmoles each of glucose-^{14}C and acetate, acetate-^{14}C, and acetate-^{14}C and glucose, with 95% O_2 and 5% CO_2 as gas phase. Other experimental details are given in text. Each value recorded below is the average ± S.E. of the results of triplicate determinations on 4 to 7 experiments with tissues from separate mice and is presented as mμmoles of added substrate taken up per mg of protein.

Kind of mammary tissue	Glucose uptake in presence of acetate	Acetate uptake in presence of:	
		No added substrate	Glucose
Normal gland	556 ± 32	357 ± 32	492 ± 24
Normal outgrowth	624 ± 45	323 ± 32	355 ± 43
HAN outgrowth	627 ± 43	373 ± 17	432 ± 25
Adenocarcinoma	453 ± 20	313 ± 13	307 ± 20

Table 3

Incorporation of labeled glucose and acetate into fatty acids and CO_2 by slices of normal, preneoplastic, and neoplastic mammary tissues from lactating mice

Twenty mg of slices were incubated at 37° for 3 hr in 0.2 ml of Krebs-Henseleit bicarbonate buffer (pH 7.3) containing 2.0 μmoles of the labeled and unlabeled substrates recorded below. Gas phase was a mixture of 95% O_2 and 5% CO_2. Each value is the average ± S.E. of results from 10 to 12 experiments with different mice and is presented as mμmoles of added labeled compound converted to CO_2 and fatty acids per mg of protein.

Substrates		Normal lactating mammary gland		Outgrowths of: Normal mammary gland		Outgrowths of: HAN		Mammary adenocarcinoma	
Labeled	Unlabeled	CO_2	Fatty acids	CO_2	Fatty acids	CO_2	Fatty acids	CO_2	Fatty acids
Glucose-1-^{14}C	Acetate	200.0 ±15.1	104.0 ± 3.2	123.7 ±15.0	66.7 ±11.8	104.2 ± 6.8	67.8 ±11.0	41.3 ± 6.0	4.0 ±0.6
Glucose-6-^{14}C	Acetate	57.9 ± 4.8	219.0 ±18.3	43.0 ± 5.4	90.3 ±15.0	48.3 ± 4.2	89.8 ±18.6	20.0 ± 3.3	4.7 ±0.6
Glucose-6-^{3}H	Acetate		71.4 ± 7.2		32.3 ± 8.6		39.0 ±10.2		2.0 ±0.2
Acetate-1-^{14}C	None	118.3 ±10.3	13.5 ± 5.6	153.8 ±25.8	7.5 ± 1.1	195.8 ±11.9	8.5 ± 1.7	105.3 ±14.0	2.0 ±0.1
Acetate-1-^{14}C	Glucose	96.8 ± 5.6	174.6 ±12.7	124.7 ±20.4	62.4 ± 8.6	159.3 ± 7.7	61.0 ±13.6	84.0 ±14.0	8.0 ±0.7

Table 4

Incorporation of ^{14}C-labeled glucoses into lactate by slices of normal, preneoplastic, and neoplastic mammary tissues from lactating mice

See Table 3 for experimental details. Each value recorded below is the average ± S.E. of results from 7 to 9 experiments with different mice and is presented as mμmoles of added labeled glucose converted to lactate per mg of protein.

Labeled substrate	Normal lactating mammary gland	Outgrowths of: Normal mammary gland	Outgrowths of: HAN	Mammary adenocarcinoma
Glucose-1-^{14}C	32.5 ± 8.0	240.9 ± 21.5	194.9 ± 11.0	168.0 ± 16.0
Glucose-6-^{14}C	73.8 ± 21.4	310.8 ± 24.8	255.1 ± 14.4	182.6 ± 20.6

As in the presence of glucose, the conversion of acetate to fatty acids by slices from normal, lactating glands exceeded that by the outgrowths by 2-fold. In the absence of glucose, slices of the adenocarcinoma converted about 25% as much acetate to fatty acids as did the normal or HAN outgrowths.

When no glucose was added to the incubation medium, the conversion of ^{14}C of acetate-1-^{14}C to CO_2 by the normal lactating gland slices was about the same as that in the presence of glucose (Table 3). The oxidation of this acetate carbon by slices prepared from the normal outgrowth and the

HAN outgrowth were similar to one another but significantly higher than by the other 2 tissues. Upon the addition of glucose to the incubation medium, these comparative relationships were maintained, as the conversion of the carboxyl carbon of acetate to CO_2 decreased to a similar extent in all tissues studied.

Conversion of Acetate to Fatty Acids and CO_2 by Tissues from Pregnant Mice. The incorporation of the isotope of acetate-1-^{14}C in the presence of glucose into CO_2 and fatty acids by all tissues collected from mice in the last trimester of pregnancy was lower than that from the same tissue types from lactating mice (Table 5). In these pregnant mice, the slices of the normal mammary tissue and the HAN outgrowth had virtually identical patterns of incorporation, that into CO_2 being 3- to 4-fold that into fatty acids. The recovery of labeled CO_2 and fatty acids from the tumor was much lower than in the other 2 tissues, as was found in the studies with lactating mice. In this case, the incorporation into lipid was less than 10% that in the other 2 tissues. The HAN outgrowth and the adenocarcinoma did respond to the onset of lactation by increasing synthesis of fatty acids from acetate by 3- to 4-fold (Tables 3 and 5). The magnitude of this increase was similar to that observed in normal mammary gland. A similar response to the onset of lactation was observed in enzymatic studies previously reported from this laboratory (23, 24) with homogenates prepared from HAN outgrowths and adenocarcinomas excised from pregnant and lactating C3H mice.

Table 5

Incorporation of acetate-1-^{14}C into fatty acids and CO_2 by slices of normal, preneoplastic, and neoplastic mammary tissues from pregnant mice

One hundred mg of slices were incubated at 37° for 3 hr in 1 ml Krebs-Henseleit bicarbonate buffer (pH 7.3 to 7.4) containing 10 μmoles of labeled acetate and 10 μmoles of glucose. Gas phase was 95% O_2 and 5% CO_2. Each value is the average ± S.E. The number of experiments is given in parentheses. The units are the same as those in Table 3.

Tissue	CO_2	Fatty acids
Normal (5)	109 ± 8.1	33 ± 4.0
HAN outgrowth (3)	112 ± 10.3	27 ± 0.3
Adenocarcinoma (2)	32	2

The Type of Lipids Synthesized from Acetate. The predominant type of lipid synthesized from acetate-1-^{14}C *in vitro* by slices of HAN outgrowth from pregnant mice was identical with that by normal glandular tissue from the same mice (Table 6). Triglycerides constituted 40 to 50% of the labeled lipid; diglyceride and cholesterol together made up about 25%, and phospholipid constituted about 11%.

When the normal and preneoplastic tissues were taken from lactating mice, the synthesis of triglyceride from acetate was twice that observed in tissues from pregnant mice. Concurrently, incorporation of acetate carbon into the other lipid classes either decreased ore remained unchanged. Whether the normal tissue was suckled or not made no difference as to the type of lipid synthesized.

Length of the Carbon Chain of Fatty Acids Synthesized from Acetate. Slices of the glands of normal pregnant mice synthesized more fatty acids of medium chain length, C_{12} and C_{14}, than did slices of HAN outgrowths, but the differences were not outstanding (Table 7). During lactation, the fatty acids synthesized *in vitro* from acetate by normal glands were predominantly 10 to 12 carbons long, whereas those by outgrowths of HAN's were the same as those synthesized by the preneoplastic outgrowths in pregnant mice. In this case, the HAN outgrowth did not respond to lactation as did the normal tissue. While the nonsuckled mammary gland excised from lactating mice synthesized less C_{10} and more C_{16} than suckled tissue from the same animal, the pattern of fatty acids synthesized by the nonsuckled tissue was clearly different from that of its preneoplastic counterpart. The pattern of synthesis of fatty acids by the adenocarcinomas was similar to

Table 6

Incorporation of acetate-1-^{14}C into various classes of lipids by normal and preneoplastic mammary gland tissues from pregnant and lactating mice

The various tissues were incubated with labeled acetate and unlabeled glucose as described in Tables 3 and 5. The lipids were separated by means of thin-layer chromatography and identified, and then isotopic content was assayed as described in the text. The results are expressed as the percentage of radioactivity in each lipid class. Each value is the mean of the number of experiments listed in parentheses.

	Pregnant mice		Lactating mice		
Lipid class	Normal gland (13)	Outgrowth of HAN (3)	Suckled gland (4)	Nonsuckled gland (2)	Outgrowth of HAN (2)
Cholesterol ester	0.2	0.2	0.1	0.2	0.3
Triglyceride	46.8	40.2	82.3	86.2	79.9
Fatty acid	5.6	5.4	10.3	4.9	10.1
Diglyceride and cholesterol[a]	25.7	30.6	5.0	5.7	7.3
Monoglyceride	10.4	12.2	1.3	0.5	1.6
Phospholipid	10.9	11.4	1.0	2.3	0.8

[a] About 95% of the ^{14}C-activity of this fraction was in the form of diglyceride when separated by thin-layer chromatography in chloroform:benzene:ethyl acetate:acetic acid (150:50:25:4).

Table 7

Incorporation of acetate-1-^{14}C into individual fatty acids by normal, preneoplastic, and neoplastic mammary gland tissues from pregnant and lactating mice

The various tissues were incubated with labeled acetate and unlabeled glucose as described in Tables 3 and 5. The fatty acids were separated by means of gas-liquid chromatography and collected and assayed for their isotopic content as described in the text. The results are expressed as the percentage of the total ^{14}C recovered in each fatty acid fraction.[a] Each value is the mean of the number of experiments listed in parentheses.

	Pregnant mice			Lactating mice			
Chain length of fatty acid	Normal gland(6)	Outgrowth of HAN (4)	Tumor (1)	Suckled gland (4)	Nonsuckled gland (2)	Outgrowth of HAN (2)	Tumor (3)
8	0.7	2.5	0	2.0	1.0	1.1	0.1
10	3.7	1.4	0.3	25.7	13.8	6.9	2.6
12	11.2	6.0	1.4	29.0	20.8	9.0	6.5
14	18.5	8.6	13.6	19.1	19.3	14.8	13.5
16	45.8	52.6	57.2	16.3	29.0	54.5	54.3
18	13.6	14.3	15.0	4.6	8.8	9.2	16.5
>18	5.7	14.2	12.5	3.3	5.7	4.3	6.5

[a] In some cases, a small amount of ^{14}C activity was recovered prior to collection of caprylic acid so that the percentages may not add up to 100.

Table 8

Incorporation of acetate-1-^{14}C into the carboxyl carbon of fatty acids compared to that into the total fatty acid molecule by normal and preneoplastic mammary gland tissues from lactating mice

Tissues were incubated with labeled acetate and unlabeled glucose as described in Tables 3 and 5. The fatty acids were decarboxylated and the released $^{14}CO_2$ and the total fatty acid-^{14}C were assayed as described in the text. Each value is the mean of number of determinations listed in parentheses.

	Total ^{14}C in carboxyl carbon (%) (A)	Ratio of total ^{14}C to carboxyl-^{14}C (B)	Median length of ^{14}C-labeled fatty acids calculated from: Data in Table 7 (C)	Column B, if *de novo* (D)
Normal gland (3)	19.3	5.18	12.5	10.4
Nonsuckled gland (2)	25.4	3.94	14.5	7.9
Outgrowth of HAN (2)	23.4	4.26	15.7	8.5

that of the HAN outgrowths and, hence, was distinctly different from that of any normal glandular tissues. The physiological (hormonal) state of the mouse had little effect on the individual fatty acids synthesized by the adenocarcinoma.

Pathway of Incorporation of Acetate Carbon into Fatty Acids. The extent of acetate incorporation into fatty acids in the presence of glucose was less in the nonsuckled tissue, whether normal or preneoplastic outgrowth, than in suckled tissue (Table 3). Hence, the question arose: is the mode of synthesis the same in all 3 tissues? This question was answered by comparing the distribution of isotope in the fatty acids synthesized from acetate-1-^{14}C. If it was evenly distributed in alternate carbon atoms, the mode of synthesis was *de novo* (23). If a predominant portion of the isotope was in the carboxyl carbon of the fatty acids, elongation of an existing long-chain fatty acid accounted for the majority of the acetate converted to fatty acids (18, 19, 26, 31). Ratios of total ^{14}C to carboxyl-^{14}C in fatty acids formed *in vitro* from acetate in the presence of glucose by normal tissues, both suckled and nonsuckled, and by outgrowths of HAN are shown in Table 8. If all fatty acid synthesis were by the *de novo* pathway, twice these ratios would equal the median length of the synthesized fatty acids (Table 8, Column D). The median length of fatty acids containing ^{14}C, calculated from the values in Table 7, are listed in Table 8, Column C. The agreement between the actual and the theoretical values of the type of fatty acids synthesized by suckled, lactating gland makes it clear that almost all the acetate was incorporated via the *de novo* pathway. The discrepancy between these 2 values (Table 8, Columns C and D) in the nonsuckled normal tissue and the HAN outgrowth demonstrates that the mode of synthesis in these tissues must have been via both the *de novo* and the elongation pathways.

DISCUSSION

Glucose Stimulation of Acetate Incorporation into Fatty Acids. The effect of glucose on the conversion of acetate to fatty acids by normal mammary gland slices prepared from lactating and nonlactating rats has been studied by many workers (1, 4, 20). In studies with lactating glands, glucose increased incorporation of ^{14}C of acetate-1-^{14}C about 3-fold, whereas its effect in nonlactating glands was only about 3-fold (1). In the present studies, the presence of glucose caused a 13-fold stimulation of lipogenesis from acetate in the normal gland. The extent of the stimulatory effect was slightly less in the outgrowths, namely, 8-fold with the normal outgrowth and 7-fold with the HAN outgrowth. The adenocarcinoma did respond to the addition of glucose, but the extent was one-third that in the normal mammary gland.

Clearly, preneoplastic tissue and even neoplastic tissue can alter its lipogenic capacity in response to the glucose concentration. The fact that the extent of the stimulatory effect varied between the tissues might be related to the metabolic basis for this response. It has been suggested that glucose diverts acetyl units from oxidation toward fatty acid biosynthesis (4, 20). This possibility was examined in each tissue by calculating the total conversion of carbon to CO_2 and to fatty acids. The calculation was made by doubling the values for the incorporation of glucose-6-^{14}C in the presence of acetate and adding this figure to that obtained from labeled acetate in the presence of glucose. The correlation between the total conversion to CO_2 or to fatty acids and the extent of glucose stimulation was calculated for all tissues. In each case, the ability of glucose to divert acetyl units toward fatty acids was related (*a*) directly to the lipogenic capacity of the tissue as gauged by the total incorporation into fatty acids and (*b*) indirectly to the oxidative capacity as measured by the total incorporation of both substrates into CO_2.

While we cannot predict the precise basis for this effect, the availability of oxalacetate as a carrier of acetyl units out of the mitochondria in the form of citrate (6) could influence both oxidation and lipogenesis. Whatever the mechanism, it is operable in both preneoplastic and neoplastic tissues.

Pathways of Glucose Utilization. Of the various pathways of glucose catabolism studied, 2 have been most intensively investigated in mammalian tissues. The 1st is the Embden-Meyerhof pathway, whereby glucose is converted to triose phosphate via hexokinase, phosphohexose isomerase, phosphofructokinase, and aldolase, and the other is the PPC, in which the hexose is oxidized to pentose phosphate and CO_2 via hexokinase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, and the pentose phosphate is subsequently converted to hexose phosphate and triose phosphate. For the most part, only these 2 pathways of carbohydrate utilization have been demonstrated in mammary glands and tumors (1, 2).

The incorporation of tritium on C-6 of glucose into fatty acids relative to that of ^{14}C was the same in the preneoplastic tissue; the neoplastic tissue; and the normal, lactating gland. Such a finding provides evidence that not only is glucose catabolized to triose phosphate via the same pathways but similar mechanisms operate at each step in all 3 tissues. Since there was no evidence of preferential discrimination or exchange of tritium in any 1 tissue, the mechanisms at each step in glucose catabolism are probably the same in all 3 tissues.

That there is a difference between the tissues in the utilization of the triose phosphate is obvious from the amount of lactate that accumulated in all tissues except the normal suckled gland. It is well known that lactate production is high in many tumors, so it may not be surprising that preneoplastic tissue shows the same characteristic. The normal, nonsuckled tissue in the lactating mouse shows the same tendency toward lactate production. Hence, increased lactate production cannot be used as a criterion of neoplasia in mammary gland.

An estimate of the extent of operation of the major pathways of glucose utilization can be made from the data in Table 3. C-1 of glucose is converted to CO_2 in the PPC and in the Embden-Meyerhof pathway via the tricarboxylic acid cycle, whereas C-6 of glucose is converted to CO_2 solely in the tricarboxylic acid cycle. Hence, a comparison of $^{14}CO_2$ derived from C-1 in the various tissues indicates the relative extent of PPC activity, (22) since except for the adenocarcinoma the $^{14}CO_2$ yield from C-6 was similar in all tissues examined. Clearly, the 2 outgrowths appear to have very similar pathways of glucose utilization. The normal lactating gland appears to have more extensive PPC activity than these 2 tissues, and the tumor appears to have less PPC activity.

The proportion of glucose carbon appearing in fatty acids and lactate after traversing the PPC can also be estimated from the present data (1) if certain assumptions are made (21). The same pattern is revealed. Similar paths of conversion of glucose carbon to fatty acids and lactate operate to similar extents in both outgrowths; the normal suckled gland has the higher PPC contribution, and the adenocarcinoma has the lower.

Pathways of Fatty Acid Synthesis. Evaluation of the extent of *de novo* synthesis in normal gland, both suckled and nonsuckled, and in HAN outgrowths again revealed no differences between the preneoplastic tissue and its nonsuckled, normal counterpart. In the nonsuckled tissues, whether preneoplastic or normal, acetate was converted to fatty acid by the *de novo* and elongation pathways. The low contribution of *de novo* synthesis in these tissues is probably related to their low activity of acetyl-CoA carboxylase and fatty acid synthetase (J. C. Bartley, S. Smith, and S. Abraham, unpublished observations). Indeed, the low activity of acetyl-CoA carboxylase probably dictates chain elongation via condensation of acetyl-CoA (19) rather than malonyl-CoA (18, 26, 31), with existing long-chain, fatty acid acyl-CoA derivatives. *De novo* synthesis was by far the major pathway in suckled, normal tissue.

Length of Carbon Chain of Fatty Acids Synthesized from Acetate. Although the mode of synthesis was the same in nonsuckled, normal gland and in the outgrowth of the HAN's collected from lactating animals, the lengths of the carbon chains of fatty acids formed by slices of these tissues was clearly different. Furthermore, the onset of lactation resulted in little change in the pattern of fatty acids synthesized by the

HAN outgrowth, whereas there was a distinct difference between the fatty acids synthesized by normal, pregnant gland and those synthesized by lactating gland, whether suckled or not.

The hyperplastic tissue has lost the ability to make the unique pattern of fatty acids characteristic of normal glands, predominantly medium-chain fatty acids. We consider this deletion a deviation from the normal toward neoplasia, since the pattern of fatty acids formed by the HAN outgrowth is more like that of the adenocarcinoma than of normal tissue. In all other metabolic parameters examined, activity of individual enzymes (23, 24), glucose uptake, incorporation of glucose and acetate into various metabolic products, the extent of lipogenic stimulation by glucose, the pathways of glucose utilization, and the pathway of fatty acid synthesis, the normal, nonsuckled tissue and the outgrowth from the HAN's appeared to be similar.

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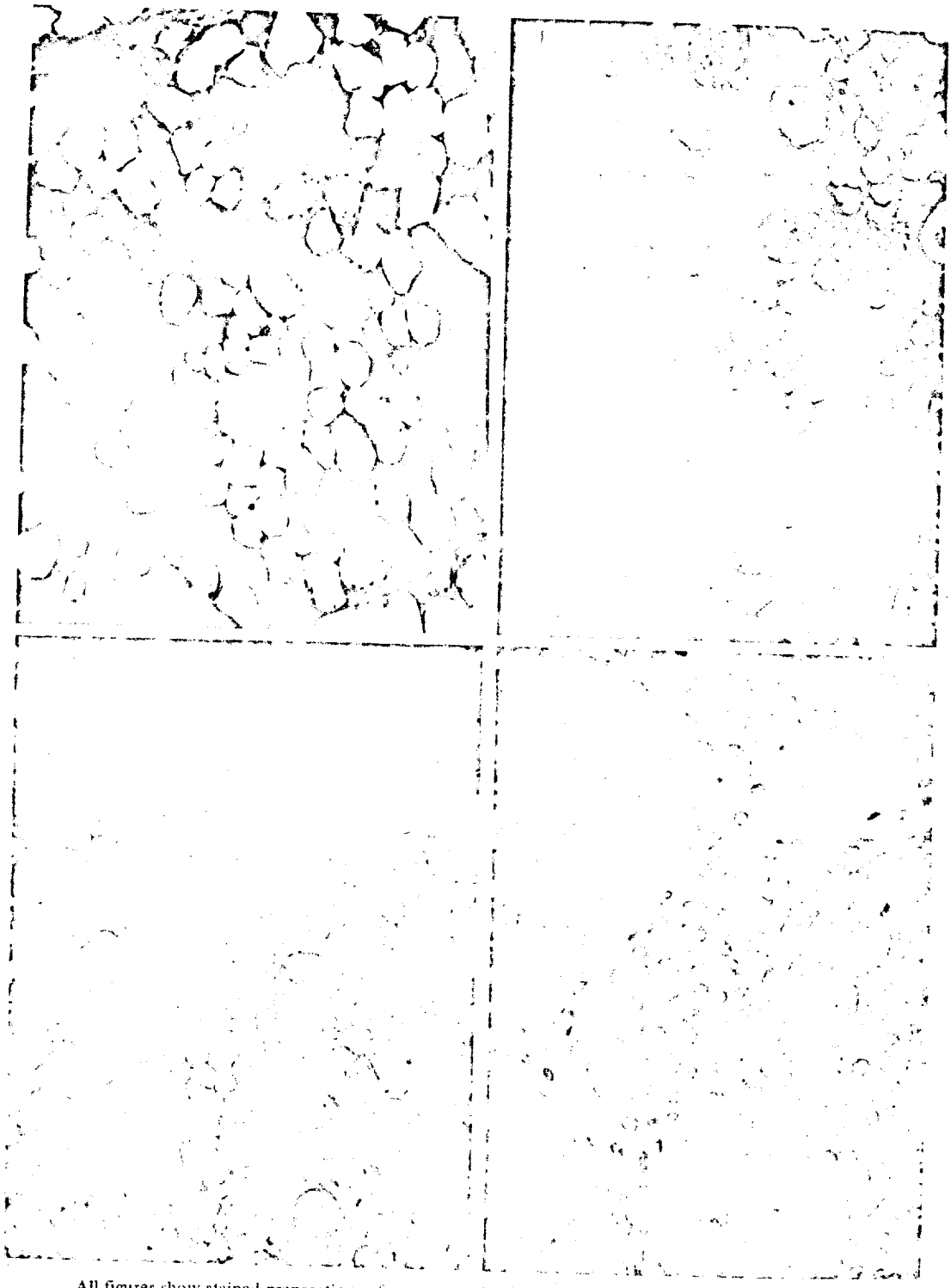

All figures show stained preparations of mammary gland tissues taken from female C3H/Crgl mice.
Fig. 1. Section of HAN outgrowth, subline 1-P, from a 12-week-old virgin mouse. H & E. × 200.
Fig. 2. Section of HAN outgrowth, subline 1-P, from a 9-day lactating mouse. H & E. × 200.
Fig. 3. Section of outgrowth of mammary gland from a 9-day lactating mouse. H & E. × 200.
Fig. 4. Section of a spontaneous mammary adenocarcinoma from a virgin mouse. H & E. × 500.

The Toxic Effect of Acids of the Formic Series in Relation to Adaptation of the Organism

by J. Belehradek & F. Schwarz

Introduction

The effect of organic acids on living protoplasm has been the subject of much research, and theories are not lacking to explain the effects of the acids by various physical-chemical principles. Specifically, it has been shown several times that protoplasm, according to its origin, shows differing sensitivity to organic and mineral acids. For plant cells on which the most research has been done (see the work of Lepeschkin [1]), research has shown that the protoplasm of bacteria and mold spores resists the effect of acids much more than the protoplasm of higher plants. In animals, several species are known to be particularly resistant to acid surroundings, but quantitative detailed research is lacking.

We proposed to examine the resistance of Anguillula of vinegar to various organic acids, which adapted to acid milieux lends itself particularly well to such a study. As subjects for comparison, we chose Tubifex (rivulorum?), Daphnia and tadpoles of Rana fusca.

Experiments and Results

The anguillula aceti, cultivated in a decoction of apples was washed several times in distilled water with centrifugation. The Tubifex and the Daphnia gotten from outside were maintained in ordinary water. The tadpoles came from eggs laid and hatched in the laboratory. The animals were placed in a small crystallizer filled with acid solution and covered with a glass plate to prevent evaporation. The animals were

observed closely so that the time necessary for the complete disappearance of all movement was determined. The acids used were all products of Kahlbaum.

The numerical results are given in Tables IV-XV at the end of this article. Represented graphically, these results give even curves. Their mathematical analysis shows that they are general parabolas with the formula:

$$T = \frac{A}{C^b} \qquad (1)$$

where C is the concentration of the acid, T the time of maximum survival at this concentration, A and b are constants. In the logarithmic form, the formula becomes:

$$\log T = \log A - b \log C \qquad (2)$$

in other words, if the concentrations and the corresponding survival times are put into logarithmic scale, a straight line is formed.

Actually, the constant A represents the length of survival at a concentration of 1 p. 100 of a specific acid. It can therefore serve to indicate the absolute resistance of the organism to the solution having a concentration of 1 p. 100 of that acid. Here are the values for the animals and acids studied:

Table I

(Time of maximum survival in seconds in acid solution of concentration 1 p. 100 = A of the formula)

2 Acides	1 Temps de survie pour 3 Têtards	*Daphnia*	*Tubifex*	*Anguillula*
4 Formique	—	400	270,0	16×10^3
5 Acétique	100	480	59,8	262×10^6
6 Propionique	—	—	23,6	145×10^5
7 Butyrique	—	—	22,4	12×10^4
8 Valérique	—	—	636,0	—

1. Survival times for
2. Acids
3. Tadpoles
4. Formic
5. Acetic
6. Propionic
7. Butyric
8. Valeric

The constant b indicates the way the survival time diminishes when the acid concentration increases. By knowing A for an animal and for a given acid, we can calculate by using b, the survival of this animal at any concentration. If we try to rank the acids, according to their toxicities, it can be seen that the order varies when the concentration of the acid in the solutions is changed. Thus, for example, (Figure II), Tubifex is more sensitive to formic acid than to acetic at concentrations of less than 1 p. 100, while for higher concentrations, the relationship is reversed. When the effect of a toxic substance on the protoplasm is studied, it is not sufficient to determine survival time at only one concentration; it is necessary to determine the survival times at several different concentrations.

Here is a table showing b values:

Table II

b values for acids of the Formic Acid Series

1 Acides	2 Têtards	*Daphnie*	*Tubifex*	*Anguillula*
3 Formique	—	0,80	0,52	1,21
4 Acétique	1,27	1,14	1,18	3,52
5 Propionique	—	—	1,11	3,22
6 Butyrique	—	—	1,10	2,02
7 Valérique	—	—	1,24	—

1. Acids
2. Tadpoles
3. Formic
4. Acetic
5. Propionic
6. Butyric
7. Valeric

In the graphic representation in logarithmic scale, b is identical with the tangent of the angle made by the line and the x-axis. Its value increases as the slant of the line increases. The graphs translating our numerical results show that Anguillula has a resistance to acids of the formic acid series generally, but that it is especially resistant to acetic and propionic acids. Further research will show whether this animal has a special resistance to toxic substances in general.

Discussion and Results

It is well known that acids which are slightly dissociated are more toxic for protoplasm than acids having strong ionic dissociation. Thus, Jacques Joeb (2) has shown that butyric acid damages the egg of the sea-urchin 100 times more quickly than hydrochloric acid. Recently, Traube and Somogyi (3) have found that caprylic acid while slightly dissociated kills staphylococcus and coli bacilli more energetically than either hydrochloric or sulfuric acids. It is not only the hydrogen ions which determine the method of attacking the acids in general; we must look for other intervening factors.

Wo. Ostwald (5) did research on the toxicity of some solutions for

the crustacean, Gammarus and found that the survival time T depended on the concentration C according to the formula:

$$TC^m = K \qquad (3)$$

where m and K are constants. The same formula expresses as well the relationship between the degree of adsorption and the concentration of the substance. Wo. Ostwald's theory leads one to think that toxic substances exert their effect in proportion to the adsorption of the protoplasmic surfaces.

At what point do our results confirm this hypothesis?

If Ostwald's formula is written another way:

$$T = \frac{K}{C^m} \qquad (4)$$

it can be seen that it is identical to our equation (1), except that the constants are designated by other letters.

Notwithstanding the identity of the mathematic formulas, it could be useful to explain the biological significance of formulas discovered empirically. It seems that similar mathematical equations generally have value in biology. It should be recalled that Porodko (6) derived similar formulas for the survival of protoplasm at high temperatures and for the relationship between the intensity of the stimulant and that of the chemical-tropic response of vegetable roots. Also, one of us (7) established that the relationship between temperature and the speed of most biological reactions can be expressed by a similar equation. However, we are not entitled to assume similarity in the nature of two processes only because of the similarity of their mathematical expressions. In our particular case, adsorption alone does not suffice to explain the effect of acids of the formic series, because there is a disagreement

between the absolute value of the exponent b of the two phenomenon. In the formula for adsorption, the exponent m must always be less than 1. As is seen in Table II, the value of b is less than 1 only for formic acid and only for the Tubifex and the Daphnia.

For the Anguillula the value for this same acid is greater than 1, and all the other acids for all the animals studied are characterized by a b which is always greater than 1. For this reason, it can only be stated that the toxic effect of acids of the formic series is determined by adsorption.

Czapek (4) put forth a hypothesis analogous to Ostwald's, and postulated that the toxicity of fatty acids, completely dependant on the degree of adsorption, is determined by their capillary action. In Table III, the A and b constants of our experiments are juxtaposed to values indicating the surface tension of fatty acids, measured against their own vapors (8).

Table III

1 Acides	2 Tension superficielle en dynes	Tubifex A	Tubifex b	Anguillule A	Anguillule b
3 Formique.....	37.47	270	0,52	16×10^3	1,21
4 Acétique.....	23,46	59.8	1,18	262×10^6	3,52
5 Propionique.....	26.57	23,6	1,11	145×10^5	3,22
6 Butyrique.....	26.74	22,4	1,10	12×10^4	2,02

1. Acids
2. Surface Tension in dynes
3. Formic
4. Acetic
5. Propionic
6. Butyric

It can be seen that the resistance of the animals is as much the weaker as the surface tension of the acids is higher, which evidently contradicts Czapek's hypothesis. Further, Traube and Somogyi, in work already cited (3), state that the surface tension is not the only factor

which determines the toxicity of acids.

Table IV

1 *Anguillula aceti.* — Acide formique.2

3 Concentration en p. 100 de poids	4 Temps de survie en minutes et secondes
46,6	2 min.
30.6	3 min 10 sec.
15.8	7 min. 15 sec
10.6	12 min. 30 sec.

Table V

1 *Anguillula aceti.* — Acide acétique.5

6 Concentration p. 100	Survie 7
69,4	1 min. 2 sec.
66,4	1 min. 35 sec.
55,7	3 min. 45 sec.
42,1	7 min. 15 sec.
33.8	16 min.
28.3	30 min.

Table VI

1 *Anguillula aceti.* — Acide propionique.8

6 Concentration p. 100	Survie 7
68,6	0 min. 15 sec.
65,9	1 min. 30 sec.
56,1	2 min. 22 sec.
42	5 min. 30 sec.
32.8	11 min.
27.3	14 min. 30 sec.

Table VII

1 *Anguillula aceti.* — Acide butyrique.9

6 Concentration p. 100	Survie 7
33.5	1 min. 30 sec.
19,2	5 min. 13 sec.
13.8	8 min.
10,4	14 min. 30 sec.

1. Anguillula aceti
2. Formic acid
3. Concentration in p. 100 weight
4. Survival time in minutes and seconds
5. Acetic acid
6. Concentration p. 100
7. Survival
8. Propionic acid
9. Butyric acid

Table VIII

10 *Tubifex*.— Acide formique. 2

6 Concentration p. 100	Survie 7
2.29	2 min. 45 sec.
1.14	4 min. 12 sec.
0,572	5 min. 25 sec.
0,286	8 min. 55 sec.
0,143	10 min.
0,143	10 min. 25 sec.
0,0715	18 min. 30 sec.
0,0715	19 min. 30 sec.
0,0358	23 min.
0,0358	27 min.
0,0179	34 min.

Table IX

10 *Tubifex*.— Acide acétique. 5

6 Concentration p. 100	Survie 7
9.10	0 min. 10 sec.
4,55	0 min. 10 sec.
2,27	0 min. 15 sec.
1,14	1 min. 20 sec.
0,569	2 min.
0,284	4 min.
0.142	9 min. 30 sec.
0.0711	20 min.
0,0711	26 min. 26 sec.

Table X

10 *Tubifex*. — Acide propionique. 8

6 Concentration p. 100	Survie 7
8,67	0 min. 8 sec.
4,33	0 min. 12 sec.
2.17	0 min. 10 sec.
1.08	0 min. 24 sec.
0,542	0 min. 32 sec.
0.271	1 min. 45 sec.
0,135	4 min.
0,0677	13 min. 30 sec.
0.0338	17 min.

2. Formic acid
5. Acetic acid
6. Concentration p. 100
7. Survival
8. Propionic acid
10. Tubifex

Table XI

10 *Tubifex.* — Acide butyrique. 9

6 Concentration p. 100	Survie 7
1,83	0 min. 10 sec.
0.965	0 min. 17 sec.
0,965	0 min. 20 sec.
0,482	0 min. 50 sec.
0.241	2 min. 4 sec.
0.121	3 min. 48 sec.
0,0603	8 min.
0,0301	18 min.
0,0151	41 min.

Table XII

10 *Tubifex.* — Acide valérique. 11

6 Concentration p. 100	Survie 7
1,87	6 min. 15 sec.
0,933	7 min. 30 sec.
0,933	12 min. 50 sec.
0,467	19 min.
0,467	26 min. 15 sec.
0,233	73 min. 30 sec.

Table XIII

12 Larves de *Rana fusca.* — Acide acétique. 5

6 Concentration p. 100	Survie 7
9.10	0 min. 6 sec.
4.55	0 min. 14 sec.
2.27	0 min. 38 sec.
1,13	2 min. 24 sec.
0,568	3 min. 20 sec.
0,284	7 min.
0,142	11 min.

Table XIV

13 *Daphnia.* — Acide formique. 2

6 Concentration p. 100	Survie 7
1,14	6 min 40 sec.
0,57	11 min. 40 sec.
0.29	20 min.
0.14	35 min.

2. Formic acid
6. Concentration p. 100
8. Propionic acid
10. Tubifex
12. Rana fusca larvae
5. Acetic acid
7. Survival
9. Butyric acid
11. Valeric acid
13. Daphnia

Table XV

13 *Daphnia*.— Acide acétique 5

6 Concentration p. 100	7 Survie
1,05	7 min.
0,52	19 min.
0,26	38 min.

5. Acetic acid
6. Concentration p. 100
7. Survival
13. Daphnia

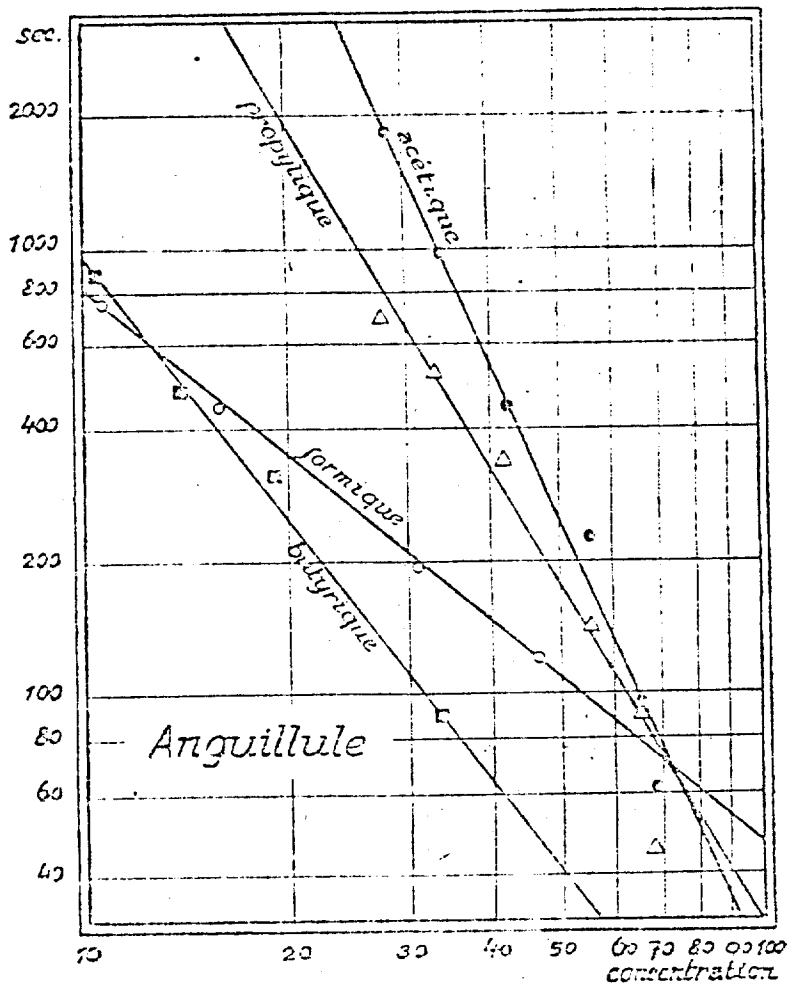

Fig. I -- Survival time of Anguillula aceti in acid solutions of the formic series at varying concentrations. Abscissas: Concentrations, expressed as logarithmic values. Ordinates: survival time in seconds, logarithmic scale.

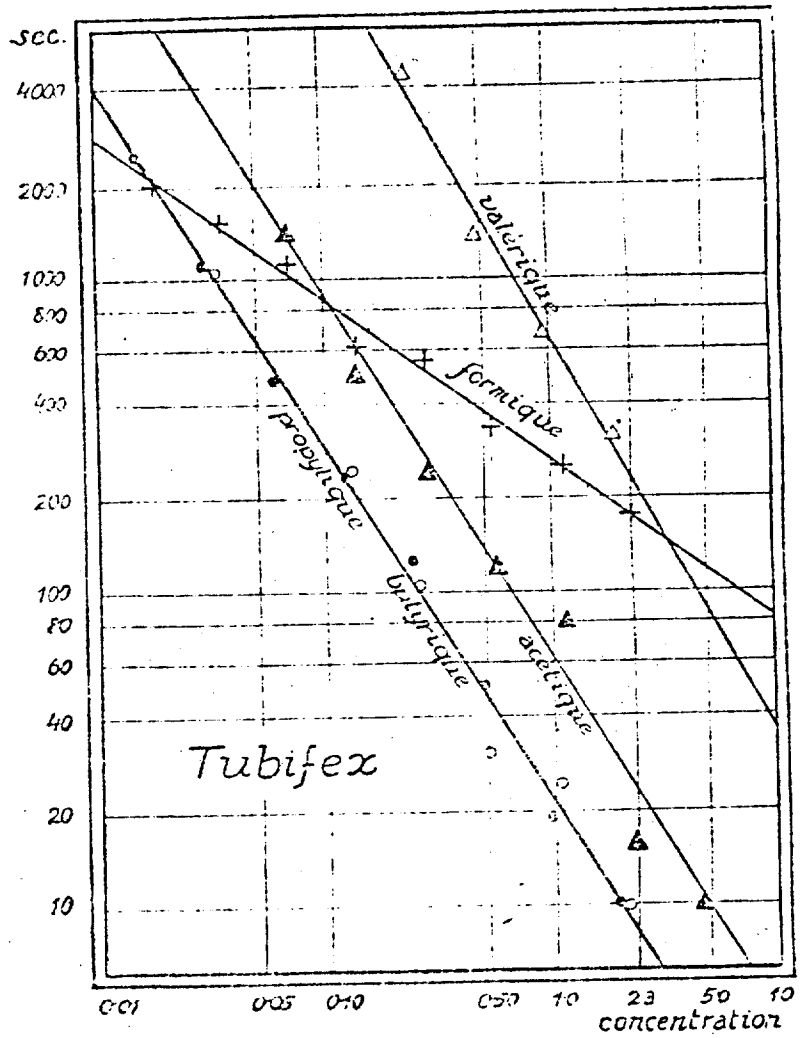

Fig. II -- Survival time of Tubifex (rivulorum?) in acid solutions of the formic series at varying concentrations. Abscissas and ordinates as in Figure I.

Conclusions

1. The survival time T for tadpoles, Tubifex, Daphnia and Anguillula of vinegar, placed in aqueous solutions of formic, acetic, propionic, butyric, valeric acids at different concentrations (C), is expressed by the equation:

$$T = \frac{A}{C^b}$$

in which A and b are constants.

2. In spite of the resemblance of the formula to the adsorption equation, the toxic effect of the acids studied is not determined by adsorption alone, the exponent b of the formula being in the majority of cases greater than 1.

3. There exists no relationship between the toxicity of the acids studied and their surface tensions.

4. The order of the acids, ranked according to their toxicity is not the same for the different concentrations of the acid solutions used in the experiments.

5. Anguillula of vinegar shows a greater resistance to acids of the formic series than the other animals studied. It was also particularly resistant to acetic and propionic acids.

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L'action toxique des acides de la série formique en relation avec l'adaptation de l'organisme,

par MM. J. BĚLEHRÁDEK et F. SCHWARZ.

(*Mémoire présenté à la séance du 25 mai 1928*).

Introduction.

L'action des acides organiques sur le protoplasma vivant a fait l'objet de nombreuses recherches et il ne manque pas de théories pour expliquer l'effet des acides par divers principes physico-chimiques. En particulier on a plusieurs fois constaté que le protoplasma, suivant son origine, manifeste une sensibilité différente à l'égard des acides organiques et minéraux. Pour les cellules des végétaux, sur lesquelles ont été faits la plupart des travaux (consulter l'ouvrage de LEPESCHKIN, [1]), ces recherches ont démontré, entre autres, que le protoplasma des bactéries et des spores de moisissures résiste beaucoup plus à l'action des acides que le protoplasma des végétaux supérieurs. Pour les animaux, on connait quelques espèces particulièrement résistantes aux milieux acides, mais les recherches quantitatives détaillées font défaut.

Nous nous sommes proposé d'examiner la résistance à divers acides organiques de l'Anguillule du vinaigre, qui, adaptée aux milieux acides, se prêtait particulièrement à pareille étude. Comme sujets de comparaison, nous avons choisi le *Tubifex* (*rivulorum ?*), la *Daphnie* et des Têtards de *Rana fusca*.

Expériences et résultats.

L'*Anguillula aceti*, cultivée au laboratoire dans une décoction de pommes, fut lavée plusieurs fois à l'eau distillée avec centrifugation. Les *Tubifex* et les *Daphnies*, apportés du dehors, étaient maintenus dans l'eau ordinaire. Les *Têtards* provenaient d'œufs pondus et éclos au laboratoire. Les animaux ont été mis dans de petits cristallisoirs remplis de solution acide et couverts d'une plaque de verre pour éviter l'évaporation. Les animaux étant observés sur

fond blanc, on déterminait le temps nécessaire à la disparition complète de leurs mouvements. Les acides employés étaient tous des produits Kahlbaum.

Les résultats numériques sont consignés dans les tableaux IV-XV à la fin de ce mémoire. Représentés graphiquement, ces résultats donnent des courbes régulières. Leur analyse mathématique montre qu'elles sont des paraboles générales de la formule :

$$T = \frac{A}{C^b} \qquad (1)$$

dans laquelle C est la concentration de l'acide, T le temps de survie maximum à cette concentration, A et b des constantes. Dans la forme logarithmique, la formule devient :

$$\log. T = \log. A - b \log. C \qquad (2)$$

c'est-à-dire, que, si les concentrations et les temps de survie correspondants sont portés en échelles logarithmiques, on obtient une ligne droite.

Pratiquement, la constante A représente la durée de survie à la concentration de 1 p. 100 d'un acide déterminé. Elle peut donc servir à indiquer la résistance absolue de l'organisme vis-à-vis de la solution à 1 p. 100 de cet acide. Voici ses valeurs pour les animaux et les acides examinés :

TABLEAU I.

(Temps de survie maximum en secondes, dans des solutions acides à 1 p. 100 = constantes A de la formule).

Acides	Temps de survie pour Têtards	*Daphnia*	*Tubifex*	*Anguillula*
Formique	—	100	270,0	16×10^3
Acétique	100	490	59,8	262×10^6
Propionique	—	—	23,6	115×10^5
Butyrique	—	—	22,4	12×10^4
Valérique	—	—	635,0	—

La constante b indique la façon dont le temps de survie diminue, lorsque la concentration en acide s'élève. En connaissant A pour un animal et pour un acide donnés, on peut donc calculer, au moyen de b, la survie de cet animal à n'importe quelle concentration de cet acide. Si l'on essaie de ranger les acides suivant leur toxicité, on s'aperçoit que leur ordre varie lorsque change la con-

centration en acide des solutions. Ainsi, par exemple (figure II), le Tubifex est plus sensible à l'acide formique qu'à l'acide acétique à des concentrations inférieures à 1 p. 100, tandis que, pour des concentrations plus élevées, le rapport est renversé. Quand on étudie l'action d'une substance toxique sur le protoplasma, il ne suffit donc pas de déterminer le temps de survie à une seule concentration ; il est nécessaire de déterminer les temps de survie à plusieurs concentrations différentes.

Voici le tableau des valeurs de b :

TABLEAU II.

Valeurs de b pour les acides de la série formique.

Acides	Têtards	*Daphnia*	*Tubifex*	*Anguillula*
Formique	—	0,80	0,52	1,21
Acétique	1,27	1,14	1,18	3,52
Propionique	—	—	1,11	3,22
Butyrique	—	—	1,10	2,02
Valérique	—	—	1,24	—

Sur la représentation graphique en échelles logarithmiques, b est identique avec la tangente de l'angle compris entre la ligne et l'axe x. Sa valeur est d'autant plus grande que la ligne est plus escarpée. Les graphiques traduisant nos résultats numériques font voir que l'Anguillule a une résistance élevée à l'égard des acides de la série formique en général, mais qu'elle résiste surtout à l'acide acétique et à l'acide propionique. Des recherches ultérieures montreront si cet animal est doué d'une résistance particulière vis-à-vis des substances toxiques en général.

Discussion des résultats

C'est chose bien connue que les acides faiblement dissociés sont plus toxiques pour le protoplasma que les acides à forte dissociation ionique. Ainsi Jacques Loeb [2] avait constaté que l'acide butyrique endommage l'œuf d'Oursin cent fois plus vite que l'acide chlorhydrique. Récemment, Traube et Somogyi [3] ont trouvé que l'acide caprylique, quoique très faiblement dissocié, tue les staphylocoques et les bacilles coli plus énergiquement que ne le font les acides chlorhydrique et sulfurique. Ce ne sont donc pas unique-

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[illegible] l'attaque des [illegible] il faut chercher d'autres facteurs intervenant.

[illegible] [5] a recherché la toxicité de quelques solutions pour [illegible] et a trouvé que les temps de survie T dépendent de la concentration C suivant la formule :

$$T\,C^m = K \qquad (3)$$

où m et K sont des constantes. Or, la même formule exprime le rapport entre le degré d'adsorption et la concentration de la substance. La théorie de Wo. Ostwald conduirait donc à penser que les substances toxiques exercent leur action à mesure de l'adsorption des surfaces protoplasmiques.

A quel point nos résultats confirment-ils cette hypothèse ?

Si l'on écrit la formule de Wo. Ostwald [3] d'une autre façon :

$$T = \frac{K}{C^m} \qquad (4)$$

on voit qu'elle est identique à notre équation (1), seulement les constantes sont désignées par d'autres lettres.

Malgré cette identité de la forme mathématique, il faut être très prudent pour expliquer la signification biologique des formules trouvées empiriquement. Il semble que des équations du même type mathématique se fassent assez généralement valoir en biologie. Rappelons pourtant que Borodko [6] a trouvé des formules du même type pour la survie du protoplasma à hautes températures et pour le rapport entre l'intensité de l'excitant et celle de la réponse chimio-tropique des racines végétales. De même, l'un de nous [7] a établi que le rapport entre la température et la vitesse de la plupart des réactions biologiques peut s'exprimer par une équation comparable. On n'est donc pas autorisé à décider de la similitude de nature de deux processus par la seule similitude de leurs expressions mathématiques. Dans notre cas particulier, l'adsorption seule ne suffit pas à expliquer l'action des acides de la série formique, parce qu'il y a un désaccord entre la grandeur absolue de l'exposant b des deux phénomènes. Dans la formule d'adsorption, l'exposant m doit toujours être inférieur à 1. Comme on le voit dans notre Tableau II, la valeur de b est inférieure à 1 seulement pour l'acide formique et seulement pour le Tubifex et pour la Daphnie. Pour l'Anguillule, la valeur de ce même acide est supé-

rieure à 1, et tous les autres acides pour tous les animaux examinés sont caractérisés par un b qui est toujours plus grand que 1. Pour cette raison, on ne peut admettre que l'action toxique des acides de la série formique soit déterminée uniquement par une adsorption.

Czapek [4] a émis une hypothèse analogue à celle de Wo. Ostwald et a imaginé que la toxicité des acides gras, tout en dépendant du degré d'adsorption, est déterminée par leur activité capillaire. Dans le Tableau III, les constantes A et b de nos expériences se trouvent juxtaposées aux valeurs indiquant la tension superficielle des acides gras, mesurée contre leurs propres vapeurs [8].

TABLEAU III.

Acides	Tension superficielle en dynes	*Tubifex* A	*Tubifex* b	*Anguillule* A	*Anguillule* b
Formique.....	37,47	270	0,52	16×10^3	1,21
Acétique.....	23,46	59,8	1,18	262×10^6	3,52
Propionique....	26,57	23,6	1,11	145×10^5	3,22
Butyrique.....	26,74	22,4	1,10	12×10^4	2,02

On voit que la résistance des animaux est d'autant plus faible que la tension superficielle des acides est plus élevée, ce qui contredit évidemment l'hypothèse de Czapek. D'ailleurs, Traube et Somogyi, dans le travail cité déjà [3], admettent que la tension superficielle n'est pas le seul facteur qui détermine la toxicité des acides.

TABLEAU IV.

Anguillula aceti. — Acide formique.

Concentration en p. 100 de poids	Temps de survie en minutes et secondes
46,6	2 min.
30,6	3 min. 10 sec.
15,8	7 min. 15 sec.
10,6	12 min. 30 sec.

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TABLEAU V.

Anguillula aceti. — Acide acétique.

Concentration p. 100	Survie
69,1	1 min. 2 sec.
66,4	1 min. 35 sec.
55,7	3 min. 45 sec.
42,1	7 min. 15 sec.
33,8	16 min.
28,3	30 min.

TABLEAU VI.

Anguillula aceti. — Acide propionique.

Concentration p. 100	Survie
68,6	0 min. 45 sec.
65,9	1 min. 30 sec.
56,1	2 min. 22 sec.
42	5 min. 30 sec.
32,8	11 min.
27,3	14 min. 30 sec.

TABLEAU VII.

Anguillula aceti. — Acide butyrique.

Concentration p. 100	Survie
33,5	1 min. 30 sec.
19,2	5 min. 12 sec.
13,8	8 min.
10,4	14 min. 56 sec.

TABLEAU VIII.

Tulipe. — Acide formique.

Concentration p. 100	Survie
2,29	2 min. 45 sec.
1,44	4 min. 12 sec.
0,572	5 min. 25 sec.
0,286	8 min. 55 sec.
0,143	10 min.
0,143	10 min. 25 sec.
0,0715	18 min. 30 sec.
0,0715	19 min. 30 sec.
0,0358	23 min.
0,0358	27 min.

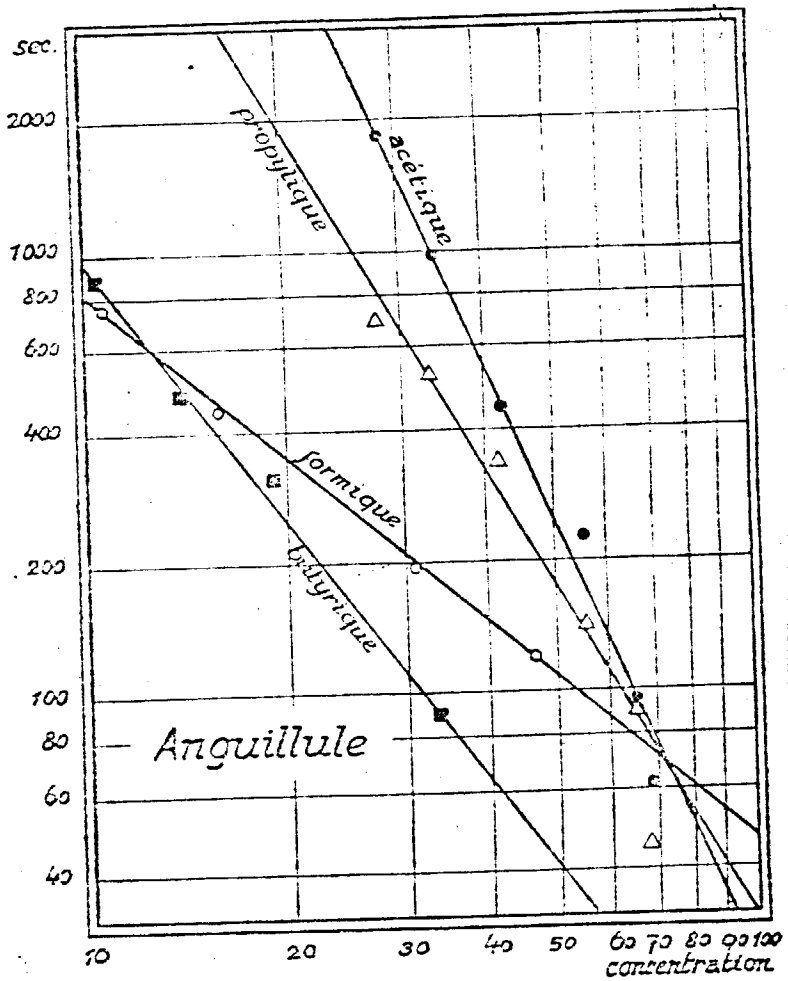

Fig. I. — Temps de survie de l'*Anguillula aceti* dans des solutions d'acides de la série formique à des concentrations différentes. En abscisses : concentrations, en valeurs logarithmiques. En ordonnées : temps de survie en secondes, échelle logarithmique.

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TABLEAU IX

Tubifex. — Acide acétique.

Concentration p. 100	Survie
9.10	0 min. 10 sec.
4.55	0 min. 10 sec.
2.27	0 min. 15 sec.
1.14	1 min. 20 sec.
0.569	2 min.
0.284	4 min.
0.142	9 min. 30 sec.
0.0711	20 min.
0.0711	26 min. 26 sec.

TABLEAU X.

Tubifex. — Acide propionique.

Concentration p. 100	Survie
8.67	0 min. 8 sec.
4.33	0 min. 12 sec.
2.17	0 min. 19 sec.
1.08	0 min. 24 sec.
0.542	0 min. 32 sec.
0.271	1 min. 45 sec.
0.135	4 min.
0.0677	13 min. 50 sec.
0.0338	17 min.

TABLEAU XI.

Tubifex. — Acide butyrique.

Concentration p. 100	Survie
1.93	0 min. 10 sec.
0.965	0 min. 17 sec.
0.965	0 min. 20 sec.
0.482	0 min. 50 sec.
0.241	2 min. 4 sec.
0.121	3 min. 48 sec.
0.0603	8 min.
0.0301	18 min.

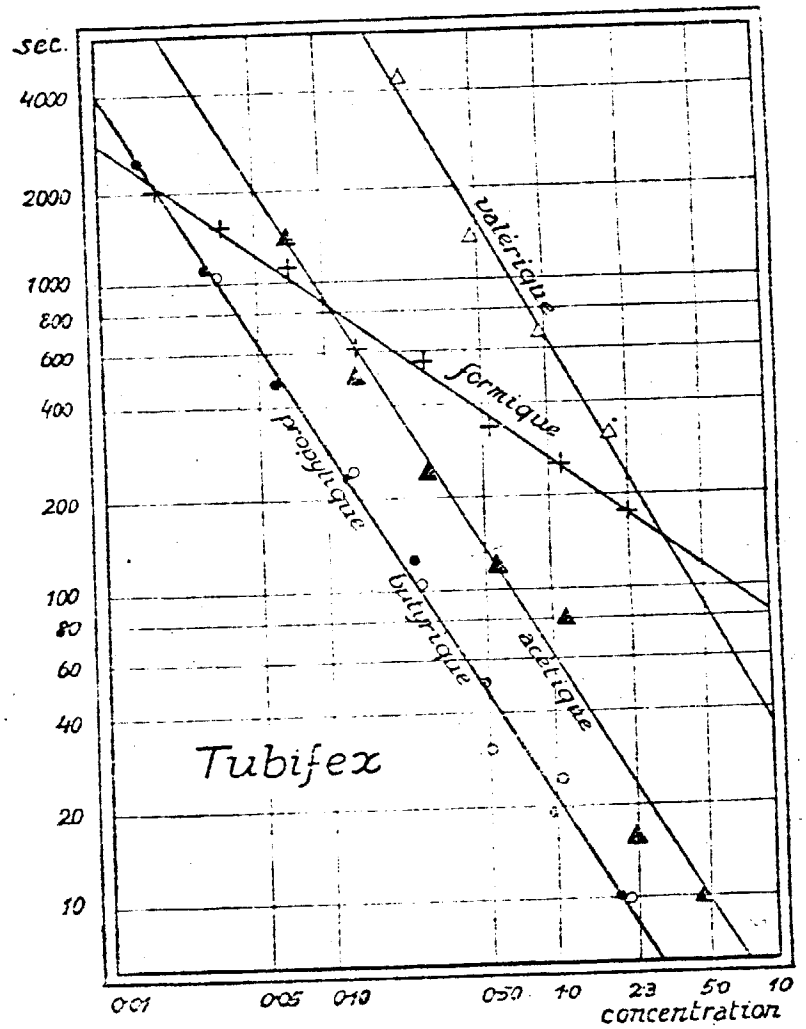

FIG. II.— Temps de survie du *Tubifex rivulorum* ?) dans des solutions d'acides de la série formique à des concentrations variées. Abscisses et ordonnées comme dans la Fig. I.

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TABLEAU XII.

Tubifex. — Acide valérique.

Concentration p. 100	Survie
1.87	6 min. 15 sec.
0.933	7 min. 30 sec.
0.933	12 min. 50 sec.
0.467	19 min.
0.467	26 min. 15 sec.
0.233	73 min. 30 sec.

TABLEAU XIII.

Larves de *Rana fusca*. — Acide acétique.

Concentration p. 100	Survie
9.19	0 min. 6 sec.
4.55	0 min. 14 sec.
2.27	0 min. 58 sec.
1.13	2 min. 24 sec.
0.568	3 min. 20 sec.
0.284	7 min.
0.142	11 min.

TABLEAU XIV

Daphnia. — Acide formique.

Concentration p. 100	Survie
1.14	6 min. 40 sec.
0.57	11 min. 40 sec.
0.29	20 min.
0.14	35 min.

TABLEAU XV.

Daphnia. — Acide acétique.

Concentration p. 100	Survie
1.05	7 min.
0.52	19 min.
0.26	38 min.

Conclusions.

1° Le temps de survie T des Têtards, des Tubifex, des Daphnies et des Anguillules de vinaigre, placés dans des solutions aqueuses d'acides formique, acétique, propionique, butyrique, valérique, à des concentrations (C) différentes, est exprimé par l'équation :

$$T = \frac{A}{C^b}$$

dans laquelle A et b sont des constantes.

2° Malgré la ressemblance de cette formule avec l'équation de l'adsorption, l'action toxique des acides examinés n'est pas déterminée par l'adsorption seule, l'exposant b de la formule étant, dans la majorité des cas, plus grand que 1.

3° Il n'y a pas de rapport entre la toxicité des acides examinés et leur tension superficielle.

4° L'ordre des acides, rangés suivant leur toxicité, n'est pas le même pour les concentrations différentes des solutions acides expérimentées.

5° L'Anguillule de vinaigre présente une résistance plus grande, à l'égard des acides de la série formique, que les autres animaux examinés. Elle résiste particulièrement bien à l'acide acétique et à l'acide propionique.

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EXPERIMENTAL INHIBITION OF TUMOUR INDUCTION BY MUSTARD GAS AND OTHER COMPOUNDS.

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Previous investigations.

[illegible] communication (Berenblum 1929) it was shown that the [illegible] of warts in mice by the repeated application of tar could be almost [illegible] prevented by the addition to the tar of 0·1 per cent. mustard [illegible] The same effect was obtained when the tar and a 0·1 per cent. solution [illegible] in acetone were applied separately to the same area of skin [illegible] intervals of 3 and 4 days. It was therefore suggested that the [illegible] due to some direct effect of the mustard gas on the animal, [illegible] the latter from responding to the carcinogenic tar, and not to [illegible] of the tar itself. This explanation has since been amply [illegible] by the production of a similar inhibition with mustard gas when [illegible] anthracene was used as the carcinogenic agent, and by the [illegible] that no chemical interaction takes place between the two [illegible] under normal conditions.

[illegible] it was shown (Berenblum 1931) that the "anticarcinogenic" [illegible] mustard gas is local, that it is limited to the period of time during [illegible] the mustard gas is acting, becoming manifest very soon after the [illegible] applied and subsiding almost as soon as the mustard gas to it [illegible] continued, and that once a wart has appeared, its continued [illegible] is not significantly affected by subsequent applications of [illegible] mustard gas. [illegible] higher concentrations, the ulcerative [illegible] mustard gas comes into play, and then a fully developed wart may [illegible] be caused to disappear.

New experiments.

[illegible] present investigations are concerned with a study of the [illegible] tumour induction of a selected group of compounds [illegible] related to mustard gas. The fact that many of them [illegible] known to be skin irritants necessitated a further enquiry [illegible] possible anti-carcinogenic activity of other substances [illegible] which were not chemically related to mustard [illegible] which were known to possess some irritative action on the

* An unpublished experiment [illegible] the author.

At first, each compound was added to the tar and the [illegible] applied once a week to the skin of 60 mice. These early experiments proved to be worthless since it was soon found that some of the compounds were liable to be decomposed by the tar. The results described below have all been obtained from subsequent experiments in which this complication was overcome by applying the various compounds separately from the tar on different days of the week. The compounds were dissolved in dry acetone and the dilutions were made up freshly each time before application, thereby avoiding any risk of decomposition.

Each compound in any one dilution was tested on 50 or 60 mice. Six to 16 such tests were carried out at a time and these constituted a series. Each series included one tar control group of 50 or 60 mice in which acetone was applied in place of the solution of the compound to be tested. The tar was applied to a small area of skin in the region of the shoulder blades with a camel hair brush; the acetone solutions were applied by touching the skin with the end of a thick-walled capillary tube containing a small quantity of the appropriate solution. A careful note was taken of the survival rate in each group and of the time of appearance of warts. The experiments were continued for 16 or 20 weeks except in those particular groups in which 50 per cent. of survivors had already developed warts before that time.

In four separate control groups this 50 per cent. level was reached after 16, 14½, 16 and 14½ weeks respectively. In some of the experimental groups the 50 per cent. level was never reached, only 3 or 4 out of the 50 mice developing any warts at all. This constitutes a very marked degree of inhibition (+++++ in tables I and II). A delay of 4-6 weeks in reaching the 50 per cent. level (++ or +++) can be accepted as definite evidence of inhibition, but a slight delay of 2 or 3 weeks (+) is probably not significant.

Preliminary tests were undertaken on small groups of mice to determine the degree of irritation produced by the various compounds in different concentrations. Each dilution was applied 3 times at weekly intervals to the skin of 4 mice, and a week after the last application the skins were examined both macroscopically and microscopically. Certain values were then given for evidence of thickened skin, loss of hair, ulceration, subcutaneous inflammation and fibrosis, etc. By this means it was possible to grade the compounds according to the severity of irritation produced. Thus the figures 10-12 represent gross ulceration; 6-7 indicate marked thickening of the skin with loss of hair but little or no evidence of ulceration, while 2-4 denote minor degrees of stimulation (tables I and II).

The compounds [illegible] to mustard gas (table I, [illegible]

TABLE I.

Effect on tumour induction of mustard gas and related compounds.

		Number of animals used.	Survivors after 15 weeks.	Total number of animals with warts after 15 weeks.*	Time taken for 50 per cent. of survivors to develop tumours.	Inhibition of tumour induction.	Irritant action.
Series A.							
Control		60	44	19	16 weeks	...	...
Di-chloro-diethyl sulphide	0·1 per cent.	60	29	3 (5)	...	+ + + + +	7
Di-chloro-diethyl sulphoxide	0·1 ,,	60	52	17	16½ weeks	—	0
Di-chloro-diethyl sulphone	0·1 ,,	60	43	23	16 ,,	—	2
Thiodiglycol	0·1 ,,	60	41	29	16 ,,	—	0
Di-bromo-diethyl sulphide	0·1 ,,	60	39	1 (3)	...	+ + + + +	9
Di-bromo-diethyl sulphone	0·1 ,,	60	51	18	16½ weeks	—	1
Di-iodo-diethyl sulphone	0·1 ,,	60	47	17	16 ,,	—	0
Series B.							
Control		50	44	24	14½ weeks	...	...
Di-chloro-diethyl sulphide	0·01 per cent.	50	42	14	17½ ,,	[illegible]	2
Di-bromo-diethyl sulphide	0·01 ,,	50	38	5 (15)	19½ ,,	[illegible]	6
Thiodiglycol	1 ,,	50	41	19	17 ,,	[illegible]	0
Di-chloro-diethyl sulphoxide	1 ,,	50	41	22	15 ,,	[illegible]	4
Di-chloro-diethyl sulphone	1 ,,	50	38	5 (16)	...	[illegible]	10
Ethylene-bis-chloro-ethyl sulphide	0·01 ,,	50	42	3 (6)	...	[illegible]	6
,, ,, ,, ,, ,,	0·1 ,,	50	42	3 (5)	...	[illegible]	10
Di-bromo-diethyl sulphone	1 ,,	50	38	6 (13)	...	[illegible]	6
Di-iodo-diethyl sulphone	1 ,,	50	43	15	15½ weeks	[illegible]	2

* Figures in brackets represent the total numbers of animals with warts after 19 weeks.

Table II.

Effect on [illegible] of [illegible] of [illegible]

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Series C.	[illegible] per cent.	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Series D.	[illegible] per cent.	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

* [illegible]

This may have been due partly, if not entirely, to the occasional occurrence of minor degrees of ulceration in this group, following self-inflicted scratches.

It would appear, therefore, that the association between irritant action and inhibition of tumour induction, which seems to exist in the case of the mustard gas group of compounds, is no longer demonstrable when other irritants are tested in the same manner.

Discussion.

The original object of these investigations was to determine whether certain compounds chemically related to mustard gas were capable of acting like mustard gas in inhibiting the induction of tar tumours. It was hoped that an analysis of the results obtained might furnish a clue to the relationship between the chemical structure of mustard gas and its power of inhibiting tumour induction.

The actual choice of the particular compounds to be tested was based on a consideration of the physical, chemical and biological properties of mustard gas and of some of the theories put forward to account for its irritant action.

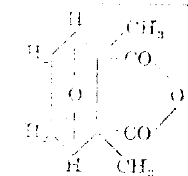
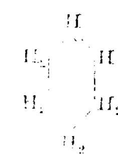
Mustard gas—$\beta\beta'$ di-chloro-diethyl sulphide—is an oily liquid, readily soluble in fats and fat solvents, but only sparingly soluble in water. In the presence of water it is decomposed fairly rapidly into di-hydroxy-diethyl sulphide (thiodiglycol) and hydrochloric acid. It has been suggested by Lynch, Smith and Marshall (1918-19) and others that the irritant action of mustard gas is dependent on its high lipoid-water distribution coefficient and that the damage is actually due to the liberation of free hydrochloric acid inside the cell. In support of this "acid theory" is the fact that di-chloro-diethyl sulphoxide, which is not hydrolysed by water, has no apparent action on the skin. But the theory has been opposed by several workers who investigated the distribution coefficient (Lawson and Lawson 1924) and the rate of hydrolysis (Peters and Walker 1923) of a number of compounds closely related to mustard gas, but failed to find any association between these properties and the power of producing skin irritation.

Mustard gas is readily oxidised into di-chloro-diethyl sulphoxide and ultimately into the sulphone. It has been suggested that this can also occur in the body (Flury and Wieland 1921), but the possibility of this reaction playing a part in the process of cell irritation is rendered unlikely by the fact that, whereas the sulphone, like mustard gas, is a severe irritant, the sulphoxide, as already stated, has no such action. Finally the reaction of mustard gas with amines and amino acids (Cashmore and McCombie 1928, and others) suggests the possibility that an interaction with the protein

[illegible]

TABLE III.

Chemical formulae, rates of hydrolysis, irritant action and anti-carcinogenic effect of the various substances tested.

	Formula.	Rate of hydrolysis. (i)	Irritant action. (ii)	[illegible]
		Mustard gas group.		
Ethylene-bis-chloro-ethyl sulphide	$CH_2-S-CH_2-CH_2Cl$ $CH_2-S-CH_2-CH_2Cl$	20 min.	v. marked (0·005 per cent.)	v. [illegible]
Di-bromo-diethyl sulphide	CH_2-CH_2Br S CH_2-CH_2Br	5 "	marked (0·01 per cent.)	[illegible]
Di-chloro-diethyl sulphide	CH_2-CH_2Cl S CH_2-CH_2Cl	5 "	marked (0·05 per cent.)	[illegible]
Di-chloro-diethyl sulphone	O CH_2-CH_2Cl S O CH_2-CH_2Cl	1 hour	moderate (0·2 per cent.)	[illegible]
Di-bromo-diethyl sulphone	O CH_2-CH_2Br S O CH_2-CH_2Br	20 min.	moderate (0·5 per cent.)	[illegible]
Di-iodo-diethyl sulphone	O CH_2-CH_2I S O CH_2-CH_2I	over 1 hour	? slight (1 per cent.)	[illegible]
Di-chloro-diethyl sulphoxide	CH_2-CH_2Cl O = S CH_2-CH_2Cl	no hydrolysis	none	[illegible]
Thiodiglycol	CH_2-CH_2OH S CH_2-CH_2OH	"	none	none
Di-chloro-diethyl ether	CH_2-CH_2Cl O CH_2-CH_2Cl	"	none	[illegible]
		Other irritants.		
Cantharidin		30 min.	marked (0·1 per cent.)	marked
Croton oil	...	no hydrolysis	moderate (0·5 per cent.)	none
Iodoacetic acid	$CH_2I-COOH$	"	moderate (1 per cent.)	none
Trichloracetic acid	$CCl_3 . COOH$	"	moderate (5 per cent.)	none
Acetic acid	CH_3-COOH	"	moderate (5 per cent.)	none
"Lysol"	...	"	mild (10 per cent.)	none
Cyclohexene		"	mild (20 per cent.)	slight
Furocoumarine	...	"	mild (50 per cent.)	none

(i) Time taken for 50 per cent. of hydrolysis to occur when 1 mg. is suspended in [illegible] Ringer solution [illegible] C.

(ii) The figures in [illegible] a mouse [illegible]

[illegible] the fact remains that it seems [illegible] probably different modes of action. It is not impossible, therefore, that irritation does play an essential part in the process of anti-tumour induction, *provided the irritation be of a particular kind.*

But even if this were true it still does not [illegible] the anti-carcinogenic action is directly dependent on the irritation. A more likely interpretation of the result would be that the tumour inhibition, while not dependent on such irritation, is [illegible] the result of some common reaction in the tissues. In other words, the pathological change produced in the tissues [illegible] responsible for the manifestations of irritation, may also be [illegible] of the inhibition of tumour induction.

The physical properties of the various substances must also be taken into consideration, since the [illegible] such as tumour induction and tumour inhibition, [illegible] be influenced by such factors as lipoid and [illegible] solubility. It is [illegible] that a substance may be potentially capable of inhibiting tumour induction and yet fail to produce this effect in practice because it cannot penetrate the cell [illegible]. While [illegible] of solubility is probably [illegible] in the case of the rather insoluble [illegible] substances), it is extremely doubtful if this [illegible] for the results obtained in the present [illegible]. Above all, it has been shown that, at least [illegible] irritation is concerned, the lipoid-water distribution [illegible] determining factor.

There is the possibility that the observed [illegible] of anti-carcinogenic action with irritation is [illegible]. While this is very unlikely, it [illegible] very much larger series of substances has been [illegible] by the methods adopted in these investigations.

Summary.

Several compounds closely related to mustard gas were tested in conjunction with a carcinogenic tar, in order to determine whether they possessed the power of inhibiting tumour induction. Some of these compounds produced pronounced inhibition while others had no such effect.

Among a group of other irritants not related to mustard gas, methyl iodide was found to possess the property of inhibition to a marked degree.

The possible relationship of tumour inhibition to irritant action is discussed.

The [illegible] of the [illegible] of hydrolysis of the various compounds [illegible] and for hydrolysis we are Dr L. P. [illegible]

Kendal. The manometric method used by Warburg for the measurement of lactic acid production by surviving tissues was adapted for the purpose. The course of acid production during hydrolysis was studied by observation of the rate at which carbon dioxide was liberated from the bicarbonate solution. I wish to express my indebtedness to Dr Kendal for carrying out the estimations.

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070

Uue of Glacial Acetic Acid for killing Parasitic Nematodes for Collection Purposes

MANY parasitic nematodes have a tendency to coil considerably when placed in water or cold-fixing fluids such as alcohol or formalin. Ideally, in order to facilitate the subsequent microscopic examination, nematodes should die in an extended position. This is best achieved by the use of 70 per cent alcohol or Looss' fluid (9 parts 70 per cent alcohol and 1 part glycerol), near boiling temperature, in which nematodes uncoil and become fixed rapidly. However, hot alcohol is rarely available during field work, and a simple alternative method for killing and fixing nematodes in an extended position is likely to be desired by other workers.

In the summer of 1960, parasitic nematodes from the proventricle of the fulmar (*Fulmarus glacialis*) were fixed in glacial acetic acid by mistake instead of cold 70 per cent alcohol, to which they were transferred a few minutes later. The fixed specimens were found to be remarkably straight, and the idea of using glacial acetic acid on purpose presented itself. More specimens from the fulmar were collected; some were fixed in cold 70 per cent alcohol, and others in glacial acetic acid. Those fixed in alcohol were found to be rather wavy and bent, while those treated with glacial acetic acid straightened almost immediately. After a few minutes, the majority of the latter were transferred to cold 70 per cent alcohol, while a few were allowed to remain in the glacial acetic acid. It has not been possible to make a specific determination of the specimens from the fulmar, but they belong to the superfamily Spiruroidea, sub-family Acuariinae, and appear to be generically related to *Seuratia* Skrjabin, 1916.

Several specimens of *Contracaecum aduncum* (Rudolphi, 1802), a common parasite of the digestive tract of the cod and other marine teleosts, have been treated with glacial acetic acid as described here. The specimens were found first to move vigorously, then they uncoiled and died in a matter of seconds. Small specimens were found to straighten more rapidly than larger ones. Larval ascarids, *Anisakis* sp. larvæ and *C. aduncum* larvæ, liberated from their capsules, have been treated with glacial acetic acid with equally good results. The nematodes were kept in the glacial acetic acid for up to 10 min., whereupon they were transferred to and stored in 70 per cent alcohol. Before fixation, the nematodes were washed in water in order to clean them from mucus and debris.

A mixture of three parts 70 per cent alcohol and one part glacial acetic acid did not kill as rapidly as the undiluted acid, nor did the specimens straighten so well in it. When the glacial acetic acid becomes diluted with water after repeated use, the killing effect is retarded and the specimens do not straighten so well in it. The acid should therefore be used undiluted. However, by adding various amounts of absolute alcohol to the glacial acetic acid, specimens of *C. aduncum* were killed even more rapidly, but they did not uncoil as well as in the glacial acetic acid alone. The advantage of adding absolute alcohol is that the freezing-point of the glacial acetic acid is lowered, thus avoiding solidification at low temperatures. A mixture of one part of glacial acetic acid and one part of absolute alcohol was found to remain fluid at −32° C. This mixture may be useful for field work in extreme low temperatures, but at higher temperatures the amount of glacial acetic acid should be increased.

Admittedly, this simple method for killing nematodes seemed promising, but it remained to be seen whether or not the specimens had been damaged by this treatment. Specimens were cleared in the usual media, lactophenol and creosote, in which they cleared very rapidly. On microscopical examination, they were found to be in good condition. When cleared in lactophenol cuticular structures, such as lips, papillæ and striation were found to be very clearly visible. Internal organs were also seen clearly.

After having been kept in the glacial acetic acid for six months, some of the specimens from the fulmar were transferred directly to lactophenol and examined microscopically. The specimens were found to be in good condition.

It thus appears that glacial acetic acid with subsequent transfer to 70 per cent alcohol may be used for killing and fixing nematodes.

Acanthocephalans (*Echinorhynchus gadi*) and trematodes (*Hemiurus communis*) from the cod have also been treated with glacial acetic acid. Before killing in acid, the acanthocephalans had to be left for some time in fresh water in order to extrude their evertible hooked proboscis. No movement of the specimens was observed during fixing. They cleared quickly in lactophenol. The trematodes were washed in 1 per cent saline solution before fixing. Whole trematodes were found to stain fairly well in carmalum.

Manuals on microscope technique[1,2] do not recommend fixing in pure glacial acetic acid for histological work, although it is known to kill with the utmost rapidity. However, as nematodes rarely have to be sectioned, the method described may be useful, at least when it is imperative that they should die and remain extended. However, for histological work it is perhaps better to avoid prolonged stay in glacial acetic acid; the specimens should preferably be transferred to 70 per cent alcohol as soon as possible. It is possible that this method may be regarded as a two-step modification of Carnoy's.

Although nematodes in general are rather difficult to stain, transverse sections of *C. aduncum* killed in glacial acetic acid were stained in haemalum for 1-5 hr., and overnight in eosin, with quite good results. Even the nuclei stained well. Carmalum or paracarmine alone stained poorly.

In conclusion, it is suggested that the use of glacial acetic acid, followed by transfer to 70 per cent alcohol, is satisfactory for fixing nematodes in an extended position. No adverse effect on the specimens was observed after this treatment. In addition, the anatomy may be studied [illegible] in stained sections. However, it is not known [illegible] effect, if any, the glacial acetic acid treatment [illegible] on the finer histological or cytological details.

BJØRN [illegible]

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German Translation

Esophageal Burns Caused by Vinegar Essence (Concentrated Acetic Acid) - Study of the Decrease of Burn Sequelae with Unfavorable Prognosis (see Note)*

M. Birke and B. Mros (From the City Clinic Berlin-Buch (Medical Director: Chief Medical Councillor Dr. O.F. Dudzus), the Throat, Nose and Ear Clinic (Chief Physician: Dr. med. habil. W. Kup), and the Charité Throat, Nose and Ear Clinic of Humboldt University (Director: Prof. Dr. Dr. med. habil. I. Keiser-Meinhardt)).

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The constantly occurring burns and scalding of the oral cavity, throat and esophagus, which sometimes have a rather complicated course and even a fatal outcome, have prompted us to tabulate corresponding groups of patients with such injuries treated at several city throat, nose and ear clinics and also at the Charité Throat, Nose and Ear clinic during the period 1963 to 1967 (Table 1). Special consideration was given in this case to burns caused by concentrated acetic acid, the so-called vinegar essence.

Burns due to vinegar essence were found in 40 cases (34.4%); this figure does not include 7 cases (3 with a fatal outcome) in which this product was used for suicidal purposes.

* Note: From a condensed report presented on November 30, 1968 at the Charité Throat, Nose and Ear Clinic during a conference of the Berlin Throat, Nose and Ear Association.

The remaining 69 patients included mainly cases of accidental drinking of various products such as, for example, industrial cleaning agents, disinfectant solutions, fire extinguishing fluids, fluorine-containing insulation agents, and other solutions (Table 2).

Table 1. In patients with esophageal burns or scalding, admitted during 1963-1967.

1 HNO-Klinik[1] *	Anzahl der Fälle — 2
Charité	45
Städt. Klinikum Buch	37
Prenzlauer Berg	16
Friedrichshain	10
Weißensee	8
Total — Gesamt:	116

1. Throat, nose and ear clinic
   *Note: We wish to thank the chief physicians Dr. R. Krienitz, Dr. G. Rumpf and Dr. P. Strumpel for providing us with the patient histories and the medical facts).

2. Number of cases

Table 2.

1 — Verwendetes Mittel	Anzahl der Fälle - 2
3 - Essigessenz	40
4 Essigessenz aus suizidaler Absicht	7
5 Verbrühungen	14
6 Salzsäure	6
7 Desinfektions- und Seifenlösungen	6
8 Lötwasser	5
9 Kalium-Permanganat-Granula	5
10 Kalilauge	2
11 Formalin	2
12 Übrige Mittel (Feuerlöschflüssigkeit, Haarwasser u. a.)	29
Total — Gesamt:	116

1. Product used
2. Number of cases
3. Vinegar essence
4. Vinegar essence for suicidal purposes
5. Scalding
6. Hydrochloric acid
7. Disinfectants and soap solutions
8. Soldering fluid
9. Granulated potassium permanganate
10. Caustic potash
11. Formalin
12. Other products (fire extinguishing fluids, hair lotions, etc.)

The majority of patients consisted of children up to 10 years old; here, in 14 cases alone, concentrated acetic acid was the corrosive agent. The frequency of cases in different age groups is shown in Table 3.

Years	Patients
0— 5 Jahre	56 Patienten
6—10 Jahre	9 Patienten
11—20 Jahre	11 Patienten
21—30 Jahre	13 Patienten
31—40 Jahre	6 Patienten
41—50 Jahre	5 Patienten
over → über 50 Jahre	16 Patienten

The immediate treatment used is the same at all clinics; it has been generally adopted on an international scale and consists in the simultaneous administration of corticosteroids and penicillin. The diagnosis and estimate of local events is based on esophagoscopy, and the time at which it is first performed is estimated differently for each individual, and is carried out as a rule when the general condition of the patient allows to do so. In the group of patients studied by us, esophagoscopy was performed, on the average, on the fifth day after the corrosive product was ingested, regardless of the individual findings noted in the mouth and throat area.

From a prognosis view point, the following can be stated in regard to the above treatment (therapy): a stenosis requiring treatment was observed in only 2 cases out of the total number of patients (excluding the cases with a fatal outcome). All other patients, after undergoing a final esophagoscopy, swallowing mush (brei) and getting an X-ray check, as well as making subjective statements on undisturbed food intake, were released as recovered in the sense of complete restoration of health. The average period of bed confinement per

patient was 19 days.

Of interest to us is the question whether a decrease in the number of burn cases can be observed as a result of joint efforts made by pediatricians, throat, nose and ear specialists and industry representatives, aimed at the possible replacement of 80% vinegar essence by the almost harmless 40% essence. Table 4 shows the number of burn cases caused by 80% vinegar essence during the above years.

Table 4.

Jahr / Year	Anzahl der Fälle, die eine Verätzung durch 80%ige Essigessenz erlitten hatten —①
1963	10
1964	8
1965	10
1966	9
1967	3

1. Number of burn cases caused by 80% vinegar essence.

It was shown that a definite decrease of burn cases was observed after introduction of a 40% vinegar essence. During the first 6 months of 1968, no more burn cases due to 80% concentrated acetic acid were admitted at the Berlin-Buch and Charité clinics. We hope that no further poisoning cases will occur after all remaining vinegar essence in households is used up, and that in all other burn cases mentioned above the number of burns will be further reduced as a result of sufficient explanations given by parents and a careful identification of bottle contents, especially when using beer and lemonade bottles.

Discussion of Results

After it was established that a 40% vinegar essence would not

cause any substantial injury (Burian, Kup, Dietzel, Gerth and Krichel-dorff), the removal of the 80% vinegar essence could be tackled as a propylactic task. This became possible on the territory of the German Democratic Republic (East Germany) as the result of a collaboration between pediatricians, throat, nose and ear specialists and the Ministry of Trade. Whereas we felt obliged recently to devote a publication to the removal of 80% vinegar essence, we can state the following today:

1. No 80% vinegar essence is available any longer at the appropriate stores.
2. The number of burn cases caused by vinegar essence appears to be declining.
3. Only low-and medium-degree burn cases are admitted for treatment.

Thus, during the first 6 months of 1968, no admission cases of such type have been recorded at two Berlin hospitals, namely the Charité Throat, Nose and Ear Clinic and the Berlin-Buch clinic. Similarly, we can state on the basis of the patient group studied by us that the well-known and good results of corticosteroid therapy with simultaneous penicillin protection (Burian, Werner, Schobel and Zippel) afford a favorable prognosis in light and medium severe cases of burns due to vinegar essence. In spite of this happy fact, the total number of burn cases, viewed as a whole, is still too high, in spite of the favorable developments concerning vinegar essence. Since the principal cause of such burns lies in the confusion (mistaking) of bottle contents, it is primarily the task of parents, schools, etc. to contribute, by providing proper information, to a further decrease

of other burn cases.

Summary

On the basis of 116 burn cases recorded during 1963-1967 at 5 Berlin throat, nose and ear clinics, it is shown that the number of burn cases due to 80% vinegar essence has dropped. The fact that the sale of 80% vinegar essence is prohibited can be considered as the decisive cause of the fact that only burn cases of slight to medium severity are now predominantly noted. Burn sequelae are prevented to a great extent by means of corticosteroid therapy and antibiotics protection. The importance of careful storage of caustic liquids and proper instructions to the population is pointed out.

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ıus dem Städtischen Klinikum Berlin-Buch (Ärztlicher Direktor: Obermedizinalrat Dr. O. F. Dudzus);
:als-Nasen-Ohren-Klink (Chefarzt: Medizinalrat Dr. med. habil. W. Kup)
nd der Hals-Nasen-Ohren-Klinik (Charité) der Humboldt-Universität
)irektor: Prof. Dr. Dr. med. habil. I. Kaiser-Meinhardt)

ıpeiseröhrenverätzungen mit Essigessenz[1])

Eine Untersuchung über den Rückgang prognostisch ungünstiger Verätzungsfolgen)

on M. Birke und B. Mros

Z. Aerztl Forthild (Jena)
63: 877-9, 15 Aug. 69 (Ger)

Die immer wieder auftretenden Verätzungen oder 'erbrühungen von Mundhöhle, Rachen und Ösophagus ıit teilweise erheblich kompliziertem, wenn nicht gar ödlichem Verlauf haben uns veranlaßt, dieses entspre-hende Krankengut mehrerer städtischer Hals-Nasen-)hren-Kliniken sowie der Hals-Nasen-Ohren-Klinik der 'harité für die Jahre 1963 bis 1967 zusammenzustellen (Tabelle I). Besondere Berücksichtigung fanden dabei die Verätzungen mit konzentrierter Essigsäure, der sog. Essigessenz.

Eine Verätzung mit Essigessenz fand in 40 Fällen (= 34,4%) statt. Nicht in diese Zahl einbezogen wurden 7 Fälle, wo dieses Mittel aus suizidalen Gründen eingenommen wurde; 3 davon gingen letal aus.

Bei den restlichen 69 Patienten handelt es sich um überwiegend versehentliches Trinken von verschiedenen Substanzen wie zum Beispiel Industriereiniger, Desin-

[1]) Als Vortrag auszugsweise vorgetragen am 30. Novem-er 1968 in der Hals-Nasen-Ohren-Klinik (Charité) zur Ta-ıng der Berliner hals-nasen-ohren-ärztlichen Gesellschaft.

Table 1.

Tabelle 1 Stationär aufgenommene Patienten der Jahre 1963 bis 1967 mit Verätzung oder Verbrühung des Ösophagus

HNO-Klinik[1]) *	Anzahl der Fälle — (2)
Charité	45
Städt. Klinikum Buch	37
Prenzlauer Berg	16
Friedrichshain	10
Weißensee	8
Total- Gesamt:	116

Note. *[1]) Für die Überlassung der Krankengeschichten bzw. Zurverfügungstellen der Fakten danken wir den Herren Chefärzten Dr. R. Krienitz, Dr. G. Rumpf und Dr. P. Strümpel.
Die HNO-Abteilungen des Volkspolizeikrankenhauses und des Krankenhauses in Kaulsdorf konnten uns keine Angaben überreichen.

Table 2

Tabelle 2

(1) — Verwendetes Mittel	Anzahl der Fälle — (2)
(3) Essigessenz	40
(4) Essigessenz aus suizidaler Absicht	7
(5) Verbrühungen	14
(6) Salzsäure	6
(7) Desinfektions- und Seifenlösungen	6
(8) Lötwasser	5
(9) Kalium-Permanganat-Granula	5
(10) Kalilauge	2
(11) Formalin	2
(12) Übrige Mittel (Feuerlöschflüssigkeit, Haarwasser u. a.)	29
Total- Gesamt:	116

Table 3

Tabelle 3

0— 5 Jahre	56 Patienten	
6—10 Jahre	9 Patienten	
11—20 Jahre	11 Patienten	Patients
21—30 Jahre	13 Patienten	
31—40 Jahre	6 Patienten	
41—50 Jahre	5 Patienten	
over → über 50 Jahre	16 Patienten	

Table 4

Tabelle 4

Jahr Year	Anzahl der Fälle, die eine Verätzung durch 80%ige Essigessenz erlitten hatten — (1)
1963	10
1964	8
1965	10
1966	9
1967	3

fektionslösungen, Feuerlöschflüssigkeit, fluoridhaltigem Isolierungsmittel und anderen Lösungen (s. Tabelle 2).

Der Hauptanteil der Betroffenen besteht aus Kindern bis zum Alter von 10 Jahren; allein 14mal war hier konzentrierte Essigsäure das Ätzmittel.

Die Häufigkeit der Fälle in den verschiedenen Altersgruppen wird aus der Tabelle 3 ersichtlich.

Übereinstimmend ist die in den Kliniken sofort eingeleitete Therapie; sie hat sich allgemein international durchgesetzt und besteht in der Verabreichung von Kortikosteroiden und gleichzeitiger Gabe von Penizillin. Die Diagnose bzw. die Beurteilung des lokalen Geschehens erfolgt durch die Ösophagoskopie. Der Zeitpunkt der ersten Ösophagoskopie wird individuell unterschiedlich beurteilt; sie wird in der Regel dann durchgeführt, wenn es der allgemeine Zustand des Patienten erlaubt. Sie erfolgte im untersuchten Krankengut unabhängig vom jeweiligen Befund im Mund- und Rachengebiet durchschnittlich am 5. Tag nach Einnahme des Mittels.

Prognostisch kann mit der angeführten Therapie folgendes zum Ausdruck gebracht werden: vom gesamten Krankengut (exklusive der letal ausgegangenen Fälle) blieb in nur 2 Fällen eine behandlungsbedürftige Stenose zurück. Alle anderen Patienten konnten, belegt durch abschließende Ösophagoskopie, Breischluck und Röntgenkontrolle sowie subjektive Angaben über ungestörte Nahrungsaufnahme, als geheilt im Sinne einer restitutio ad integrum entlassen werden.

Die durchschnittliche Liegedauer pro Patient betrug 19 Tage.

Interessant erscheint uns die Frage, ob nach gemeinsamen Bemühungen der Pädiater und Hals-Nasen-Ohren-Ärzte in Verbindung mit dem Handel, wonach die 80%ige Essigessenz durch die kaum schädigende 40%ige ersetzt werden konnte, ein Rückgang dieser Verätzungen zu beobachten war. Aus der Tabelle 4 werden die Zahlen der Verätzungen mit 80%iger Essigessenz für die genannten Jahre ersichtlich.

Es zeigt sich, daß nach Einführung der 40%igen Essigsäure ein deutlicher Rückgang der Fälle zu verzeichnen ist. In Berlin-Buch und in der Charité wurden in den ersten 6 Monaten des Jahres 1968 keine Verätzungen mit konzentrierter 80%iger Essigsäure mehr aufgenommen. Hoffen wir, daß durch Verbrauch aller Reste in den Haushalten keine weiteren Fälle mehr auftreten werden und daß in allen übrigen obengenannten Verätzungsfällen durch hinreichende Aufklärung der Eltern und der Propagierung einer sorgfältigen Kennzeichnung von Flascheninhalten, insbesondere bei Benutzung von Bier- und Limonadenflaschen, eine weitere Verringerung der Zahl von Verätzungen möglich wird.

Diskussion der Ergebnisse

Nachdem festgestellt worden war, daß eine 40%ige Essigessenz keinen wesentlichen Schaden hinterlassen würde (Burian, Kup, Dietzel, Gerth, Krichel-dorff), konnte als Aufgabe der Prophylaxe die Abschaffung der 80%igen Essigessenz in Angriff genommen werden. Auf dem Gebiet der Deutschen Demokratischen Republik konnte dieses im Gefolge einer Zusammenarbeit zwischen Pädiatern, Hals-Nasen-Ohren-Ärzten und dem Ministerium für Handel erreicht werden. Während wir noch vor kurzem uns veranlaßt sahen (Mros), der vollständigen Abschaffung der 80%igen Essigessenz eine Publikation zu widmen, können wir heute feststellen, daß

1. in den einschlägigen Geschäften keine 80%ige Essigessenz mehr angeboten wird,

2. die Zahl der Verätzungen mit Essigessenz im Abnehmen begriffen zu sein scheint,

3. daß überwiegend nur noch Verätzungen geringen und mittleren Grades zur Aufnahme kommen.

So konnten in zwei Berliner Krankenhäusern (HNO-Klinik der Charité und Berlin-Buch) in den ersten 6 Monaten des Jahres 1968 überhaupt keine Aufnahmen dieser Art mehr registriert werden. Desgleichen kann an Hand unseres untersuchten Krankengutes gesagt werden, daß die bekannten guten Ergebnisse der Therapie mit Kortikosteroiden bei gleichzeitigem Penizillinschutz (Burian, Werner, Schobel, Zippel) bei leichten und mittelschweren Verätzungsfällen mit Essigessenz eine günstige Prognose erfahren haben. Ungeachtet dieser erfreulichen Tatsache sind insgesamt gesehen die Verätzungszahlen trotz der erfreulichen Entwicklung in bezug auf die Essigessenz noch immer zu hoch. Da es sich vorwiegend um Verwechslungen von Flascheninhalten handelt, ist es vor allem Aufgabe der Eltern, der Schulen usw., durch Aufklärung mit dazu beizutragen, ein weiteres Senken der übrigen Verätzungen zu erreichen.

Zusammenfassung

An Hand von 116 Verätzungsfällen der Jahre 1963 bis 1967, die in fünf Berliner HNO-Abteilungen registriert wurden, wird der Nachweis geführt, daß die Zahl der Verätzungen mit 80%iger Essigessenz zurückgegangen ist. Hierbei wird die Tatsache, daß ein Verbot des Verkaufs von 80%iger Essigessenz durchgesetzt werden konnte, als entscheidende Ursache dafür gesehen, daß überwiegend nur noch gering- bis mittelgradige Verätzungen dieser Art beobachtet werden konnten. Mit Hilfe der Kortikosteroidtherapie unter Antibiotikaschutz konnten die Verätzungsfolgen weitgehend vermieden werden. Auf sorgfältige Aufbewahrung ätzender Flüssigkeiten und Aufklärung der Bevölkerung wurde hingewiesen.

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ACETATE AS A PRECURSOR OF AMINO ACIDS OF CASEIN IN THE INTACT DAIRY COW*

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INTRODUCTION

Acetate is normally produced in large amounts by the rumen microflora and is an important metabolite for ruminants[1]. Its role as a precursor of the major organic constituents of milk has been investigated in the intact cow[2]. In these studies it was shown that carbon from acetate-^{14}C was transferred mainly to milk fat but 1% to 2% of the injected carbon-14 was recovered in casein during the first 33 hours after injection.

Preliminary studies with ^{14}C-labeled short chain fatty acids indicated that in the tissues of the cow, carbon from acetate was transferred in significant amounts only to the non-essential** amino acids in casein[3]. The present paper reports on a more extensive study of the relationship between acetate and amino acids in the intact cow. Amino acids were recovered from twenty casein samples collected from five cows at intervals after injection of acetate labeled with ^{14}C on either carbon atom. In each case it was found that carbon from acetate was transferred most efficiently to glutamic acid. Glucose, which was studied earlier[1], was a better precursor of serine and alanine. The differences between glucose and acetate as precursors of amino acids are discussed.

METHODS

Twenty casein samples were prepared from milk collected at intervals after injecting five normal, lactating cows with a single dose of 4 to 5 millicuries of acetate-^{14}C. The methods for these procedures have been described previously[5]. Data concerning the cows used for these studies are listed in Table I.

The casein samples were hydrolyzed and amino acids separated on ion-exchange columns of Dowex-50 and IR4 B[5]. The amino acids isolated from the columns were converted to their free base form with propylene oxide and then crystallized from solution according to methods described previously[5]. Our procedure for recovery of alanine was modified when we observed that

TABLE I

DATA ON EXPERIMENTAL ANIMALS USED IN ACETATE TRIALS

Trial No.	*Cow No.*	*Breed*	*Cow's wt kg*	*Millicuries injected*	*Compound injected*
AC III	965	Jersey	414	4.7	Acetate-1-^{14}C
AC VI	962	Jersey	499	5.5	Acetate-1-^{14}C
AC VII	1099	Holstein	700	4.0	Acetate-1-^{14}C
AC II	805	Jersey	500	5.0	Acetate-2-^{14}C
AC X	947	Jersey	465	3.80	Acetate-2-^{14}C

* This investigation was supported by grants from the National Science Foundation and the U.S. Atomic Energy Commission.

** Glutamic and aspartic acids, alanine, serine, glycine, proline, and arginine.

occasionally alanine recovered directly from the Dowex-50 column, used for the initial hydrolysate fractionation, was contaminated with small amounts of glycine even after crystallization. The contaminating glycine was eliminated by adding the alanine to a Dowex-50 column and eluting with ammonium formate buffer (pH = 3.2) according to the method of HIRS, MOORE AND STEIN[6]. Most of the buffer could be removed from the recovered alanine by sublimation; the last traces of buffer were removed by adding the alanine sample to a small Dowex-50 column and eluting with 1.3 N HCl.

The method for combustion of amino acids, planchet preparation and carbon-14 assay were described in an earlier publication[1].

RESULTS AND DISCUSSIONS

The specific activities of the amino acids are expressed in terms of the relative injected dosages, that is, microcuries per gram atom carbon/microcuries injected per kilogram body weight. Specific activity expressed in terms of the relative injected dose has the advantage that results obtained with animals of different body size which receive different amounts of carbon-14 are more directly comparable.

The specific activities of amino acids recovered from casein after intravenous injection of acetate-1-^{14}C and acetate-2-^{14}C are listed in Tables II and III, respectively. In each trial the carbon from acetate was recovered in significant quantities only in the seven non-essential amino acids, glutamic and aspartic acids, alanine, serine, glycine, proline and arginine.

Lysine was the only essential amino acid recovered in this study since previous work has indicated that essential amino acids are not formed by tissues of the cow[3,4]. Using lysine as a representative of the essential amino acids, these results with acetate ^{14}C corroborate our earlier conclusion[3] that only the non-essential amino acids are formed in appreciable quantities by the tissues of the cow.

Glutamic and aspartic acids

Glutamic acid had the greatest specific activity among the amino acids for each casein sample after injection of both acetate-1-^{14}C and acetate-2-^{14}C. The specific activity of aspartic acid was smaller than that of glutamic but was generally greater than that of any of the other amino acids. From these results it may be concluded that the carbon from acetate is transferred most efficiently, that is with least dilution, to glutamic and aspartic acids, indicating that, among the amino acids, the most direct pathway from acetate is to the dicarboxylic amino acids. One explanation for these results is the transfer of acetate carbon to amino acids via the Tricarboxylic Acid Cycle (TCA) as shown in Fig. 1.

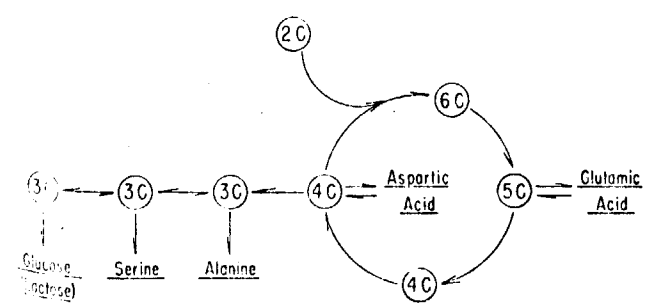

Fig. 1. Abbreviated schematic diagram of the TCA cycle. This diagram shows the transfer of carbon-14 from acetate (2C) via the TCA cycle to α-ketoglutarate (5C) and, after transamination, to glutamate; from (5C) the isotope passes to four carbon intermediates of the cycle (4C) and to aspartic acid; or by decarboxylation of a (4C) dicarboxylic acid to produce three carbon (3C) intermediates which serve as precursors of alanine, serine, and glucose (lactose).

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TABLE II

SPECIFIC ACTIVITY OF LACTOSE AND AMINO ACIDS FROM CASEIN AFTER INTRAVENOUS INJECTION OF ACETATE-1-^{14}C

Trial	Hours after injection	Glutamic acid	Aspartic acid	Alanine	Serine	Lactose	Glycine	Proline	Arginine	Lysine
Acetate III	3	2.55*	1.31	0.59	0.56	0.56	0.45	0.46	—	0.00
	10	1.20	0.81	0.57	0.00	0.68	0.08	0.31	0.42	0.01
	22	0.39	0.22	0.30	0.18	0.24	0.26	0.12	0.13	0.008
	34	0.17	0.075	0.073	0.078	0.00	0.11	0.055	0.038	0.005
Acetate VI	3	3.77	2.00	0.78	—	0.00	0.33	—	0.60	0.01
	10	0.886	0.500	0.306	0.318	0.44	0.356	0.341	0.226	0.00
	22	0.173	0.116	0.101	0.005	0.08	0.136	0.095	0.104	0.05
	34	0.091	0.058	0.049	0.050	0.04	0.077	—	0.055	0.03
Acetate VII	3	3.19	1.91	0.574	0.502	0.36	0.474	0.45	0.423	0.01**
	10	2.49	1.51	0.582	0.058	0.67	0.518	0.48	0.43	0.005
	22	1.86	1.14	0.51	0.55	0.50	0.46	0.37	0.37	0.01
	34	0.400	0.209	0.184	0.214	0.17	0.224	0.144	0.16	0.04

* Specific activity of 2.55 μc gram atom carbon = 1020 net c.p.m. in our counting system with $BaCO_3$ of "infinite thickness".

** Leucine collected instead of lysine.

TABLE III

SPECIFIC ACTIVITY OF LACTOSE AND AMINO ACIDS FROM CASEIN AFTER INTRAVENOUS INJECTION OF ACETATE-2-^{14}C

Trial	Hours after injection	Glutamic acid	Aspartic acid	Alanine	Serine	Lactose	Glycine	Proline	Arginine	Lysine
Acetate II	3	3.16	2.05	1.12	1.01	1.03	0.457	1.04	0.49	0.00
	10	2.49	1.06	1.00	1.06	1.09	0.548	0.88	0.46	0.01
	22	0.68	0.73	0.55	—	0.43	0.34	—	—	—
	34	0.426	0.25	0.22	0.23	0.20	0.18	0.17	0.14	0.02
Acetate X	3	2.36	1.38	0.80	1.50	1.30	0.506	0.390	0.263	0.00
	10	2.10	1.47	1.08	1.50	1.06	1.00	0.427	0.446	0.00
	22	0.662	0.503	0.651	0.775	[illegible]	[illegible]	[illegible]	0.222	[illegible]
	34	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Serine, alanine and lactose

The specific activities of alanine and serine were approximately equal for each casein sample and were very close to the specific activities of lactose recovered from the same milk sample. These results suggest that, in the intact cow, carbon from acetate either passes to a common precursor for alanine, serine, and lactose, or, more probably, passes to the three precursors of these compounds with almost no change of specific activity (see Fig. 1). The latter possibility would indicate that there are no large pools of intermediates on the pathway between precursors of alanine, serine, and lactose or that there is rapid isotope equilibrium between the precursor pools.

Several studies have demonstrated a relationship between glycine and serine in animal tissues[7,8,9]. Previous studies with the intact cow have indicated that carbon from glucose was primarily transferred along a pathway that may involve serine as a precursor of glycine[1] and ARNSTEIN has reached a similar conclusion from studies with rats[10]. Our results obtained with acetate-^{14}C (Tables II and III) support the same pathway. The specific activity of serine always exceeded that of glycine initially (at 3 hours) and then decreased relative to glycine at later time periods. These changes of specific activities would be expected if serine were the precursor of glycine but would not occur if glycine were the precursor of serine.

Proline and arginine

There is evidence that glutamic acid, proline, and arginine are interconvertible in animal tissues[11,12]. The most reasonable explanation for the data in Tables II and III would involve a pathway for the transfer of acetate carbon to glutamic acid and then to proline and arginine. If this were the major pathway involved then one would expect the ratio of specific activities for these amino acids to remain constant. The ratios of mean specific acitivites for glutamic acid/proline have been calculated from the data in Tables II and III to be 4.2, 5.0, and 5.0 for trials III, VII, and X, respectively. Similar calculations of the glutamic acid/arginine ratio were 3.7, 5.1, and 4.8 for trials VI, VII, and X, respectively. Since there was not a large variation between these ratios for the amino acids from different cows, our results suggest a relationship between glutamic acid, arginine, and proline in the intact cow. These ratios indicate that a minimum of about 20 to 25% of proline and arginine in casein may arise from glutamic acid.

Acetate vs. glucose as precursor of amino acids

The relative specific activities of the amino acids after injecting acetate-^{14}C differed markedly from that previously observed[1] when glucose-^{14}C was injected into cows. These differences are brought out by the curves in Fig. 2 and 3 which show the change of specific activity of glutamic acid and serine as a function of time after injecting glucose-^{14}C or acetate-^{14}C.

The specific activity of serine (and alanine*) was 2 to 3 times as great as that of glutamic (and aspartic*) acid when glucose-^{14}C was injected. These results indicate that glucose is a more direct precursor for the three carbon amino acids than for the dicarboxylic amino acids. When acetate was injected, on the other hand, (see Fig. 3)

* The specific activities for these amino acids were very nearly the same so that the curve for one represents the change of specific activity for both.

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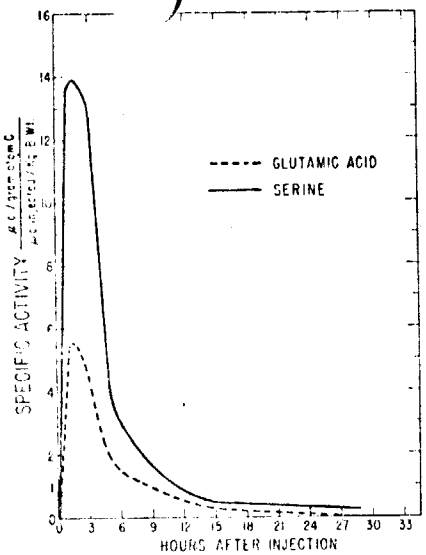

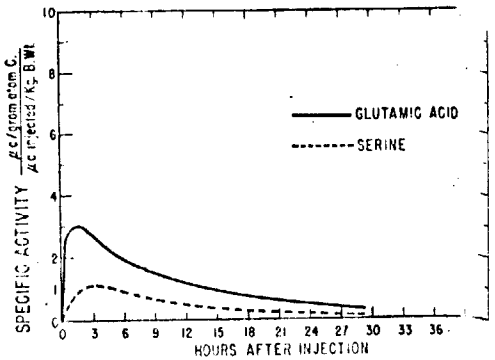

Fig. 3. ^{14}C in glutamic acid and serine after intravenous injection of acetate-^{14}C. The specific activities are plotted as the mean result for all of the acetate-1-^{14}C and acetate-2-^{14}C trials. The specific activities of serine, alanine and lactose were approximately equal so that the curve for serine represents the change of specific activity for all three compounds.

Fig. 2. ^{14}C in glutamic acid and serine after intravenous injection of uniformly labeled glucose. The curve for serine also represents the change of specific activity for alanine since these amino acids had approximately the same specific activities throughout the experimental period. The curve for glutamic acid represents the change of specific activity in aspartic acid, as well, for the same reason.

the reverse relationship was observed; the specific acitivity of glutamic acid was 2 to 3 times as great as that of serine (alanine and lactose). These results may be interpreted as indicating that the precursor of glutamic acid is "closer" to the "acetate pool" than the precursors of the three carbon amino acids (see Fig. 1). This relationship suggests that, quantitatively, acetate is a more important precursor for glutamic acid than for the three carbon amino acids.

The fact that the specific activity curves for serine and glutamic acid maintain their same relative position throughout the experimental period for both glucose-^{14}C and acetate-^{14}C suggests that there is not extensive mixing of carbon between the "acetate" and "glucose" pools. Otherwise one would expect the curves would coincide or even cross over at later intervals.

For each amino acid, except arginine, the specific activity per unit injected dose was greater after glucose than after acetate in the initial sample collected three hours after injection. This result could be explained by the postulate that in the cow the acetate pool is larger than the glucose pool. This condition would result in a greater dilution of the injected acetate-^{14}C and correspondingly lower specific activities in the amino acids. The specific activity of the amino acids at later time intervals was reduced to between 1/5 and 1/10 for acetate while for glucose the final specific activity was 1/5 to 1/80 of the initial value. These results indicate that the smaller glucose pool is turning over more rapidly than the larger acetate pool.

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SUMMARY

Five lactating dairy cows were injected intravenously with acetate-1-^{14}C or acetate-2-^{14}C. Eight amino acids, recovered from casein collected at 3, 10, 22, and 34 hours after acetate-^{14}C injection, were assayed for carbon-14.

Carbon from acetate was transferred most efficiently to glutamic and aspartic acids and in lesser amounts to alanine, serine, glycine, proline, and arginine. Lysine did not contain significant amounts of ^{14}C.

The labeling of amino acids from acetate-^{14}C differed markedly from that previously observed for glucose-^{14}C. Carbon from uniformly labeled glucose was transferred most efficiently to alanine and serine and in smaller amounts to glutamic and aspartic acids, glycine, proline, and arginine.

The specific activities of alanine, serine, and lactose were quite similar after acetate-^{14}C injection suggesting a close relationship between the precursors of the three carbon amino acids and lactose.

The distribution of ^{14}C among the amino acids formed by the intact cow was consistent with results that would be expected if the TCA cycle and the glycolytic pathway were the pathway for transfer of carbon from acetate to amino acids of casein.

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RUSSIAN TRANSLATION

THE LYMPHATIC SYSTEM OF THE HUMAN LIVER DURING POISONING WITH SOME TOXIC SUBSTANCES

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There are indications in the literature on a relationship between lymph formation and the dynamics of pathological processes and on changes of the initial lymphatic system (network) depending upon functional disorders and morphological changes in the liver (D. A. Zhdanov, 1940, 1952, 1964, 1965; M. A. Dolgova; Yu F. Vikalyuk; I. Rusn'yak et al.) A. V. Kraev, while studying the lymphatic system of the liver during poisoning of animals with carbon tetrachloride, noted a 3-5-fold increase in the diameter of superficial and deep lymphatic capillaries during the period of acute injury. Later, the appearance of protein thrombi (clots) in lymphatic capillaries and vessels was noted. During prolonged administration of the toxic compound, the above author observed the multi-layered nature of the network of lymphatic capillaries in the swollen peritoneum of the liver and in the hypertrophic interlobular connective tissue.

We have set ourselves the task of checking the reaction of the lymphatic system of the human liver under conditions of the toxic action of some toxic products. It is known that a sharply expressed dystrophy of hepatocytes and a disturbance of hepatic blood circulation are observed in people poisoned by dichloroethane (F. F. Bryzhin; A. V. Rusakov, 1949),

acetic acid and caustic soda (A. I. Shibkov, A. V. Rusakov, 1930; G. A. Mishin, G. G. Zakharov).

We have investigated the livers of 21 people who died during the first few hours and in the following 19 days after poisoning by acetic acid (9), caustic soda (5) and dichlorethane (7); for comparison purposes, we studied the liver in 4 cases of acute toxic dystrophy as an outcome of Botkin's disease. A 0.25% $AgNo_3$ solution or Guerot's (?) mass was injected into the lymphatic capillaries and vessels of the liver by the modified Zhdanov method. The histotopography of the injected lymphatic capillaries in the liver peritoneum was studied on plane total or split layer-by-layer peritoneum preparations. The histological sections were stained with hematoxylin-eosin, according to van Gieson, or with Sudan III, orsein, resorcinol-fuchsin, and Aniline Blue; the impregnation was done according to Foot and Snesarev.

In people who died during the first 24 hours after dichloroethane poisoning, the liver was enlarged, its tissue was flaccid, and its cross-section showed a red-brown color. Histologically, a dilatation of blood vessels and a capillarostasis were detected. Erythrocytes are often found in Disse's spaces. The hepatic cells are in a state of turbid swelling, and an unsharply expressed, small-and large-drop fatty degeneration of the liver is noted. Isolated necrotic foci of the parenchyma can be seen, and the swollen bundles of collagen fibers of the stroma are distended (drawn apart) by edematous fluid. The intralobular reticular fibers are clearly impregnated; lymphatic capillaries and vessels are dilated, and probein coagulants are found in isolated lymphatic capillaries.

In acetic acid poisoning, the liver is not enlarged, but is flaccid and its cross-section exhibits red-colored foci. A thrombosis of hepatic veins can be detected under the microscope. The endothelium of blood vessels is desquamated, the sinusoids are dilated and sometimes filled with erythrocytes. Hepatic cells show symptoms of granular dystrophy and small-drop fatty degeneration, and foci of hemorrhages, necrobiosis and necrosis are found. The zone of necrotic injury is usually located along the periphery of Rappaport's acinus, but does not have a sharp boundary. The collagen fiber bundles are swollen, without a clear-cut structure. As in dichloroethane poisoning, the lymphatic capillaries and vessels are dilated and contain isolated protein coagulants.

Regardless of the kind of poison, a monotype (single-type) reaction on the part of the lymphatic system of the liver was observed during the first 24 hours in all fatal cases. Already during the first few hours after poisoning, an extremely light exit of the injection mass beyond the limits of lymphatic capillaries and vessels was noted, regardless of the magnitude of the injection pressure (Fig. 1). The size of the superficial lymphatic capillaries of the liver is 0.015-0.045 mm, which is greated than under normal conditions. A considerable number of capillaries with an irregular, notched contour (profile) is observed. In one case (Fig. 2), already during the first few hours after dichloroethane poisoning a particularly great dilatation (expansion) of the lymphatic capillary network was detected; however, as in other observations, no rearrangement of the network of lymphatic capillaries and vessels was noted. In the study of plane total and layer-by-layer split preparations of the liver peritoneum, it was noted that the lymphatic capillary network is located exclusively

in the deep collagen-elastic layer. When a 0.25% $AgNo_3$ solution is injected into lymphatic capillaries and vessels, the intensity of impregnation of intercellular boundaries of the endothelium is higher than under normal conditions. The intercellular boundaries impregnated with silver have a uniform width.

FIGURE 1

Lymphatic capillary network in the liver peritoneum of a 17-year old man. Escape (exit) of small lumps of dye through the walls of lymphatic vessels and capillaries. Death 7 hours after dichloroethane poisoning. Guerot (?) mass injection. X32.

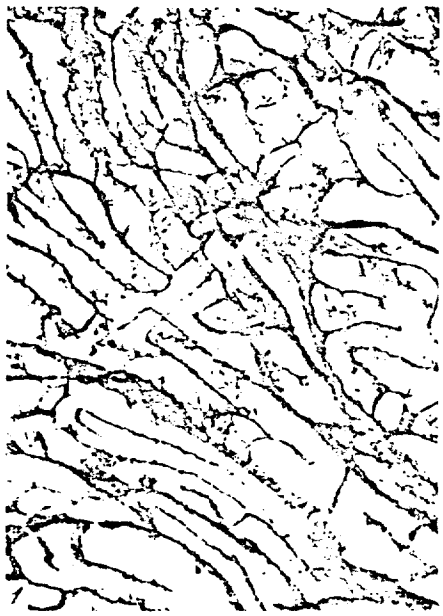
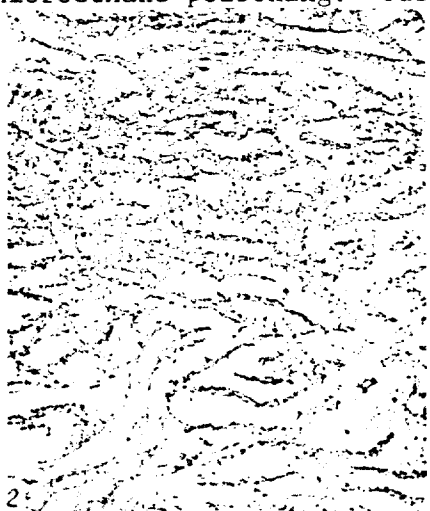


FIGURE 2

Lymphatic capillary network in the liver peritoneum of a 35-year old man. Dilated lymphatic vessels and capillaries. Death 4 hours after dichloroethane poisoning. Guerot (?) mass injection. X32.



Deep lymphatic capillaries also have an irregular diameter (cross section), and some have a notched profile. Numerous lacunae are found at the fusion (junction) points of capillaries. On the basis of an investigation of clarified preparations with injected deep lymphatic capillaries and vessels and a study of control serial histological sections across doubtful areas, we have also arrived at the conclusion that lymphatic capillaries are located in interlayers of the connective tissue, containing collagen fibers. No lymphatic capillaries were detected inside the lobules, and no direct connections between Disse spaces and lymphatic capillaries could be found.

In those cases when patients died on the second day after poisoning and later, the growth of dystrophic changes in hepatic cells and marked symptoms of a disturbed blood circulation were found. In dichloroethane poisoning, stagnation and stases were detected microscopically. The sinusoids are partly dilated, partly present in a collapsed state, and in some spots are filled with erythrocytes. A marked discomplexion of trabeculae, a turbid swelling of hepatic cells and a different degree of fatty infiltration are noted. Bile pigment deposits were found in the cytoplasm of some hepatic and Kupffer cells. Necrosis foci of hepatic cells are frequently observed, and the liver stroma is edematous, with a found cell infiltration.

In acetic acid poisoning, the liver is swollen, with a brown-colored cross section. Thrombosis of hepatic veins is detected histologically. No capillarostasis and diapedesis of erythrocytes could be detected. The sinusoids are either strongly dilated, or, on the contrary, are in a

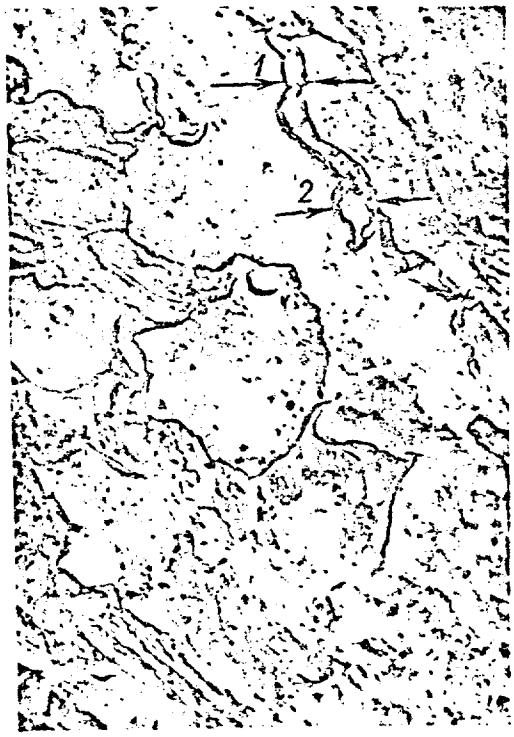
collapsed state; hyperplasia of Kupffer's cells is noted. The hepatic trabeculae are discomplexed, and extensive foci of small- and large-drop fatty degeneration of the liver are found. In 2 cases, a picture of a sharply expressed diffuse fatty degeneration of the liver was noted. The collagen fiber bundles are swollen, and the lymphatic capillaries and vessels in the periportal fields and in the peritoneum of the liver are dilated.

An extensive growth and restructuring of the superficial lymphatic capillaries in the liver is noted. On the 7th to 12th to 18th day after poisoning, the presence of lymphatic capillaries directly under the boundary (limiting) membrane can be seen in plane preparations of the liver peritoneum. A corresponding rearrangement of collagen fiber bundles in the surface layers of the outer and inner collagen can be observed. While studying plane preparations of the liver peritoneum in an edematous state, we cannot agree with M. S. Ignashkina, who believes that, during edema, the lymphatic capillaries are compressed by the swollen collagen fiber bundles. The surface (superficial) lymphatic capillaries of the liver are dilated 3-5 times in comparison with the norm, and are closed up (joined) into a fine-loop mesh (network) with loops of oval or circular shape. The lymphatic capillaries lying in the liver peritoneum above the deep collagen-elastic layer are often closed (joined) into loops of rectangular or oval shape, and form the surface (superficial) capillary lymphatic network of the peritoneum. In these sectors of the liver peritoneum, a clearly expressed three-dimensional or two-layered network of lymphatic capillaries can be noted.

When a 0.25% $AgNo_3$ solution was injected into superficial lymphatic capillaries of the liver, we observed swollen endothelial cells with a less contrasting contour (profile). The intervals (spaces) between cells, filled with an argyrophil substance, are dilated up to 8-14 microns (Fig. 3). In these intervals, clearly expressed "openings" are found, which are not filled with the argyrophil substance. In some spots, fragmented sectors of argyrophil substance can be detected. We also observed a similar picture, during silver nitrate injection, in 4 cases of acute toxic liver dystrophy with Botkin's disease outcome. Casley-Smith, during an electron microscope study of lymphatic capillaries, reached the conclusion that, even in case of an insignificant thermal injury, their endothelial cells are separated from each other as a result of the tightening, arising during edema, of connective tissue fibers attached to these cells. We believe that the expansion of intercellular boundaries of the endothelium cannot be explained only on the basis of this mechanism. Already during the first 24 hours after poisoning, we observed a sharply expressed edema without any sign (pattern) of a great expansion of interendothelial boundaries, and such a pattern can be observed only during later periods. Apparently, the great expansion of interendothelial intervals (spaces) can be linked not only with the edema of the connective tissue, but also with an injury (damage) of the endothelial cells themselves, and with some sort of change of the intercellular substance (matter), caused by a disturbance of tissue metabolism.

FIGURE 3

Reaction of endothelial cells of a lymphatic vessels in the liver peritoneum of a 42-year old man 3 days after dichloroethane poisoning. Swelling of endothelial cells.



1-Sharp expansion of intercellular boundaries
2-Formation of intervals, not filled with argyrophil substance
Impregnation with silver. X280.

The lymphatic capillaries of the deep lymphatic system of the liver are dilated (Fig. 4) 3-5 times in comparison with the norm. A large number of capillaries with an irregular diameter (cross section) are present, in which dilatations alternate with sharp constrictions (stenosis). At the points where lymphatic capillaries are fused (joined) together, wide lacunae are formed, with dimensions of 0.06 x 0.18 and 0.105 x 0.12 mm. The lymphatic capillaries are closed up (joined) into small-loop, sheath-like networks, which are formed (shaped) in the connective tissue at the level of the axis of Rappaport's acinus. In spite of the fact that we studied the lymphatic system under conditions of high permeability of lymphatic capillaries and vessels, we are unable to detect any kind of passages or ducts (B. V. Ognev) connecting Disse's spaces with lymphatic capillaries.

FIGURE 4

Initial networks of lymphatic capillaries and vessels in the periportal connective tissue of the liver of a 40-year old man, who died 10 days after dichloroethane poisoning. Injection with Guerot's (?) mass. X16.

SUMMARY

On the basis of the date obtained, we believe that the changes in the structure and shape of lymphatic capillaries and in the architectonics of initial lymphatic networks indicate a significant disturbance of the function of the lymphatic system of the liver caused by the toxic action of the above mentioned poisons. We agree with D. A. Zhdanov, who believes that, in spite of the formation of openings in the wall of lymphatic capillaries, the ability of the latter to absorb water and to resorb proteins from tissues is impaired, since a necessary condition for the resorption from tissues of proteins and other colloids by the roots of the lymphatic system is the biological activity of a

whole (undamaged) and continous endothelial wall of lymphatic capillaries. In our opinion, the swelling of endothelial cells and the smoothing of their contour (profile), the expansion of intercellular intervals in the endothelium, the presence of openings in the walls of lymphatic capillaries and vessels all constitute a morphological expression of the resorptive insufficiency of the lymphatic system under conditions of the toxic action of poisons. The increased number and dilatation of lymphatic capillaries, the restructuring of the network of lymphatic capillaries and vessels in later periods after poisoning together with the growth of dystrophic and necrobiotic changes, the increased disruption of blood circulation combined with regeneration processes--all these factors can be considered as an adaptation of the roots of the lymphatic system of the liver to pathologically altered conditions, under which the liver performs its function.

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ЛИМФАТИЧЕСКАЯ СИСТЕМА ПЕЧЕНИ ЧЕЛОВЕКА ПРИ ОТРАВЛЕНИИ НЕКОТОРЫМИ ЯДАМИ

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В литературе имеются указания на связь лимфообразования с динамикой патологических процессов и на изменения начальной лимфатической сети в зависимости от функциональных расстройств и морфологических изменений в печени (Д. А. Жданов, 1940, 1952, 1964, 1965; М. А. Долгова; Ю. Ф. Викалюк; И. Русньяк с соавторами). А. В. Краев, изучая лимфатическую систему печени при отравлении животных четыреххлористым углеродом, отметил в период острого поражения увеличение диаметра поверхностных и глубоких лимфатических капилляров в 3—5 раз. В дальнейшем в лимфатических капиллярах и сосудах возникали белковые тромбы. При длительном введении яда автор наблюдал в утолщенной брюшине печени и в гипертрофированной междольковой соединительной ткани многослойность сети лимфатических капилляров.

Мы поставили перед собой задачу проследить реакцию лимфатической системы печени человека в условиях токсического действия некоторых ядов. Известно, что резко выраженная дистрофия гепатоцитов и нарушение кровообращения печени развиваются у людей при отравлении дихлорэтаном (Ф. Ф. Брыжин; А. В. Русаков, 1949), уксусной кислотой и каустической содой (А. И. Шибков, А. В. Русаков, 1930; Г. А. Минин и Г. Г. Захаров).

Исследована печень от 21 человека, умершего в первые часы и в последующие 19 дней после отравления уксусной кислотой [9], каустической содой [5], дихлорэтаном [7]; для сравнения исследована печень в 4 случаях острой токсической дистрофии печени в исходе болезни Боткина. Лимфатические капилляры и сосуды печени инъецировались 0,25% раствором азотнокислого серебра или массой Герота в модификации Жданова. Гистотопографию инъецированных лимфатических капилляров в брюшине печени исследовали на плоскостных тотальных и послойно расщепленных препаратах брюшины. Гистологические срезы окрашивали гематоксилин-эозином, по ван Гизону, суданом III, орсеином, резорцин-фуксином, анилиновым синим; производили импрегнацию по Футу и Снесареву.

У лиц, умерших в первые сутки после отравления дихлорэтаном, печень увеличена, ткань ее дряблая, на разрезе красно-коричневого цвета. Гистологически обнаружены расширение кровеносных сосудов, капилляростаз. В пространствах Диссе нередко содержатся эритроциты. Печеночные клетки находятся в состоянии мутного набухания; отмечается нерезко выраженное мелко- и крупнокапельное ожирение клеток. Видны единичные некротические очаги паренхимы. Набухшие пучки коллагеновых волокон стромы раздвинуты отечной жидкостью. Внутридольковые ретикулярные волокна импрегнируются отчетливо. Лимфатические капилляры и сосуды расширены. В единичных лимфатических капиллярах встречаются белковые коагулянты.

При отравлении уксусной кислотой печень не увеличена, дряблая, на разрезе с очагами красного цвета. Микроскопически обнаруживается тромбоз печеночных вен. Эндотелий кровеносных сосудов слущен, синусоиды расширены, иногда заполнены эритроцитами. Печеночные клетки с явлениями зернистой дистрофии и мелкокапельного ожирения,

встречаются очаги кровоизлияний, некробиоза и некроза. Зона некротического поражения обычно расположена по периферии ацинуса Раппапорта, но не имеет четкой границы. Пучки коллагеновых волокон набухшие, без четкой структуры. Как и при отравлении дихлорэтаном, лимфатические капилляры и сосуды расширены, с единичными белковыми коагулятами.

Независимо от вида яда во всех случаях смерти больных в первые сутки наблюдалась однотипная реакция со стороны лимфатической системы печени. Уже в первые часы после отравления отмечен чрез-

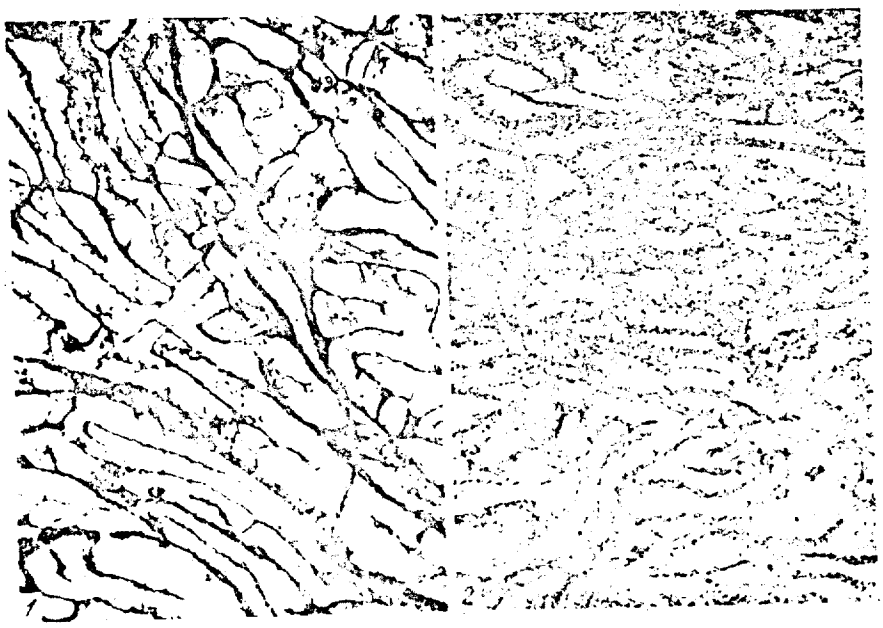

Рис. 1. Сеть лимфатических капилляров в брюшине печени мужчины 17 лет. Выход глыбок краски через стенки лимфатических сосудов и капилляров. Смерть через 7 часов после отравления дихлорэтаном.

Инъекция массой Герота. × 32.

Рис. 2 Сеть лимфатических капилляров в брюшине печени мужчины 35 лет. Расширенные лимфатические сосуды и капилляры. Смерть через 4 часа после отравления дихлорэтаном.

Инъекция массой Герота. × 32.

вычайно легкий выход инъекционной массы за пределы лимфатических капилляров и сосудов независимо от величины инъекционного давления (рис. 1). Размер поверхностных лимфатических капилляров печени 0,015—0,045 мм, что больше, чем в норме. Выявляется значительное количество капилляров с неровными, зазубренными контурами. В одном случае (рис. 2) уже в первые часы после отравления дихлорэтаном обнаружено особенно значительное расширение сети лимфатических капилляров, однако, как и в других наблюдениях, перестройки сети лимфатических капилляров и сосудов не выявлено. При исследовании плоскостных тотальных и послойно расщепленных препаратов брюшины печени отмечено, что сеть лимфатических капилляров залегает исключительно в глубоком коллагеново-эластическом слое. При инъекции в лимфатические капилляры и сосуды 0,25% раствора азотнокислого серебра интенсивность импрегнации межклеточных границ эндотелия выше, чем в норме. Импрегнированные серебром межклеточные границы неравномерной ширины.

Глубокие лимфатические капилляры также имеют неравномерный поперечник, некоторые из них с зазубренными контурами. В местах слияний капилляров много лакун. На основании исследования просветленных препаратов с инъецированными глубокими лимфатическими капиллярами и сосудами и изучения контрольных серийных гистологических срезов сомнительных участков мы также пришли к выводу, что лимфатические капилляры располагаются в прослойках соединительной ткани, содержащей коллагеновые волокна. Внутри долек лимфатические капилляры не обнаруживались. Непосредственных сообщений пространств Диссе с лимфатическими капиллярами выявить не удалось.

Рис. 3. Реакция эндотелиальных клеток лимфатического сосуда в брюшине печени мужчины 42 лет через 3 суток после отравления дихлорэтаном. Набухание эндотелиальных клеток.

1 — резкое расширение межклеточных границ; 2 — образование промежутков, не выполненных аргирофильным веществом.
Импрегнация серебром. × 280.

В случаях, когда больные погибли на 2-е сутки после отравления и позже, обнаружены нарастание дистрофических изменений печеночных клеток и выраженные признаки нарушения кровообращения. Микроскопически при отравлении дихлорэтаном выявлены застой и стазы. Синусоиды частью расширены, частью находятся в спавшемся состоянии, местами заполнены эритроцитами. Отмечаются выраженная дискомплексация балок, мутное набухание печеночных клеток и различная степень жировой инфильтрации. В цитоплазме некоторых печеночных и купферовских клеток обнаружено отложение желчного пигмента. Нередко встречаются очаги некроза печеночных клеток. Строма печени отечна, с круглоклеточной инфильтрацией.

При отравлении уксусной кислотой печень набухшая, на разрезе коричневого цвета. Гистологически обнаружен тромбоз печеночных вен. Капилляростаза и диапедеза эритроцитов не выявлено. Синусоиды или сильно расширены, или, напротив, в спавшемся состоянии. Гиперплазия клеток Купфера. Печеночные балки дискомплексированы. Встречаются обширные очаги мелко- и крупнокапельного ожирения печеночных клеток. В 2 случаях наблюдалась картина резко выраженного диффузного ожирения печеночных клеток. Пучки коллагеновых волокон набухшие. Лимфатические капилляры и сосуды в перипортальных полях и брюшине печени расширены.

Отмечаются бурный рост и перестройка поверхностных лимфатических капилляров печени. На 7—12—18-й день после отравления на плоскостных препаратах брюшины печени выявляются лимфатические капилляры непосредственно под пограничной мембраной. Соответственно видна перестройка пучков коллагеновых волокон поверхностных слоев наружного и внутреннего коллагена. Изучая плоскостные препараты брюшины печени в состоянии отека, мы не можем согласиться с М. С. Игнашкиной, считающей, что при отеке лимфатические капил-

ляры сдавливаются набухшими пучками коллагеновых волокон. Поверхностные лимфатические капилляры печени расширены в 3—5 раз по сравнению с нормой, замыкаются в мелкопетлистую сеть с петлями овальной или округлой формы. Лимфатические капилляры, лежащие в брюшине печени выше глубокого коллагеново-эластического слоя, часто замыкаются в петли прямоугольной или овальной формы и образуют поверхностную капиллярную лимфатическую сеть брюшины. В этих участках брюшины печени отмечается ясно выраженная трехмерная или двухслойная сеть лимфатических капилляров.

При инъекции 0,25% раствора азотнокислого серебра в поверхностные лимфатические капилляры печени мы наблюдали набухшие эндотелиальные клетки с менее контрастными контурами. Промежутки между клетками, выполненные аргирофильным веществом, расширены до 8—14 мк (рис. 3). В этих промежутках встречаются ясно выраженные «отверстия», не выполненные аргирофильным веществом. Местами обнаруживаются фрагментированные участки аргирофильного вещества. Подобные картины при инъекции азотнокислого серебра мы наблюдали и в 4 случаях острой токсической дистрофии печени в исходе болезни Боткина. Casley-Smith, исследуя электронномикроскопически лимфатические капилляры, пришел к выводу, что даже при незначительном тепловом повреждении их эндотелиальные клетки отделяются друг от друга в результате возникающего при отеке натягивания прикрепленных к ним соединительнотканных волокон. Мы думаем, что расширение межклеточных границ эндотелия нельзя объяснить только этим механизмом. Уже в первые сутки после отравления мы наблюдали резко выраженный отек без картин сильного расширения межэндотелиальных границ, и они выявляются лишь в более поздние сроки. Очевидно, можно связать сильное расширение межэндотелиальных промежутков не только с отеком соединительной ткани, но и с поражением самих эндотелиальных клеток и каким-то изменением межклеточного вещества, обусловленным нарушением тканевого метаболизма.

Рис. 4. Начальные сети лимфатических капилляров и сосудов в перипортальной соединительной ткани печени мужчины 40 лет. Смерть через 10 суток после отравления дихлорэтаном. Инъекция массой Герота. × 16.

Лимфатические капилляры глубокой лимфатической системы печени расширены (рис. 4) в 3—5 раз по сравнению с нормой. Имеется множество капилляров с неравномерным поперечником, расширения сменяются резкими сужениями. В местах слияний лимфатических капилляров образуются широкие лакуны размером 0,06×0,18 и 0,105×0,12 мм. Лимфатические капилляры замыкаются в мелкопетлистые футлярообразные сети, которые сформированы в соединительной ткани на уровне оси ацинуса Раппапорта. Несмотря на то что мы иссле-

довали лимфатическую систему в условиях повышенной проницаемости лимфатических капилляров и сосудов, выявить какие-либо ходы (Б. В. Огнев), связывающие пространства Диссе с лимфатическими капиллярами, нам не удалось.

Заключение

На основании полученных данных мы полагаем, что изменения строения и формы лимфатических капилляров и архитектоники начальных лимфатических сетей свидетельствуют о значительном нарушении функции лимфатической системы печени при токсическом действии перечисленных выше ядов. Мы согласны с Д. А. Ждановым, который полагает, что, несмотря на образование отверстий в стенке лимфатических капилляров, страдает способность капилляров всасывать воду и резорбировать белки из тканей, так как обязательным условием резорбции корнями лимфатической системы из тканей белков и других коллоидов является биологическая активность цельной и сплошной эндотелиальной стенки лимфатических капилляров. По нашему мнению, набухание эндотелиальных клеток, сглаживание их контуров, расширение межклеточных промежутков эндотелия, наличие отверстий в стенках лимфатических капилляров и сосудов являются морфологическим выражением резорбционной недостаточности лимфатической системы в условиях токсического действия ядов. Увеличение числа лимфатических капилляров, их расширение, перестройку сети лимфатических капилляров и сосудов в поздние сроки после отравления при нарастании дистрофических, некробиотических изменений, усилении нарушения кровообращения в сочетании с процессами регенерации можно считать адаптацией корней лимфатической системы печени к патологически измененным условиям, в которых осуществляется функция.

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THE LYMPHATIC SYSTEM OF THE HUMAN LIVER IN POISONING WITH SOME POISONS

E. G. Boiko, G. A. Frank

Summary

On the basis of studying the liver obtained from 21 persons who died within the first hours and the subsequent 19 days after poisoning with acetic acid, caustic soda and dichlorethane, the authors consider that the changes of the structure and the shape of lymphatic capillaries, architectonic of the initial lymphatic networks pointed to a considerable functional disturbance of the lymphatic system of the liver. Swelling of endothelial cells, smoothening of their contours, dilatation of the intercellular endothelial spaces, openings in the walls of lymphatic capillaries and vessels served as a morphological manifestation of resorptive insufficiency of lymph outflow. Increase of the number of lymphatic capillaries, their dilatation, reconstruction of the network of lymphatic capillaries and vessels may be considered as a compensatory adaptation of the roots of hepatic lymphatic system due to the pathologically changed conditions and functions.

LAB BENCH

Determination of Acetic Acid in Vinegar Using Gravimetric Titrimetry

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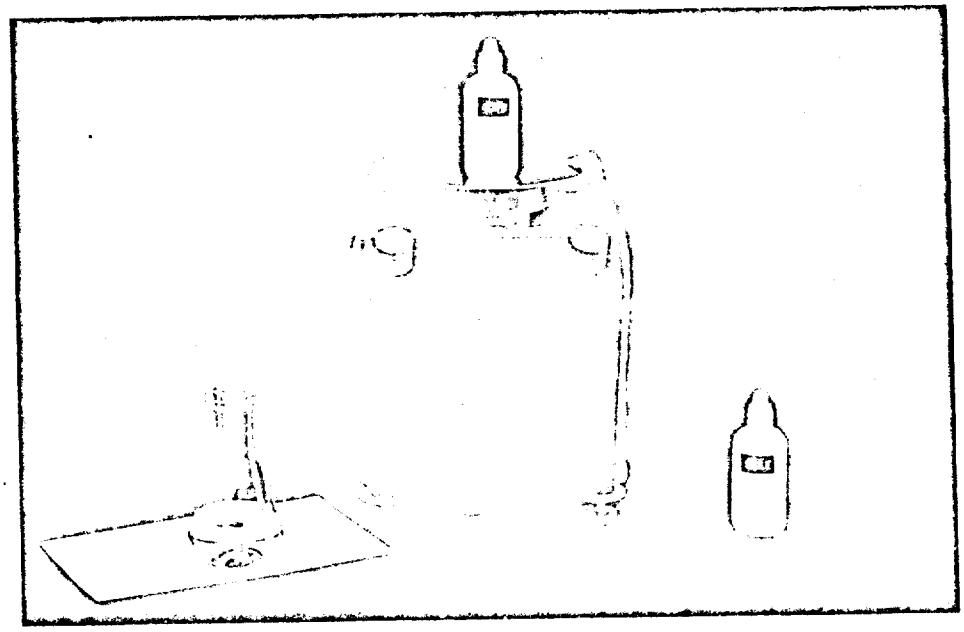
For generations, familiarity with titration analysis has been a part of every student's experience in general chemistry. Titration is commonly a process of determining the volume of a solution needed to react with a given weight, or volume, of a sample. This process is used to study quantitatively the reaction between an acid and a base, or a redox reaction, as well as certain other types of reactions. In the following experiment, an unusual titration technique is used to determine the acidity of a sample of vinegar, using phenolphthalein as the indicator. The phenolphthalein signals the end point of the titration by converting from a colorless molecular form to a red-colored ionic form that exists in a basic solution.

Until recently, most titrations were done volumetrically because gravimetric titrations were time-consuming. However, in 1955, the first top loading semi-analytical balance was marketed in this country, and the time necessary to complete a weighing readable to ±10 mg. or better was drastically reduced. This rapid method of weighing makes practical the technique of gravimetric titration, and has been employed successfully in quantitative analysis and general chemistry courses.

Experimental

The following apparatus and materials are required for this experiment: top loading balance with readability of ±0.01 gram (Mettler P-1200 or equivalent); 125-ml. Erlenmeyer flask; polyethylene wash bottle containing distilled water; two polyethylene dropping bottles (squeeze-type) having a 2-ounce capacity; clear household vinegar; phenolphthalein; and 100 grams of NaOH solution, 1.00 weight molar. Because weight molarity is defined as moles of solute per kilogram of solution, a 1.00 weight molar solution of standard NaOH can be prepared by taking 7.77 grams of commercially available concentrated NaOH solution and adding water to a final solution weight of 100.00 grams. This is accomplished easily and quickly on a top loading balance. The choice of the 7.77 grams of NaOH is determined by the actual assay on the bottle of concentrated NaOH solution. In this case,



Gravimetric titration assembly

the NaOH assayed 51.5%, by weight.

If a 50% commercial NaOH solution is not available, solid reagent grade NaOH can be used after Na_2CO_3 is removed and the solution is standardized. This procedure is necessary because reagent grade solid NaOH is not available in pure form. It is always contaminated by moisture and up to 3% of Na_2CO_3. Purification and standardization procedures can be found in most textbooks of quantitative analysis.

Procedure

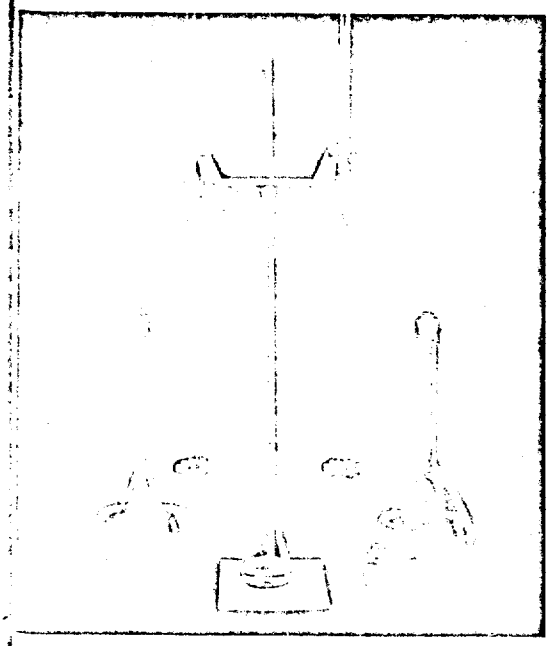
Obtain 50 ml. of the 1.00 weight molar solution of NaOH. Place in a dropping bottle, label, and weigh the bottle and contents. Fill a second dropping bottle with vinegar. Label and weigh. These two bottles constitute the weight burets.

Squeeze approximately 20 grams of the NaOH solution into the flask and add a few drops of phenolphthalein. Swirl to mix. Titrate to the phenolphthalein end point with the vinegar. The vinegar may be run in quickly for the first trial, and, if the end point is overrun, the solution may then be backtitrated with the NaOH solution to a sharp end point. Swirl constantly and observe the end point against a white paper background. While titrating, wash down the interior wall of the flask with distilled water from the wash bottle. This prevents any unreacted drops of titrant from clinging to the wall.

When a sharp end point is obtained, weigh the vinegar buret and the NaOH buret again. The weight of each reagent dispensed is found by subtracting the final weights from the original weights of the filled burets. Empty the contents of the flask, rinse, and repeat the weighings and titrations using different weights of NaOH each time until a constant ratio of reacting weights is established.

Discussion

Assuming 23.97 grams of vinegar are necessary to neutralize 20.05 grams of standard NaOH solution, we can conclude that vinegar is less concentrated than NaOH because a greater weight of vinegar was used. Thus, to find the concentration of the vinegar, multiply the concentration of the standard by a fraction *less* than one.



Typical volumetric titration assembly

The concentration of the vinegar is calculated:

$$\frac{20.05\text{ g.}}{23.97\text{ g.}} \times 1.00\text{ wt. molar NaOH} = 0.836\text{ wt. molar vinegar}$$

The above relationship is valid because 1 mole of NaOH reacts with exactly 1 mole of acetic acid, as shown by the balanced equation for the reaction.

$$NaOH + HC_2H_3O_2 \longrightarrow NaC_2H_3O_2 + H_2O$$

If all the acidity is due to acetic acid, $HC_2H_3O_2$, the 0.836 weight molarity can be interpreted to mean 8.36×10^{-2} mole acetic acid per 100 grams of vinegar solution, by our definition of weight molarity (moles of solute per kilogram of solution). Since $HC_2H_3O_2$ weighs 60.0 grams per mole, we have:

$$8.36 \times 10^{-2}\text{ mole/100 g. of soln.} \times 60.0\text{ g./mole } HC_2H_3O_2 = 5.02\text{ g. } HC_2H_3O_2\text{/100 g. soln.}$$

The acetic acid concentration is commonly expressed as per cent (grams of acetic acid per 100 ml. vinegar). The density of commercial vinegar averages 1.004 grams per ml.; therefore, 100 ml. of solution would weigh 100 grams.

Thus, within the limits of uncertainty, our results would correspond to a percentage of 5.02. Legal requirements specify that vinegar must contain a minimum of 4% acetic acid, and most household vinegar should assay close to 5%.

There are certain advantages to titrations based on weight rather than volume: Unlike the volumes, the mass of reacting solutions does not vary with temperature; drainage and buret reading errors are eliminated; there is no meniscus to interpret; fragile burets are replaced with inexpensive polyethylene squeeze bottles from which a drop or a stream of titrant can be dispensed easily; and highly viscous solutions, impossible to drain from conventional burets, can be titrated.

Suggested Further Experiments. Household ammonia is an aqueous solution of NH_3. This can be titrated gravimetrically using a 3.00 weight molar HCl solution and methyl orange as the indicator. Since the efficacy of household ammonia as a cleanser is a function of the NH_3 concentration, several brands of clear (nondetergent) ammonia can be compared using the gravimetric titration technique to determine their NH_3 concentration.

Many other titrations, traditionally done volumetrically, could be attempted on a gravimetric basis.

If the reader is mathematically inclined, he can undertake an analysis of the uncertainties associated with a volumetric titration and compare these with the uncertainties involved in a gravimetric titration to ascertain which of the methods can be made the most accurate.

Suggested Reading

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Energetics of Sheep Concerned with the Utilization of Acetic Acid [1,2]

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ABSTRACT The efficiency of utilization for growth-fattening, of the energy of diets resulting in high (5.4:1) and low (3.1:1) ratios of acetic acid to propionic acid in the ruminal ingesta was determined in 24 intact male and 24 female sheep by means of a slaughter-analysis experiment. To establish the chemical composition and energy value of the body at the beginning of the feeding period, nine other animals of each sex were analyzed. Each of two basal diets (pelleted, ground hay (H), and a pelleted mixture of the same hay and corn meal (HC)) was supplemented singly with triacetin (Ac^3) and glycerol (G). A given animal was fed continuously one of the four diets at one of two levels of intake (i.e., approximately 1 or 2.2 times the maintenance level) during the 175-day feeding period. For an increment of intake equivalent to the maintenance intake of gross energy (GE), the metabolizable energy (ME) value of the diets declined by the following rates: (in %) H + Ac^3, 3.5; H + G, 3.0; HC + Ac^3, 2.1; and HC + G, 3.6. The rate of depression in the digestible energy (DE) value was considerably greater than that of the ME value; thus, with increasing intake the energy loss in urine and methane declined. The amount of ME required to maintain body energy equilibrium was 87.1 kcal/(kg of body weight)$^{0.73}$ per day, as the mean for all diets and both sexes; this value was not significantly different ($P > 0.1$) from that for any diet or either sex. The mean rates with which the ME ingested above the maintenance level of intake was utilized for body energy gain, were: (in %) H + Ac^3, 59.9; H + G, 59.5; HC + Ac^3, 63.7; and HC + G, 61.8 ($P > 0.3$). Ignoring the kind of basal diet, the utilization rates were 62.0 and 61.2% for the ME provided by the diets containing triacetin and glycerol, respectively. The mean pooled net utilization of ME for body-energy gain by females (65.5%) was markedly greater ($P < 0.01$) than that by males (57.6%). In a series of respiration-calorimetric experiments, the net utilization of ME provided by the acetic acid moiety of triacetin was 76.4%, between days 50 and 70 of continuous feeding. On days 3, 15 and 30 after animals were changed abruptly from the continuous feeding of the H + G diet for 70 days to the H + Ac^3 diet, the net utilization of the ME of the acetic acid was 47.9, 77.0, and 85.4%, respectively. These observations, in concert with those made in the 175-day slaughter-analysis experiment, suggest that the sudden influx of a high level of acetic acid results in a high heat increment soon after the change, but that this subsides by day 15 of continuous feeding.

The absorbed volatile fatty acids (VFA), lactic acid, and sugars resulting from the digestion of mainly or solely carbohydrates are quantitatively important nutrients, but the degree of their significance depends on the kind of animal involved. As a consequence, attention has been given to the possibility that differences in the amounts and kinds of metabolites absorbed might explain the recognized differences in energetic efficiency among animals having different gut architectures and digestive agents. In ruminants, the relative amounts of the various metabolites produced and absorbed are associated with the nature of the diet. Since 1950, the viewpoint has developed that dietary treatments resulting in a ruminal mixture high in acetic acid and low in propionic acid effect a low energetic efficiency of lipogenesis, and vice versa. Recent evidence, however, including that to be reported here, questions the relatively high heat increment of acetic acid as an unequivocal phenomenon.

In acute, short-term studies in which individual VFA, lactate or glucose was infused intravenously into curarized, fasting rabbits (1–4) or was abruptly fed as a meal to fasting dogs (5), the heat increment of acetic acid or acetate was higher than that of the other metabolites studied. This observation is the historical basis of more recent studies with the ruminant.

For approximately a century the cause of the considerably higher heat increment of feeding in ruminants than in simple-gutted animals has been sought, but has remained a mystery. Von Mering and Zuntz (6) and Kellner (7) attributed the ruminant's high heat increment to the "work of digestion." Although early attempts to associate the high heat increment with the metabolism of short-chain fatty acids produced in the rumen were repelled, Marston (8) in 1948 revived this viewpoint, and McClymont (9) amplified its bases, proposing reasons why the metabolism of acetic and butyric acids might be the major cause. This hypothesis led to the magnificent series of experiments by Armstrong and Blaxter (10–16), the first of which was published in 1956.

In their experiments (14–16) concerned with lipogenesis, they infused acetic acid, propionic acid, or butyric acid (singly or mixed) continuously during 7-day periods into the rumen of sheep fed a maintenance ration, and the calorimetric efficiency of lipogenesis was determined by respiratory-exchange measurements and carbon-nitrogen balances determined during the final 4 or 5 days of the period. The average net efficiencies with which the metabolizable energy (ME) of the VFA infused singly was utilized for fattening, were: (in %) acetic acid, 32.9; propionic acid, 56.3; and *n*-butyric acid, 61.9. The ME provided by mixtures of acetic, propionic, and butyric acids in the molar proportions of 75:15:10 and 25:45:30 had a net efficiency of 31.8 and 58.1%, respectively. In similar experiments, Armstrong and Blaxter (14) infused glucose into the rumen, abomasum, or a jugular vein and observed that the net efficiencies with which the administered ME was utilized for body-energy gain were 54.5, 71.5, and 72.8%, respectively.

These findings strongly incriminated acetic acid as the VFA contributing to the high heat increment of the ruminant, and suggested that the absorption of VFA resulting from rumen microbial action on carbohydrates causes a lower lipogenic efficiency than that which is associated with the absorption of glucose per se. It was suggested further that the discrepancy in the net energy value of the digested nutrients between feeds, which Kellner (7) attempted to rectify by the application of his "fiber correction factor," might be attributable to differences in the absorbed products of carbohydrate digestion.

Nevertheless, it is difficult to reconcile these observations (14–16) with those made in other experiments. The efficiency with which the energy provided by acetic acid was utilized for body-energy gain was similar to that provided by glucose to rats (McManus et al. (17)) or rabbits (Crasemann and Schneeberger (18)), and by propionate or butyrate to sheep (Elliott et al. (19); Ørskov et al. (20–23)). The criteria of response in the experiments of Ørskov and co-workers (20–23) were body-weight changes, carcass specific gravity and the fat concentration of the loin cut; however, we arrived at the same conclusion by estimating body energy storage (24) from their data on empty-body weight changes.

There are several obvious differences among the various experiments (14–23): a) the kinds of animals involved, b) the chemical form and manner in which the VFA were administered, c) the method by which energetic efficiency was determined, and d) the length of the time period during which the metabolite under study was administered prior to or during the estimation of energy storage. In the experiments of Armstrong and his associates (14–16), the determination of heat production was begun after only 2 or 3 days of the continuous ruminal infusion of the metabolite had elapsed. In the other studies (19–23) with sheep, energy storage was estimated during feeding periods of 50 to 122 days. Whether the apparent discrepancy among the various experiments is attributable to the length of the period during which the metabolites were administered has not been ascertained.

The objectives of the present study were: a) to examine the energetic efficiency o maintenance and body gain in sheep fe diets resulting in ruminal mixtures hig

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[2] The data presented here are a part of the Ph.D. thesis by L. S. Bull to the Graduate School, Cornell University, 1969.
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in acetic acid or propionic acid during a long-term feeding period, and b) to investigate the possibility that sheep might adapt metabolically to the abrupt influx of large amounts of acetic acid which might explain the low energetic efficiency of this acid as observed in short-term ruminal infusion experiments.

EXPERIMENTAL PROCEDURES

The studies to be reported here consisted of: a) a long-term, body-energy balance experiment, and b) a series of short-term, respiration-calorimetric experiments. The design and procedures employed in the two kinds of experiments will be described in the following paragraphs.

Long-term, body-energy balance experiment

Animals. The experimental subjects were 66 purebred Southdown lambs (33 rams and 33 ewes). During a 2-month period following their procurement and prior to their allotment to the experiment, all animals were fed a late-cut, mixed-hay diet ad libitum, were deparasitized, accustomed to the quarters, weighed at intervals and ranked according to body weight within sex. The age of the lambs at the beginning of the experimental feeding period ranged from 6 to 7 months.

Of the 33 animals of each sex, nine, representing the entire body-weight range (14.2 to 27.1 kg) at uniform intervals, were slaughtered at the beginning of the experiment. Their minced ingesta-free, wool-free bodies were analyzed for water, ether extractives, protein, ash and heat of combustion. The remaining 24 lambs of each sex were sorted into three body-weight subgroups (designated as light, medium and heavy) of eight animals each. The members of each subgroup were randomly allotted to the eight treatments of the factorial experiment described below.

In order to distribute the work load at the end of the experiment the three replicates (each of eight males and eight females) were begun at different times; 1 week intervened the beginning of replicates 1 and 2, and 2 weeks separated the beginning of replicates 2 and 3. The males and females of a given replicate were begun on consecutive days. (On the day of assignment of the eight weight-sex, subgroup animals, their corresponding three mates were killed to determine the beginning body composition and energy value.) This allotment plan made it possible to impose a feeding period of 175 days on all animals, and it required that only eight animals be killed and processed on a given day at the end of the experiment.

Experimental design and dietary treatments. The feeding experiment was factorially designed as outlined in figure 1, with two basal diets × two additives (i.e., triacetin [5] and glycerol) × two levels of energy input × two sexes × 3 replications. The two basal diets consisted of a) pelleted, finely ground (3.1 mm sieve) Cayuga alfalfa hay harvested on May 25 near Ithaca, N. Y., partially dried in the field and finished by heat drying without weather damage, and b) a pelleted mixture containing 50% of the same hay and 50% of yellow corn meal.

[5] Triacetin, $(CH_3COO)_3C_3H_5$, with boiling point 152–154°/22 mm, was obtained from Distillation Products Industries, Rochester, N. Y.

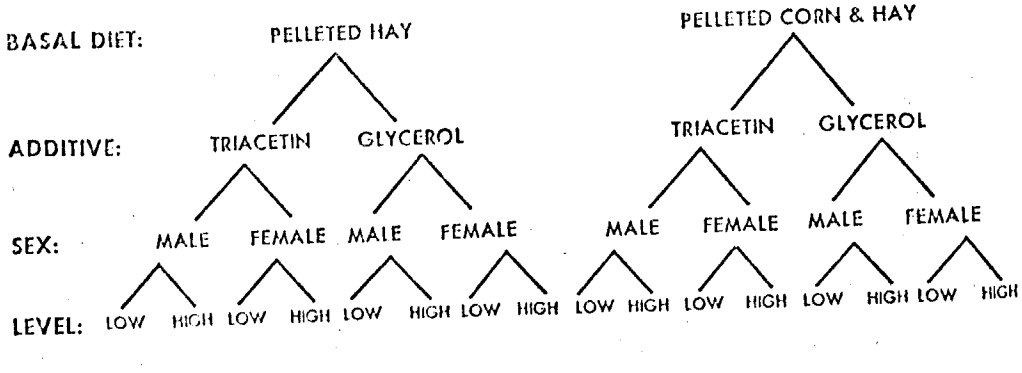

Fig. 1 Design of the long-term (175-day) experiment.

Triacetin, employed as a source of acetic acid, was added to the basal diets in sufficient quantity to comprise 10% of the total dietary dry matter. Preliminary study had revealed that some hydrolysis of triacetin (as evidenced by the presence of free acetic acid) occurs at room temperature within several days after mixing triacetin with feed pellets; as a consequence, the triacetin was stored at about 4° and the feed containing it was prepared on the day during which it was fed. After the daily feed was prepared, each animal's daily allowance was weighed and stored at 4° until fed. The daily allowance of feed was provided in three meals (8 AM, 1:30 PM, and 7 PM).

An amount of glycerol equivalent to that in the triglyceride, triacetin, was added to the other diets. Table 1 summarizes the proximate chemical composition and gross energy values of the basal diets and table 2 shows the proportions of energy ingested, supplied by the various components of the four diets (i.e., two basal diets × two additives).

Each of the four diets was imposed at each of two levels of intake designated as follows: a) high, approximately 2.2 times the maintenance level of metabolizable energy (ME) input, and b) low, about 1.1 times the maintenance input of ME. Throughout the 175-day feeding period, each animal received a constant amount of feed per unit of metabolic size (i.e., body weight $kg^{0.73}$). The feed allowances were adjusted weekly in accordance with the body weight changes. Since the diets had different ME values, the dry matter allowances of the four diets were adjusted accordingly to provide approximately the same amounts of ME at each of the two intake levels.

Samples of the basal diets were taken each day for the determination of moisture and the dried samples were then compounded for 7-day periods and prepared for proximate chemical and energy analyses. Although the method of rationing, in which the animals on the high level of intake were fed an amount slightly less than an ad libitum intake, largely obviated the refusal of feed, any refused feed was collected daily and analyzed.

Management and housing of animals. Each of the 18 animals employed to establish the initial body composition and energy value was shorn on the day prior to slaughter. Each of the 48 animals in the 175-day feeding experiment was shorn both on the

TABLE 1

Proximate composition and heat of combustion value of basal diets used in feeding experiment [1]

Basal diet	Protein	Ether extract	Ash	Carbohydrate	Gross energy
	%	%	%	%	*kcal/g*
Hay	24.48	2.71	8.99	63.82	4.546
Corn and hay	18.02	3.29	5.23	73.46	4.532

[1] All values expressed on the absolute dry basis.

TABLE 2

Proportions of gross energy supplied by basal diets and additives

Diet	Component		
	Basal diet	Acetic acid	Glycerol
	% of gross energy		
Hay + triacetin	89.8	8.4	1.8
Hay + glycerol	98.3	—	1.7
Corn + hay + triacetin	89.8	8.4	1.8
Corn + hay + glycerol	98.2	—	1.8

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day prior to the beginning, and on that prior to the end, of the feeding period, which was the day before slaughter. The wool and minced body tissue representing the total ingesta-free body were analyzed separately for moisture, ether extractives, nitrogen, ash and heat of combustion by methods previously outlined (25, 26).

During the feeding period all animals were housed individually in a battery of comfortable metabolism cages each of which has 1 m^2 of floor space and provides freedom of movement of the animal within this area at all times. Each unit has an expanded metal floor and is equipped with devices for the separation and quantitative collection of the urine and feces and for the quantitative feeding of the individual animal.

Metabolism trials. Urine and feces were collected totally, but separately, from each of the 48 animals during 7-day periods of months 2, 4 and 6 of the 175-day feeding period. For 14 days prior to, and during, the 7-day collection period, the feed intake of each animal was held constant. Aliquots of feces were analyzed for the proximate chemical constituents and heat of combustion, and samples of urine were analyzed for nitrogen. Urinary energy was estimated from urinary nitrogen as outlined by Paladines et al. (27), and the amount of energy represented by the methane loss was computed from the amount of digestible dry matter in accordance with the procedure outlined by Swift and French (28).

Slaughter and whole-body analysis. After an animal was killed by exsanguination, the total ingesta-free, wool-free body with the blood added was minced by means of a high-capacity grinder. Regrinding the total mass six times produced a very homogeneous sample as determined by previous studies of sampling errors. Duplicate 1-kg samples were weighed into tared pans covered with aluminum foil and stored at −10° until they were freeze-dried. After drying and the determination of the moisture content (based on a constant weight on 2 consecutive days), the duplicate dry samples were broken into pieces, pooled, mixed with crushed dry ice and ground through a 0.16 cm screen of a Wiley mill. After the dry ice had sublimated, the sample was mixed and a subsample was stored at −10° until analyzed for moisture (by freeze-drying), ether extractives, nitrogen, ash and heat of combustion according to methods described previously (25, 26).

Measurement of energy storage in the body and of energetic efficiency. The body-energy gain of each of the 48 animals in the feeding experiment was determined as the difference between the amounts of body energy at the beginning and at the end of the feeding period plus the amount of energy represented by the wool grown during the 175-day period. Body energy at the end was determined by direct analysis while that at the beginning was estimated by means of equations (table 3) relating the empty-body weight to the energy value of the body as derived from the data obtained for the nine animals of each sex killed at the beginning of the experiment.

The net energetic efficiency of body energy gain was computed by regressing body-energy gain on the metabolizable energy (ME) intake with both variables expressed in kilocalories/day per kilogram of metabolic size (i.e., body weight $kg^{0.73}$). Since the feeding of even the lower level of all diets resulted in a positive energy balance, the coefficient of X in the regression equations × 100 represents the percentage of ME ingested above maintenance

TABLE 3

Relationship between the energy value and weight of wool- and ingesta-free body of the sheep at the beginning of the experiment

Sex	Regression equations [1]	$S_{y \cdot x}$ [2]	R [3]
		Mcal	
Males	Y = 3.56 X − 23.6	3.94	0.922
Females	Y = 4.47 X − 32.9	3.77	0.947

[1] Y = Energy value (Mcal) of wool- and ingesta-free body, and X = weight (kg) of wool- and ingesta-free body.
[2] $S_{y \cdot x}$ = Standard error of estimate.
[3] R = Correlation coefficient.

that was stored (table 5). Extrapolation of the same relationships to the zero energy balance derives the amount of ME required for maintenance. Such values were employed to compare the four dietary treatments and the two sexes of sheep.

Statistical analysis. Both linear and nonlinear regression analyses were applied to certain relationships examined in this study. Appropriate tests of differences between slopes, intercepts and predicted values were applied. A factorial analysis of variance was employed to test the differences between means of the several factors and variables studied. All methods used here are in accordance with those described by Steel and Torrie (29).

Respiration-calorimetric experiments

Soon after the completion of the slaughter-analysis experiment, it became apparent that the observed energetic efficiency of acetic acid for body energy storage was different from that of previous experiments (14–16) of short term in which respiration calorimetry was the method employed. As a consequence, a series of respiration experiments was conducted to determine whether adaptive behavior might account for the discrepancy.

Animals. Six purebred Southdown rams, constituting two groups of three rams each, were employed in this experiment. They were approximately 1 year of age at the beginning and weighed 30 to 46 kg. When the animals were not in the respiration chamber, they were housed in the metabolism cages described above. Each animal was shorn twice during the 100-day experiment.

Diets and design. The diets studied consisted of a) pelleted alfalfa hay plus triacetin, and b) pelleted alfalfa hay plus glycerol. These diets were fed in amounts which provided the following quantities of gross energy per (kilogram of body weight)$^{0.73}$ per day: a) 227 kcal as alfalfa hay and 24.4 kcal as triacetin (i.e., 20.3 kcal as acetic acid and 4.1 kcal as glycerol); or b) 227 kcal as alfalfa hay and 4.1 kcal as glycerol. (The total amounts of energy ingested as well as the proportion supplied by acetic acid were similar to those imposed in the ruminal infusion studies of Armstrong and Blaxter (14–16). Each of the diets was fed continuously to three rams during a 70-day period. At the end of 70 days, the three rams on each diet were changed abruptly to the opposite diet and provided the amounts of energy listed above for an additional period of 30 days.

Heat-production measurements. During the first 50 days, each animal was confined in the respiration chamber several times for 2-day periods. When an animal was in the chamber (constructed of transparent acrylic plastic, 95 mm thick), he could see another animal facing him about 2 m away. The latter animal then replaced the former in the chamber and another animal was brought in turn to face him. During these periods, the calorimeter was operated as it would be during experimental periods in order to accustom the animals to the experimental conditions and operations.

During two intervals between days 50 and 70, and during days 3, 15, and 30 after the reversal of the diets, the heat production of each animal was determined. Heat production was determined indirectly from the respiratory exchange as measured in an open-circuit respiration chamber. Normal "outside" air was drawn into the sealed chamber, the outgoing air was exhausted through duplicate gas test meters, and continuous aliquots were taken by means of two constant-filling, sampling spirometers. Samples of both the incoming and outgoing air were analyzed for oxygen, carbon dioxide and methane. Oxygen and carbon dioxide were determined by the Haldane method as modified by Carpenter et al. (30) and methane was determined by a gas chromatographic method (31). Based on the amount of oxygen consumed, the amount of carbon dioxide and methane produced, and on the amount of nitrogen excreted in the urine, the amount of heat produced was computed using the equation reported by Brouwer (32).

Feces and urine collections were made during metabolism trials conducted as described above for the body-balance experiment.

The heat increment attributable to acetic acid was computed as the difference between the amounts of heat produced by the animals receiving the two diets before

the diet reversal (i.e., between days 50 and 70). The heat increment of acetic acid after the reversal of diets (between days 71 and 100) was computed as the difference in heat production between-diets and within-animal. The calorimetric efficiency of acetic acid was considered to be: 100% minus the percentage of the ME of acetic acid determined as heat.

RESULTS AND DISCUSSION

Long-term, body-energy balance experiment

Metabolizability of dietary energy. The data obtained in the 144 individual metabolism trials (i.e., three trials with each of 48 animals or 36 observations for each diet) were employed to determine the influence of the various dietary factors on the metabolizability of the energy, and ultimately to derive the metabolizable energy (ME) intake of each animal. The mean digestible energy (DE) and ME values of the four diets at each of two intake levels are summarized in table 4 as percentages of the gross energy (GE) ingested. Ignoring level of input and kind of additives, the mean DE value of hay + corn diets (79.4%) was 8.2 percentage-units higher than that of the hay diets (71.2%), and ignoring level of input and kind of basal diet, the DE value of diets containing triacetin (76.1%) was 1.6 percentage-units higher than that of the diets containing glycerol (74.5%). Digestibility of the energy at the low level of intake (77.9%) was 5.2 percentage-units greater than that at the high level of intake (72.7%), disregarding kind of basal diet and kind of additive. A factorial analysis of variance revealed that all of these differences are highly significant statistically ($P < 0.005$). In addition, a significant interaction ($P < 0.01$) was found between kind of basal diet and level of intake which is attributable to a greater rate of depression in the digestibility of the energy of corn + hay diets with increasing intakes than in that of the hay diets. No differences were observed between the sexes ($P > 0.1$).

The relationship between the DE value of each diet and the GE intake was examined further in a regression analysis. The relative digestibility of GE (i.e., the DE expressed as a percentage of the DE value of a given diet at the maintenance level of GE input) was regressed on the GE intake expressed as a multiple of the maintenance intake. This revealed that the relative digestibility of GE of the four diets declined at the following rates for each increment of GE intake equivalent to that of maintenance: (in %) hay + triacetin, 4.5; hay + glycerol, 4.3; corn + hay + triacetin, 5.5; and corn + hay + glycerol, 6.2.

The relative metabolizability of the GE (i.e., the ME expressed as a percentage of the ME value of each diet at the maintenance level of GE intake) was regressed on the GE intake expressed as a multiple of the maintenance intake. For an increment of GE intake equivalent to the maintenance intake, the relative metabolizability of the GE of the four diets declined at the following rates: (in %) hay + triacetin, 3.5; hay + glycerol, 3.0; corn + hay + triacetin, 2.1; and corn + hay + glycerol, 3.6. A comparison of these rates with the rates of depression in the DE value that accompany increasing GE intakes indicates that the increasing loss of energy in the feces is partially compensated by a decreasing loss of energy in the urine and digestive gases. With increasing intake, the urinary energy loss changed considerably more than did the estimated methane loss.

Intake of metabolizable energy. The ME intake, in kcal/(kilogram of full-body weight)$^{0.73}$ per day was regressed on the GE intake (kcal/(kilogram of full-body weight)$^{0.73}$ per day). Equations relating these two variables for each of the four diets are summarized in table 5. Although separate equations were determined for the rams and ewes, they did not differ significantly ($P > 0.1$). The equations shown in table 5 were employed to determine the mean daily ME intake of each animal during the 175-day feeding period. The ME intakes so derived were used to compute the mean ME intakes within level and diet as summarized in table 6, with the corresponding GE intakes.

Relationship between body energy gain and the metabolizable energy intake. In order to determine the maintenance requirement and the net efficiency of utilization of ME for body-energy gain, the body-energy gain (including the wool-energy gain) was regressed on the intake of ME of each of the four diets. These equations, representing the data for both sexes, the pooled data for both basal diets and sexes, the pooled data for all diets for males and females separately, and the pooled data for all diets and both sexes, are summarized in table 7.

By solving the equations in table 7 for $Y = 0$, the intakes of ME required to maintain body-energy equilibrium were derived. These values, summarized in table 7, are

TABLE 4

DE and ME values of the diets at each of two intake levels

Diet	GE[1] intake	DE[2]	ME[3]
	kcal/day/body weight$^{0.73}_{kg}$	*% of gross energy*	
Hay + triacetin	158	73.8	55.9
	342	69.9	53.6
Hay + glycerol	161	72.2	53.0
	346	68.6	51.2
Corn + hay + triacetin	144	83.1	65.3
	310	77.1	63.6
Corn + hay + glycerol	147	82.1	64.0
	324	74.9	60.9

[1] GE = Gross energy.
[2] DE = Digestible energy.
[3] ME = Metabolizable energy.

TABLE 5

Relationship between the ME and GE intakes

Diet	Regression equations[1,2]	S y · x[3]
		kcal/day/body weight$^{0.73}_{kg}$
Hay + triacetin	Y = 0.517 X + 6.6	3.8
Hay + glycerol	Y = 0.497 X + 5.4	3.3
Corn + hay + triacetin	Y = 0.619 X + 5.1	2.9
Corn + hay + glycerol	Y = 0.580 X + 9.1	5.7

[1] Y = Metabolizable energy intake $\left(\text{kcal/day/body weight}^{0.73}_{kg}\right)$, and X = gross energy intake $\left(\text{kcal/day/body weight}^{0.73}_{kg}\right)$.
[2] Since the relationships for males were not different from those for females ($P > 0.1$), the data for the two sexes were pooled; the slopes of the regression lines were significantly ($P < 0.05$) different between basal diets but were not significantly different ($P > 0.1$) between triacetin and glycerol within basal diet.
[3] S y · x = Standard error of estimate.

TABLE 6

Average intakes of GE and ME during the 175-day feeding period

Diet	Level of intake	GE[1]	ME[2]
		kcal/day/body weight$^{0.73}_{kg}$	
Hay + triacetin	Low	144	80
	High	336	181
Hay + glycerol	Low	151	80
	High	329	169
Corn + hay + triacetin	Low	132	86
	High	301	192
Corn + hay + glycerol	Low	134	86
	High	317	195

[1] GE = Gross energy.
[2] ME = Metabolizable energy.

TABLE 7

Relationship between the ME intake and the gain in body and wool energy

Diet and sex	Maintenance requirement for ME	Prediction equation [1]	$S_{y \cdot x}$ [2]
	kcal/body weight$_{kg}^{0.73}$ /day		*kcal/body weight$_{kg}^{0.73}$ /day*
Hay + triacetin [3]	88.5	Y = 0.599 X − 53.0	4.7
Hay + glycerol [3]	86.4	Y = 0.595 X − 51.4	6.9
Corn + hay + triacetin [3]	87.8	Y = 0.637 X − 55.9	4.8
Corn + hay + glycerol [3]	85.1	Y = 0.618 X − 52.6	5.5
Triacetin (both basals)	88.3	Y = 0.620 X − 54.7	4.8
Glycerol (both basals)	86.1	Y = 0.612 X − 52.7	6.1
All diets (males)	88.0	Y = 0.576 X − 50.7	4.7
All diets (females)	86.6	Y = 0.655 X − 56.7	4.3
All diets (both sexes)	87.1	Y = 0.616 X − 53.7	5.4

[1] Y = Body energy + wool energy $\left(\text{kcal/body weight}_{kg}^{0.73}\text{/day}\right)$, and X = ME intake $\left(\text{kcal/body weight}_{kg}^{0.73}\text{/day}\right)$.
[2] $S_{y \cdot x}$ = Standard error of estimate.
[3] Including both sexes.

not significantly different from each other or from the mean requirement of 87.1 kcal of ME/(kilogram of body weight)$^{0.73}$ per day, representing all data ($P > 0.1$). Also, this value closely agrees with those obtained with hay + concentrate diets by Marston (8), 86 kcal; Burton,[6] 87 kcal; and Paladines et al. (33), 82 kcal; and with pelleted, ground hay of good quality by Blaxter and Graham (34), 89 kcal (our calculations based on all positive balances); and Paladines et al. (33), 87 kcal. For the same hay fed in chopped form, the latter workers obtained a value of 91 kcal/(kilogram of body weight)$^{0.73}$ per day.

The slopes of the regression lines (i.e., the coefficients of X in the equations shown in table 7) multiplied by 100 represent the net efficiencies (%) with which the ME ingested above the maintenance level of intake was utilized for body gain and the incidental wool growth. The mean efficiency of utilization of the ME provided by the diets containing triacetin (62.0%) was not different ($P > 0.1$) from that of the ME of the control diets containing glycerol (61.2%).

The rumen ingesta of the animals fed the triacetin diets contained a considerably higher proportion of acetic acid ($P < 0.05$) and a lower proportion of propionic acid ($P < 0.05$) than that of the sheep fed the glycerol-containing diets. The average molar ratios of acetic acid to propionic acid in the ruminal ingesta of animals fed the hay basal diet supplemented with triacetin and glycerol were 5.35:1 and 3.25:1, respectively. The corresponding ratios for the ingesta of animals fed the corn + hay basal diet were 5.25:1 and 2.88:1, respectively. These differences between additives within basal diet were highly significant ($P < 0.01$). Thus, the ME from ruminal mixtures of metabolites high in acetic acid (or low in propionic acid) was not utilized any less efficiently than mixtures low in acetic acid (or higher in propionic acid).

This observation, made as the result of a 175-day body-energy balance experiment, agrees with the apparent results of Elliot et al. (19) and our calculations based on the studies of Ørskov and co-workers (20–23), both of which involved long-term feeding periods with sheep. However, our observation is distinctly different from the results of Armstrong and his associates (14–16) obtained in short-term ruminal infusion experiments with sheep fed maintenance rations.

The mean pooled net utilization of ME was 59.6% for the hay basal diet and 62.8% for the corn-plus-hay basal diet, ignoring additives. This difference was not statistically significant ($P > 0.3$).

Difference in energetic efficiency between the sexes. The digestibility and metabolizability of energy were not differ-

[6] Burton, J. H. 1967 The effects of age and weight on the body composition of sheep. M.S. thesis, Cornell University, Ithaca, N. Y.

ent between the males and females ($P > 0.1$). Also, the amount of ME required for the maintenance of body energy equilibrium was the same, 88.0 and 86.6 kcal/metabolic size (kg) per day, for males and females, respectively. However, as shown in table 7, the efficiency with which ME was utilized for body-energy gain was decidedly greater in females than in males for each diet studied ($P < 0.01$). Ignoring the kinds of basal diet and additives, the mean pooled net efficiencies were 65.5 and 57.6% for females and males, respectively.

For some time it has been believed that the ME ingested above the maintenance level is utilized by cattle more efficiently for milk production (70%) than for body gain (58%) (see review of this subject by Reid (35)). For the most part, the data from which such estimates have been made represent those for milk production by cows only, and those for body gain by steers mainly. Thus, the results of the present study raise the question whether the apparent difference in energetic efficiency between these body functions in cattle might be the result of the confounding of body function with sex. On the other hand, in recent short-term experiments with female goats Armstrong and Blaxter (36) observed efficiencies of utilization of ME of the order of 50 and 70% for fattening of nonlactating goats and for milk secretion at zero energy balance, respectively. In addition, their studies indicated that the energetic efficiency of body fattening occurring simultaneously with milk production is similar to the energetic efficiency of milk production.

In the present study, the average rates of feed conversion were 6.28 ± 0.52 and 5.85 ± 0.42 kg of dry matter per kilogram of body weight gain by females and males, respectively. Although the difference between the sexes was not significant ($P > 0.1$), this is in marked contrast with the pronounced sex difference in the net utilization of ME for body-energy gain. This peculiarity reflects the decided differences in body composition between the females and males. From the lowest to the highest body weights involved in this study, the females contained 30 to 35% more fat, 5 to 16% less water and 3 to 15% less protein, but 17 to 19% more energy, than the males.

Net energy value of diets. The net energy (NE) value (for fattening) of each diet studied (table 8) was computed at the maintenance and 2.5 × maintenance levels of intake for each sex, employing the data summarized in tables 5 and 7. These data show the markedly higher NE value of each diet for females than for males, and the decidedly lower NE value of each diet ingested at a production level of intake than at the maintenance level. The diminishing NE value with increasing intake level is the result chiefly of the increasing fecal energy loss discussed above. The NE value of corn was derived by difference. Its value (211 Mcal/100 kg of dry matter) at the maintenance level of

TABLE 8

Net energy value of each diet at two intake levels by each sex

Diet	Intake level [1]	Net energy value for Males	Net energy value for Females
		Mcal/100 kg dry matter	
Hay + triacetin	1.0	141	165
Hay + triacetin	2.5	134	158
Hay + glycerol	1.0	129	157
Hay + glycerol	2.5	124	151
Corn + hay + triacetin	1.0	185	194
Corn + hay + triacetin	2.5	178	187
Corn + hay + glycerol	1.0	170	197
Corn + hay + glycerol	2.5	160	184
Corn [2]	1.0	211	236
Corn [2]	2.5	196	217

[1] Expressed in multiples of the maintenance requirement for GE (i.e., the GE intake at zero energy balance is equivalent to the multiple of 1.0).
[2] Values for corn calculated by difference.

intake for males is similar to that (208 Mcal/100 kg of dry matter) recorded by Morrison (37). The mean NE value of acetic acid, constituting 8.4% of the GE intake, was 259 ± 25 Mcal/100 kg. The values summarized in table 8 reflect the exceptionally high quality of the hay employed.

Short-term, respiration-calorimetric experiment

Calorimetric efficiency of acetic acid. The mean outputs of heat by the three rams on each diet during the five periods are summarized in table 9. Trials 1 and 2 were conducted between days 50 and 70 of a 70-day period during which the same diet was fed continuously to a given ram. The heat production values recorded for trials 3, 4, and 5 were determined during days 3, 15 and 30, respectively, after each animal was changed to the opposite diet. The differences between the two treatments (i.e., triacetin- and glycerol-containing diets) was the GE increment provided by acetic acid (20.3 kcal/body weight $kg^{0.73}$ per day). Based on the data obtained in the metabolism trials conducted during the long-term feeding experiment described above, it was estimated that the metabolizability of the GE of acetic acid was 86.4 ± 6.6%. Thus, the ME increment provided by acetic acid in the respiration experiments was 17.3 kcal/body weight $kg^{0.73}$ per day.

Based on the mean heat outputs during trials 1 and 2, the mean heat increment attributable to the input of 17.3 kcal of ME was 4.1 kcal/body weight $kg^{0.73}$ per day. This indicated that the mean net efficiency with which the ME of acetic acid was utilized for body energy gain is 76.4% after a prolonged feeding period (i.e., 50 to 70 days). Although this value is close to the theoretical net efficiency (77%) with which acetic acid is utilized to synthesize stearic acid, it is a little higher than the net efficency (71.2%) of the ME of acetic acid as predicted from the data obtained in the 175-day, body-energy balance experiment described above.

Based on the within-animal differences between the mean heat increment measured during trials 1 and 2, as the base-

TABLE 9

Heat production during respiration-calorimetric trials [1]

Preliminary period [2]			After diet change [3]			
Diet	Trial 1	Trial 2	Diet	Trial 3	Trial 4	Trial 5
Hay + triacetin	113.0 ± 0.3	112.5 ± 5.6	Hay + glycerol	111.1 ± 2.0	109.0 ± 3.8	105.8 ± 5.9
Hay + glycerol	112.0 ± 4.1	105.4 ± 3.6	Hay + triacetin	117.7 ± 2.3	112.7 ± 2.6 [4]	111.2 ± 2.7 [4]

[1] Heat production in kcal/day/body weight$^{0.73}$ ± SD.
[2] Trials 1 and 2 conducted between days 50 and 70 of the preliminary period.
[3] Trial 3 was conducted 3 days after the diet change; trial 4 at 15 days, and trial 5 at 30 days, after the change.
[4] Data from two of the three sheep; one animal lost.

line, and each of those determined during trials 3, 4, and 5, the mean energetic efficiencies of the ME of acetic acid were derived for days 3, 15, and 30, respectively, after the dietary change. These were 47.9, 77.0, and 85.4%, respectively. On day 3 after the abrupt addition of triacetin to the diet, the energetic efficiency was considerably lower ($P = 0.06$) than that observed after the continuous feeding period of 50 to 70 days. By day 15 of feeding, the energetic efficiency was similar to that determined after the prolonged feeding period. The change in efficiency between days 15 and 30 was not significant ($P > 0.1$).

Although these data are limited and the magnitude of the ME increment as acetic acid was small (and as a consequence varied appreciably), they do support the observation of Armstrong and co-workers (14–16), made in 7-day infusion experiments, that the heat increment of acetic acid is relatively high. In addition, these data in concert with those obtained in the 175-day slaughter-analysis experiment suggest that the sheep might undergo a metabolic adaptation to accommodate, at least within 15 days, the abrupt influx of an increased amount of acetic acid.

GENERAL DISCUSSION

Cellular-level investigations of the apparent adaptive behavior observed in these experiments have not been made. However, several cardinal metabolic reactions would seem to be worthy of attention.

It has been suggested that acetic acid may uncouple oxidative phosphorylation and result in wasteful heat production (38). Also, Armstrong and Blaxter (14) emphasized that in order for acetyl CoA to be converted to fatty acids (de novo) via the scheme proposed by Wakil (39), reduced nicotinamide adenine dinucleotide phosphate (NADPH) is an obligatory cofactor. The major source of NADPH is the hexose monophosphate shunt (40), a side pathway of glucose oxidation extramitochondrially. Employing rat epididymal fat pads in in vitro experiments, Flatt and Ball (41) showed that when glucose and acetate were present together, the flow of glucose carbon through the pentose cycle was increased by 83% and the total synthesis of fatty acids was increased by 60% over that obtained with glucose alone. It seems likely that if a sudden increase in the level of acetic acid to be metabolized is not accompanied by a concomitant increase in pentosephosphate shunt activity, the acetic acid might be channeled into oxidative pathways rather than utilized for the synthesis of long-chain fatty acids, until NADPH is generated in adequate amounts.

Another explanation of the apparent adaptation might concern the step in fatty acid biosynthesis between acetyl CoA and malonyl CoA which is catalyzed by acetyl CoA carboxylase. This reaction is generally considered to be the rate-limiting step in fatty acid synthesis and requires biotin, HCO_3^-, ATP, Mn^{2+}, Mg^{2+} and a Krebs cycle intermediate as cofactors (42). A lag in the activity of this system with an increased input of acetic acid, could cause a shift in acetic acid metabolism toward oxidation, independently of NADPH generation. This possibility would seem to be in accord with the results of the experiments reported here since in both kinds of experimentation employed, glycerol was added to the diets. In addition to the glycerol, some propionic acid, a glucogenic substance in ruminants, was produced as a result of ruminal fermentation of the basal diet. As a consequence, it seems unlikely that the amount of glucose available for pentosephosphate shunt activity was deficient.

In addition to certain dissimilarities cited earlier in this report, the experiments of Armstrong and co-workers (14–16) were different from those reported here with respect to the feeding patterns and meal frequencies imposed. In their experiments the basal, maintenance diets were fed in two or four meals daily and the fatty acid solutions were infused continuously into the rumen at a steady rate throughout the period. In our studies, triacetin or glycerol was provided as a part of the total diet in three and two meals per day in the slaughter and calorimetric experiments, respectively.

The influence of meal frequency on lipogenesis and energetic efficiency varies

among animal species. In sheep, the net energetic efficiency is higher under a frequent meal (eight/day) regimen than it is when an equicaloric amount of the same diet is fed in one meal per day (43, 44). Thus, it seems unlikely that meal frequency could account for the difference in the energetic efficiency of acetic acid as observed between the Scottish and present experiments. On the other hand, rats fed two meals per day stored much more fat and were decidedly more efficient energetic- and were decidedly more efficient energetically during periods of 10 to 22 days' durasame amount of energy under ad libitum (i.e., "nibbling") conditions.[7] This is in accord with the observations made earlier that hyperlipogenesis can be induced by an infrequent meal regimen (45, 46, 47). Associated with the body composition changes is the adaptive behavior of certain enzymes significant to carbohydrate metabolism and lipogenesis. The activity of three NADPH-generating enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP-malate dehydrogenase) in liver or adipose tissue or both (46, 48, 49), and of acetyl CoA carboxylase in adipose tissue (50) was greatly increased in rats fed one meal per day as compared with "nibblers." Although similar observations have been made in the chicken (51, 52), neither the enzyme activity of adipose tissues nor the apparent composition of the bodies of pigs differed between "nibblers" fed ad libitum and pigs fed one meal per day (53).

Recently, on the basis of an experiment designed similarly to that of McManus et al. (17), Vermorel (54) presented prima-facie evidence that rats fed a glycerol-containing diet stored 13.4% more body energy than did their mates pair-fed isocaloric amounts of a diet in which triacetin provided 25.9% of the gross energy during a 30-day body-balance experiment. The triacetin diet, however, was fed ad libitum and the glycerol diet was restricted to an intake of the same amount of ME. It seems possible that the feeding method employed might have resulted in some degree of "nibbling" of the triacetin diet and of "meal feeding" of the glycerol diet. As a consequence, the greater storage of energy by rats on the glycerol diet might be a reflection more of the effect of "meal feeding" than of a difference in the energetic efficiency between the metabolites absorbed from the two diets. Vermorel (54) also observed a 14.1% greater retention of nitrogen by rats fed the triacetin diet than by those ingesting the glycerol. An increased nitrogen retention has been a characteristic of "nibbling" rats.

Whether the feeding patterns for the two diets were different in the experiment of McManus et al. (17) is not clear from their paper. However, during the respiration experiments, each of the pair-fed rats was provided a meal of 24.7 kcal of ME (ca. 6 g of feed) and was allowed only 40 to 60 minutes to ingest it. Beginning 0.5 hour after the meal had been ingested, oxygen consumption and carbon dioxide output were measured for 5 to 6 hours. During the periods in which the ME was determined, meals of both diets were provided at 3- to 4-hour intervals.

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Dissolution Rates of Enamel and Dentin in Acid Buffers

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It is generally agreed that acid action is a major factor in caries, and lactic acid has been considered primarily responsible. Analyses of plaques, saliva, and carious material, however, have revealed the existence of many acids, any or all of which may contribute to the dissolution of teeth.[1-8] These acids represent a range of chemical properties which may influence the rate and extent of enamel and dentin decalcification, depending on the type of acid or acids involved. Johnston,[9, 10] for instance, has reported that the capacity of organic acids to dissolve synthetic calcium phosphates is considerably influenced by their structure. Because hydrogen ion is common to all acids, variations in solubility effects observed by Johnston can be related to differences in the chemical structure of the anions of the acids.

There is considerable evidence that the structure of anions is extremely important biologically, as, for example, the effect of the anions derived from citric and ethylenediaminetetraacetic acids in increasing the solubility of calcified structures through complex formation. Little attention, however, has been given to the possibility that specific anions may influence the acid dissolution of enamel and dentin. Stralfors[11] has recently shown that buffers of different acids vary in their capacity to dissolve intact enamel surfaces, but he did not attempt to relate these variations to the structure of the acids. Among his buffers, both the anion and the undissociated acid concentration differed, with pH the only common factor. Under such conditions it is difficult to separate effects on solubility which may be due to differences in amount of undissociated acid from those due to differences in the amount or chemical structure of the anions.

In the present investigation, acids were compared for their ability to decalcify enamel and dentin as simple buffers, wherein the pH and anion concentration were equated. Differences among the buffers, therefore, were essentially in the amount of undissociated acid and the kind of anions present. Under these conditions the finding of a definite correlation between undissociated acid concentration and extent of dissolution would be meaningful and suggestive of the absence of specific anion effects. On the other hand, deviations from such a solubility trend line could be indicative of specific anion effects.

MATERIALS AND METHODS

Nine chemically pure, fluoride-free acids were used in this investigation. To eliminate the pH variable, simple buffers of sufficient capacity to resist change in pH during the reaction time were employed. So that the same amount of anions was compared for

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each acid, a given amount of base was titrated with a solution of each acid to pH 4.0. To prepare the buffers, a quantity of standardized sodium hydroxide solution, sufficient to produce a molarity of 0.043 when made up to a final volume of 1000 cc., was added to 850 cc. of distilled water. This solution was then titrated with a solution of known concentration of each acid to pH 4.0, using a meter* to follow the course of the titration. The volume was then brought to the desired level, and the pH rechecked and adjusted if necessary. For proprionic acid an additional buffer was prepared, using half the afore-mentioned amount of sodium hydroxide to give an anion concentration of 0.022 M. This method of preparing the buffers produces solutions having the same pH, acid anion, and sodium ion concentrations, since the total amount of base is completely neutralized in each instance. For other tests in which aspartate and acetate buffers alone were compared, decalcifying solutions of 0.2 M acid concentration were made in the conventional manner by titration of acid with base to pH 4.0. In equating pH and acid anion concentration for the buffers, the concentration of undissociated acid present in each buffer must necessarily be different and directly proportional to the pK of the acid. Except for the anions of the amino acids which are polyvalent, the anions of the other acids are monovalent, and, since the undissociated acid molecules have no charge, their buffers have approximately the same ionic strength. The amino acids, however, exist as zwitterions, and in this paper the natural zwitterionic form is designated as "undissociated acid." The dissociated and undissociated forms of the amino acids are polyvalent and therefore contribute to a higher ionic strength for these buffers. The enamel and dentin powders used were prepared according to the method of Manly and Hodge[12] as modified by Burnett and Battistone.[13]

In one series of tests the dissolving rates of enamel and dentin were determined by mechanically shaking 50-mg. samples with 100 cc. of the buffer solutions for 30 minutes on a wrist-action shaking machine.† In another series, 100-mg. samples were used with 50 cc. of the 0.2 M buffers. In still another series of tests, 50-mg. tissue samples were employed with 100 cc. of buffers which were 0.1 M with respect to the anion concentration. These buffers were used in tests with anorganic enamel prepared according to the method of William and Irvine[14] by taking 200–270-mesh enamel and treating with ethylene diamine to remove organic matter. The resulting enamel has about the same particle-size distribution as the smaller than 270-mesh untreated enamel. The solutions were filtered through selas filtering crucibles of 5-μ maximum pore diameter, and the filtrates were analyzed for calcium and phosphorus. Phosphorus was determined by the method of Quinlan and DeSesa,[15] and calcium was analyzed by the method of Schwarzenback.[16] Both methods were modified as outlined by Koulourides[17] to include the use of perchloric acid to oxidize the organic acids present which interfere in the analysis. At least duplicate runs were made for each buffer, and the runs were repeated when the analytical findings did not check within 5 per cent. All tests were conducted at 25 ± 1° C. Changes in pH were generally within 0.1 of a unit following reaction.

RESULTS

Table 1 shows the results of treating 50 mg. of 120–200-mesh enamel for 30 minutes with 100 cc. of each of the pH 4.0 buffer solutions. Dissolution of enamel was measured

* Beckman Model G, Beckman Instrument Co., Pasadena, California.

† Burrill Corp., Pittsburgh, Pennsylvania.

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in milligrams of calcium and phosphate dissolved. Under the heading "Initial undissociated acid molarity," are the concentrations of the buffers with respect to the undissociated acid as determined from titration figures. The amounts of undissociated acid present in the buffers ranged from 0.016 M for formic acid to 0.281 M for propionic acid and bore no definite relationship to the amount of enamel dissolved. This is clearly shown in comparing the low solubility in the aspartic buffer with the high value obtained in the lactate buffer, which had the same concentration of undissociated acid, and is also evident from the result with the glutaric acid buffer, which had about one-third the amount of undissociated acid of the acetate buffer, yet dissolved approximately the same quantity of enamel. Similarly, a comparison of the results with glutaric and glycolic acids leads to the same conclusion. There nevertheless appeared to be a tendency

TABLE 1

SOLUBILITY OF 50 MG. SAMPLES OF 120-200 MESH ENAMEL SHAKEN FOR 30 MINUTES IN 100 CC. OF pH 4 BUFFERS OF SAME ANION CONCENTRATION

(Phosphorus and Calcium Analysis of Filtrates)

Buffer	Initial Undissociated Acid Molarity	Final pH	Mg. Dissolved		Per Cent Difference from Aspartate
			Ca	P	
Formic	0.016	4.15	10.8	5.0	+ 64.5
Glycolic	0.024	4.11	12.5	5.6	+ 88.0
Aspartic	0.030	4.05	6.6	3.0	.....
Lactic	0.030	4.12	11.3	5.3	+ 72.7
Succinic	0.050	4.04	11.8	5.5	+ 75.3
Glutaric	0.065	4.09	12.3	6.1	+ 92.0
Glutamic	0.086	4.04	8.6	3.9	+ 29.5
Acetic	0.197	4.05	13.5	6.2	+104.2
Propionic	0.281	4.05	12.1	5.9	+ 88.0
Propionic*	0.152	4.05	11.9	5.6	+ 82.1

* 0.022 anion concentration.

for the buffers of the weaker acids to dissolve more enamel than the buffers of the stronger acids. This can be related to the larger amounts of undissociated acid present in the buffers of the weaker acids which provided a greater diffusion potential of undissociated acid from the bulk solution to the surface of the enamel particles, to replenish acid which was used up in the dissolution reaction. The availability of greater numbers of acid molecules for reaction with enamel could be expected to increase the dissolution rate. In line with these considerations are the results with the propionic acid buffers, wherein doubling the anion and free acid resulted in a 6 per cent increase in the amount of enamel dissolved. This result, however, also indicates that large increases in undissociated acid concentration had relatively little effect on dissolution capacity, at least for proprionic acid at the concentration levels used.

The most significant observation in these tests was the unexpected low dissolution demonstrated by the aspartate and glutamate buffers. The large percentage differences in dissolving capacities between the aspartate buffer and the other acid buffers are shown in the last column of Table 1. The comparable dissolving capacities of the aspartate and glutamate buffers are not surprising, in view of the chemical similarity between these two acids.

If the reason for the low solubility of enamel in the aspartate and glutamate buffers is due to factors inherent in the buffers rather than in the enamel, then comparable patterns of dissolution should prevail for dentin, since the dissolving inorganic phase is chemically similar to that in enamel. The results of the solubility tests using 80-120-mesh dentin are shown in Table 2. Contrary to expectations and to their behavior shown with enamel, all buffers showed similar dissolving capacities for dentin. Since the different buffers had the same pH and anion concentration, it would appear that these factors, rather than the kind of anion and/or difference in the amounts of undissociated acid dominated the dissolution of dentin under the experimental conditions.

The difference in solubility between the glutamic acid buffer, which gave the lowest solubility, and the acetic acid buffer, which showed the highest solubility with dentin, was 15 per cent. This difference is small compared with the 104 per cent solubility difference in the enamel series between the aspartate buffer, which gives the lowest solubility, and the acetate buffer, which gives the highest. This indicates that the low enamel solubility in the amino acid buffers was not due to any inherent lack of capacity of these buffers to dissolve apatite. The results of tests to determine whether the difference in solubility behavior of enamel and dentin could be related to the surface areas of these materials are shown in Table 3, along with those for a well-characterized sample of synthetic hydroxyapatite.* Although enamel and dentin solubility increased in both buffers as particle size decreased, the differences in the amounts of enamel dissolved by the aspartate and acetate buffers for each mesh size were approximately the same. Hence increases in enamel surface area, within the limits of the particle sizes used, had relatively little effect on the magnitude of the difference between the dissolving capacities of the aspartate and acetate buffers. Dentin behaves essentially as hydroxyapatite, which showed no difference in solubility in the two buffers.

TABLE 2

SOLUBILITY OF 50 MG. SAMPLES OF 80-120 MESH DENTIN SHAKEN FOR 30 MINUTES IN 100 CC. OF pH 4 BUFFERS OF 0.043 ANION CONCENTRATION

(Phosphorus and Calcium Analysis of Filtrates)

Buffer	Initial Undissociated Acid Molarity	Final pH	Mg. Dissolved		Per Cent Difference from Aspartate
			Ca	P	
Formic	0.016	4.15	11.4	5.3	−1.4
Glycolic	0.024	4.10	12.0	5.4	+2.4
Aspartic	0.030	4.08	11.5	5.4	.....
Lactic	0.030	4.13	11.9	5.0	0.0
Succinic	0.050	4.05	12.3	5.5	+5.0
Glutaric	0.065	4.08	11.7	5.4	+1.1
Glutamic	0.086	4.03	11.3	5.0	−3.8
Acetic	0.197	4.05	12.4	6.1	−9.3
Propionic	0.281	4.06	11.7	5.7	+1.1
Propionic*	0.152	4.05	11.4	5.3	−1.8

* 0.022 anion concentration

* Victor Chemical Co., New York, New York

Table 4 shows a comparison of the solubility of enamel treated with ethylene diamine to increase surface area with intact enamel and dentin in acetate and aspartate buffers, both 0.1 M with respect to anion concentration. The acetic acid buffer at this anion concentration contained an undissociated acid concentration of more than twice that present in the 0.2 M acetic acid buffer (Table 3). With the aspartate buffers, however, there was relatively little difference in undissociated acid concentration. Hence one might expect a tendency toward greater dissolution in the acetate buffer, thus favoring

TABLE 3

DISSOLUTION OF 100 MG. OF ENAMEL, DENTIN, AND SYNTHETIC APATITE BY 50 CC. OF 0.2 M pH 4 ASPARTIC AND ACETIC ACID BUFFERS IN 30 MINUTES

(as Mg. P)

Buffer	Enamel			Dentin		Synthetic Hydroxyl-apatite
	120–200 Mesh	200–270 Mesh	<270 Mesh	80–120 Mesh	120–200 Mesh	
1. Aspartate	3.4	4.9	6.5	6.1	8.7	14.8
2. Acetate	7.3	9.4	10.7	7.3	8.7	14.8
Difference 2 over 1	3.9	4.5	4.2	1.2	0	0

TABLE 4

SOLUBILITY OF 50 MG. OF ENAMEL, DENTIN, AND ANORGANIC ENAMEL, EXPRESSED AS MG. PHOSPHORUS IN 100 CC. OF pH 4 BUFFERS OF 0.1 M ANION CONCENTRATION FOR 30 MINUTES

Buffers	Enamel (200–270 Mesh)	Anorganic Enamel (270 Mesh)	Dentin (120–200 Mesh)
1. Aspartic	2.6	6.0	3.5
2. Acetic	6.7	8.8	4.4
Difference 2 over 1	4.1	2.8	0.9

a larger difference between it and the aspartate buffer than that shown in Table 3. This appeared to be the case with 120–200-mesh dentin but not with enamel, where the 4.1-mg. difference in solubility was of the same order as previously found. This 4.1-mg. difference in enamel solubility, measured as phosphate dissolved between the aspartate and acetate buffers, was reduced to 2.8 mg. with anorganic enamel.

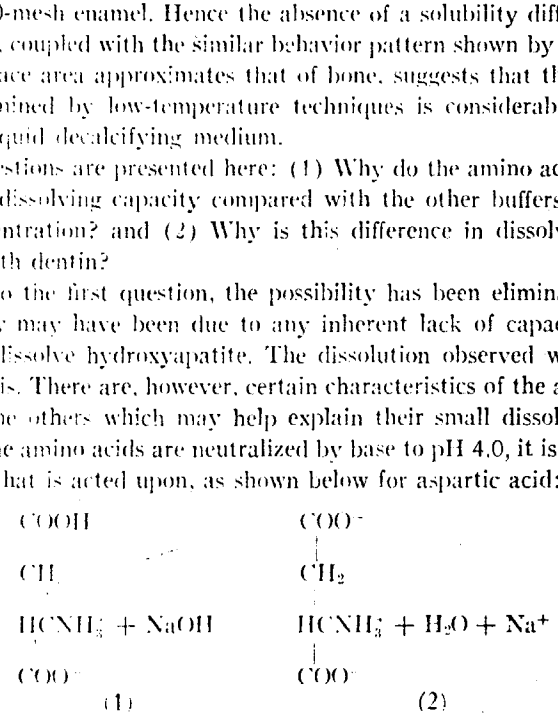
DISCUSSION

The results of this study indicate that differences in the rate of enamel dissolution in buffers at constant pH 4.0 are dependent on the kind of acid used, and not on the anion concentration or to a particular solid/solution ratio (data of Table 1 compared with data of Tables 3 and 4). Dentin, on the other hand, seems to dissolve to about the same ex-

tent in all the buffers. The similarity in behavior patterns of dentin and synthetic apatite, as manifested by the absence of a difference in solubility in acetate and aspartate buffers (Table 3), suggests that the particle size of dentin does not reflect available surface area. Although surface-area measurements were not made on our dentin and enamel powders, they probably would show areas similar to those reported by McCann and Bullock.[18] These authors report surface areas for 325-mesh enamel as being about double those of 200–230-mesh dentin. On this basis it might have been expected that the difference between the amounts of 120–200-mesh dentin dissolved in the acetate and aspartate buffers would be at least as large as the difference obtained by using the smaller than 270-mesh enamel. Hence the absence of a solubility difference with 120–200-mesh dentin, coupled with the similar behavior pattern shown by synthetic apatite, whose large surface area approximates that of bone, suggests that the surface area of dentin as determined by low-temperature techniques is considerably less than that available to a liquid decalcifying medium.

Two basic questions are presented here: (1) Why do the amino acid buffers show a reduced enamel-dissolving capacity compared with the other buffers at the same pH and anion concentration? and (2) Why is this difference in dissolving capacity not demonstrated with dentin?

With regard to the first question, the possibility has been eliminated that the low enamel solubility may have been due to any inherent lack of capacity of the amino acid buffers to dissolve hydroxyapatite. The dissolution observed with dentin leaves little doubt of this. There are, however, certain characteristics of the amino acid buffers different from the others which may help explain their small dissolving capacity for enamel. When the amino acids are neutralized by base to pH 4.0, it is the undissociated carboxyl group that is acted upon, as shown below for aspartic acid:



At the pH 4.0 level, more than half the initial acid 1 (which in this study is called "undissociated acid") has been neutralized to the form 2 (which is termed the "acid anion"). Forms 1 and 2 are in equilibrium with each other. With the non-amino acids the undissociated acid molecules have no charge, whereas the aspartic acid molecule is doubly charged. Moreover, all the anions of the non-amino acids are singly charged, while the anions of aspartic acid have a triple charge. It is apparent, therefore, that, in contrast to the other buffers, the aspartic and glutamic acid buffers contain relatively large numbers of multivalent undissociated acid molecules and anions. Because of the presence of these highly charged ionic species, buffers have a significantly higher ionic strength than the buffers of the other acids.[8] This factor, however, is not involved in the

The ionic strength contribution of the highly charged amino acid ions is probably not so great as it could be if the charges were of the same sign. The proximity of positive and negative charges in the same molecule causes partial internal neutralization, which may reduce their net contribution to ionic strength.

reduced ability of the amino acid buffers to dissolve enamel, inasmuch as it would tend to influence the results in the direction of increased dissolution.[19] For instance, it is known that, within certain limits, increases in the ionic strength of a dissolving medium will result in an increased solubility of a sparingly soluble substance. Although these considerations are derived for equilibrium conditions and not specifically for rate studies, the ionic strength factors leading to an increase in equilibrium solubility are operative at all times in the dissolving medium. Hence in the present study the moment-to-moment operation of the ionic strength factors should express itself in an increased enamel dissolution. Evidence to support these considerations has already been reported from our laboratories.[17] The reduced solubility, therefore, found with the amino acid buffers is probably related to the charges of the amino acid ions but independent of their ionic strength contribution.

Two possibilities may be considered to account for the manner in which the high charges might bring about a reduced enamel solubility. These possibilities are based on the fact that the acid dissolution of enamel probably involves the diffusion of undissociated acid molecules to the enamel surfaces from the solution and the diffusion in the opposite direction of reaction products. Let us consider the first possibility. There is evidence that in solutions of the sodium salts of aspartic and glutamic acids large ionic aggregates exist.[20] These ionic aggregates and/or the anions themselves may be attracted to, or adsorbed on the surface of, the enamel crystallites to form a sort of ionic barrier, or surface-bound complexes. An ionic barrier or shell could conceivably be composed of anions or ionic aggregates having their negative charges oriented toward the enamel particle and their positive charges directed toward the solution. The net effect of such an arrangement would be to retard the diffusion of undissociated acid molecules to the apatite surface to provide hydrogen ion for decalcification and, in addition, retard the diffusion of dissolution products to the bulk solution, hence slowing down dissolution rates. The presence of surface-bound complexes would involve, of course, similar considerations to account for the low dissolution rates in the amino acid buffers.[17]

The second possibility is based in part on certain aspects of diffusion as outlined by Scheiner.[21] Although the crystallites of enamel are densely packed, there nevertheless exist small spaces between the crystallites which may limit the diffusion of the multicharged undissociated amino acid ions but not the uncharged acid molecules of the other buffers. Because of this, the amount of enamel surface accessible for dissolution in the amino acid buffers may be considerably less than that exposed in the other buffers. The important point here is that, while an increase in external surface area is obtained by reducing the mesh size, even in these smaller enamel particles many spaces exist between the crystallites which may be less accessible to the "undissociated" amino acid ions than to uncharged undissociated acid molecules. It is apparent, however, that, by progressively decreasing particle size, the ratio of accessible to inaccessible surface must increase, which should result in progressively smaller differences between the solubility of the enamel in the amino and non-amino acid buffers. In our tests this tendency was, however, not associated with reductions in mesh size but was evident with enamel treated with ethylene diamine, which apparently increases surface area

considerably more than reduction in particle size from 120-200 to smaller than 270 mesh. The persistence of a comparatively large difference between the solubility of anorganic enamel in the aspartate and acetate buffer (Table 4), however, indicates that the surface available for reaction is nevertheless considerably smaller than that for dentin and synthetic hydroxyapatite.

This brings us to the second basic question regarding why dentin does not show a reduced solubility in the amino acid buffers compared with the other buffers. It is apparent from the results that if charge factors are involved in the reduced solubility with enamel, they are operating differently with dentin. In view of the chemical similarity of the inorganic phases of enamel and dentin, it seems logical to consider that physical differences between these substances may be responsible for their different solubility behavior. One physical characteristic of dentin that is different from enamel is the loose packing and random distribution of considerably smaller crystallites in the former. Consequently, the charged "undissociated" acid ions from the amino acids can diffuse between the crystallites as readily as the uncharged undissociated molecules of the other acids. Solubility rates among the buffers should therefore be similar, other things being equal. Another characteristic of dentin that is different from enamel is the greater amount and kind of organic matter present, which presumably surrounds the apatite crystallites. The existence of this organic matter may prevent, by chemical or physical means, the formation of an ionic shell or surface-bound complexes at the crystallite surfaces to retard dissolution, as postulated for enamel.[17]

The demonstration of specific ion effects as evidenced by the lower solubility rates of enamel in the amino acid buffers compared with the others appears to be a function of both the structural characteristics of the acid ions used and the structural characteristics of the enamel. The finding that certain ionic species can reduce the rate at which an acidic environment dissolves enamel may have particular significance with respect to the initiation and spread of caries in enamel. For instance, it has been previously reported by this author[22] that the addition of aspartic acid to decalcifying buffers, such as lactic, acetic, and pyruvic, reduces the rate of enamel dissolution in these buffers. Moreover, it is interesting to note that the solubility-reducing effect is not related to any rise in the pH of the decalcifying environment. It is conceivable, therefore, that the presence of certain ionic species may have similar effects on the decalcifying rates of the acidic buffer systems of plaques and other areas of caries attack toward enamel. In vitro support for this idea is suggested by the results of Koulourides[19] and Koulourides and Buonocore,[23] which show that anions derived from citric and tartaric acids and lysine, when added to a decalcifying medium, render it less effective in dissolving enamel. In addition, Osborn[24] and more recently Jenkins[25] have reported that certain organic phosphates are capable of similar solubility-reducing effects. Jenkins, in fact, suggests the idea that anticaries action of fluoride may be the result of enolase inhibition in the glycolytic cycle, which may bring about an accumulation of 2-phosphoglyceric acid, which in turn reduces the effectiveness of the lactic acid in dissolving enamel. These investigations, therefore, lend support to our own findings that the decalcifying potential of an acid medium to decalcify enamel can be considerably influenced by the kind of acid or acids present therein.

In contrast to the sensitivity of enamel to the composition of the decalcifying environment, the dissolution of dentin appears to be independent of the type of acid.

Hence, if effects of the sort presented herein occur in vivo, they would be of particular significance with respect to the rate of initiation and spread of caries in enamel but not in dentin.

SUMMARY

A comparison of the rates of solution of enamel and dentin in simple pH 4 buffers of nine acids (acetic, lactic, glycolic, aspartic, glutamic, glutaric, succinic, propionic, formic) indicated that the rate of enamel dissolution was influenced by the kind of acid used. The rate of dentin solubility, on the other hand, which is approximately the same in all the buffers, appears to depend not on the kind of acid but on the pH and anion concentration of the buffer.

The solubility of enamel was lowest in the buffers of the amino acids, with the solubility in the aspartic acid buffer ranging from about 40 to 50 per cent less than in the other buffers.

It has been postulated that the multicharged ions derived from the amino acids, by forming surface-bound complexes with the enamel particles, retard dissolution rates. With dentin the presence of the organic matter may interfere with the formation of surface complexes.

Effects of this sort occurring in vivo could have significant bearing on the rate of initiation and spread of caries in enamel.

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A COMPARISON OF THE TOXICITY OF SOME COMMON INDUSTRIAL WASTE COMPONENTS TESTED INDIVIDUALLY AND COMBINED

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KNOWLEDGEABLE PEOPLE IN THE FIELDS OF WASTE DISPOSAL AND WATER MANAGEMENT are well aware that the components of an effluent may interact so that their collective toxic effect on aquatic organisms is substantially different from their individual toxicities. These interactions may be either additive, in which case the collective toxicity is the sum of the individual toxicities; antagonistic, in which case one component of the waste interacts with another component to reduce toxicity; or synergistic, in which case the interaction of the components produces a toxicity that is greater than additive. The classic example of synergistic interaction is the one reported by Doudoroff (1952) for copper and zinc. He found that although fish could survive for 8 hours in water containing 8 milligrams per liter of zinc alone, and for 8 hours in water containing 0.2 milligram per liter of copper alone, most fish died within 8 hours when exposed to a mixed solution containing only 1.0 milligram per liter of zinc and 0.025 milligram per liter of copper. The possibility of waste component interactions creates a nightmarish problem for regulatory agencies, as not only are qualitative and quantitative variations in the effluent of a single industry very likely, but the materials from several such industries entering a stream within a short distance often create regulatory problems of incredible complexity. There is also a possibility that the waste from an industry may interact with materials deposited in a streambed in bygone days and recently exposed by dredging or some change in the flow patterns of the stream.

Although an understanding of the interactions of the various components of a waste under both laboratory and field conditions is essential to enlightened water management programs, surprisingly little work has been done in this area. The purpose of the experiments reported in this paper was to contrast the response of fish to some compounds common to many industrial effluents both individually and in mixtures. Although this report covers only a few of an almost infinite number of possible interactions, we trust that it will provide an insight into one aspect of this critical and important problem.

Methods and Procedures

The methods and procedures used follow those suggested by Doudoroff and co-writers (1951) for acute toxicity tests.

All work on this project was done in the Limnological laboratories of the Academy of Natural Sciences of Philadelphia. Experimental work was conducted under U.S. Public Health Service Grant RRG-3905; this paper was prepared under U.S. Public Health Service Grant EF-00266, National Institutes of Health.

The fish used in these experiments were bluegills (Lepomis macrochirus). The synthetic dilution water simulated a typical soft water from the Middle Atlantic States. We chose to use a snythetic dilution water because it could be duplicated by others more readily than a natural water. The constituents of this synthetic water are listed in table 1. Each chemical was pipetted separately from a concentrated stock solution into sufficient distilled water to reach a total volume of 18 liters for each test container. This solution was then aerated until the dissolved oxygen concentration was between 5 and 9 p.p.m., and this concentration was maintained throughout the test period. Temperature was maintained at 18° ± 2° C. Containers used for these experiments were 5-gallon glass jars fitted with an aeration tube (7-millimeter outside diameter) which extended the length of the jar, and a short air-vent tube that also served as an aperture through which a short piece of tygon plastic tubing could be inserted for periodic sampling. Chemicals used were A.C.S. grade.

All fish were acclimatized for 2 weeks to the synthetic dilution water maintained at 18° ± 2° C. During the acclimatization period the maximum permissible mortality was 5 percent. If more mortality than this occurred, the entire group of fish was discarded. All fish were fed daily with chopped, freshly cooked shrimp during the period of acclimatization, but they were not fed for 36 hours before the tests began or during the test period. Total length of of the fish, including the tail, ranged from 5.3 to 7.2 centimeters; weight of the fish ranged from 3.5 to 3.9 grams. These figures were obtained from measurements of 100 fish selected at random from all test fish. In any one experimental container, no single fish differed more than 50 percent in total length from any other fish (recommendation of Hart et al., 1945).

TABLE 1.--Composition of synthetic dilution water

Chemical	Grams per liter	Molarity
KCl	0.02	2.68×10^{-4}
Na_2SiO_3	.02	1.63×10^{-4}
$NaHCO_3$	.04	4.76×10^{-4}
$MgSO_4 \cdot 7H_2O$	.04	1.62×10^{-4}
$Ca(NO_3)_2$	.03	1.82×10^{-4}
$CaCO_3$	.01	1.00×10^{-4}
K_2HPO_4	.01	5.73×10^{-5}
Fe^{+++} (as ferric citrate)	.0004	----------

The pH and dissolved oxygen concentration were determined at the beginning of each experiment, before the introduction of the fish, and at the end of every 24-hour period during these tests. The pH of the solution was determined with a Beckman pH meter that was equipped with external electrodes. The dissolved oxygen concentration was determined by the sodium azide modification of the Winkler method (American Public Health Association et al., 1955). After these preliminary determinations indicated that the environment was of appropriate quality, test materials were placed in the containers and mixed thoroughly with the synthetic dilution water. Then 10 bluegills were placed in the containers. One control container was maintained for each five experimental containers. Mortality counts were made at the end of every 24-hour period: the cessation of gill movements and a failure to respond to mechanical stimulus were used as evidence of death. As our primary interest was the response of the fish to the test materials at a specific point in time rather than the ultimate effect of these materials on the fish, we chose the 96-hour TL_m, that is, the 50-percent survival concentration, for this purpose. This is a bit longer than most acute bioassays but not of sufficient duration to be considered chronic exposure. The latter area deserves attention but was beyond the scope of this particular series of experiments.

Compounds tested were the following: Acetaldehyde, acetic acid, acetone, potassium cyanide, potassium dichromate, copper, naphthenic acids, and zinc chloride. Results for these tested individually are shown in table 2, and results for four

TABLE 2.--96-hour results for constituent chemicals in mixtures

Chemical	100% survival concentration			50% survival concentration			No survival concentration		
	p.p.m. introduced	p.p.m. analyzed at 0 hour	p.p.m. analyzed at 96 hours	p.p.m. introduced or interpolated	p.p.m. analyzed at 0 hour	p.p.m. analyzed at 96 hours	p.p.m. introduced	p.p.m. analyzed at 0 hour	p.p.m. analyzed at 96 hours
Acetaldehyde------	28.0	21	0.3	[1]53.0	-----	-----	65	63	0.4
Acetic acid-------	56.0	[2]21	0	75.0	[2]38	[2]24	100	[2]63	[2]40
Acetone----------	3,700	3,000	1,500	8,300	-----	-----	11,500	11,400	9,400
CN^-----------	.07	.064	.01	[1].18	-----	-----	.24	.20	.10
Cr----------	48.4	---------	--------	113	-----	-----	176.8	---------	--------
$(K_2Cr_2O_7)$[3]--------	(137)	---------	--------	(320)	-----	-----	(500)	---------	--------
Cu^{++}----------	.75	.63	.48	[1]1.25	-----	-----	2.10	1.97	1.0
Naphthenic acids--	3.5	---------	--------	5.6	-----	-----	7.0	---------	--------
Zn^{++}----------	2.4	2.20	1.56	4.2	3.78	2.86	5.9	5.8	4.8

[1] Interpolated result.
[2] p.p.m. acidity.
[3] Figures in parentheses are the entire $K_2Cr_2O_7$ molecule for the Cr ion listed above.

mixtures containing two or more of these compounds are shown in table 3. The ratios for mixtures 1, 2, and 3 were based on results of diatom bioassays made by Patrick (1960). The base concentrations for mixtures 1 and 3 (each with three compounds) were one-third of those concentrations individually producing a 5-day TL_m for diatoms, and one-half for mixture 2 (with two compounds). These concentrations were then increased without changing the basic ratio so that enough of each was present to kill bluegills in less than 96 hours. Tests were then made to determine the TL_m of the mixture. The ratio for mixture 4 was selected arbitrarily.

Chemical analyses were made occasionally for the substances being tested during the experiments. The procedures used were as follows:

TABLE 3.--96-hour results for mixtures

[All concentrations in p.p.m. introduced]

Base mixture	100% survival	50% survival (TL_m)	No survival
	[1](0.238)	(0.274)	(0.313)
No. 1			
17.3 p.p.m. Naphthenic acids--	4.12	4.74	5.41
.096 p.p.m. CN^-----------	.023	.026	.030
.07 p.p.m. Cr----------	.017	.019	.022
(or 0.20 p.p.m. as $K_2Cr_2O_7$)-----	(.05)	(.06)	(.06)
	(0.38)	(0.61)	(0.8)
No. 2			
6.39 p.p.m. Zn^{++}----------	2.43	3.90	5.11
.432 p.p.m. CN^-----------	.16	.26	.35
	(0.6)	(0.745)	(0.925)
No. 3			
7.6 p.p.m. Naphthenic acids--	4.56	5.66	7.03
.32 p.p.m. CN^-----------	.19	.24	.296
.268 p.p.m. Cr----------	.161	.200	.248
(or 0.76 p.p.m. as $K_2Cr_2O_7$)-----	(.46)	(.57)	(.70)
	(0.18)	(0.52)	(0.87)
No. 4			
50 p.p.m. Acetic acid----------	9.0	26.0	43.5
10 p.p.m. Acetaldehyde----------	1.8	5.2	8.7
10 p.p.m. Acetone----------	1.8	5.2	8.7
2 p.p.m. Cu^{++}----------	.36	1.04	1.74

[1]The upper number in parentheses is multiplied by the base mixture concentrations to obtain the test concentrations listed beneath it.

1. KCN. Tartaric acid distillation procedure, followed by the titration method using p-dimethylaminobenzalrhodanine as an indicator. American Public Health Association et al., 1955.

2. Zn^{++}. Colorimetric determination of zinc and copper with 2-carboxy-2'-hydroxy-5'-sulfoformazylbenzene. Rush and Yoe, 1954.

3. Acetic acid. Total acidity. American Public Health Association et al., 1955.

4. Acetaldehyde. Sodium bisulfite method. Siggia and Maxcy, 1947.

5. Acetone. Iodometric titration with sodium thiosulfate. Mitchell et al., 1953.

Discussion of Results

For mixture 1, the TL_m appeared to be due almost entirely to the presence of naphthenic acids, as there was no evidence of toxicity from the mixture until its concentration was increased to a point where the naphthenic acids alone were at a concentration high enough to produce a TL_m. The 96-hour TL_m was 5.6 p.p.m. for naphthenic acids alone (table 2), and at the 96-hour TL_m for mixture 1 the concentration of naphthenic acids was 4.73 p.p.m. (table 3). Though this is somewhat lower than the individual response point for naphthenic acids, the difference is small enough to be within the normal variation expected in bioassay tests. It is also possible that some slight additive interaction with one of the other components occurred. In any event, standards based on individual results for these compounds, appropriately corrected to give biologically safe concentrations (Doudoroff et al., 1951), would adequately protect against fish kills.

Mixture 2 had a 96-hour TL_m concentration for cyanide (table 3) which was somewhat above the 96-hour TL_m concentration of 0.18 p.p.m. when cyanide was tested alone (table 2). The TL_m concentration for Zn^{++} at 3.9 p.p.m. in mixture 2 (table 3) was somewhat below its comparable threshold when tested individually at 4.2 p.p.m. (table 2). This suggests a slight antagonistic interaction between these two ions, probably because the zinc complexed with the cyanide and thereby slightly reduced the toxicity of the cyanide.

Mixture 3 reached a 96-hour TL_m concentration at a point where the individual toxicities of both naphthenic acids and cyanide were each high enough to produce individually a 96-hour TL_m. It is possible that the toxicity of the cyanide was somewhat reduced by the presence of chromium, but, if so, the reduction was not particularly substantial.

The results for mixture 4 also suggest no substantial interaction between the components, since the concentration of copper is clearly within the toxic range; however, the copper appears to be slightly more toxic in the mixture than when tested individually, so there is a possibility of some slight interaction with one of the other components. Possibly the presence of acetic acid caused a slightly greater amount of copper to remain in solution for the mixture test--although, with a limited number of test organisms per container, this is well within the range of variation that might be expected for acute bioassays.

Conclusions

No dramatic interaction between the various components of the mixtures tested was evident. We have often noted, however, interactions nearly as dramatic as those reported by Doudoroff (1952) when testing industrial wastes. It would be most discouraging to us if the reader were to interpret our results as an indication that synergistic effects are rare and improbable. We believe that the significance of this work is that it supports the practice of segregating certain waste components for intensive treatment before disposal or compositing with other wastes. For many industrial effluents, this might provide more effective treatment at less cost.

Acknowledgments

We are indebted to Dr. Ruth Patrick, curator and chairman of the Department of Limnology, Academy of Natural Sciences of Philadelphia, for her continuing

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During the FAO World Scientific Conference on the Biology and Culture of Shrimps and Prawns, held in Mexico City, June 1967, Dr. S. W. Ling--FAO/UNDP Regional Fish Culturist for Asia and the Far East--presented a detailed account of methods for rearing and culturing the large fresh-water shrimps of the genus Macrobrachium. Dr. Ling described and explained each step in spawning and hatching, care and feeding, disease control, and preparing facilities for their culture.

Dr. Ling's paper, which was available at the Conference in processed form as FR:BCSP/67/E/31, "Methods of Rearing and Culturing Macrobrachium rosenbergii (de Man)," will be published in the transactions of the meeting.

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0002

ACETIC ACID

$$CH_3\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}OH$$

Acetic acid is a clear, colorless liquid with an acrid taste and odor. Acetic was probably the first acid known and is presently one of the most important large-volume, synthetic organic acids.

Celanese acetic acid is of top quality meeting the specification of the U.S.P. (XVII) and the Federal Specifications O-C-275 & O-A-76C. It is used in the manufacture of cellulose acetate fibre and plastics, vinyl acetate monomer, ester solvents, dyes, metal salts and many other chemicals.

SPECIFICATIONS

Acetic acid, wt %, min	99.85
Freezing point, °C, min	16.35
Specific gravity @ 20/20°C	1.0505-1.0520
Distillation range, °C, max	1.0
Initial b.p., °C, min	117.3
Dry point, °C, max	118.3
Color, platinum-cobalt units, max	10
Water, wt %, max	0.15
Formic acid, wt %, max	0.05
Aldehydes, as acetaldehyde, wt %, max	0.05
Iron, ppm by wt, max	1
Heavy metals, ppm by wt, max	0.5
Chlorides, ppm by wt, max	1
Sulfates, ppm by wt, max	1
Sulfurous acid, ppm by wt, max	1
Permanganate time (ACS Test) min	2 hrs

2.

PHYSICAL PROPERTIES

Appearance	Clear, colorless
Auto ignition temperature, °C	565
Boiling point at 760 mm Hg, °C	117.9
Coefficient of cubical expansion at 20°C	1.071×10^{-3}
Critical pressure, atmos	57.2
Critical temperature, °C	321.6
Explosive limits in air: lower limit, vol %	4
Explosive limits in air: upper limit, vol %	11.5
Electrical conductivity at 25°C, MHOS/cc	1.12×10^{-8}
Flash point, Tag open cup, °F	130
Heat of combustion, k cal/mole	−209.4
Heat of formation, k cal/mole	−116.2
Heat of fusion, cal/g at 16.7°C	44.7
Heat of solution, k cal/mole at 18°C	+0.375
Heat of vaporization, cal/g	96.8
Ionization constant at 25°C	1.753×10^{-5}
Melting point, °C	16.6
Molecular weight	60.052
Odor	Pungent
Refractive index n_D^{20}	1.3718
Solubility of water in	Infinite
Solubility in alcohol, benzene, ether, water	Infinite
Solubility in carbon disulfide	Insoluble
Specific heat, cal/g/°C at 0°C	0.468
Specific gravity at 20/4°C	1.0492
Specific gravity at 20/20°C	1.0507
Surface tension at 20°C, dynes/cm	27.6
Vapor density, (air=1.0)	2.07
Vapor pressure at 20°C, mm Hg	11.7
Viscosity at 20°C, cp	1.22
Weight, pounds/gallon at 20°C	8.74

USES

The largest use for acetic acid is in the production of acetic anhydride which is used in the manufacture of cellulose acetate, plasticizers, and pharmaceuticals.

Large quantities of acetic acid are used to manufacture esters of acetic acid such as amyl acetates, propyl acetates, ethyl and methyl acetate. These solvents find general application in the lacquer, plasticizer, and pharmaceutical fields.

Acetic acid is a basic raw material used in the production of vinyl acetate monomer, the base for various polymers utilized in the paint, adhesive, plastics & textile industries.

Chloroacetic acid is manufactured from acetic acid and is the starting point for the production of a series of herbicides, prime examples of which are 2,4 – dich-

lorophenoxyacetic acid and the isopropyl ester of 2,4,5, – trichlorophenoxyacetic acid. Chloroacetic acid has other uses including the manufacture of sodium carboxymethylcellulose (CMC).

Textile finishing operations require considerable quantities of acetic acid. This chemical is also used in the manufacture of nylon and of some acrylic fibres and in the production of various dyestuffs and pigments.

The pharmaceutical industry consumes acetic acid in the manufacture of vitamins, antibiotics, hormones, and other products. Other products made from acetic acid are salts of acetic acid, peracetic acid, and various rubber and photographic chemicals.

CHEMICAL REACTIONS

1. Anhydride Formation

$$2\,CH_3COOH \longrightarrow (CH_3CO)_2O + H_2O$$

Large quantities of the anhydride are used as an acetylating agent chiefly in the production of cellulose acetate.

2. Ester Formation

$$CH_3COOH + ROH \longrightarrow CH_3COOR + H_2O$$

Of particular importance are the lower aliphatic acetates for use as solvents.

3. Salt Formation

$$CH_3COOH + NaOH \longrightarrow CH_3COONa + H_2O$$

The salts of many mono-and polyvalent metal ions are used extensively.

4. Halogenation

$$CH_3COOH + Cl_2 \longrightarrow CH_2ClCOOH + HCl$$

Monochloroacetic acid (MCA) is used in the production of 2,4 – D.

5. Amide Formation

$$CH_3COOH + NH_3 \longrightarrow CH_3CONH_2 + H_2O$$

6. Nitrile Formation

$$CH_3COOH + NH_3 \longrightarrow CH_3CN + 2H_2O$$

7. Ketene Formation

$$CH_3COOH \xrightarrow{600^\circ C} CH_2=C=O + H_2O$$

8. Vinyl Acetate monomer

$$CH_3COOH + CH\equiv CH \longrightarrow CH_3COOCH=CH_2$$

TOXICITY INFORMATION

Acetic Acid in higher concentrations is potentially a highly hazardous acid. It may produce severe burns to the skin and eyes plus damage to any other tissue with which it is in contact. In the event of contact, the area should be flushed copiously with water immediately and medical attention should be obtained promptly. Since the vapor of strong Acetic Acid is irritating to the eyes and to the respiratory tract, inhalation of the vapor or mist should be avoided and adequate ventilation should be provided at all times. The suggested Threshold Limit Value for Acetic Acid vapor is 10 ppm based on 8 hrs/day and 40/hrs week.

Should accidental oral ingestion occur, it is desirable to have the person, if conscious, drink a large quantity of milk or water and immediately obtain medical care.

Goggles, rubber gloves and other rubber protective clothing should be worn whenever there is a possibility of contact. An approved type respirator should be used whenever exposure to the vapor of Acetic Acid is unavoidable.

SHIPPING AND HANDLING

This material is not classified as a flammable liquid by ICC regulations; however, it requires the normal handling precautions which would be accorded any organic compound of comparable flash point and vapor pressure. Recommended instructions for unloading Acetic Acid are outlined in Chemical Safety Data Sheet SD-41. In general, the procedures recommended in Manual Sheet TC-4, "Unloading Flammable Liquids from Tank Cars", and Safety Guide SG-3, "Flammable Liquids: Storage and Handling of Drum Lots and Smaller Quantities", are also applicable. These publications are available from the Manufacturing Chemists Association, 1825 Connecticut Avenue, N.W., Washington, D.C., 20009.

Material is available in tank car, tank truck, and drum quantities. For details contact the nearest Celanese Chemical Company District Sales Office.

NOTE:

To the best of our knowledge, the information contained herein is accurate. However, neither Celanese Corporation nor any of its affiliates assumes any liability whatsoever for the accuracy or completeness of the information contained herein. Final determination of the suitability of any information or material for the use contemplated, the manner of use and whether there is any infringement of patents is the sole responsibility of the user.

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Photometric Micro-analytical Estimation of Acetic Acid in Blood and Tissues

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The chemical methods which have been proposed for the estimation of acetic acid in biological materials are not specific and are, in consequence, of restricted value: Treatment of acetic acid with cupric oxide yields oxalic acid, and this reaction was utilized by Mugdan & Wimmer (1933) for the estimation of acetic acid; but, under their conditions tartaric, glycollic and the lower fatty acids also yield oxalic acid. Pyrolysis of calcium acetate yields acetone, but since the calcium salts of other monocarboxylic acids also yield carbonyl compounds under these conditions, the method of Dulian (1939), which is based on this reaction, is inadequate. A photometric method based on the lanthanum reaction of Kruger & Tschirch (1929) has recently been developed by Hutchens & Kass (1949), but since propionic acid also gives the blue colour their method lacks specificity.

Procedures dependent on the physical properties of acetic acid can be classified into: (i) Distillation methods: Duclaux (1900), Dyer (1917), Olmsted, Whitaker & Duden (1929), McClendon (1944) and Black (1949). (ii) Partition methods: Behrens (1926), Werkman (1930*a*, *b*, *c*; 1931). Friedemann (1938) devised a method which is a combination of both the distillation and partition methods. (iii) Chromatographic methods: Ramsey & Patterson (1945, 1948), Elsden (1946), Ramsey (1948), Moyle, Baldwin & Scarisbrick (1948) and Peterson & Johnson (1948). Of all these procedures only those involving chromatography can make any real claim either to specificity or to precision.

Acetic acid, however, does differ from the other members of the homologous series in its stability to the potassium dichromate-sulphuric acid reagent; but at the same time it is completely oxidized by treating it with a silver dichromate-sulphuric acid mixture (Simon, 1922*a*, *b*). Cordebard & Michl (1928) used this difference in behaviour as the basis of a quantitative method for the estimation of acetic acid in known mixtures; their method was adapted to the micro-estimation of acetic acid by Cavelli & Ciaranfi (1942). In this procedure acetic acid was separated from its higher homologues by conversion

to the methyl ester and subsequent distillation. The interfering compounds which distilled with methyl acetate were allowed for by treating with potassium dichromate-sulphuric acid prior to oxidation with the silver dichromate-sulphuric acid reagent. This method, whilst useful for known mixtures, was not directly applicable to the estimation of acetic acid in biological materials. It has, however, proved possible to modify the technique in such a way as to permit the photometric estimation of acetic acid in blood and tissues.

METHOD

Principle. Methyl acetate (b.p. 57°) boils at a lower temperature than methanol (b.p. 65°), whereas the methyl esters of the other members of the homologous series have a higher boiling point; consequently by esterifying a mixture of fatty acids in excess methanol and subsequent distillation it is possible to effect a considerable separation of acetic acid from the remainder. After one such distillation and with the relative recovery of acetic acid placed at 100, the recoveries of other acids are as follows: formic, 87; propionic, 63; butyric, 14; valeric, 8; hexanoic, 3; heptanoic, 1. The recovery of formic acid is not quantitative as it is partially decomposed during esterification in the presence of H_2SO_4. H_2S, produced from cystine and related compounds, is converted to methyl mercaptan and distils under these conditions. By repeated distillation methylmercaptan, methyl formate and methyl acetate are separated from the methyl esters of the higher acids, and saponification of the esters in the final distillate yields the sodium salts of H_2S, formic and acetic acids. The first two are readily oxidized by treatment with the $K_2Cr_2O_7$-H_2SO_4 reagent, whereas acetate is oxidized only in the presence of Ag^+.

Under standard conditions the extra dichromate used following treatment with $Ag_2Cr_2O_7$ is related to the amount of acetate present. The dichromate may be estimated either photometrically, using a filter absorbing at 450 mμ., or titrimetrically.

Reagents. Methanol (A.R.). Traces of volatile acids are removed by adding 2 g. of NaOH/l. of methanol and distilling from an all-glass apparatus. Conc. H_2SO_4 (sp.gr. = 1·84). NaOH (10%, w/v, and 1·25%, w/v). Saturated aqueous solution of $AgNO_3$. 0·05N-$K_2Cr_2O_7$.

Apparatus. (*a*) Distillation units, seven of which are required. These are constructed of 8 mm. bore tubing and are of the micro-Kjeldahl type as modified by Embden. The ascending limb is 15 cm. long with a bulb of 3 cm. diameter situated 5 cm. above the standard joint. The oblique limb is 20 cm. long and the descending limb 40 cm.; the first 18 cm. of the latter is water-jacketed.

(*b*) A supply of 50 and 100 ml. Kjeldahl flasks fitted with standard joints. The 50 ml. flasks carry a 12 ml. calibration mark.

(*c*) Two Schiff-type reflux condensers 80 cm. long and fitted with standard joints.

(*d*) A Coleman spectrophotometer model S-14 with round cuvettes (19 × 105 mm.) or a Lumetron photoelectric colorimeter model 400-A.

Extraction. Blood. Blood (5 ml.) is placed in a centrifuge tube containing 40 ml. of cold methanol, stirred thoroughly with a glass rod and centrifuged at 5000 rev./min. for 5 min. in a refrigerated centrifuge. The supernatant is transferred to a measuring cylinder and the volume noted. It is filtered into a 100 ml. Kjeldahl flask and both cylinder and filter paper are washed with a few ml. of methanol, the washings being added to the Kjeldahl flask.

Tissues. A known weight of tissue (approx. 1 g. when using liver, kidney or muscle) is placed in a cooled glass mortar and ground thoroughly with powdered quartz sand; 5 vol. of cooled methanol are then added and stirred with a glass rod. The mixture is transferred to a centrifuge tube and the mortar washed with a further 5 vol. of cooled methanol and the washing added to the centrifuge tube. The contents are made alkaline with 0·2 ml. of 40% (w/v) NaOH and procedure continued as for blood.

Analytical procedure. The Kjeldahl flask containing the methanolic extract is attached to a distillation unit and the contents boiled gently in order to minimize frothing. When the volume has been reduced to about 3 ml. the flask is disconnected and the contents taken to dryness in a bath of boiling saturated $CaCl_2$, the vapours being removed with a water pump. The flask is then cooled and 15·0 ml. methanol and 1·0 ml. conc. H_2SO_4 added. The ground-glass joint is lubricated with a few drops of conc. H_2SO_4 and the flask attached to a second distillation unit. Methanol (4·0 ml.) is added to a 50 ml. Kjeldahl flask, which serves as a receiver, and which is so placed that the tip of the condenser dips below the surface of the methanol. It is distilled gently until the volume in the receiver is 12 ml.; the tip of the condenser is rinsed with 4·0 ml. methanol which are added to the rinsings in the receiver. During this step the fatty acids present in the extract are esterified and the esters distilled over. The methyl acetate is next separated from the esters of the higher acids by repeated distillation: in all, five such distillations are required for complete separation (Table 1). In each case distillation is stopped when the volume in the 50 ml. Kjeldahl flask is 12 ml. and the tip of the condenser washed down with 4 ml. methanol. The final distillate is

Table 1. *Elimination of C_3–C_7 fatty acids by successive distillations of their methyl esters*

Acid	Amount added (mg.)	Amount* (mg.) recovered after					
No. of distillations	...	1	2	3	4	5	6
Formic	1·09	0·88	0·96	0·90	0·84	0·85	0·90
Acetic	1·09	0·98	1·05	1·02	1·00	1·01	1·03
Propionic	1·09	0·63	0·41	0·29	0·15	0·04	— †
Butyric	1·09	0·14	0·22	0·11	—	—	—
Valeric	1·09	0·08	—	—	—	—	—
Hexanoic	1·09	—	—	—	—	—	—
Heptanoic	1·09	—	—	—	—	—	—

* The values for formic, acetic and propionic acids in the table are the average of three determinations varied within ±5% of the mean.

† — signifies that the fatty acid cannot be detected in the sample.

collected in 4·0 ml. of 1·25% (w/v) NaOH instead of methanol. A glass bead is added and the mixture set aside for 30 min. to ensure complete hydrolysis of the esters. The contents of the flask are then taken to dryness in the boiling $CaCl_2$ bath. The dry sodium salts are next treated with the oxidizing agents as follows. 0·05 N-$K_2Cr_2O_7$ (7·0 ml.) is added followed by 7·0 ml. conc. H_2SO_4, the latter being added in such a way as to moisten the standard joint. The flask is attached to a reflux condenser and the contents boiled for 30 min. When the flask has cooled 4·0 ml. of the contents are removed with a pipette and delivered into a colorimeter tube containing 6·0 ml. water; this sample is called 'K unknown'. Two drops of saturated $AgNO_3$ and 3·0 ml. conc. H_2SO_4 are added to the remaining liquid in the flask and the contents boiled under a reflux condenser for

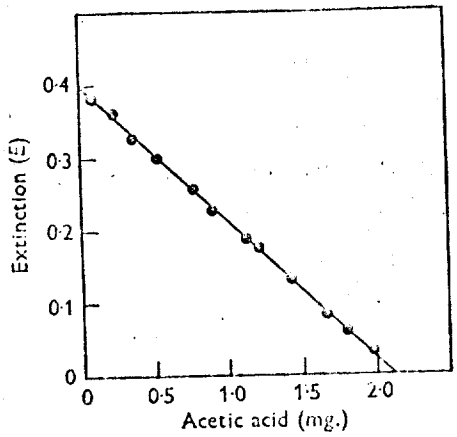

Fig. 1. Calibration curve obtained by estimating samples of standard acetic acid solution, dried together with 4·0 ml. of 1·25% (w/v) NaOH.

1 hr. When cool, a second 4·0 ml. sample is transferred to a colorimeter tube containing 6·0 ml. water; this sample is called 'Ag unknown'. Whilst carrying out the oxidation of the unknown a reagent blank is performed and the two 4·0 ml. samples taken are referred to as 'K blank' and 'Ag blank' respectively. The amount of dichromate present in each sample is then determined spectrophotometrically. If a suitable spectrophotometer is used such as one of those specified it is possible to correct for the blanks directly on the instrument. In all there are three corrections to be made, the 'K blank' and the 'Ag blank' and, in addition, that due to materials present in the distillate which are oxidized by dichromate in the absence of Ag^+, i.e. formate and sulphide.

If the amount of dichromate taken has been sufficient to oxidize all the acetate present then, by setting the 'Ag blank' at an extinction of 0·4 the 'Ag unknown' will give a reading of between 0·4 and 0·0. If this is the case, the 'K unknown', which corresponds to zero acetate concentration, is set at an extinction of 0·4 and the extinction of the 'K blank' determined. This is equal to or greater than 0·4 depending on the amount of easily oxidizable material (formate and sulphide) present in the sample. The reading of the 'K blank' so obtained is used as the arbitrary zero against which the extinction of the 'Ag unknown' is measured. Fig. 1 gives the calibration curve of the method showing that there is a linear relationship between acetic acid and extinction over the range 0·0–2·3 mg. acetic acid.

Titrimetric method. If a photometer is not available a titrimetric procedure similar to that of Caselli & Ciaranfi (1942) may be used. In addition to the apparatus and reagents specified for the photometric procedure the following solutions are required:

0·05 N-$FeSO_4(NH_4)_2SO_4.6H_2O$ in 5% (v/v) H_2SO_4,

0·05 N-$KMnO_4$.

The extraction and distillation procedures are the same as those outlined above. However, the final distillate, which is collected in 4·0 ml. 1·25% (w/v) NaOH solution, is treated differently. The distillate is divided into two equal parts each of which is transferred to a 50 ml. Kjeldahl flask. After allowing 30 min. to complete the hydrolysis of the esters the contents of each flask are taken to dryness. To one flask is added 5·0 ml. 0·05 N-$K_2Cr_2O_7$ and 5·0 ml. conc. H_2SO_4 and to the other 5·0 ml. 0·05 N-$K_2Cr_2O_7$, two drops saturated $AgNO_3$ and 5·0 ml. conc. H_2SO_4. Each flask is then boiled for 1 hr. under a reflux condenser and, when cool, 5·0 ml. of 0·05 N-ferrous ammonium sulphate added, and the contents titrated with 0·05 N-$KMnO_4$. In addition, both a 'K blank' and an 'Ag blank' are determined. When acetate is oxidized under these conditions, 1 ml. of 0·05 N-dichromate is equivalent to 0·375 mg. acetic acid. The amount of acetic acid present in half the sample of blood or tissue taken is equal to $[(A - a) - (K - k)]$ 0·375 mg., where A, a, K and k are the volumes of 0·05 N-$KMnO_4$ required for the 'Ag unknown', 'Ag blank', 'K unknown' and 'K blank' respectively.

RESULTS

Recoveries of known amounts of acetic acid added to various tissues as determined by the photometric and the titrimetric procedures are given in Tables 2 and 3 respectively; Table 4 gives the results of estimations of the acetic acid content of blood, liver,

Table 2. *Recovery of acetic acid estimated photometrically*

('Found' values are corrected for acetic acid present in controls.)

Material	Acetic acid Added (mg.)	Acetic acid Found (mg.)
Blood (human)	0·13	0·13
	0·26	0·24
	0·39	0·36
	0·78	0·84
	1·17	1·15
	1·30	1·24
	1·43	1·36
	1·95	1·90
Muscle (rat)	0·37	0·40
	0·74	0·72
Kidney (rat)	0·70	0·72
	1·11	1·08
Kidney (guinea pig)	0·30	0·32
	0·39	0·38
	0·56	0·53
	1·26	1·21
Liver (guinea pig)	0·39	0·39
	0·61	0·58

Table 3. *Recovery of acetic acid estimated volumetrically from human blood*

('Found' values are corrected for acetic acid present in controls.)

Acetic acid	
Added (mg.)	Found (mg.)
0·13	0·14
0·22	0·23
0·25	0·25
0·50	0·52
0·51	0·48
0·53	0·51
0·54	0·58
0·56	0·55
0·63	0·61
1·00	0·94

Table 4. *Acetic acid determinations in blood and tissues*

		Acetic acid found (mg./100 ml. for blood, mg./100 g. for tissues)		
	Material	Mean	Range	No. of determinations
Blood	Human venous*	4·8	3·2–7·2	10
	Sheep arterial*	7·1	4·6–8·8	3
	Guinea pig arterial†	10	8·2–12·0	3
	Rat arterial†	9	6·1–12·0	3
Liver	Guinea pig†	61	35–98	5
	Rat†	54	40–69	3
Kidney	Guinea pig†	47	27–71	3
	Rat†	35	20–52	3
Muscle	Guinea pig†	23	11–36	3
	Rat†	16	10–22	3

* 6 hr. after feeding. † 1 hr. after feeding.

kidney and muscle. Reid (1950) determined the acetic acid content of sheep blood using the chromatographic methods of Elsden (1946) and of Moyle *et al.* (1948), and our figures for sheep blood are in agreement with those obtained by this author. On the other hand, McClendon (1944), in his paper on the micro-estimation of total volatile fatty acids in biological materials, states that the venous blood of both normal humans and dogs is 0·0003 N in respect of these acids; our results for acetic acid alone are substantially higher. Unfortunately, data are lacking for the acetic acid content of other tissues using other methods, and we are thus unable to obtain a comparison of our method.

SUMMARY

1. Acetate, extracted from blood or tissues with methanol, is dried and transformed into methyl acetate. By repeated distillations the C_3–C_7 fatty acid esters are removed. The ester is saponified and the resulting sodium acetate brought to dryness. Formic acid and hydrogen sulphide, which cannot be eliminated in such a way, are oxidized by potassium dichromate-sulphuric acid, the acetic acid remaining unchanged. Then acetic acid is fully oxidized by increasing the sulphuric acid concentration and by adding silver ions. The reduction of dichromate is measured photometrically and the acetic acid is estimated by the difference in the extinction.

2. This method permits the estimation of acetic acid in blood and tissues (0·1–2·3 mg.) with sufficient accuracy for biological purposes. The error is always less than ± 10 %.

3. The concentrations of acetic acid under physiological conditions, in human blood and in the blood, liver, kidney and muscle of the guinea pig and rat are discussed.

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

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SUMMARY

Blood flow from the coeliac artery, which is distributed to the rumen, reticulum, omasum, abomasum, and the spleen, and eventually passes through the gastrosplenic vein, was estimated in seven calves anesthetized with nembutal. The abdominal cavity was entered through an incision along the line of the 12th rib. Erythrocytes tagged with P^{32} served as markers, and the isotope dilution method was used to determine the rate of blood flow. The tagged erythrocytes were injected into the coeliac artery and collected from the gastrosplenic vein. The first radioactivity appeared in the gastrosplenic vein 1 to 2 sec. after the injection, and the maximum concentration occurred in 12.1 sec. The average time required for all the injected, labelled erythrocytes to pass through the gastrosplenic vein was 31.3 sec. The mean rate of gastric blood flow was calculated to be 764 ml. per minute per 100 lb. of body weight. Based on the analysis of serial blood samples obtained from a calf fitted with a polyethylene catheter into the gastrosplenic vein, it was calculated that approximately 63 g. of acetic and 25 g. of propionic acid were absorbed for each pound of dry feed (a 2:1 mixture of alfalfa hay and grain concentrate) consumed during a 24-hr. period.

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

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

EXPERIMENTAL PROCEDURES AND RESULTS

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

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

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

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

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

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

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

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

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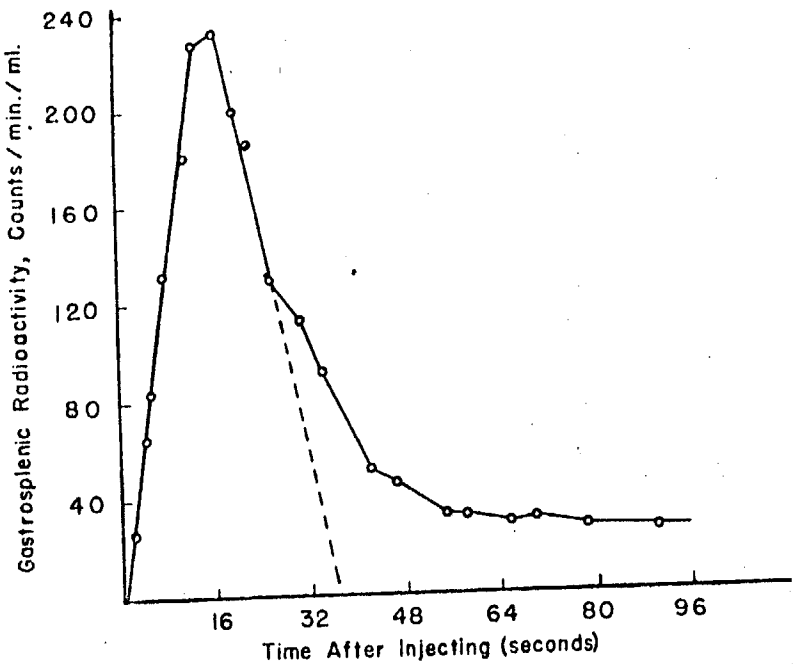

FIG. 1. Radioactivity curve of Calf 412, showing the appearance of tagged erythrocytes in blood drawn from the gastrosplenic vein, in relation to time after instantaneously injecting in the coeliac artery. Broken line shows method of extrapolating, to estimate area under curve had there been no recirculation.

TABLE 1

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

Calf No.	180	189	189	254	254	411	412	244	Mean
Age *(mo.)*	5	6	6	4	4	7	7	5	
Trial No.	1	1	2	1	2	1	1	1	
Time after injection required to reach maximum radioactivity in the gastrosplenic vein *(sec.)*	12	13	11	13	12	12	15	11	12.4
Time required for all tagged erythrocytes to pass through the gastrosplenic vein *(sec.)*	29	37	36	30	24	24	36	34	31.3
Blood flow *(ml/min/100 lb. body wt.)*	780.2	872.2	856.1	865.9	878.1	741.1	365.0	751.0	763.7
Total blood volume *(% body wt.)*	[a]	6.6	6.6	6.6	6.6	5.7	5.1	[a]	6.0[b]
Transfused blood administered during surgery *(ml/100 lb. body wt.)*	410	205	205	360	360	220	300	340	300

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

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

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

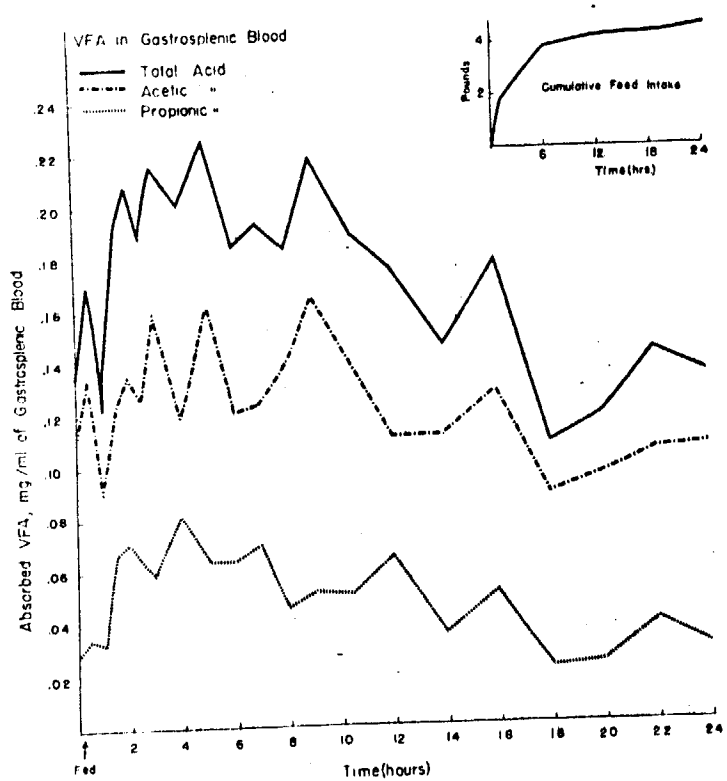

FIG. 2. The concentration of volatile fatty acids (acetic, propionic, and traces of butyric) in the gastrosplenic blood corrected for arterial VFA, and the cumulative feed intake of a 5-mo.-old Jersey calf during the 24-hr. period after allowing it free access to a fresh fe of high roughage pellets (two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

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

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

TABLE 2

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

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

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

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

DISCUSSION OF RESULTS

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

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

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

ACKNOWLEDGMENT

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GASTRIC MUCOSAL INJURY BY FATTY AND ACETYLSALICYLIC ACIDS

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

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

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Materials and Methods

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

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

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

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

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

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

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

Results

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

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

TABLE 1

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

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

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

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

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

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

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

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

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

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

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

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

TABLE 2

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

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

Solutions	n	H^+, net	Na, net	Na, in	K, net	Cl,[d] net	H_2O, net
Glycine controls[a]	20	56	153	−32	17	175	1.0
		±17	±7	±4	±2		±0.2
Acetate[b]	4		49	−39	21	409	1.1
			±43	±8	±2	±66	±0.4
HCl postacetate[c]	4	90	79	−5	8	109	1.3
		±21	±4	±4	±2	±26	±0.2
Propionate[b]	4		161	−24	6	261	0.4
			±44	±13	±2	±63	±0.3
HCl postpropionate[c]	4	−80	184	0	4	181	1.9
		±49	±11	±5	±2	±97	±0.7
Butyrate[b]	4		193	−35	12	244	0.2
			±15	±4	±2	±25	±0.4
HCl postbutyrate[c]	4	86	162	−2	9	194	1.9
		±116	±47	±9	±1	±65	±0.3

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

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

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

[d] Number of observations less than n given.

TABLE 3

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

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

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

[a] 100 mN of HCl, 15 mN of NaCl, 78 mN of mannitol.
[b] 20 mN of acetylsalicylic acid, 100 mN of HCl, 15 mN of NaCl, 78 mN of mannitol.
[c] 20 mN of acetylsalicylic acid exactly neutralized with TRIS, 15 mN of NaCl, 250 mN of glycine.
[d] Physically impossible positive value not different from zero probably resulting from error in volume measurement.
[e] + designates faintest detectable tinge of blood; ++++ designates "coffee grounds."

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

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

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

Discussion

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

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

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

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

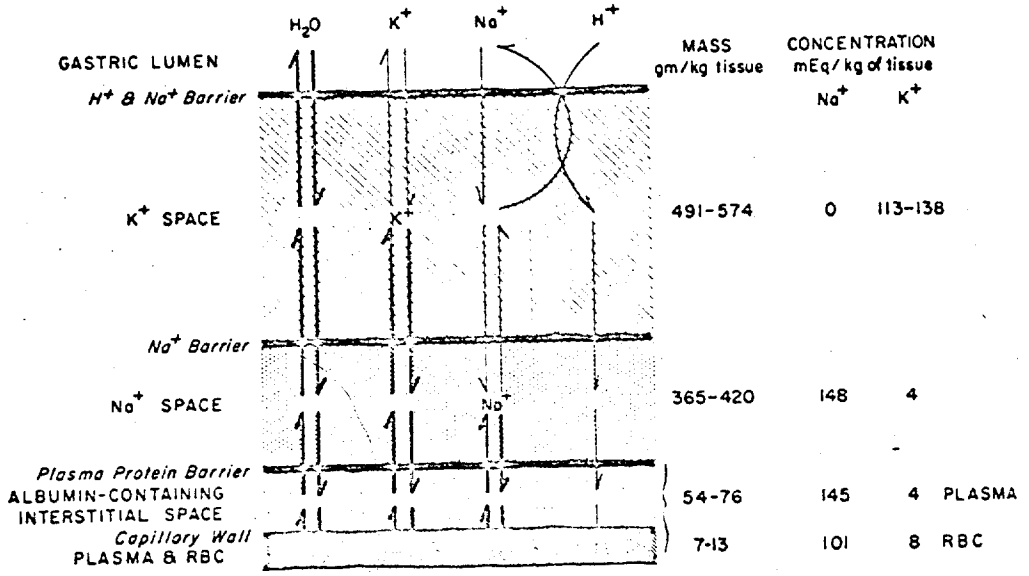

FIG. 1. Electrolyte distribution and barriers of the canine gastric mucosa. See text for references and for discussion of the assumptions upon which the calculations are based. The figure is purely schematic, and it is not meant to imply that all movement from blood to lumen need be through the potassium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

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

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

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

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

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

This description leaves many things

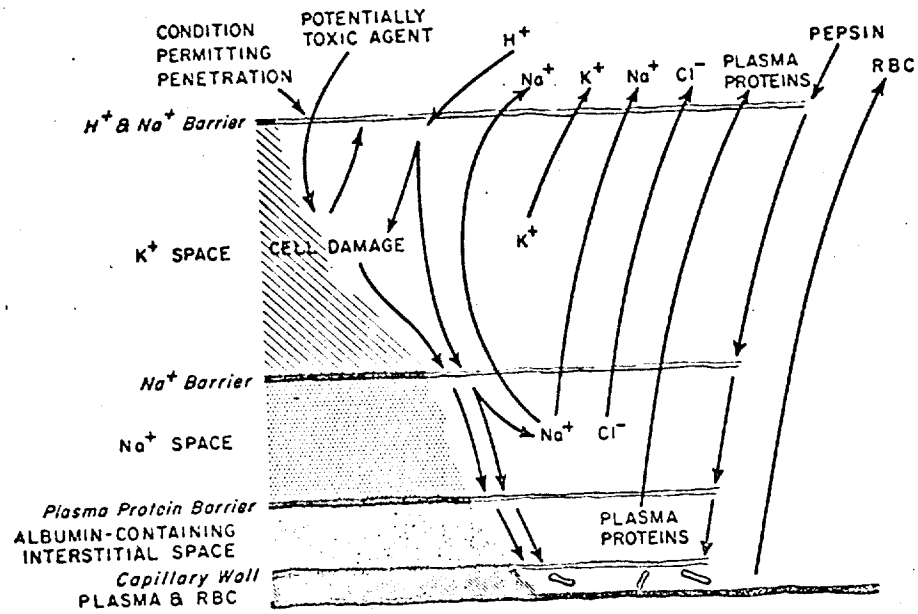

FIG. 2. Scheme of the sequence of events occurring during chemical injury to the gastric mucosa.

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

Summary

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

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

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

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FLUID PRODUCED BY THE GASTRIC MUCOSA DURING DAMAGE BY ACETIC AND SALICYLIC ACIDS

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The otherwise unstimulated mucosa of the oxyntic gland area of the stomach, when damaged by salicylates or fatty acids, produces a large volume of fluid. For example, the volume contained in a separated pouch of the dog's stomach irrigated with 20 mM salicylic acid in 100 mM HCl may increase from 35 to 55 ml within 30 min.[1]

The four possible reasons for increased volume output are: (a) Damage, acting through some local humoral or nervous mechanism, may stimulate secretion of juice containing H^+, K^+, pepsin, and mucus. (b) Filtration may be increased as the result of elevated capillary pressure. Fluid entering the lumen would be derived from an ultrafiltrate of plasma; its protein content would depend upon the natural permeability of the mucosal capillaries and of the mucosa to protein. (c) If capillary permeability is increased by damage, an increased amount of plasma proteins, in addition to other plasma components, may be shed into the lumen. (d) Gross destruction of capillaries would result in frank bleeding, with hemoglobin in the gastric contents as the distinguishing component.

This paper describes an attempt to identify and quantitate the contribution of the several sources of fluid to the volume output of the damaged gastritic mucosa.

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Materials and Methods

Four dogs with vagally denervated, separated pouches of the oxyntic gland area of the stomach were used. The animals were in excellent 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's showing signs of discomfort. Before each test the dogs were deprived of food but not of water for 20 hr.

Chemical analytical methods, counting procedures, and calculations have been previously described.[1,2] Additional methods are described in appropriate sequence. Stimulation, collection, and irrigation schedules are presented in each section under Results.

The following solutions were used: The 100 mM HCl test solution contained 100 mM HCl, 15 mM NaCl, and 78 mM mannitol. The 1 mM HCl test solution contained 1 mM HCl, 15 mM NaCl, and 278 mM mannitol. Salicylic acid was added to either solution by weighing out the quantity required to make a 20 mM solution

and dissolving it in the test solution by heating. Salicylic acid solutions were kept at 37 C at which 20 mM salicylate remains in solution. The 100 mM acetic acid test solution contained 100 mM acetic acid, 15 mM NaCl, and 78 mM mannitol. The glycine test solution contained 250 mM glycine, 15 mM NaCl, and 28 mM mannitol, and its pH ranged from 5.2 to 6.4.

Histamine dihydrochloride was used, but its quantity is always reported as weight of the base. The weight of bethanechol is reported as that of the chloride.

Results

Plasma protein output measured with albumin-Cr^{51}. Common methods for measuring plasma protein could not be used. Because the solution in the pouch, especially when the mucosa was damaged, contained other proteins, measurement of total protein could not be used. Because the solutions were acid and contained pepsin, digestion of plasma proteins doubtless occurred. Radioiodinated serum albumin (Risa) is useful only when conditions can be carefully controlled to prevent digestion.[5] In addition, any radioactive iodide liberated by the inevitable destruction of Risa elsewhere in the animal is selectively secreted by the gastric mucosa,[6] and counting gastric contents gives a falsely high estimate of the amount of Risa in them. Polyvinylpyrrolidone labeled with I^{131} is far worse than Risa, for its liberation of free iodide is rapid.[7] When only small quantities of plasma protein were shed, the blue color of intravenously administered T-1824 (Evan's blue) could be seen in the secretions collected from the damaged pouch, but the small amount of dye could not be separated from mucus and debris for quantitative measurement. Consequently, human serum albumin labeled with Cr^{51} was used.[8] This compound, obtained from E. R. Squibb and Sons, is chemically very stable, for dialysable Cr^{51} is liberated at less than 0.05% per day.[9] Preparations available are inhomogeneous whether or not they are biologically screened, and they must contain radically altered albumin. When albumin-Cr^{51} is injected into a human subject there is a rapid fall in plasma radioactivity in the first 4 days to about 6% of the initial level; thereafter there is a steady logarithmic decay of plasma radioactivity with a half-life of 8.5 days.[8]

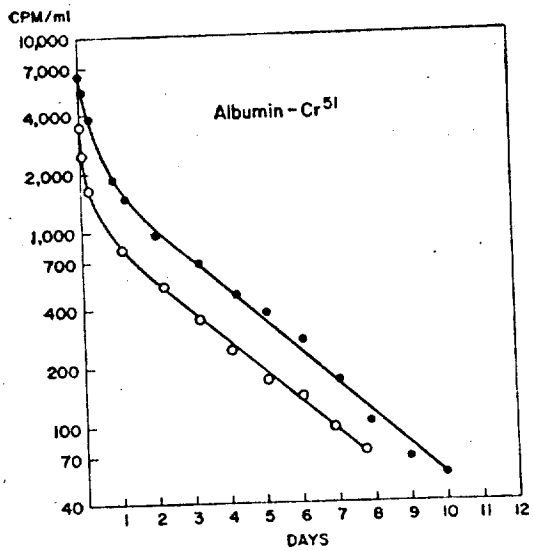

FIG. 1. Plasma radioactivity in counts per minute per milliliter following intravenous injection of approximately 30 μc of albumin-Cr^{51} into two dogs.

In figure 1 the plasma radioactivity of two dogs in counts per minute per milliter is plotted against time following intravenous doses of approximately 30 μc of albumin-Cr^{51}. Results on the other two dogs were similar. The rapid phase of disappearance occupied only 1 day, and thereafter plasma radioactivity decayed with a half-life of 2 days. All experiments reported were done at least 1 day after the compound was given.

Output of plasma by the undamaged mucosa is shown in figure 2. Juice collected from the pouch before stimulation contained radioactivity corresponding to the output of less than 0.1 ml of plasma in 15 min, and it had a barely detectable fleck of hemoglobin in its mucus. The dog was then given histamine, and juice was collected in four 15-min periods. The pouch was irrigated for 30 min with 154 mM NaCl, and after this fluid had been removed, the pouch was again stimulated by

histamine injection. Although there were large changes in volume output, plasma protein output remained essentially constant at a low value. Similar experiments with both histamine and bethanechol stimulation on two other dogs gave the same results.

Output of plasma protein by the damaged mucosa is shown in figure 3. In this experiment juice collected from the unstimulated pouch contained only small amounts of plasma protein and hemoglobin. When the pouch was stimulated by three successive doses of bethanechol, volume output but not plasma protein or hemoglobin output increased. The pouch was then irrigated for 30 min with 20 mM salicylic acid in 100 mM HCl test solution. During irrigation plasma protein loss from the mucosa

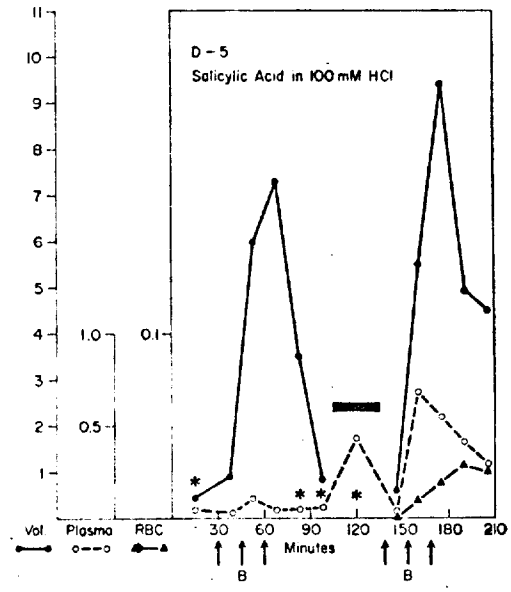

FIG. 3. Volume, plasma, and red blood cell outputs by pouch of dog D-5 in milliliters per 15 min. Plasma output calculated from albumin-Cr^{51} content and red blood cell output calculated from hemoglobin content. *Asterisks* denote detectable but unmeasurable amounts of hemoglobin. Juice collected for 30 min before stimulation and in five 15-min periods after beginning of bethanechol stimulation. Pouch then irrigated with 20 mM salicylic acid in 100 mM HCl as shown by *bar*. Pouch drained and juice collected in 15-min periods after beginning of second stimulation by bethanechol. Bethanechol chloride dose of .02 mg at each *arrow*.

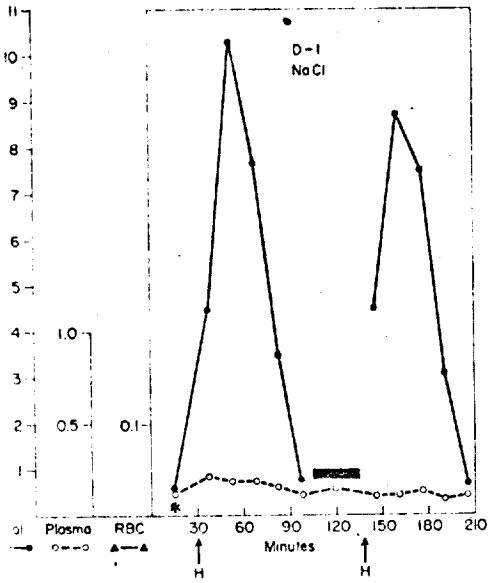

FIG. 2. Volume, plasma, and red blood cell outputs by pouch of dog D-1 in milliliters per 15 min. Plasma output calculated from albumin-Cr^{51} content and red blood cell output calculated from hemoglobin content. Juice collected for 30 min before stimulation and in 15-min periods after subcutaneous injection of 0.228 mg of histamine. Pouch then irrigated for 30 min with 40 ml of 154 mM NaCl as shown by the *bar*. Pouch drained and juice collected in 15-min periods after second injection of 0.228 mg of histamine. *Asterisk* denotes only detectable hemoglobin in any sample.

increased. The pouch was drained, and bethanechol stimulation was resumed. There was a relatively large output of plasma protein early in the response, but thereafter the amount of plasma protein in the juice decreased. On the other hand, red blood cell output increased steadily in the first four periods of collection. In all periods proportionately more plasma than red blood cells crossed the mucosa; the maximal V_c (the hematrocrit expressed as a decimal) of 0.1 was obtained in the last period of collection, and in all other periods it was far lower. This means that much of the plasma crossing the mucosa came from capillaries which, although perhaps abnormally permeable, were able to retain red blood cells. Repetition of the experiment in

another dog gave quantitatively similar results.

The experiment of damaging the mucosa with 20 mM salicylic acid in 100 mM HCl test solution was repeated in two dogs with the difference that histamine was used to stimulate secretion. In each instance plasma protein output was greater after damage than it was before, but the maximal output of plasma protein from the damaged mucosa during histamine stimulation was only one-fifth as great as it was during bethanechol stimulation. Likewise, there was far less hemoglobin in the samples obtained under the influence of histamine. It is possible that bethanechol stimulation increases bleeding from a damaged mucosa through its stimulation of muscular contraction and consequent elevation of venous and capillary pressure.[1] Elevation of capillary pressure could also account for the greater output of plasma during bethanechol stimulation.

In two experiments the pouches of dogs whose plasma contained albumin-Cr^{51} were irrigated for two successive 30-min periods with 100 mM HCl test solution, then for two 30-min periods with the same solution to which 20 mM salicylic acid had been added, and finally for two periods with the 100 mM HCl test solution. Volume increments and the amounts of plasma and red blood cells entering the irrigation fluid are given in table 1. These data show that no more than a third of the increased volume output occurring after damage can be accounted for as plasma and that much more plasma than whole blood escapes from the injured mucosa.

Glucose in gastric juice after histamine and bethanechol stimulation. The appearance of glucose in gastric juice was studied in order to estimate the amount of interstitial fluid appearing in samples before and after damage. Glucose is suitable for this purpose, because its plasma (and therefore interstitial) concentration remains reasonably constant, it has a small molecular weight and it can be measured by a highly specific enzymatic method (Worthington Glucostat).

Glucose was first measured in samples of juice obtained from undamaged pouches after stimulation with histamine or bethanechol. Juice was collected for four 30-min periods. Histamine was given subcutaneously five times at 15-min intervals in a dose of 0.114 mg. Bethanechol chloride was given six times in a dose of 0.2 mg. There was no detectable hemoglobin in any sample. The samples collected during bethanechol stimulation contained abundant mucus which on microscopic examination was found to be full of leucocytes but to contain no surface epithelial cells. The dogs' plasma glucose concentrations were 103 to 105 mg per 100 ml.

In six experiments glucose was detectable

TABLE 1. *Volume, plasma, and red blood cell outputs of unstimulated, vagally denervated pouches of dogs D-1 and D-6 during six consecutive 30-min periods of irrigation*[a]

Test solution	Dog D-1			Dog D-6		
	Volume increment	Plasma	Red blood cells	Volume increment	Plasma	Red blood cells
		ml/30 min			*ml/30 min*	
100 mM HCl	0.2	0.15	0	0.6	0.11	0
	1.1	0.22	0	1.3	0.04	0
20 mM salicylic in 100 mM HCl	3.3	0.31	0	1.8	0.47	0
	1.2	0.43	0.004	1.5	0.45	0
100 mM HCl	6.6	0.70	0.011	4.9	0.46	0
	1.6	0.93	0.020	1.3	0.18	0

[a] Dogs were irrigated twice with 100 mM HCl test solution, twice with 20 mM salicylic acid added to the same solution, and twice more with 100 mM HCl test solution.

in all of the samples collected during the first 30 min after injection of stimulants, and its concentration ranged from 2 to 17 mg per 100 ml. It was below the limits of detectability in all subsequent histamine-stimulated samples. It was detectable at concentrations of 1, 1, 3, and 7 mg per 100 ml in four out of 11 subsequent bethanechol stimulated samples. Some glucose, corresponding to that in not more than 0.25 ml of interstitial fluid, appears to be washed out at the beginning of stimulation; thereafter the amount of glucose in gastric juice is negligible.

Glucose in gastric juice after damage. Results of a characteristic experiment are shown in figure 4. Juice was collected for 30 min before stimulation was begun. (In this instance not enough juice was collected to permit analysis.) Then a large dose of histamine was given, and juice was collected for five 15-min periods. The pouch was irrigated for 30 min with 40 ml of 100 mM acetic acid test solution, and after this solution had been removed histamine stimulation and juice collection were repeated. In the first episode of stimulation glucose output fell as volume and acid outputs rose; in the second episode glucose output rose with volume output. Essentially identical results were obtained in many repetitions of this type of experiment with this and two other dogs and also with bethanechol stimulation and with damage with salicylic acid in 100 mM HCl.

The relation between Na^+ and glucose outputs in one dog before and after damage are shown in figure 5. Data from the other dogs gave similar figures. The shaded area covers the values to be expected if Na^+ and glucose had crossed the mucosa at rates proportional to their concentrations in interstitial fluid. The upper edge of the *shaded area* was determined by drawing a line from the origin through the point glucose = 760, Na^+ = 145, and the lower edge was determined by drawing a line from the origin through the point glucose = 1030, Na^+ = 145. This dog's plasma glucose ranged from 760 to 1030 μg per ml, and its plasma Na^+ was 145 μeq per ml. Since in the case of Na^+ corrections for plasma water content and the Donnan distribution across capillary walls nearly cancel each other out, these concentrations can be taken as representing those of Na^+ and glucose in interstitial fluid. If a certain volume of interstitial fluid enters the pouch, and if Na^+ and glucose enter only as components of that fluid, points obtained on analysis of gastric juice should fall within the shaded area. For the undamaged stomach, the data show that far more Na^+ than glucose appears in the juice; in relation to Na^+ the passage of glucose across the mucosa is highly restricted. The points obtained on analysis of juice secreted by the damaged mucosa show that the ratio between Na^+ and glucose more nearly approaches the ratio of their concentrations in interstitial fluid. Nevertheless, Na^+ crosses the damaged mucosa at about twice the rate of glucose. Data for exact specification of the electrochemical gradient along which Na^+ moved are not available, and further analysis in terms of permeability coefficients is impossible.

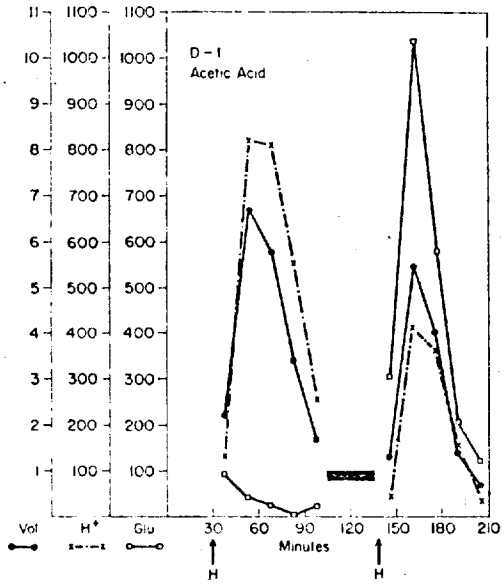

FIG. 4. Volume, acid, and glucose outputs from pouch of dog D-1 before and after irrigation with 100 mM acetic acid as shown by the *bar*. Volume in milliliters per 15 min; acid in μeq per 15 min; glucose in micrograms per 15 min. Histamine in dose of 0.228 mg given at each *arrow*.

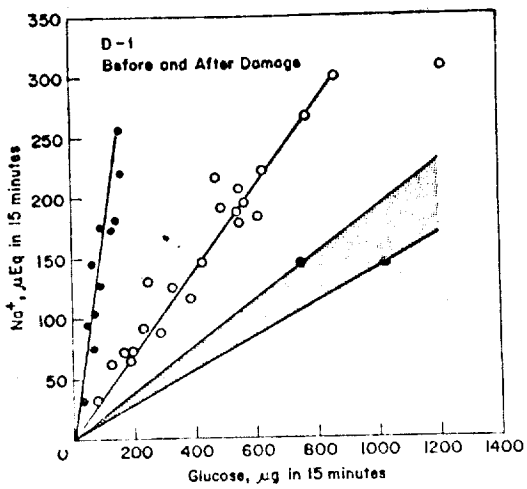

FIG. 5. Rate of output of Na^+ in gastric juice of dog D-1 plotted against rate of output of glucose before and after damage by salicylic or acetic acids. Secretion stimulated by histamine or bethanechol chloride. *Closed circles* represent data obtained before damage; *open circles* represent data obtained after damage. The *shaded area* is that in which points would fall if rates of output were proportional to concentrations of Na^+ and glucose in interstitial fluid. See text for its method of construction.

When data for K^+ are plotted in the same way, the ratio between its rate of output and that of glucose is found to be close to their ratio in interstitial fluid. Since K^+ comes from cells as well as from interstitial fluid,[1] the observed relation is probably fortuitous.

Glucose in irrigation fluids after damage. An unstimulated pouch was irrigated for 30 min with 100 mM HCl test solution and then for two periods with the same solution in which 20 mM salicylic acid had been dissolved. Finally the pouch was irrigated for two more periods with the 100 mM HCl test solution. The solutions were analyzed for glucose as well as for their other constituents, and the volume increment was measured. Volume increments and glucose outputs are given in table 2.

The clearance of glucose, i.e. its rate of entry into the irrigation fluid or gastric juice divided by its plasma concentration, was calculated for these experiments and for those described in the previous section. For damaged mucosa, the glucose clearance was never greater than 20% and it was usually less than 10% of the volume output. These percentages describe the maximum contribution of unmodified interstitial fluid to the fluid produced by the mucosa. The rest must be modified interstitial fluid and secretions.

Stimulus for fluid production. Throughout this work the magnitude of fluid production by the damaged mucosa has been observed to correlate with diffusion of acid into the mucosa. The following sections of this paper offer evidence to support the arguments: (a) that acid itself is the primary stimulus for fluid production; (b) that acid acts by liberating histamine within the mucosa; and (c) that histamine, if it is present within the interstitial fluid of the damaged mucosa, causes it to shed plasma and interstitial fluid and to secrete acid.

The results of 18 experiments which demonstrate that acid in contact with the normal mucosa causes fluid production are presented in table 3. In these experiments pouches of the oxyntic gland area were irrigated for five consecutive 30-min periods, first with 100 mM HCl test solution, twice with 250 mM glycine test solution whose *p*H was initially greater than 5, and finally twice with the 100 mM HCl solution. Outputs of fluid during the periods in which the *p*H of the contents of

TABLE 2. *Volume increment and glucose output of unstimulated, vagally denervated pouches of dogs D-5 and D-6 during five consecutive 30-min periods of irrigation*

Test solution	Dog D-5		Dog D-6	
	Volume increment	Glucose output	Volume increment	Glucose output
	ml/30 min	*μg/30 min*	*ml/30 min*	*μg/30 min*
100 mM HCl	4.8	242	−0.5	0
20 mM salicylic in 100 mM HCl	4.6	524	1.8	337
	9.7	1001	2.4	266
100 mM HCl	9.1	941	2.2	265
	9.0	896	2.4	229

the pouches was greater than 5 were significantly smaller ($P < 0.01$) than outputs in any of the acid periods.

Fluid production is increased when diffusion of acid into the mucosa is accelerated by damage.[4] Further evidence for this is contained in experiments in which volume output was carefully measured. At the beginning of these experiments the dogs were given a subcutaneous injection of 0.05 mg of bethanechol chloride, and the dose was repeated at 15-min intervals for a total of 12 times. This dose was just at threshold, for although it did not in itself cause an increase in volume output, juice collected from the pouches increased slowly in acidity. The reason for giving bethanechol injections was to provide a background of cholinergic stimulation with which any histamine liberated during irrigation could act in a synergistic fashion, thereby increasing the sensitivity of the test.

The irrigation apparatus was attached to the cannula draining the pouch, and the pouch was filled with 40 ml of 154 mM NaCl. At the end of 15 min the pouch and irrigation apparatus were emptied as completely as possible into a graduated cylinder. The tubing from the syringes was disconnected from the cannula in order to allow collection of any fluid draining from the pouch. Drainage was continued for 5 min, and fluid collected was added to that in the cylinder. The volume was read, and the difference from 40 ml was recorded as the volume output in 20 min. This technique eliminated reliance on estimation of residual volume which was part of the earlier observations.

TABLE 3. *Mean increases in volume of contents of unstimulated, vagally denervated pouches of oxyntic gland area of dogs irrigated for five consecutive 30-min periods, once with 100 mM HCl test solution, twice with 250 mM glycine (pH > 5) and twice with 100 mM HCl test solution*

Period	Solution	$\bar{x}$	SD
		ml/30 min	
1	HCl	1.6	±1.2
2	Glycine	0.4[a]	±0.8
3	Glycine	0[a]	±1.1
4	HCl	2.4	±1.7
5	HCl	1.5	±1.0

[a] Significantly smaller than first, fourth, or fifth periods, $P < 0.01$; N = 18.

TABLE 4. *Volume increments in milliliters per 20 min during irrigation of vagally denervated pouch of oxyntic gland area of dog D-5; three consecutive periods with 154 mM NaCl, three with test solutions named, and three with NaCl*[a]

Test Solution	NaCl	Test	NaCl
154 mM NaCl	2.3±1.6	2.3±1.1	2.7±1.3
100 mM HCl	1.5±0.7	2.9±1.2	3.5±2.0
100 mM acetic acid	1.3±1.7	4.3±1.3	3.5±0.8
20 mM salicylic, 1 mM HCl	2.3±1.5	3.3±0.7	3.6±1.9
20 mM salicylic, 100 mM HCl	1.3±1.2	4.9±1.6	3.6±1.6

⟵ $P < 0.01$ ←- - $P < 0.02$

[a] Background stimulation by subcutaneous injection of 0.05 mg of bethanechol chloride every 15 min. *Arrows* indicate degree of statistical significance of difference between pairs of results; no other differences reached the 0.05 level.

In the control series of observations the process of filling the pouch with 40 ml of 154 mM NaCl was continued for a total of nine periods. In the experimental series the pouch was filled in the fourth, fifth, and sixth periods with one of four test solutions. Irrigation with 154 mM NaCl was resumed in the seventh, eighth, and ninth periods. Control and experimental series were repeated three times. Data obtained in the first, second, and third periods were pooled as were those in the fourth, fifth, and sixth periods and from the seventh, eighth, and ninth periods. Those from dog D-5 are given in table 4. Volume increase was uniform throughout the control periods when the pouch was irrigated with NaCl.

When salicylic acid in 1 mM HCl was used, changes did not reach the level of statistical significance. When acetic acid or 100 mM HCl together with salicylic acid were used the greatest increases in volume occurred; it is from these solutions that acid rapidly diffuses into the mucosa.[1]

The whole series of experiments was repeated on another dog. Although the general trend of the data was the same, statistically significant increases occurred only when the pouch was irrigated with acetic acid.

Histamine in gastric contents after damage. The most cogent evidence for histamine liberation within the mucosa would be provided by demonstration of increased histamine content of lymph or venous blood immediately draining the stomach. Neither gastric lymph nor gastric venous blood could be sampled in these experiments, and histamine was sought in gastric contents. The molecular weight of histamine is less than that of glucose and, if histamine is liberated into interstitial fluid during damage, it, like glucose, should be able to diffuse into gastric contents. Consequently, detection of histamine in gastric contents would be presumptive evidence that it is present in interstitial fluid during damage. On the other hand, the mucosa is normally so impermeable to histamine[10] that failure to find it in gastric contents would not rule out its presence in interstitial fluid.

In order to obtain flow of fluid into the pouch as well as to provide opportunity for diffusion, the dog was given a subcutaneous dose of 0.4 mg of bethanechol chloride, and the dose was repeated at 15-min intervals for a total of seven times. Twenty minutes after the first dose the dog's pouch was washed out with 154 mM NaCl. It was filled with 30 ml of 154 mM NaCl which was allowed to remain in the pouch for 30 min. The fluid was completely removed and saved for analysis. The pouch was filled with 30 ml of one of four test solutions: 100 mM HCl, 100 mM acetic acid, 20 mM salicylic acid in 100 mM HCl, or 20 mM salicylic acid in 1 mM HCl. At the end of 30 min this fluid was removed and replaced by another 30 ml of the same solution. After 30 min this solution was removed and replaced by 30 ml of 154 mM NaCl which remained in the pouch for a final 30-min period. A 20-ml sample of each solution was analyzed for histamine by the bioassay method described in detail by Code and McIntire[11] using the terminal ileum of the guinea pig, and the total quantity in each volume of fluid was calculated. The potassium content of the final extracts was always below that which in itself would cause contraction of the ileum. The minimum quantity of histamine detectable was 0.03 μg per sample. Percentage recovery of histamine from 100 mM HCl carried through the entire procedure was 95 and 98%. Recovery from NaCl was 75 and 82% and from acetic acid was 53 and 86%. Results were not corrected for incomplete recovery.

The experiments were repeated three times on two dogs with each of the four test solutions; 94 samples were analyzed, and two were lost. In 23 initial periods of irrigation with NaCl, histamine was present in small amounts (0.05 to 0.11 μg) seven times and was undetectable 16 times. In 17 periods during and following irrigation with 100 mM HCl, histamine was found only five times (0.03 to 0.25 μg), and it was found five out of 18 times (0.03 to 0.05 μg) during and following irrigation with acetic acid. These data provide no evidence that histamine is liberated by HCl or acetic acid. Histamine was found in amounts ranging from 0.03 to 0.49 μg (mean, 0.20 ± 0.15 SD) in 13 out of 18 samples obtained during and following irrigation with 20 mM salicylic acid in 100 mM HCl. During and following irrigation with 20 mM salicylic acid in 1 mM HCl, a procedure which causes mild and transitory increase in mucosal permeability,[1] histamine was found (0.03 to 0.25 μg) in eight out of 18 samples. These data show that histamine can appear in gastric contents during one form of damage, but they do not prove its simultaneous presence in interstitial fluid.

Effects of topical application of histamine. If histamine liberation into mucosal interstitial fluid is the immediate cause of some of the consequences of damage, histamine experimentally delivered to the inter-

stitial fluid should mimic and exacerbate these effects. The appropriate route of histamine administration to test this hypothesis is by topical application, not intravenous or intra-arterial injection. The normal gastric mucosa is almost totally impermeable to histamine.[10] Histamine applied to the mucosa in concentrations up to 1 mg per ml can be shown to stimulate a small amount of secretion only when topical application is combined with subcutaneous injection of histamine.[12] If, however, glucose can cross the damaged mucosa, histamine, whose molecular weight is less than that of glucose, should be able to cross it as well. Therefore, topical application of histamine to the damaged mucosa in a concentration which has no effect upon the normal mucosa should have readily detectable effects.

Juice was collected for 30 min from the pouch of a fasted dog. If the rate of secretion was negligible, the pouch was irrigated for 30 min with a test solution. For control experiments this was the 100 mM HCl test solution; for the damage experiments it was 100 mM acetic acid test solution. After the test solution had been removed, juice was collected for 65 min. In the control experiments little or nothing was obtained. After acetic acid irrigation thick opaque mucus in volume 2 to 5 ml drained from the pouch in the last half of the period. This mucus, on microscopic examination, was found to contain large numbers of desquamated epithelial cells. Toward the end of the collection period the dog was given an intravenous injection of T-1824 (Evan's blue) in a dose of 5 ml of a 0.5% solution. The pouch was then irrigated for 30 min with a solution of histamine dissolved in 154 mM NaCl, and at the end of the period the fluid was removed, measured, and analyzed for pH, glucose, and T-1824. (There was so much T-1824 in the solution obtained after damage that colorimetric estimation on diluted samples was easy.) A blood sample was obtained, and after immediate separation the plasma was analyzed for glucose and T-1824. Finally, juice samples from the pouch was collected for six 10-min periods; these samples were analyzed for pH, glucose, and T-1824. Results obtained in three pairs of experiments on one dog are given in table 5. (Data obtained during the preliminary periods of irrigation and during the 65-min period of drainage before topical application of histamine are omitted. Likewise, results from the last three 10-min periods of collection, since they added nothing, are not given.) The histamine concentration used was 0.1 mg per ml. A lower concentration was not tested. Results obtained in three pairs of experiments on another dog with a concentration of 1 mg per ml (which had no effect on the control series) were qualitatively similar.

When the preliminary irrigation fluid had been 100 mM HCl there was little or no fluid production during irrigation with histamine, and in the subsequent hour little fluid flowed from the pouch. The concentration of glucose in these samples was within the range previously encountered in this dog during control irrigations or in collections made without exogenous stimulation. The amount of plasma contained in the solutions, judged by their T-1824 content, was little if at all greater than would be expected on the basis of results obtained with albumin-Cr^{51} in the same dog.

The results were spectacularly different when the pouch damaged by preliminary irrigation with 100 mM acetic acid was irrigated with histamine solution. A large volume output occurred, and almost all of the increment was plasma. The irrigation solution became blue within 5 min, and its color steadily deepened. There were also a substantial volume output following histamine application, and much, but not all, of the fluid could be identified as plasma or interstitial fluid. There was no hemoglobin in any sample.

Stimulation of secretion by topical histamine. If, in the experiments described, histamine applied to the damaged mucosa stimulated secretion of acid, the acid was diluted or neutralized by the large volume of plasma and interstitial fluid which histamine also caused to be shed by the mucosa. However, secretion can be demonstrated by trapping the acid with glycine.[3] In five control experiments on one dog the pouch was irrigated for 30 min with 100

TABLE 5. *Results obtained in three pairs of experiments with irrigation of the separated, vagally denervated pouch of dog D-1 with 40 ml of 0.1 mg of histamine per ml in 154 mM NaCl after preliminary irrigation for 30 min with either 100 mM HCl or with 100 mM acetic acid*

Preliminary irrigation			100 mM HCl test solution				100 mM acetic acid test solution			
Collection	Time	Experiment no.	Volume	pH	Glucose	Plasma[a]	Volume	pH	Glucose	Plasma[a]
	min		*ml*		*mg/100 ml*	*ml*	*ml*		*mg/100 ml*	*ml*
Histamine in NaCl	30	1	42	ND[b]	3	ND	57	6.95	27	14
		2	40	ND	1	<0.5	51	6.95	24	15
		3	40	4.94	3	1.0	49	6.69	17	8
Collection 1	10	1	1.1	6.08	17	ND	5.7	7.26	58	3.6
		2	0.9	4.35	4	<0.05	6.1	6.93	68	1.1
		3	1.0	3.50	11	0.1	3.5	7.20	49	2.1
2	10	1	0.5	6.96	36	ND	2.4	7.36	73	1.6
		2	0.2	4.75	8	<0.02	2.5	7.53	73	0.9
		3	0.9	4.37	24	0.2	1.9	7.35	63	0.6
3	10	1	0.4	—[c]	—[c]	ND	1.6	7.61	71	0.9
		2	0.2	4.95	6	<0.02	0.8	8.82	79	0.3
		3	1.2	1.58	25	0.2	0.9	7.68	65	0.3

[a] Milliliters of plasma containing the quantity of T-1824 found in contents or juice.
[b] ND = Not determined.
[c] Combined with previous sample for analysis. Plasma glucose ranged from 76 to 83 mg per 100 ml.

TABLE 6. *Volume increment and acid trapped in glycine during irrigation of vagally denervated pouch of dog D-8 previously damaged by irrigation with 100 mM acetic acid test solution*[a]

Controls	N	Volume increment	H^+ trapped
		ml/30 min	*μeq/30 min*
Glycine			
1	5	0	9 ± 12
2	5	0	46 ± 23
Histamine			
Histamine in glycine	5	10 ± 6	169 ± 150
Glycine	6	5 ± 1	492 ± 99

[a] In control experiments pouch was irrigated for two consecutive 30-min periods with 250 mM glycine test solution. In the histamine experiments the glycine solution in the first period contained 1 mg of histamine per ml. Means and standard deviations.

mM acetic acid test solution and allowed to drain for 65 min. Then the pouch was irrigated for two 30-min periods with the 250 mM glycine test solution. Acid output was measured by titrating the samples obtained at the end of irrigation to their initial pH. Results given in table 6 show that little acid was secreted. The experiments were repeated six times with the difference that after preliminary irrigation with acetic acid and drainage the pouch was irrigated for 30 min with a solution of 1 mg of histamine per ml dissolved in the glycine solution. After this solution had been removed the pouch was irrigated for another 30 min with the glycine solution alone. The results show that acid secretion could be detected after irrigation with histamine. If acid were secreted by the oxyntic cells at a concentration of about 150 mM, secretion accounted for about 11% of the volume increment during histamine irrigation and for 65% of the volume increment in the next 30 min.

Discussion

Figure 6 presents a summary of my current concepts of the causes of fluid production from the oxyntic gland area during injury.

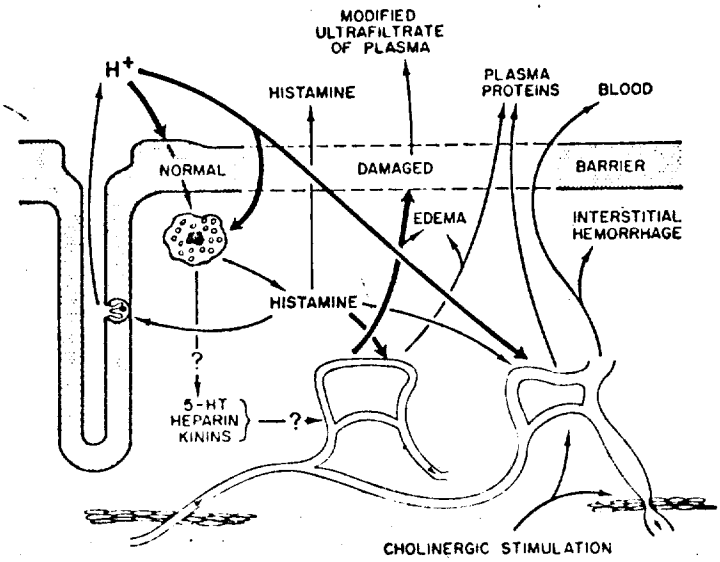

FIG. 6. A scheme of events leading to fluid production by the damaged gastric mucosa.

Acid in the lumen diffuses slowly through the normal mucosa, the rate depending upon the natural permeability of the mucosa. Acid diffuses much more rapidly through the mucosa whose permeability has been increased by damage, and it causes liberation of histamine into interstitial fluid. (Acid may also liberate 5-hydroxytryptamine, heparin, and other products of cellular injury such as kinins,[13] but we do not yet have evidence on this point.) Some histamine appears in gastric contents if the mucosa is sufficiently permeable, some stimulates acid secretion and some increases the permeability of mucosal capillaries with the result that plasma proteins enter the interstitial fluid in abnormal amounts. At the same time capillary pressure may increase as the result of arteriolar dilation with or without some obstruction of venous outflow. Decreased colloid osmotic pressure difference across the capillary wall and increased hydrostatic pressure cause net flow of fluid into the mucosa, and edema results. Because mucosal permeability is increased, interstitial fluid enters the lumen. This fluid is not simply an ultrafiltrate of plasma; its sodium exceeds its glucose content, and it contains protein. Fluid entering the lumen neutralizes and dilutes acid. Histamine and other humoral agents do not in themselves cause capillary rupture, but acid rapidly diffusing through the mucosa does. Interstitial hemorrhage and appearance of blood in the lumen follow. Bleeding is promoted by concurrent administration of a cholinergic drug, perhaps as the result of venous congestion caused by contraction of gastric smooth muscle.

There are two serious weaknesses in the scheme. The lesser is the postulation of hemodynamic changes beyond detection by our present methods of study. The more serious is placing histamine liberation by acid in the central position. The evidence adduced to support this idea is that histamine can sometimes be found in gastric contents following damage and that topical histamine promotes fluid production whose characteristics are an exaggerated version of those produced by acid. The presence of histamine in interstitial fluid must be deduced from the histamine content of fluid in the lumen, but the two fluids are separated by a barrier whose permeability to histamine is unknown. Other evidence is required. Johnson,[14] working in this laboratory, has shown that histamine appears in the gastric contents

of pylorus-ligated rats when their stomachs are irrigated with 100 mM acetic acid or 20 mM salicylic acid in 100 mM HCl, and that the histamine content of the oxyntic glandular area simultaneously decreases. The fall in mucosal histamine does not occur when the stomach is irrigated with 154 mM NaCl or with 100 mM HCl.

If the scheme is correct, the question arises at what point normal shades into abnormal function. Acid does diffuse into the normal mucosa;[15] histamine does appear in normal gastric juice;[16] plasma proteins are present in normal gastric secretions[5] and in normal gastric interstitial fluid;[17] and small amounts of blood do leak from the apparently normal mucosa. It is possible that figure 6 describes the normal as well as the pathological situation, the differences between them being only quantitative.

Summary and Conclusions

The oxyntic gland area of the gastric mucosa, when damaged by acetic or salicylic acids, frequently puts out a large volume of fluid. Sources and causes of this fluid production were sought in four healthy, unanesthetized dogs provided with separated, vagally denervated pouches of the oxyntic gland area. Plasma output was measured using albumin-Cr^{51}, and interstitial fluid output was estimated from glucose and sodium outputs.

A small amount of plasma crossed the normal unstimulated mucosa, but its output was not increased by histamine or bethanechol stimulation. Plasma output was increased by damage, but it accounted for no more than one-third the total volume. Plasma output from the damaged mucosa was greater when secretion was stimulated by bethanechol than by histamine. During frank bleeding, plasma output was greater than erythrocyte output, the maximal decimal hematocrit being about 0.1. Only minute amounts of glucose crossed the normal mucosa, but large amounts appeared in gastric contents after damage. Proportionately more sodium than glucose left the damaged mucosa.

Volume output occurred when the mucosa was exposed to acid but not to neutral solutions. Histamine was detected in gastric contents during and following salicylate damage more often than in control experiments. Topical irrigation of the damaged mucosa with histamine caused a large output of plasma but not of erythrocytes and a small stimulation of acid secretion.

It is tentatively suggested that acid itself is the initiating stimulus for fluid production and that histamine liberation by damaging agents is an important mediator.

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ON THE ALLEGED CONVERSION OF FAT TO CARBOHYDRATE.

I. THE METABOLISM OF ACETIC ACID.*

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INTRODUCTION.

No convincing experimental evidence that the mammal is able to convert the fatty acid portion of the fat molecule into carbohydrate has been put forward. In spite of this, a large school of physiologists in this country and abroad has accepted such a physiological transformation as a proved fact.

There is general agreement that glycerol acts as a glucogenetic agent. Cremer (1) and Lüthje (2) were able to recover 40 per cent of the glycerol ingested by diabetic animals as extra sugar in the urine while Chambers and Deuel (3) observed a practically complete conversion of this alcohol to glucose in two phlorhizinized dogs. On the other hand, the bulk of experimental evidence indicates that the fatty acid gives rise to β-hydroxybutyric acid and not to sugar. Lusk (4) has found no increase in the D:N ratio of phlorhizinized dogs in which the fat metabolism has been largely increased either by mechanical work or by shivering. Not only was there no excretion of urinary sugar which might be traced to a synthesis from fatty acids but even that which one would expect from the glycerol metabolized did not appear in the urine. Lusk[1] has suggested that when glycerol is metabolized with the fatty acid chain in phlorhizinized animals, it is oxidized completely to CO_2 and H_2O.

* A preliminary report of this work was published in *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 667.

[1] Personal communication from Professor Lusk.

If the fatty acid having an even numbered carbon chain were capable of conversion into glucose, it is probable that its oxidation products would be the intermediates in this transformation. As a result of β-oxidation of the fatty acid, it is generally believed that acetic acid may be produced which ultimately would be oxidized to CO_2 and H_2O. If fat is a potential glucose former, then acetic acid should serve as a source of glucose. Ringer and Lusk (5) have reported one experiment in which 10 gm. of sodium acetate (equivalent to 4.41 gm. of glucose) were given to a phlorhizinized dog without any change in the D:N ratio or any increase in the glucose excretion being observed. On the contrary, Geelmuyden (6) has reported several experiments on phlorhizinized dogs which he believes are proof of the formation of glucose from this acid. His results are not convincing and do not offer strong support for the contention that such a synthesis may occur. His experimental animals received a constant carbohydrate-containing diet (resulting in the basal D:N ratio of from 6.0 to 7.0 or higher) and the increased sugar excretion was not greater after the administration of sodium acetate than on some of the control days. In one experiment on which his conclusions are based, the total urinary glucose alone is determined without any regard being taken of the nitrogen excretion or of the D:N ratio. Geelmuyden has suggested that acetic acid is not immediately converted into glucose in some cases but that the increased glucose excretion occurs on the day following its administration. That the conversion into glucose of a substance so rapidly metabolized as sodium acetate should be so retarded is without precedent when compared with other intermediate substances capable of yielding glucose.

Thunberg (7) has postulated that acetic acid is convertible into succinic acid in the following manner in the animal organism.

$$2\ CH_3COOH \rightarrow \begin{array}{l} CH_2COOH \\ | \\ CH_2COOH \end{array} + H_2O.$$

If such a transformation is possible in the mammal, it would be suggestive of the probable intermediate reactions involved in the change from fatty acid to sugar since it has been shown by Ringer, Frankel, and Jonas (8) that succinic acid is a glucose former. However, Chatterjee (9) using the minced tissues of rabbits in

some recent work has been unable to confirm the results of Thunberg.

Acetic acid has been identified as a normal constituent in horse urine (10) and in human urine (11). Schotten (10) recovered considerable amounts of acetate in the urine after giving 25 gm. doses to dogs but noted a large increase in the Na_2CO_3 content of the urine which would indicate that the larger portion of the ingested sodium acetate was oxidized to CO_2. Dakin (12) has obtained similar results and has shown that acetic acid is not changed to oxalic acid in the course of its transformation to carbonic acid.

In view of the contradictory evidence as to the fate of acetic acid and because of its importance as a possible intermediate product of fat oxidation, the following experiments were carried out to throw further light on its metabolism.

General Procedure.

Fasting female dogs were used in the experiments reported here. Phlorhizin was administered by the technique usually followed in this laboratory (13). The urine was collected from the cages and by catheter in 24 hour periods until the animal was completely diabetic, after which time 8 to 12 hour samples were taken before and after the administration of CH_3COONa or glucose.

Since it was found in preliminary experiments that the oral administration of sodium acetate often produces a violent catharsis which may prevent the quantitative absorption of the salt, the acetate was injected subcutaneously or intraperitoneally in amounts from 14 to 27.4 gm. dissolved in 80 to 140 cc. of water. Sterile technique was employed throughout.

To prove that any glucose formed by the metabolism of the injected acetate would be eliminated in the urine and result in an increased D:N ratio, control experiments were carried out on the same animals in which glucose was administered orally or subcutaneously whereby it was demonstrated that such glucose would be excreted approximately quantitatively in the urine.

Total nitrogen was determined by the usual macro-Kjeldahl technique. The Bertrand method was used for the quantitative determination of urinary glucose. In order to ascertain whether any of the injected sodium acetate was excreted by the kidney

unoxidized, the amount of volatile acids in the urine was determined as follows: To 150 cc. of the urine, 20 cc. of concentrated H_2SO_4 were added and this mixture was distilled until 130 cc. of distillate were obtained. After the distillate had been made up to 200 cc., the acid content was determined on an aliquot portion by titration with 0.1 N alkali, phenolphthalein being used as an indicator. By this procedure with known solutions of sodium acetate, 95 to 96 per cent of the added substance was recovered. Quantitative results were obtained when sodium acetate was added to normal or diabetic urine in which the volatile acid had previously been determined on an aliquot portion. Urine of normal fasting animals was found to contain a small amount of volatile acid, while much higher values were obtained from the urine of diabetic animals. The presence of chlorides in sufficient amount will result in the distillation of appreciable quantities of HCl, thus giving an erronous value when the volatile acid is considered wholly as acetic acid. This difficulty was minimized in our experiments since the chloride excretion was reduced to a low figure by fasting. Likewise we were interested in the increase of the volatile acid excretion after the acetic acid administration, the calculation of which would not be influenced by a small constant quantity of NaCl present.

In the following experiments the extra sugar in the urine has been calculated in each case by considering the prevailing D:N ratio for the acetate period as the average of the fore and after periods. The nitrogen of the experimental period multiplied by the prevailing ratio gives the glucose to be expected from the protein metabolism of the time. The glucose actually found in excess of this is calculated as extra sugar.

EXPERIMENTAL.

Cathartic Action of Sodium Acetate.—It is not generally recognized that sodium acetate may act as a violent purge. This fact soon became evident when we attempted to administer this salt by the oral route in the early experiments which are not recorded here. The injection of this substance was always followed within a remarkably short time by the elimination of a large amount of fluid from the bowel. In five later experiments with three normal dogs which had been fed on the previous night, the oral introduction of 27 gm. of sodium acetate in 250 to 300 cc. of H_2O was followed in 11 to 26 minutes (average 19 minutes) by the ejection of a large amount of fluid from the intestines. Vomiting also

TABLE I.

Effect of Administration of Sodium Acetate on Metabolism of a Phlorhizinized Dog.

Dog 175. Weight 12.9 kilos.

Date.	Length of period.	Glucose for period.	Nitrogen.		D:N	Glucose ingested or glucose equivalent.	Extra glucose.	Volatile acid (0.1 N).		Remarks.
			Period.	Hourly.				Period.	Hourly.	
Mar., 1917	*hrs.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*cc.*	*cc.*	
3	15.53	30.94	6.370	0.410	4.86			2412	155	Phlorhizin daily from Feb. 28.
3	8.70	15.94	3.580	0.411	4.45			1500	172	
4	15.30	25.76	6.186	0.404	4.16			2400	158	
4	6.00	10.28	2.480	0.413	4.10			1224	204	
4	6.38	12.19	2.612	0.409	4.74			990	155	
5	11.62	21.84	5.250	0.452	4.16	12.01	0	2412	207	27.24 gm. $NaCH_3CO_2$ subcutaneously.
5	6.00	10.51	2.920	0.488	3.60			948	158	
5	6.00	9.73	2.714	0.452	3.59			768	128	
6	12.35	20.58	4.935	0.406	4.17	7.05	2.27	2616	212	16.0 gm. $NaCH_3CO_2$ intraperitoneally.
6	5.67	8.86	2.310	0.407	3.83			822	145	
6	6.00	8.66	2.383	0.397	3.63			660	110	
7	12.22	28.05	3.950	0.323	7.10	14.00	13.99	954	78	14.0 gm. glucose by stomach tube.
7	11.71	13.51	3.870	0.331	3.49			1662	142	
8	12.40	13.48	3.725	0.304	3.62	6.39	0.26	1338	108	14.5 gm. $NaCH_3CO_2$ intraperitoneally.
8	6.31	6.61	1.835	0.291	3.60			720	114	

occurred as a usual thing. In one animal 8.18 gm. of volatile acid were found in the fecal fluid while 12.71 gm. were found in the vomitus. This indicates the extreme rapidity with which the acetate may be carried the entire length of the gastrointestinal

tract. It was obvious from these results that one may not rely on quantitative absorption of the acetate when introduced by the oral route. Gaebler (14) has obtained a similar purgative effect with potassium acetate.

Effect of Injection of Sodium Acetate on Glucose Excretion of Diabetic Dogs.—Sodium acetate was administered subcutaneously and intraperitoneally in amounts varying from 14.5 to 27.4 gm. (equivalent to 6.39 to 12.09 gm. of glucose) in twelve experiments

TABLE II.

Effect of Subcutaneous and Intraperitoneal Administration of Sodium Acetate on Glucose Excretion and D:N Ratio of Phlorhizinized Dogs.

Dog No.	Weight of dog.	D:N ratio.				Sodium acetate.	Glucose.		Remarks.
		Control period I.	Acetate period.	Control period II.	Prevailing ratio.		Equivalent introduced.	Extra glucose.	
	kg.					*gm.*	*gm.*	*gm.*	
120	5.9	3.34	2.96	3.18	3.26	15.0	6.62	0	Subcutaneously.
120	5.9	3.18	2.94	2.99	3.08	15.0	6.62	0	"
109	9.2	3.50	3.63	3.81	3.66	27.4	12.09	0	"
109	9.2	3.81	3.77	3.30	3.56	16.0	7.05	0.67	Intraperitoneally.
180	15.2	4.77	5.14	4.28	4.53	27.2	12.00	5.14	Subcutaneously.
180	15.2	4.28	4.28	3.79	4.04	27.2	12.00	2.10	"
180	15.2	3.79	3.89	3.33	3.56	27.2	12.00	2.78	Intraperitoneally.
176	8.4	3.71	3.61	3.41	3.53	15.0	6.62	0.40	Subcutaneously.
176	8.4	3.41	3.84	3.50	3.46	27.2	12.00	2.08	Intraperitoneally.
175	12.9	4.74	4.16	3.60	4.17	27.2	12.00	0	Subcutaneously.
175	12.9	3.59	4.17*	3.83	3.71	16.0	7.05	2.27	Intraperitoneally.
175	12.9	3.49	3.62	3.60	3.51	14.5	6.39	0.26	"

* Later experiment showed no extra glucose.

on five phlorhizinized dogs. A typical experiment is given in Table I which summarizes the experimental data on Dog 175. Table II contains a summary of the other experiments.

When sodium acetate was administered subcutaneously to Dog 175, no extra sugar was excreted in the urine. After the intraperitoneal injection of this salt solution, a small increase in the D:N ratio occurred; however, when this experiment was repeated several days later with the same animal, no significant

change in the glucose elimination resulted. The small increase in the glucose output observed in the first experiment in which the acetate was injected intraperitoneally probably owes its origin to a breakdown of the glycogen still present in the dog. With phlorhizinized animals having a D:N ratio indicative of a complete diabetes, the administration of adrenalin or the production of lactic acid by muscular work or shivering may result in a considerable increase in the glucose excretion. This has been regarded as a resultant of the flushing out of the glycogen reserves since a repetition of the experiment is not followed by an increased glucose excretion. The condition in the present case is an analogous one, the shock of the first injection of the hypertonic salt solution in the peritoneal cavity probably causing the flushing out of the small amount of liver glycogen while the second administration was without such an effect.

In the twelve experiments which are summarized in Table II, no extra sugar was found in four cases, 4 to 16 per cent of the glucose equivalent was noted in four other instances, 32 per cent was eliminated in the first experiment on one animal and practically none on a second test. In only one dog was a significant amount of extra glucose excreted in more than one experiment, the results being 43, 18, and 23 per cent respectively after three successive injections. Nothing approaching a quantitative conversion was found in any of the twelve experiments and in the two instances in which there was an appreciable increase in the glucose excretion after the first injection of the acetate, later experiments resulted in only a small production of extra sugar. Controls showed that if glucose were injected or given orally an approximately quantitative excretion of the carbohydrate occurred in the urine.

In some cases the excretion of volatile acids was very uniform during the control periods while in other instances quite variable results were obtained. If one may calculate the extra volatile acid excreted after the injection of acetate as the excess found during the experimental period over the average of the first and second control periods, then the excretion of unchanged acetate usually does not exceed 15 per cent of that introduced. In the experiments with Dog 175, the extra acetate eliminated amounted to 4, 13, and 0 per cent respectively in the three experiments. In the case of Dog 120 there was less than 3 per cent excreted in the

first test. With Dog 169, 11 and 19 per cent of the injected acid were accounted for in the urine while with Dog 180 the increase in urinary volatile acid corresponded with 0 and 16 per cent of that administered in the second and third experiments. In general one may say that there is usually a small increase in the value of the urinary volatile acid after the injection of acetate and a fall following the introduction of glucose in phlorhizinized dogs. Since it is not converted to sugar and since it is not excreted to an appreciable amount, the obvious conclusion remains that the bulk of the acetic acid is oxidized in these animals. Experiments to determine the effect of the acetate administration on the respiratory quotient of phlorhizinized dogs will be carried out later.

TABLE III.

Effect of Subcutaneous Administration of 27 Gm. of Sodium Acetate on Urinary Volatile Acid Excretion of Normal Fasting Dogs.

Dog No.	Body weight.	Hourly excretion of urinary volatile acids.			Extra acetate excreted.	Remarks.
		Control period I.	Acetate period.	Control period II.		
	kg.	*mg.*	*mg.*	*mg.*	*per cent*	
177	14.6	61	71	45	3.7	
177		45	60	36	4.1	
41	14.2	55	32	36	0	
41		36	46	34	2.1	
175	10.4	19	40	27	2.9	Some $NaCH_3CO_2$ leaked from area of injection; included in urine sample.
175		27	28	27	0.2	

Effect of Injection of Sodium Acetate on Volatile Acid Excretion of Normal Dogs.—Six experiments were carried out on three normal fasting female dogs to determine the effect of the subcutaneous administration of acetate on the excretion of volatile acid in the urine. The results are summarized in Table III.

The normal dog is able to utilize nearly completely fairly large amounts of sodium acetate when introduced by the subcutaneous route. Only small increases in the urinary volatile acid excretion occurred after its injection, the maximum excretion of acetate amounting to only 4 per cent of that injected. The

normal dog must oxidize this molecule as does the diabetic animal. This agrees with the conclusions of Lusk (15) which indicate that acetic acid undergoes a rapid oxidation in the normal dog.

DISCUSSION.

If the theory that fat is capable of transformation into carbohydrate is cogent, and if acetic acid is its intermediary product, then this acid should also be a glucogenetic agent. We have demonstrated that such is not the case. There is no appreciable synthesis of glucose from sodium acetate when this substance is introduced subcutaneously or intraperitoneally in phlorhizinized dogs. Control experiments with the same animals showed that the ingested glucose was excreted practically quantitatively in the urine, which would indicate that had glucose been formed from the acetate, it would also have yielded extra urinary sugar.

It might be claimed that the amounts of sodium acetate given in our experiments were unphysiological. One might maintain that small quantities of acetic acid were readily changed into glucose but that when large doses are administered, the bulk of it escapes such a fate. There are several facts, however, which would seem to indicate that the above experiments were carried out with physiological amounts.

In the first place it would seem that if the body were overwhelmed by an excessive quantity of sodium acetate the excess would be excreted in large part in the urine unchanged—a condition which we have shown does not occur. Secondly, the amount of acetate given in the experiments reported here is no greater than that usually employed in tests with phlorhizinized dogs in which the glucogenetic action of various intermediates has been tested. In the latter experiments these amounts have never been considered too great to be normally metabolized. The experimental animals have usually been able to change any glucogenetic substances completely into glucose.

A further analysis indicates that the quantities of sodium acetate administered were within the physiological range. The experiments of Anderson and Lusk (16) offer the necessary data for such an analysis. These investigators report that a dog weighing 8.75 kilos oxidized as much as 7.65 gm. of fat hourly in doing a moderate amount of work on the 3rd day of fasting (Experi-

ment 28) at a time when the non-protein respiratory quotient was that of pure fat. This would correspond with the metabolism of 13.65 gm. of acetic acid per hour. On the basis of body weight this animal oxidized much more acetic acid than any of our animals were called upon to metabolize during the period of absorption which must have required at least several hours. The results of Anderson and Lusk represent a breakdown of sufficient fat to produce more than twice the amount of acetic acid given in many of our experiments. Therefore, the quantities of acetate administered by us must be considered well within the physiological limits either for testing the capability of the organism to oxidize it or for determining the ability of the animal to convert it to sugar.

SUMMARY.

The subcutaneous or intraperitoneal injection of sodium acetate into phlorhizinized dogs did not cause a significant change in the D:N ratio, a result which would indicate, contrary to the view of Geelmuyden, that this molecule is ineffective as a glucose former. These experimental data are offered as additional evidence that the animal is unable to transform a possible intermediate of the fatty acid portion of the fat molecule into glucose.

Only small amounts of the injected acetate escaped unchanged in the urine of the phlorhizinized or the normal fasting dogs as determined by the increase in the excretion of volatile acids. This suggests that the acetate molecule is practically completely oxidized in the living organism.

Sodium acetate acts as a violent purge when introduced orally.

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TOXICITY OF SELECTED CHEMICALS TO CERTAIN ANIMALS

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The median tolerance limit, defined as that concentration of a substance which is lethal to 50 percent of test animals in an arbitrary time period and abbreviated TL_m, has many uses in physiological studies. These values are especially useful as a starting point for studies of sublethal effects of chemicals on the metabolic processes of animals. Other uses may be made of these values depending on the nature of the research involved.

During the past 19 years the Industrial Research Laboratory at Louisiana State University has measured, for various purposes, the median tolerance limits of a large number of organic and inorganic chemicals for various animals. This paper presents the TL_m values of 86 of these chemicals as tested for 9 species of animals in 3 different support media. Some of the TL_m values were determined in conjunction with other research projects and, thus, were reported with that work (1) (2) (3). All the values reported herein have been gleaned from the Progress Reports of the Industrial Research Laboratory to the Louisiana Petroleum Refiners' Waste Control Council which have a limited circulation in the Council minutes to member organizations only. Because of the varied purposes for obtaining them, there can be little correlation of these data.

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Materials and Methods

The animals used for bioassay were amphipoda (probably *Hyallela* sp.); frog eggs, *Rana* sp.; three species of fish, *Carassius carassius*, *Lepomis marochirus*, and *Mollienesia latipinna;* mosquito larvae, *Culex* sp.; planaria worms, *Dugesia* sp.; snail eggs, *Lymnaea* sp.; and water fleas, *Daphnia magna*. The amphipods, planaria, and frogs were obtained from University Lake on the campus of the University. The snails from which eggs were obtained were found in a ditch near Fountainebleau State Park, 60 miles (96.6 km) east of Baton Rouge. The goldfish, *C. carassius*, and the mollies, *M. latipinna*, were obtained from a local pet shop. The bluegills, *L. macrochirus*, were obtained from state and federal fish hatcheries. The mosquito larvae were obtained during the summer in mixed culture of mostly *Culex pipiens* from puddles in a ditch on the campus. The daphnids were cultured in the laboratory from a starting culture originally obtained from Put-In-Bay, Ohio.

Bioassays with the fish were conducted by methods outlined by Freeman (4). Bioassays with the invertebrates and the frog eggs were conducted with the methods of Anderson *et al.* (5). These methods are basically similar to each other—they differ only in the dilution series utilized.

The support media employed were Standard Reference Water (4), Reference Dilution Water (6), and water from University Lake filtered through glass wool. Standard Reference Water is described as a laboratory-prepared medium, free from organics, and containing all the major ions in concentrations and proportions of a mean surface water of the United States. Reference Dilution Water is a more easily formulated organic-free medium which will support aquatic animals longer than other artificial media tested.

Results and Discussion

The median tolerance limits determined are shown in Table I according to the chemicals tested. The animals on which the chemicals were tested, the support media employed, and the time for which the TL_m's were determined also are shown. In certain instances in the table, the same animal may be listed more than once under one chemical. This is because more than one support medium was employed. A different support medium may evoke a very different TL_m (7).

It may be noted in Table I that in the testing of combinations of chemicals, more than one concentration is given at one TL_m time period. These TL_m values are the test solution concentrations of each of the chemicals, separated by a hyphen, given in the order listed at the left side of the column.

Some values are given with the sign > (greater than) and < (less than). The TL_m values shown with either of these symbols preceding a single number, e.g., <3,750, represent either the upper or lower limit (whichever is applicable) of the most definitive test. A general rule used in the laboratory is that with TL_m values greater than 3,000 mg/l the toxicity is due to hypertonicity of the test solution. The values with two numbers and two symbols, e.g., >100 <1,000, designate the limits or "brackets" determined from preliminary tests. The TL_m of the chemical lies between the values given. The TL_m values are presented mg/l. If, at room temperature, the chemical were a solid, the measure of TL_m was by weight. If the chemical tested were a liquid at room temperature, the measure was by volume.

It generally is expected that shorter-term TL_m values will be greater than those for longer periods of time. In Table I, it may be noted that some TL_m values for longer time periods are greater than those for shorter periods. This has occurred possibly because the TL_m values for one TL_m time period may have been tested by one worker at one time and the value for another time period tested by another worker at another time. Because the methods used by all the workers in the laboratory have been the same, these differences are considered to be due to differences in the test animals obtainable at the different times of testing.

Another consequence of having different workers determine the different TL_m's at different times is a lack of strict comparability of the data presented. In most cases, these data were obtained as background material for other research problems, e.g., effects of certain chemicals on the taste of fish flesh, effects on adults emerging from larvae exposed to various chemicals, effects on the young after sublethal exposure of the eggs to certain chemicals, and toxicity levels of common solvents used for transport or dilution of other chemicals. It is felt that the use of these data lies in their being utilized for the same purpose for which they were obtained, i.e., background material for more detailed studies.

The TL_m values given for the *Lymnaea* sp. eggs are the concentrations at which 50 percent of the eggs hatched. The *Lymnaea* snails used herein lay their eggs in an orderly arrangement of 8 to 12 eggs per egg case. This egg case is more or less rigid—having the consistency of a gela-

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TABLE I. Median Tolerance Limits of Certain Chemicals for Various Animals in Different Dilution Media

Compound and Animal Tested	Medium	TL_m (mg/l) 24 hr	48 hr	72 hr	96 hr
Acetic acid					
Culex sp. larvae	RDW	1,500	1,500	—	—
D. magna	SRW	47	—	—	—
L. macrochirus	RDW	>100<1,000	—	—	—
Acetone					
D. magna	RDW	10	10	—	—
Adipic acid					
L. macrochirus	RDW	<330	—	—	—
Ammonium chloride					
C. carassius	SRW	640	—	—	—
D. magna	ULW	202	161	67	50
D. magna	SRW	—	—	—	139*
L. macrochirus	SRW	725	725	725	725
Lymnaeae sp. egg	ULW	241	173	73	70
Ammonium hydroxide					
D. magna	SRW	60*	32*	—	20*
Ammonium sulfate					
D. magna	SRW	423*	433*	—	292*
Ammonium sulfite					
D. magna	SRW	299*	273*	—	203*
Barium chloride					
Rana sp. eggs	ULW	244.3 mg/l—91.67% development 2,443.0 mg/l—89.55% development 24,430.0 mg/l— 0.00% development			
Butyric acid					
D. magna	SRW	—	61	—	—
L. macrochirus	RDW	200	—	—	—
Calcium chloride					
D. magna	SRW	3,526*	3,005*	—	—
D. magna	ULW	1,838	759	759	649*
L. macrochirus	SRW	8,350	—	—	—
Lymnaea sp. eggs	ULW	4,485	3,094	3,308	2,573
Capric acid					
L. macrochirus	RDW	I			
Caproic acid					
L. macrochirus	RDW	>150<200	—	—	—
Caprylic acid					
L. macrochirus	RDW	I	—	—	—
Chromic sulfate					
D. magna	ULW	0.1	0.03	—	—
Chromic sulfate plus sodium dichromate					
Lymnaea sp. eggs	ULW	0.17—0.22	0.17—0.22	—	—

* Denotes 25 hr when placed after a value in the 24-hr column, 50 hr when in the 48-hr column and 100 hr when in the 96-hr column.

Note:

TL_m (mg/l)—median tolerance limit.
SRW—Standard Reference Water (4).
RDW—Reference Dilution Water (3).
ULW—glass-wool filtered University Lake water.
I—chemical too insoluble in water to be toxic.

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l) 24 hr	48 hr	72 hr	96 hr
Ferric chloride					
D. magna	SRW	36*	21*	—	15*
Formaldehyde					
D. magna	RDW	>100<1,000	—	—	—
Formic acid					
L. macrochirus	RDW	175	—	—	—
Glutaric acid					
L. macrochirus	RDW	330	—	—	—
Magnesium chloride					
D. magna	SRW	3,391*	3,699*	—	3,484*
Magnesium sulfate					
D. magna	ULW	963	929	861	788
D. magna	SRW	—	—	—	3,803
L. macrochirus	SRW	19,000	—	—	—
Lymnaea sp. eggs	ULW	10,530	6,525	6,300	6,250
Malonic acid					
L. macrochirus	RDW	150	—	—	—
Phenol					
D. magna	RDW	100	100	—	—
D. magna young	SRW	17*	7*	—	—
D. magna adult	SRW	61*	21*	—	—
L. macrochirus	SRW	>10<15*	—	—	—
M. latipinna	SRW	63*	22*	—	—
Potassium chloride					
D. magna	SRW	—	—	—	679*
D. magna	ULW	343	337	117	29
L. macrochirus	SRW	5,500	—	—	—
Lymnaea sp. eggs	ULW	1,941	1,492	1,018	1,100
Potassium cyanide					
D. magna	ULW	2	2	0.7	0.4
Lymnaea sp. eggs	ULW	796	796	147	130
Potassium dichromate					
C. carassius	SRW	705	—	—	—
D. magna	SRW	—	—	—	0.4*
L. macrochirus	SRW	739	—	—	—
Potassium ferricyanide					
D. magna	ULW	905	549	0.6	0.1
Potassium nitrate					
D. magna	ULW	490	490	226	39
D. magna	SRW	—	—	—	900*
L. macrochirus	SRW	5,500	—	—	—
Lymnaea sp. eggs	ULW	1,941	1,492	1,018	1,100
Propionic acid					
Culex sp. larvae	RDW	>1,000	>1,000	—	—
D. magna	SRW	—	50	—	—
L. macrochirus	RDW	188	—	—	—
Pyridine					
D. magna	RDW	2,111	911	—	—
Sodium acetate					
L. macrochirus	RDW	5,000	—	—	—
Culex sp. larvae	RDW	7,500	7,425		—
Sodium anthraquinone alpha-sulfonate					
D. magna	ULW	186	186	186	50
D. magna	SRW		—	—	12*
Lymnaea sp. eggs	ULW	186	186	186	186

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l) 24 hr	48 hr	72 hr	96 hr
Sodium bicarbonate					
Culex sp. larvae	RDW	2,000	2,000		—
Sodium bisulfate					
Culex sp. larvae	RDW	300	300		
Sodium bisulfite					
D. magna young	SRW	116*	81*		
D. magna adult	SRW	—			102
D. magna adult	ULW	171	119	97	82
Dugesia sp.	ULW	179	179	179	179
Lymnaea sp. eggs	ULW	179	59	59	59
M. latipinna	SRW	241*	220*	—	—
Sodium bisulfite plus sodium carbonate					
D. magna	ULW	1,484–665	1,484–665	1,060–475	99–45
D. magna	SRW	—	—	—	436–85
Sodium bisulfite plus sodium chromate					
D. magna	SRW	—	—	—	67–0.278*
Sodium bisulfite plus sodium silicate					
D. magna	SRW	14,210–950	11,723–784	222–15	222–15
Sodium bisulfite plus sodium sulfate					
D. magna	SRW	—	—	—	82–3,654
Sodium bisulfite plus sodium carbonate and sodium chromate					
D. magna	SRW	—	—	—	86–441–0.354*
Sodium bisulfite plus sodium carbonate and sodium silicate					
D. magna	SRW	—	—	—	39–198–93*
Sodium bisulfite plus sodium carbonate and sodium sulfate					
D. magna	SRW	—	—	—	57–296–2,569*
Sodium bisulfite plus sodium chromate and sodium silicate					
D. magna	SRW	—	—	—	224–0.86–506*
Sodium bisulfite plus sodium chromate and sodium sulfate					
D. magna	SRW	—	—	—	78–0.32–3,443*
Sodium bisulfite plus sodium silicate and sodium sulfate					
D. magna	SRW	—	—	—	52–126–2,326*
Sodium p-bromobenzene sulfonate					
D. magna	ULW	2,347	1,943	971	809
D. magna	SRW	—	—	—	523*
L. macrochirus	SRW	<1,560	—	—	—
Lymnaea sp. eggs	ULW	2,590	2,590	2,590	2,590
Sodium butyl sulfonate					
D. magna	ULW	8,000	8,000	5,400	2,700

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l) 24 hr	48 hr	72 hr	96 hr
Sodium butyrate					
L. macrochirus	RDW	5,000	—	—	—
Sodium carbonate					
Amphipoda	ULW	360	176	67	67
Culex sp. larvae	RDW	1,820	600	—	—
D. magna	ULW	347	265	—	—
D. magna	SRW	607*	565	—	524
Dugesia sp.	ULW	384	360	360	341
L. macrochirus	ULW	385	—	—	—
Lymnaea sp. eggs	ULW	403	403	395	411
M. latipinna	SRW	405*	297*	—	—
Sodium carbonate plus sodium chromate					
D. magna	SRW	—	—	—	420–0.34*
Sodium carbonate plus sodium chromate and sodium silicate					
D. magna	SRW	—	—	—	187–0.15–88
Sodium carbonate plus sodium chromate and sodium sulfate					
D. magna	SRW	—	—	—	240–0.19–2,078*
Sodium carbonate plus sodium silicate					
D. magna	ULW	265–1,776	265–1,776	265–1,776	265–1,776
Sodium carbonate plus sodium silicate and sodium sulfate					
D. magna	SRW	—	—	—	161–76–1,396*
Sodium carbonate plus sodium sulfate					
D. magna	ULW	198–666	172–577	172–577	66–222
Sodium chloride					
C. carassius	SRW	13,750	—	—	—
Culex sp. larvae	RDW	10,500	10,200	—	—
D. magna	SRW	6,447*	5,874*	—	3,114*
D. magna	ULW	3,412	3,310	—	—
L. macrochirus	SRW	14,125	—	—	—
Lymnaea sp. eggs	ULW	3,412	3,388	—	—
M. latipinna	SRW	18,735*	16,595	—	—
Sodium p-chlorobenzene sulfonate					
D. magna	ULW	8,600	7,659	3,964	2,150
D. magna	SRW	—	—	—	2,394*
L. macrochirus	SRW	<3,219	—	—	—
Lymnaea sp. eggs	ULW	8,600	7,633	6,343	5,053
Sodium 2-chlorotoluene-4-sulfonate					
L. macrochirus	SRW	<1,374	—	—	
Sodium 2-chlorotoluene-5-sulfonate					
D. magna young	SRW	0.8*	0.6*	—	0.4*
D. magna adult	SRW	3.3*	1.3*	—	—
Lymnaea sp. eggs	SRW	30*	—	—	—
M. latipinna	SRW	115.2*	66.1*	—	—

TABLE I. *Continued*

Compound and Animal Tested	Medium	TL_m (mg/l) 24 hr	48 hr	72 hr	96 hr
Sodium chromate plus sodium silicate					
D. magna	SRW				0.21-130*
Sodium chromate plus sodium silicate and sodium sulfate					
D. magna	SRW				0.28-122-2,255*
Sodium chromate plus sodium sulfate					
D. magna	SRW	—			0.28-3,044*
Sodium 2, 5-dichlorobenzene sulfonate					
D. magna	ULW	4,931	4,931	2,490	938
D. magna	SRW	—			1,468*
L. macrochirus	SRW	<3,750	—		
Lymnaea sp. eggs	ULW	4,981	4,513	3,981	3,141
Sodium dichromate					
D. magna	ULW	22	10		
Sodium formate					
L. macrochirus	RDW	5,000	—		
Sodium nitrate					
C. carassius	SRW	12,150	—	—	
D. magna	ULW	5,980	3,581	2,125	665
D. magna	SRW	—	—	—	4,206
L. macrochirus	SRW	12,800	—	—	—
Lymnaea sp. eggs	ULW	6,375	6,460	5,950	3,251
Sodium m-nitrobenzene sulfonate					
D. magna	ULW	8,665	8,665	6,017	5,067
D. magna	SRW	—	—	—	2,235*
L. macrochirus	SRW	<1,350	—	—	—
Sodium 4-nitrochlorobenzene-2-sulfonate					
D. magna	ULW	4,698	3,483	948	948
D. magna	SRW	—	—	—	1,474
L. macrochirus	SRW	6,375	—	—	6,375*
Lymnaea sp. eggs	ULW	3,532	4,439	3,736	3,208
Sodium 4-nitrotoluene-2-sulfonate					
L. macrochirus	SRW	<1,440	—	—	—
Sodium oxalate					
L. macrochirus	RDW (without $CaCl_2$)	4,000	—	—	—
Sodium p-phenolsulfonate					
D. magna	ULW	13,510	13,510	3,494	1,471
L. macrochirus	SRW	—	—	—	19,616*
Lymnaea sp. eggs	ULW	10,700	9,122	8,828	8,828
Sodium mono-hydrogen phosphate					
D. magna	SRW	1,154*	1,089*	—	426*
Sodium mono-hydrogen phosphate plus sodium pyrophosphate					
D. magna	ULW	3,580-433	3,580-391	—	—
Lymnaea sp. eggs	ULW	2,685-63	2,954-223	2,828-60	2,685-248

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l) 24 hr	48 hr	72 hr	96 hr
Sodium phosphate					
D. magna	SRW	237*	177*	—	126*
Sodium propionate					
Culex sp. larvae	RDW	—	2,320	—	—
L. macrochirus	RDW	5,000	—	—	—
Sodium pyrophosphate					
D. magna	ULW	433	391	—	—
Sodium silicate					
Amphipoda	ULW	895	263	261	160
D. magna	ULW	575	494	413	216
D. magna	SRW	—	—	—	247
Lymnaea sp. eggs	ULW	632	630	630	632
Sodium sulfate					
Amphipoda	ULW	2,380	1,110	880	880
Culex sp. larvae	RDW	11,430	13,350	—	—
D. magna	ULW	8,384	2,564	725	630
D. magna adult	SRW	—	—	—	4,547
D. magna young	SRW	6,800	6,100	—	—
L. macrochirus	SRW	17,500	—	—	—
Lymnaea sp. eggs	ULW	5,401	5,400	5,400	3,553
M. latipinna	SRW	20,040	15,996	—	—
Sodium sulfide					
D. magna	SRW	16*	13*	—	9*
Sodium sulfite					
D. magna	SRW	299*	273*	—	203*
Sodium thiosulfate					
D. magna	SRW	2,245*	1,334*	—	805*
Sodium valerate					
L. macrochirus	RDW	5,000	—	—	—
Valeric acid					
D. magna	SRW	—	45	—	—
Xylene					
D. magna	RDW	>100<1,000	—	—	—
Zinc stearate					
L. macrochirus	RDW	I			

tin dessert which has "set." It is felt that the more or less rigid case of *Lymnaea* may offer a small amount of protection from the chemicals tested, thereby giving the TL_m values almost equivalent to the values for adults of other forms.

One very significant effect of some of the chemicals tested herein has been largely unexplored. This is the effect of pH on the TL_m values. In one of the few definitive tests of pH effect conducted it was found that a 0.1 unit increase in pH increased the toxicity of ammonium chloride tenfold and that a 0.1 unit decrease reduced the toxicity by tenfold. The concentration of ammonium chloride was held at the TL_m concentration. This variation in toxicity probably is due to the logarithmic basis for pH. The pH and some other effects of some weak acids have been studied and reported to be due to the concentration of the undissociated portion of the molecule (7).

Summary and Conclusions

The median tolerance limits (TL_m) of 86 different chemicals and combinations of chemicals, both organic and inorganic, are given for 9 different animals in 3 different support media. Determination of the TL_m of a chemical at a different time may show vari

ations from the expected because of differences in test animals available at the different times of testing. *Lymnaea* sp. eggs show TL_{50} values comparable to those of adults of other forms because of protection afforded by the egg case. Increasing or decreasing the pH of a concentration of a chemical can cause an increase or decrease in toxicity commensurate to the logarithmic basis for pH.

Acknowledgment

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MONTHLY CONTRACT AWARDS—WASTEWATER TREATMENT WORKS AND COLLECTING SEWERS AS REPORTED BY THE PUBLIC HEALTH SERVICE (MILLIONS OF DOLLARS)

Month	Treatment		Sewers		Total	
	Monthly	Cumulative	Monthly	Cumulative	Monthly	Cumulative
(1965)						
January	41.2	41.2	22.9	22.9	64.1	64.1
February	40.4	81.6	22.0	44.9	62.4	126.5
March	43.4	125.0	38.3	83.2	81.7	208.2
April	38.8	163.8	28.2	111.4	67.0	275.2
May	33.5	197.3	38.6	150.0	72.1	347.3
June	64.8	262.1	56.6	206.6	121.4	468.7
July	41.6	303.7	37.0	243.6	78.6	547.3

DESTRUCTION OF ANTIGENIC COMPLEXES BY HYDROLYSIS WITH WEAK ACETIC ACID*

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(Received 15 May 1956)

Weak acid hydrolysis is often used as a method for studying the antigenic complexes of Gram-negative bacteria. The polysaccharide, lipid and protein fractions of the antigen can be obtained from the hydrolysate. After these fractions go into solution a significant part of the antigen, the so-called residual antigen fraction, often remains. The latter is usually not analysed; sometimes the amount only is mentioned.

Table 1. Chemical characteristics of residual antigen fractions

Strain	Antigen	Residual antigen fraction	Yield in antigen (%)	In residual antigen fraction: Reducing substances (%)	Nitrogen (%)	Phosphorus (%)	Low molecular weight substances in antigen (%)
Flexner 26	Obtained after treatment with trypsin	Before dialysis	51·05	1·92	0·32	2·2	45·9
		After dialysis	6·05	8·23	0·81	Trace	
	From concentrated culture fluid	Before dialysis	37·26	2·15	0·58	2·35	27·39
		After dialysis	9·87	5·66	1·14	0	
Sonne 7380	Obtained after treatment with trypsin	Before dialysis	71·12	1·52	0·25	3·15	64·1
		After dialysis	7·02	9·03	0·78	0	
	From concentrated culture fluid	Before dialysis	66·71	0·84	0·17	1·84	50·51
		After dialysis	16·2	3·32	0·58	Trace	
S. typhi 1:3	Obtained after treatment with trypsin	Before dialysis	55·42	1·78	0·55	2·82	48·07
		After dialysis	7·35	8·45	1·2	0·23	
	From concentrated culture fluid	Before dialysis	59·41	1·52	0·24	1·78	46·76
		After dialysis	12·65	5·1	0·92	0	
S. paratyphi B 192	Obtained after treatment with trypsin	Before dialysis	45·93	2·05	0·45	2·35	36·35
		After dialysis	9·58	5·74	0·84	0·3	
	From concentrated culture fluid	Before dialysis	37·95	2·35	0·62	1·94	27·75
		After dialysis	10·2	6·1	1·08	Trace	

* Zh. mikrobiol., epidemiol. immunobiol. No. 7, 74–76, 1957. Reprint Order No. [illegible]

We have carried out a comparative study of antigenic substances obtained in various ways from bacteria of the enteric group grown on synthetic media (Dubrovskaia, Bitkova, Gostev and Mekhedov).* By well-known methods we isolated antigen preparations, and amongst these were complexes extracted from the mass of bacteria after trypsin treatment for a short time, and specific substances isolated from the cell-free culture fluid. In these preparations, which had all the properties of complete antigens, the residual antigen fraction varied from 37 to 71 per cent.

In order to analyse the residual antigen we lyophilized the solutions after removal of the polysaccaride, lipid and protein fractions of the antigen hydrolysate. The nitrogen in the white powder thus obtained was measured by the micro-Kjeldahl method, the reducing substances by the Hagedorn-Jensen and the phosphorus by Fiske and Subbarow methods. The residual antigen fraction was then dialysed for 3 days against cold tap water and for one day against distilled water. The dialysates were freeze-dried and again analysed.

The results (Table 1) showed that the residual antigen fraction consisted mainly of low molecular weight substances, easily passing through a semipermeable membrane during dialysis. These substances, which are formed during hydrolysis of the preparation, are the decomposition products of the high molecular weight components of the antigen. They possess little reducing ability and contain little nitrogen, but are rich in phosphorus.

After dialysis of the residual antigen fractions there remained a small quantity of high-molecular weight substances, the composition of which varied according to the method of isolation of the antigen. This part of the residual fraction was somewhat richer in reducing substances and nitrogen, but did not contain phosphorus.

The question arises whether the residual antigen fraction retains any specificity after removal of all the basic components of the original complex. To answer this question we studied the serological activity of the residual antigen fractions by a precipitin test with homologous antisera.

For comparison, the residual antigen fractions in preparations obtained by Boivin's method were studied.

Table 2. Serological activity of residual antigen by precipitin test

Strain	Method of preparing antigen	Serum dilution (upper line) and concentration of antigen in mg per ml saline (lower line)				
		1 : 400 10	1 : 200 5	1 : 400 2·5	1 : 800 1·25	1 : 6100 0·625
Flexner 26	Concentration of culture fluid	+	−			
	Trypsin treatment	±	−			
	Boivin	+ +	+	−		
Sonne 7380	Concentration of culture fluid	+ + + +	+ + + +	+ +	+	−
	Trypsin treatment	+ +	+	−		
	Boivin	+ + + +	+ + + +	+ + +	+	±
S. typhi 1203	Concentration of culture fluid	+ + + +	+ + + +	+ +	+ +	±
	Trypsin treatment	+ +	+	±	−	

Titres of precipitating sera were 1 : 256,000 with *S. typhi* antigens, and 1 : 400,000 with dysentery (Flexner and Sonne).

* The results obtained are in the press (2 publications).

Table 2 shows that the higher the content of high-molecular weight substances in the residual antigen fractions, the more marked are their specific serological properties.

Residual antigen fractions in a dose of 10 mg were not toxic to mice on intraperitoneal injection.

The presence of an appreciable amount of low molecular weight substances in antigen preparations after hydrolysis shows that even the relatively mild action of 0·1 N acetic acid brings about an appreciable destruction of the basic components of the specific complex.

Translated by G. Beale

COMBINED IMMUNIZATION WITH PURIFIED ADSORBED TETANUS TOXOID AND TETRAVACCINE*

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(*Received* 11 *October* 1956)

In our previous paper (Vorob'ev, Bron and Rodiakina) we have shown the high effectiveness of purified alum-precipitated tetanus toxoid given in one or two doses (75 persons). It was of importance to study the possibility of using this preparation in combination with enteric vaccines, which are usually administered together with crude tetanus toxoid. Animal experiments (Vorob'ev) have shown that combined administration of purified adsorbed tetanus toxoid and tetravaccine had no influence upon the immunogenicity of the toxoid, and gave a slower fall in the typhoid, paratyphoid and dysentery agglutinin titres as compared with combined immunization by tetravaccine and crude tetanus toxoid.

In the present paper we report the results obtained with combined immunization of human beings with purified adsorbed tetanus toxoid and tetravaccine.

We inoculated 102 women (blood donors) 19–54 years of age with the combined vaccine. The distribution of the inoculated persons into groups and according to the schemes of immunization is shown in Table 1. In all groups the interval between inoculations was 20 days.

Table 1. Distribution of inoculated persons according to groups and schemes of immunization

Group	Number of persons inoculated	First inoculation			Second inoculation		
		Tetanus toxoid (ml)		Tetravaccine (ml)	Tetanus toxoid (ml)		Tetravaccine (ml)
		Adsorbed	Crude		Adsorbed	Crude	
First	32	0·5	–	–	–	–	1
Second	25	1	–	0·5	–	–	1
Third	20	0·5	–	0·5	0·5	–	1
Fourth	25	–	1	0·5	–	2	1

* Zh. mikrobiol., epidemiol. immunobiol. No. 7, 77–84, 1957 [Reprint Order No. MIC 237]

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A Method for the Quantitative Determination of Acetic Acid

By F. Dulian

On the occasion of an investigation of preserved fodder (feed), I have used a method which is a modification of the method reported by Zeppert, Feigl and Vasquez, and which proved to be practical.

This method is based on the dry distillation of calcium acetate, formed during evaporation of acetic acid with CaO. The acetone formed in this reaction is bound by the SWR reagent, located in the receiver of the first "B"-flask, and is liberated from the reagent with H_2O_2 and fed into an alkaline potassium iodide solution; the amount of acetone or acetic acid is then calculated from the amount of iodine used (1 cc of 1/100 N-iodine solution corresponds to 0.0004 g acetic acid).

The required reagents are pulverized calcium oxide, CaO, and 3% hydrogen peroxide, H_2O_2. The SWR reagent consists of 10 g $HgCN_2$ and 180 g NaOH in 600 cc water. After cooling, 2.9 g $AgNO_3$ dissolved in 400 cc water is added dropwise and one waits until the slight turbidity has settled.

Analysis procedure: 50 cc of the aqueous extract containing acetic acid are mixed with 0.1 cc concentrated H_2SO_4 in a 100-cc flask, then sugars and proteins are removed by adding 10 cc lime and 2 cc of a 10% $CuSO_4$ solution, and the contents are allowed to stand for 1/2 hour, after filling up the flask with water up to the 100 cc mark. The liquid must

have an alkaline reaction; it is filtered, and 5 cc are placed into a small infusible glass flask.

This flask is connected to a receiver containing the SWR reagent; all water is removed from the flask by gentle heating. Only then, the flask (which we shall designate as flask A) is connected to flask B and the receiver containing the SWR reagent. Both flasks are provided with a double-perforated plug and an air stream is passed through them. To prevent the escape of gas during changes in the distillation, flask B with SWR reagent is connected in turn to a second receiver C, containing iodine in an alkaline solution of known concentration. After water has been removed from flask A, stronger heating is applied until the flask contents are carbonized and finally turn completely white. Then, flask A is removed, 5 cc of 3% H_2O_2 solution is introduced through one tube, and the mixture again heated to the boiling point. Under continuous and slight suction, all of the liberated acetone goes over into receiver C, containing J-NaOH, and is converted there to iodoform, which is decomposed by adding 3 drops of concentrated HCl, and titrated with a 1/100-N-thiosulfate solution, using starch as indicator. The tube through which acetone flows is narrowed down with fused pumice at the tip which is immersed into the reagent, in order to make the acetone flow through it as slowly as possible.

The blank test with distilled water gave a loss of 2 cc of the 1/100-N-thiosulfate solution, and this loss was always subtracted in the calculation.

Translated by
A. Schidlovsky (JITCO)
November 8, 1973

EINE METHODE DER QUANTITATIVEN BESTIMMUNG VON ESSIGSÄURE.

Von

F. DULIAN.

Bei Anlaß einer Untersuchung von konserviertem Futter habe ich eine Methode benutzt, welche eine Modifikation der von ZEPPERT, FEIGL, VASQUEZ* angegebenen Methode ist und die sich als praktisch erwies.

Die Methode beruht auf der trockenen Destillation von Calciumacetat, welches beim Abdampfen der Essigsäure mit CaO gebildet wird. Das dabei entstehende Aceton wird durch das Reagens SWR., welches sich in der Vorlage des ersten „B"-Kolbens befindet, gebunden. Daraus wird es durch H_2O_2 freigemacht und in eine alkalische Lösung von Jodkali geleitet, worauf das Aceton oder die Essigsäure berechnet wird aus der verbrauchten Jodmenge (1ccm $^1/_{100}$-n-Jodlösung entspricht 0,0004 g Essigsäure).

Die nötigen *Reagenzien* sind: Calciumoxyd pulverisiert CaO, 3% Wasserstoffsuperoxyd H_2O_2. Das SWR.-Reagens: 10 g $HgCN_2$, 180 g NaOH in 600 ccm Wasser. Nach der Abkühlung setzt man 2,9 g $AgNO_3$ in 400 ccm Wasser gelöst tropfenweise zu und wartet, bis sich die leichte Trübung abgesetzt hat.

Gang der Analyse: 50 ccm des wässerigen Extraktes, in dem sich Essigsäure befindet, werden in einem 100 ccm fassenden Kolben mit 0,1 ccm konzentriertem H_2SO_4 versetzt, dann 10 ccm Kalkmilch und 2 ccm einer 10proz. $CuSO_4$-Lösung entzuckert und enteiweißt und für eine halbe Stunde stehen gelassen, nachdem man bis zur Marke 100 mit Wasser ergänzt hat. Die Flüssigkeit muß alkalisch reagieren. Man filtriert und gibt 5 ccm in ein Kölbchen von schwer schmelzbarem Glase.

Dieser Kolben wird mit einer Vorlage verbunden, welche das SWR.-Reagens enthält. Durch gelindes Erwärmen wird aus dem Kolben alles Wasser vertrieben. Dann erst wird der Kolben — welchen wir Kolben A nennen wollen — mit dem Kolben B und dem SWR. in Verbindung gesetzt. Beide Kolben sind mit doppelt durchbohrten Stöpsel versehen und durch beide wird Luft durchgesogen. Damit bei der Änderung der Destillation kein Gas entweicht, wird von vornherein der Kolben B mit SWR. seinerseits mit der zweiten Vorlage C verbunden, die Jod in alkalischer Lösung von bekannter Konzentration enthält. Nachdem das Wasser von Kolben A vertrieben ist, beginnt man das stärkere Erwärmen, bis der Inhalt verkohlt und endlich vollkommen weiß wird. Darauf setzt man den Kolben A weg, bringt durch das eine Rohr 5 ccm Wasserstoffsuperoxydlösung 3proz. und erwärmt abermals bis zum Kochen. Nun geht unter fortwährendem, gelinden Saugen alles freigemachte Aceton in die Vorlage C, welche das J-NaOH enthält und wird dort zu Jodoform. Das Jodoform wird durch 3 Tropfen konzentrierter HCl zersetzt und unter Benutzung von Stärke mit $^1/_{100}$-n-Thiosulfat titriert. Das Rohr, welches das Aceton leitet, ist an dem Ende, welches in das Reagens taucht, durch Einschmelzen von Bimsstein verengt, damit das Aceton möglichst langsam durchgeht.

Der blinde Versuch mit destilliertem Wasser ergab einen Verlust von 2 ccm der $^1/_{100}$-n- Thiosulfatlösung, die immer abgerechnet wurden. *(Aus dem Institut für Zootechnik [Vorstand: Prof. Dr. T. Olbrycht] und dem Institut für Physiologische Chemie und allgemeine Pathologie Lwów [Vorstand: Prof. Dr. W. v. Moraczewski].)*

A Survey of Some Scandinavian Toxicologic Observations

J. Am. Vet. Med. Assoc. 110: 386-9, 1947

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FREQUENTLY, it is assumed that sudden or unexpected disease or death in domestic animals is occasioned by an obscure poison, even if it is obvious to the trained veterinarian that the trouble is not an intoxication in the strict sense of the word. Quacks often use intoxication as a diagnosis if their treatment fails. The qualified veterinarian's ambition is to diagnose intoxication only when founded upon clinical evidence and or postmortem findings and when it is possible to demonstrate the poison.

Table 1 lists a number of substances which have caused intoxications in Scandinavian domestic animals in recent years. The monetary loss occasioned by accidental poisonings may be great.

ZINC POISONING

There were 46 cases of zinc poisoning, 44 in domestic animals and 2 others. As the tissues of domestic animals normally contain zinc, diagnosis demands an exact quantitative analysis (O. Grini[1]). This report on chronic zinc intoxication is valuable to veterinarians since it is the first such case in domestic animals. The animal developed diarrhea, eventually hemorrhagic, within a few days after consumption of the poison. The purgation became sluggish and dark in the course of some few days. At the same time, posterior weakness was accompanied with stiff and unsteady gait. Later, the pulse became labored. Death occurred, in some cases, without appearance of striking symptoms. On section, gross degenerative changes of kidney, liver, and heart muscles, transudates in the abdominal cavity, and edema of striated musculature were found. Chronic zinc poisoning may last for three weeks.

A peculiar type of zinc poisoning occasioned by inhalation of zinc vapors was reported by A. Johansson[2] and E. Hoffmann.[3]

In domestic animals, only a few cases of zinc poisoning have been described. In man, on the contrary, intoxication due to inhalation of zinc vapors is well known. In these cases, the main symptoms are a transient, febrile state combined with respiratory difficulties. These symptoms are occasioned by absorption of the zinc vapors into the epithelial cells lining the alveoli of the lungs, causing precipitation of albumen which, when absorbed, results in shock.

Both cases of poisoning due to inhalation of zinc vapors occurred in barns in which welding was being done. A total of 20 cows were toxic. In both cases, the symptoms reported were transient fever, labored pulse, respiration frequent and labored, coughing, subcutaneous emphysema on breast and neck, and auscultatory symptoms indicative of interstitial emphysema of the lungs. The autopsy revealed corresponding gross lesions.

As these symptoms harmonize with those occurring in man suffering from inhalation of zinc fumes, A. Johansson[2] assayed the effect of zinc vapors experimentally inhaled by cattle. He proved that, in the course of some few minutes, inhalation of zinc vapors occasioned symptoms identical with those noted in spontaneously intoxicated animals. The experiments also revealed that the symptoms became aggravated, when the intervals between the inhalation were so short that the injuries occasioned by the first had not been repaired before reëxposure.

The fact that pregnant animals are especially disposed to intoxication with zinc vapors is explained by E. Hoffmann[3] on the basis of deeper inhalation in open than in pregnant cows. The respiratory areas, being smaller, are more quickly inactivated, functionally, in pregnant cows than in dry ones.

Treatment of this type of intoxication consists of effective ventilation of the barn, after which recovery occurs in a few days.

ARSENIC POISONINGS

Since 1935, there has been a striking increase of arsenic poisonings in Swedish animals (H. Wanntorp[4]). The reason is an

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TABLE I—Recent Toxicologic Observations in Scandinavian Countries

Poison	Author(s)	Number and kind of animals									
		H.	C.	S.	P.	G.	D.	Cat	F.	Pn.	Others
Zinc	O. Grini[1]		23		18				33		
	A. Johansson[2]		12								
	E. Hoffmann[3]		8								
	H. Wanntorp[4]		1								2
Thallium	M. Christiansen & H. C. Momberg-Jørgensen[6]				18						
	C. C. Kinslev[7]						7				
	H. Wanntorp[4]						3				
Arsenic	H. Wanntorp[4]		32			13					
	H. Wanntorp[5]		52								
Mercury	Fr. Harbitz[8]		23								
	O. Lauridsen[9]		4		17						
	H. Wanntorp[4]		5								
Lead	H. Wanntorp[4]		32								
Copper	H. Wanntorp[4]		2						80		
Sodium chloride	H. Wanntorp[4]				2						2
Chlorate	H. Wanntorp[4]		9								
Saltpeter	H. Wanntorp[4]		5								
Phosphorus	H. Wanntorp[4]		Numerous		Numerous				Numerous		
Fluorine	H. Wanntorp[4]		1		Numerous	4	6		Numerous	Numerous	
Strychnine	H. Wanntorp[4]						14		25		
Naphthalene	H. Wanntorp[4]								4		
Acetic acid	A. Johansson[12]	6									
Carbon tetrachloride	B. Voss[11]		1								
Carbon monoxide	H. Wanntorp[4]							1		29	
Frangula	N. Södermark[13]		1								
Bulbus Scilla	H. Wanntorp[4]							2	Numerous		
	M. Christiansen & H. C. Momberg-Jørgensen[6]				20						
Mould	S. Rubbarth & T. Henricson[15]	8									
	Johs. Elsness[14]		4								

H.=horse. C.=cattle. S.=sheep. P.=pig. G.=goat. D.=dog. F.=fowl. Pn.=pigeon.

increasing use of wood impregnated with arsenic.

In most cases, cows showed symptoms of intoxication after licking poles impregnated with arsenic. H. Wanntorp[5] showed experimentally that from 2 sq. meters of surface of impregnated poles, a rainfall of 22 mm. had extracted an average of 3 Gm. of arsenic. No differences occurred in poles protected against rain, even from poles coated with sublimated arsenic salt. The results show that wood impregnated with arsenic is by no means harmless, even if the arsenical compounds did not form a superficial coating.

THALLIUM POISONINGS

These occurred in 25 animals. Due to increasing use of thallium in eradicating rats, Christiansen and Momberg-Jorgensen[6] have assayed its noxious effect on 18 pigs, weighing from 6.75 to 40.5 kg. The animals received thallium dissolved in water and then mixed with milk. The dose of thallium varied from 10 to 50 mg./kg. The test showed that the lethal dose of thallium in pigs varies from 15 to 25 mg./kg. Due to the cumulative effect of thallium, repeated administration of sublethal amounts can occasion grave chronic intoxications or even death, provided the interval between subtoxic doses is not too long.

As nothing has been published about the effect of thallium, the clinical and postmortem findings published by Christiansen and Momberg-Jorgensen will be reported briefly.

The symptoms varied with the size of the dose. One large, surely lethal dose given in the course of twenty-four to forty-eight hours reduced the appetite and caused dullness and weakness. Diarrhea was variable. If it ocurred early in the disease, the purgation was thin, yellowish, and watery, sometimes tinged with blood. Vomiting occurred in some pigs. Convulsions and other nervous symptoms were never noted. The intoxication ran an acute course. Before death, coma developed. By administration of lesser but still toxic doses, the symptoms developed in the course of several days.

In chronic intoxications, a variable number of these symptoms appeared but peculiar changes occurred, especially in the skin of the loin. They were characterized by hyperemia and moist, greasy surfaces frequently brownish in color.

On section, acute cases showed changes characteristic of acute inflammation in the mucous membrane of the stomach and superficial erosions coated by either a thin, greyish, or a thick, croupous, membrane. The small intestine showed changes characteristic of acute inflammation; the colon and cecum those of croupous diphtheritic inflammation. The content of the digestive tract was thin and yellowish. Further degenerative changes of the liver, flaccid heart and, eventually, small, subendocardial hemorrhages could be noted.

C. C. Kinsley[7] reported 7 cases of thallium poisoning in dogs. Symptoms, course of the intoxication, and postmortem changes corresponded to those known from earlier publications. Treatment of the intoxication was ineffective when instituted some days after the consumption of the poison, but, if instituted immediately after consumption, treatment with tannin combined with chalk seemed efficient.

MERCURIAL POISONINGS

Poisoning occasioned by inhalation of mercurial vapors is rare. For this reason, the observation of Fr. Harbitz[8] regarding an intoxication of 23 cows poisoned by mercurial vapors from an ointment containing mercury is important. Even though the symptoms noted did not differ from those described as characteristic of mercurial poisoning, it must be pointed out that, at first, this intoxication was believed to be foot-and-mouth disease. Lauridsen[9] calls attention to a hitherto unnoticed symptom of mercurial poisoning—abortion in pregnant cattle.

CHLORAL HYDRATE

The use of chloral hydrate may give rise to intoxications even if it is, as reported by Nesse and Buer,[10] free of noxious contaminations. As chloral hydrate was mixed with sodium carbonate in the mixture used, Nesse and Bugge believed that a cleavage of chloral hydrate formed toxic compounds. Therefore Nesse and Buer warn against treatment of cattle with mixtures containing both chloral hydrate and sodium carbonate.

CARBON TETRACHLORIDE POISONINGS

This drug is used as an anthelmintic in animals. Caution must be used, however, since the individual tolerance of cattle varies greatly. Well-fed, older animals are most susceptible to the toxicity of carbon tetrachloride, but Voss[11] says young animals may be intoxicated even by small doses. He reported a case of intoxication in a 1½-year-old heifer treated with only 30 Gm. of carbon tetrachloride. In twenty-

four hours, a general paresis developed ending with death. On section, a sparse amount of serohemorrhagic liquid was found in the peritoneal cavity, as well as stasis of the liver, edematous gall bladder, acute inflammation of the duodenum, submucous, epi- and endocardial hemorrhages.

ACETIC ACID POISONINGS

Only a few cases of intoxications occasioned by acetic acid have been reported in animals. Acetic acid is generally used as rectal lavage against oxyuris in horses. Such a treatment may be noxious, however, as was noted by Johansson,[12] who found 6 horses intoxicated after each had been treated with 15 liters of a 2.5 per cent solution of acetic acid as an enema. Subsequent to treatment, the horses showed colic, tremor, foaming purgation, hyperemic and icteric conjunctivitis, and albuminuria. On section, necrotic ulcerous colitis and thrombosis of the blood vessels into adjacent tissue were found. The effect of acetic acid was tested on rabbits by administration of enemas of 0.5 to 5.0 per cent solutions. The effect depended upon the concentration of the drug, the filling of the rectum, and time of the effect. Weak solutions showed a more noxious effect than did more concentrated ones, because the vigorous tenesmus occasioned by concentrated solutions caused quick expulsion of the liquid. In rabbits, the following symptoms occurred: tenesmus, coprostasis, postmortem changes in the terminal part of the colon, with extensive necroses and inflammations.

RHAMUS CATHARTICA POISONING

This drug may intoxicate animals as was seen by Södermarks[13] in a cow which had consumed a large amount of leaf and twigs of the plant. The cow suddenly became ill, showed diarrhea, vomiting, labored pulse, and slight fever, and died in the course of two hours. On section, there was found acute gastroenteritis, and a large amount of *Rhamus frangula* in the stomach.

SCILLA MARITIMA

It is generally believed that this plant is noxious to pigs. Christiansen and Momberg-Jorgensen[6] experimentally tested the susceptibility of 20 swine, weighing from 12 to 60 kg., to the toxicity of the supposed poison. The results obtained show that, even when the animals had not been fed for twenty-four hours, they refused feed containing the poison in amounts varying from 1,000 to 3,333 mg./kg. Symptoms appeared if the poison was administered in amounts corresponding to 4,545 and 6,140 mg./kg. body weight. For that reason, it was concluded that spontaneous cases of this intoxication are rarities in swine.

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Is Penicillin Expensive?

Although the initial cost of treating mastitis with penicillin is high, Packer points out (*Successful Farming*, Nov., 1946) that other factors favor its use. For example, following injection of some drugs, the milk may not be used for human food for seven to ten days, whereas with penicillin only one or two milkings pass before the milk is again normal. Then, too, penicillin has no irritating effect on the udder. And the clinching argument is that the percentage of cows recovering is higher with penicillin than with any other drug in his experience.

C.R Acad. Sci. Paris, 271:237-240, 1970

NUTRITION - Distinct and Accumulated Effects of Acetic and Stearic Acid on the Secretion of Fatty Acids from the Goat Mammae

Observations* of Pierre-Morand Fehr and Jacques Delage (1)
communicated by Jean Roche

Acetic acid added to the diet increases not only the secretion in goat's milk of fatty acids of less than 18 carbon atoms, but also that of fatty acids of 18 carbon atoms. The addition of stearic acid to the diet increases the quantity of stearic and oleic acids secreted, but diminishes that of lower fatty acids and especially that of palmitic acid. The interaction between the effects of the addition of these two principal nutritives depends on the raw cellulose content of the diet.

In ruminants, the fatty acids of milk having 18 carbon atoms and some of the C_{16} acids, are essentially derived from the dietary lipids (2). On the other hand, the shorter chain acids and the majority of the C_{16} acids are synthesized by the mammae, above all, from acetic acid (3). Its production in the rumen is added by cellulosic substances coming principally from unpulverized fodder.

The factors which direct the synthesis of milk lipids in the ruminant towards the usage of fatty acids of one kind or another are little understood. Thus, it seemed useful to us to study whether acetic acid has an effect on the secretion of fatty acids generally considered as derivatives of the alimentary lipids and, reciprocally, whether a long chain fatty acid, such as stearic acid, influences the synthesis of shorter chain acids.

Material and Methods

In the course of two experiments, F and C, done under the standard conditions, 18 goats received during periods of three weeks, the following diets: a basic diet, or a diet to which was added sodium acetate or ethyl stearate, or a diet to which both the acetate and the stearate were added at the same time.

The basic diet of experiments F and C are similar for the daily contribution of fatty materials which is respectively an average of 24.2 and 26.2 g per goat. By contrast, they differ in the amounts of raw cellulose, because the fodder supplies 40 to 60% of the total dry material in experiment F, and 20 to 27% in experiment C where the feed is more concentrated. The actual quantities of acetate and stearate ingested whatever the diet, are respectively in experiment F, 58 and 52 g/day per goat, and in experiment C, 62 and 47 g. The rations provide equal amounts of energy and nitrogen and are calculated to meet daily requirements and the needs for the milk production of goats.

Control of the milk production and the butyraceous level along with the determination by gas chromatography of the composition of the milk fatty acids, enables the calculation of the quantity of the fatty materials secreted per day and that of the principal fatty acids. The statistical interpretation is done by the factorial analysis 2^2.

Results

The results are shown in the following table:

TABLEAU

Influence de l'acétate et du stéarate sur les productions et les quantités d'acides gras sécrétés par jour

	Expérience F							Expérience C						
	Résultats moyens selon les régimes				Signification des effets			Résultats moyens selon les régimes				Signification des effets		
	Témoin	Acétate	Stéarate	Acétate + stéarate	Acétate	Stéarate	Interaction	Témoin	Acétate	Stéarate	Acétate + stéarate	Acétate	Stéarate	Interaction
Matières grasses sécrétées en g/jour	67,4	72,5	77,1	80,8	N. S.	+ + +	N. S.	65,2	73,8	68,7	77,0	+ +	N. S.	N. S.
Taux butyreux en ‰	26,0	27,4	28,3	28,5	N. S.	+ +	N. S.	31,3	34,1	33,3	36,5	+ + + +	+ +	N. S.
Quantités d'A. G. sécrétés en g/jour :														
8 : 0	1,2	1,2	1,2	1,3	N. S.	N. S.	N. S.	1,7	1,8	1,7	2,0	N. S.	N. S.	N. S.
10 : 0	6,5	6,9	6,5	7,1	N. S.	N. S.	N. S.	7,4	8,1	6,9	7,5	N. S.	N. S.	N. S.
12 : 0	4,4	4,4	4,1	4,4	N. S.	N. S.	N. S.	3,7	3,9	3,2	3,3	N. S.	+ + +	N. S.
14 : 0	8,6	8,9	9,0	9,9	+	+	N. S.	7,3	8,2	7,2	8,1	+ +	N. S.	N. S.
16 : 0	26,3	27,0	20,9	24,7	+ +	+ + + +	+	21,3	24,4	19,0	21,0	+ + +	+ +	N. S.
18 : 0	1,7	2,3	7,0	5,9	N. S.	+ + + +	+ +	3,0	3,8	6,2	7,8	+ + + +	+ + + +	N. S.
18 : 1	8,4	9,9	16,9	15,5	N. S.	+ + + +	+ +	9,5	10,7	13,0	14,4	+ +	+ + + +	N. S.
18 : 2	1,4	1,5	1,3	1,5	N. S.	N. S.	N. S.	1,8	1,9	1,6	1,7	N. S.	N. S.	N. S.
Somme des A. G. de 8 : 0 à 16 : 0	46,8	48,4	41,4	47,5	+ +	+	N. S.	41,5	46,3	38,1	42,3	+ +	+	N. S.
Somme des A. G. en C_{18}	11,5	13,6	25,2	22,9	N. S.	+ + + +	+ +	15,1	17,0	21,4	23,8	+ + +	+ + + +	N. S.

N. S., non significatif ; + , $P < 0,1$; + + , $P < 0,05$; + + + , $P < 0,01$; + + + + , $P < 0,001$.

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Table

1 - Influence of acetate and stearate on the production and quantity of fatty acids secreted per day
2 - Experiment F
3 - Experiment C
4 - Average results following the diet
5 - Significance of effects
6 - Control
7 - Fatty materials secreted in g/day
8 - Butyraceous level in %
9 - Quantities of fatty acids secreted in g/day
10 - Sum of fatty amounts of C_8 to C_{16}
11 - Sum of C_{18} fatty acids
12 - N. S. = not significant

The addition of acetate and stearate to the two basic diets increased the butyraceous level and the quantity of fatty materials secreted daily. However, this increase is more notable for the incorporation of stearates into the diet rich in fodder (experiment F) and of acetate in the more concentrated diet (experiment C). In every case, acetate increases the quantity of short chain fatty acids in the milk. It is the same with C_{18} acids, but the result is only significant in experiment C. When stearate is added to the basic diets, the secretion of stearic and oleic acids is higher. It increases two times more in the case of the diet rich in fodder (experiment F). However, stearate tends to lower the production of fatty acids having less than 18 carbon atoms, and this effect is principally due to a decrease in the secretion of palmitic acid.

No interaction between the effect of the stearate and the acetate was shown to be significant with the more concentrated diet (experiment C). On the other hand, interactions of the secretion of stearic, oleic and to a lesser degree, palmitic acid were significant with the diet rich in fodder (experiment F).

Discussion and Conclusions

The results obtained confirm that the presence of acetate is favorable for the synthesis of fatty acids containing less than 18 carbon atoms, and the presence of stearate is favorable for the secretion of stearic and oleic acids ((3), (4)). The degree of these effects may be related to the limiting factors of the basic diet which are acetic acid in experiment C and C_{18} fatty acids in experiment F.

According to the generally accepted theory, C_{18} fatty acids are not synthesized by the mammae of ruminants (3). Also, it is surprising that their secretion increases when acetate is added to the diets. However, according to the results of Gerson and colleagues in the cow (5), it seems that a synthesis from acetate may take place in certain organs, such as the liver or in the adipose tissue, and furnish the mammae with a small amount of C_{18} fatty acids.

In our two experiments, stearate decreased the secretion of lauric acid and especially that of palmitic acid, as Steele and Moore have already noted (6). Some studies done with cows ((7), (8)) suggest that this decrease is not due to a reduction in the production of acetic acid in the rumen, resulting from the addition of stearate to the diet. It is more plausible to think that the taking up by the mammae of a large quantity of long chain fatty plasmic acids, causes a decrease in the synthesis of fatty acids which would be due to the inhibitory effect of acetyl-CoA carboxylase, as Bortz and colleagues (9) have shown in specimens of rat liver. The work of Hibbitt (10) and of Fehr and colleagues (11) obtained <u>in vivo</u> confirm this interpretation.

The interaction of the effects of the stearate and the acetate on the secretion of certain acids by the mammae seems to depend on the nature of the basic diet. The effects are complementary with the more concentrated diet (experiment C). In this case, the mammae are probably deficient in both acetic acid and C_{18} acids. On the other hand, with the diet rich in fodder (experiment F), the acetate seems to lessen the effect of the stearate on the quantity of C_{18} fatty acids, although, used alone, it has a favorable, though not significant, effect. In this last case, the mammae would essentially be deficient in C_{18} acids as shown by the slight effect of acetate on the secretion of short chain fatty acids, and the very clear increase in the production of C_{18} acids following the addition of stearate. The depressant effect of stearate on the secretion of palmitic acid is attenuated by simultaneously adding acetate, so that the partial inhibition of the synthesis of fatty acids by the mammae due to the stearate is in part lifted by a supplementary addition of acetate. It can then be surmised that resulting from this phenomenon, the secretion of C_{18} is reduced because the mammae have reached their maximum limits for synthesis, as the values of the butyraceous levels would seem to indicate.

It follows that the mammae will utilize as much acetate and stearate to synthesize the fatty material of milk as they are lacking. When the lipid secretion is limited by other factors, the utilization of these principal nutrients may depend on the relative proportion of their amounts in the plasma. It is in this direction that we will pursue further research.

(*) Meeting of July 6, 1970

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C. R. Acad. Sc. Paris, t. 271 (15 juillet 1970) Série D — 237

NUTRITION. — *Effets distincts et cumulés des acides acétique et stéarique sur la sécrétion des acides gras par la mamelle de chèvre.* Note (*) de MM. **Pierre-Morand Fehr et Jacques Delage** (1), transmise par M. Jean Roche.

L'acide acétique ajouté au régime augmente non seulement la sécrétion dans le lait de chèvre des acides gras à moins de dix-huit atomes de carbone, mais aussi celle des acides gras à dix-huit atomes de carbone. L'addition d'acide stéarique au régime favorise la quantité sécrétée d'acides stéarique et oléique, mais diminue celle des acides gras inférieurs et notamment celle de l'acide palmitique. L'interaction entre les effets des additions de ces deux principes nutritifs dépend de la composition du régime en cellulose brute.

Chez les ruminants, les acides gras du lait à 18 atomes de carbone et une partie des acides en C_{16} dérivent essentiellement des lipides du régime (2). D'autre part, les acides à plus courte chaîne et la plus grande fraction des acides en C_{16} sont synthétisés par la mamelle surtout à partir de l'acide acétique (3). Sa production dans le rumen est favorisée par les substances cellulosiques apportées principalement par les fourrages non broyés.

Les facteurs qui orientent la synthèse des lipides du lait de ruminant vers l'utilisation des acides gras de l'une ou de l'autre origine sont mal connus. Ainsi, il nous a paru utile de rechercher si l'acide acétique a un effet sur la sécrétion des acides gras considérés généralement comme dérivant des lipides alimentaires et réciproquement si un acide gras à longue chaîne comme l'acide stéarique influence la synthèse des acides plus courts.

MATÉRIEL ET MÉTHODES. — Au cours de deux expériences (F) et (C) réalisées selon le dispositif du carré latin, 18 chèvres reçoivent pendant des périodes de trois semaines, les régimes suivants : régime de base seul ou additionné d'acétate de sodium ou de stéarate d'éthyl ou à la fois d'acétate et de stéarate.

Les régimes de base des expériences F et C sont comparables pour l'apport journalier de matières grasses qui est respectivement en moyenne de 24,2 et 26,2 g par chèvre. Par contre, ils diffèrent par leur teneur en cellulose brute, car les fourrages fournissent 40 à 60 % de la matière sèche totale dans l'expérience F et 20 à 27 % dans l'expérience C où la ration est plus concentrée. Les quantités réellement ingérées d'acétate et de stéarate quel que soit le régime, sont respectivement dans l'expérience F de 58 et 52 g/jour et par chèvre et dans l'expérience C de 62 et 47 g. Les rations sont isoénergétiques et isoazotées, et calculées pour couvrir les besoins d'entretien et de production laitière des chèvres.

Le contrôle de la production laitière et du taux butyreux ainsi que la détermination par chromatographie en phase gazeuse de la composition en acides gras du lait permettent de calculer la quantité de matières grasses sécrétées par jour et celle des principaux acides gras. L'interprétation statistique est réalisée par analyse factorielle 2^2.

RÉSULTATS. — Les résultats sont rapportés par le tableau ci-contre. L'addition d'acétate et de stéarate aux deux régimes de base augmente le taux butyreux et la

TABLEAU

Influence de l'acétate et du stéarate sur les productions et les quantités d'acides gras sécrétés par jour

	Expérience I							Expérience C						
	Résultats moyens selon les régimes				Signification des effets			Résultats moyens selon les régimes				Signification des effets		
	Témoin	Acétate	Stéarate	Acétate + stéarate	Acétate	Stéarate	Interaction	Témoin	Acétate	Stéarate	Acétate + stéarate	Acétate	Stéarate	Interaction
Matières grasses sécrétées en g/jour	67,4	72,5	77,1	80,8	N. S.	+ + +	N. S.	65,2	73,8	68,7	77,0	+ +	N. S.	N. S.
Taux butyreux en ‰	26,0	27,4	28,3	28,5	N. S.	+ +	N. S.	31,3	34,1	33,3	36,5	+ + + +	+ +	N. S.
Quantités d'A. G. sécrétés en g/jour :														
8 : 0	1,2	1,2	1,2	1,3	N. S.	N. S.	N. S.	1,7	1,8	1,7	2,0	N. S.	N. S.	N. S.
10 : 0	6,5	6,9	6,5	7,1	N. S.	N. S.	N. S.	7,4	8,1	6,9	7,5	N. S.	N. S.	N. S.
12 : 0	4,4	4,4	4,1	4,4	N. S.	N. S.	N. S.	3,7	3,9	3,2	3,3	N. S.	+ + +	N. S.
14 : 0	8,6	8,9	9,0	9,9	+	+	N. S.	7,3	8,2	7,2	8,1	+ +	N. S.	N. S.
16 : 0	26,3	27,0	20,9	24,7	+ +	+ + + +	+	21,3	24,4	19,0	21,0	+ + +	+ +	N. S.
18 : 0	1,7	2,3	7,0	5,9	N. S.	+ + + +	+ +	3,0	3,8	6,2	7,8	+ + + +	+ + + +	N. S.
18 : 1	8,4	9,9	16,9	15,5	N. S.	+ + + +	+ +	9,5	10,7	13,0	14,4	+ +	+ + + +	N. S.
18 : 2	1,4	1,5	1,3	1,5	N. S.	N. S.	N. S.	1,8	1,9	1,6	1,7	N. S.	N. S.	N. S.
Somme des A. G. de 8 : 0 à 16 : 0	46,8	48,4	41,4	47,5	+ +	+	N. S.	41,5	46,3	38,1	42,3	+ +	+	N. S.
Somme des A. G. en C_{18}	11,5	13,6	25,2	22,9	N. S.	+ + + +	+ +	15,1	17,0	21,4	23,8	+ +	+ + + +	N. S.

N. S., non significatif ; +, $P < 0,1$; + +, $P < 0,05$; + + +, $P < 0,01$; + + + +, $P < 0,001$.

quantité de matières grasses sécrétées journellement. Toutefois, cette augmentation est plus marquée par l'incorporation de stéarate au régime riche en fourrages (expérience F) et d'acétate au régime plus concentré (expérience C). Dans tous les cas, l'acétate augmente la quantité d'acides gras à chaîne courte du lait. Il en est de même de celle des acides en C_{18}, mais la réponse n'est significative que dans l'expérience C. Quand du stéarate est ajouté aux régimes de base la sécrétion des acides stéarique et oléique est plus élevée. Elle s'accroît deux fois plus dans le cas du régime riche en fourrages (expérience F). Par ailleurs, le stéarate tend à abaisser la production d'acides gras à moins de dix-huit atomes de carbone et cet effet est principalement dû à une diminution de la sécrétion d'acide palmitique.

Aucune interaction entre l'effet du stéarate et celui de l'acétate ne s'est révélée significative avec le régime plus concentré (expérience C). En revanche, les interactions sur la sécrétion des acides stéarique, oléique et à un moindre degré palmitique sont significatives avec le régime riche en fourrages (expérience F).

Discussions et conclusions. — Les résultats obtenus confirment que l'acétate est favorable à la synthèse des acides gras contenant moins de dix-huit atomes de carbone, et le stéarate à la sécrétion des acides stéarique et oléique [(3), (4)]. L'amplitude de ces effets pourrait être liée aux facteurs limitants des régimes de base, qui sont l'acide acétique dans l'expérience C, et les acides gras en C_{18}, dans l'expérience F.

D'après la théorie généralement admise, les acides gras à dix-huit atomes de carbone du lait, ne sont pas synthétisés par la mamelle des ruminants (3). Aussi est-il surprenant que leur sécrétion augmente quand de l'acétate est ajouté aux régimes. Cependant, d'après les résultats de Gerson et coll. chez la vache (5), il semble qu'une synthèse à partir d'acétate pourrait s'effectuer dans certains organes, comme le foie, ou dans le tissu adipeux et fournir à la mamelle une faible fraction d'acides en C_{18}.

Dans nos deux expériences, le stéarate diminue la sécrétion d'acide laurique et surtout celle d'acide palmitique, comme l'ont déjà remarqué Steele et Moore (6). Certaines études effectuées sur vaches [(7), (8)] suggèrent que cette diminution n'est pas due à une réduction de la production d'acide acétique dans le rumen consécutive à l'addition de stéarate au régime. Il est plus plausible de penser que le prélèvement par la mamelle d'une plus grande quantité d'acides gras plasmatiques à longue chaîne, provoque une diminution de la synthèse des acides gras qui serait due à l'inhibition de l'acétyl-CoA carboxylase, comme l'ont montré Bortz et coll. (9) sur des coupes de foie de rat. Les travaux d'Hibbitt (10) et ceux de Fehr et coll. (11) obtenus *in vivo* confirmeraient cette interprétation.

L'interaction des effets du stéarate et de l'acétate sur la sécrétion de certains acides par la mamelle semble dépendre de la nature du régime de base. Leurs effets s'ajoutent l'un à l'autre avec le régime plus concentré (expérience C). Dans ce cas, la mamelle est probablement déficitaire à la fois en acide acétique et en acides en C_{18}. Au contraire avec le régime plus riche en fourrages (expérience F) l'acétate semble amoindrir l'effet du stéarate sur la quantité des acides gras en C_{18}, bien que distribué seul, il ait un effet favorable, mais non significatif. Dans ce dernier cas, la mamelle serait essentiellement carencée en acides en C_{18} comme le montrent le faible effet de

l'acétate sur la sécrétion d'acides gras courts et la très nette augmentation de la production des acides à dix-huit atomes de carbone à la suite de l'addition de stéarate. L'effet dépressif du stéarate sur la sécrétion d'acide palmitique est atténué en ajoutant simultanément de l'acétate, comme si l'inhibition partielle de la synthèse des acides gras dans la mamelle due au stéarate était en partie levée par un apport supplémentaire d'acétate. On peut alors penser que consécutivement à ce phénomène, la sécrétion des acides en C_{18} est réduite parce que la mamelle a atteint ses possibilités maximales de synthèse, comme semblent l'indiquer les valeurs du taux butyreux.

Il s'ensuit que la mamelle utiliserait d'autant mieux l'acétate et le stéarate pour synthétiser la matière grasse du lait qu'elle en est carencée. Lorsque sa sécrétion lipidique est limitée par d'autres facteurs, l'utilisation de ces principes nutritifs pourrait dépendre de la proportion relative de leur teneur dans le plasma. C'est dans cette voie que nous poursuivrons nos recherches.

(*) Séance du 6 juillet 1970.

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German Translation

Photometric Microdetermination of Acetic Acid in Urine

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In view of the importance of acetic acid in intermediate metabolism, whereby it is formed as a fragment in the breakdown of carbohydrates, fats and proteins, and takes part as a building component in many biochemical syntheses, it is necessary to develop a method which would allow the accurate determination of acetic acid in organic liquids.

The knowledge that acetic acid is a component of normal urine must be credited to the old studies of Proust (1) and Liebig (2), in which acetic acid was determined by titration following preliminary separation by steam distillation. Methods for the determination of acetic acid, which are based on the physical properties of this compound, can be subdivided as follows: distillation methods [Proust (1); Liebig (2); Duclaux (3); Welde (4); Dyer (5); Olmsted, Whitaker and Duden (6); Janke and Kropacsy (7); McClendon (8); Black (9); Brouwer and Hiykamp (10)], partition methods [Behrens (11), Werkman (12)], methods based on a combination of the above methods (Friedemann (13)], and finally chromatographic methods [Ramsey and Patterson (14); Elsden (15); Ramsey (16); Moyle, Baldwin and Scarisbrick (17); Peterson and Johnson (18)]. Only the last methods are specific enough, but they are too inconvenient for routine determinations of acetic acid in urine. Other methods are based on the chemical properties of

acetic acid. Among such methods, oxidation of acetic acid to oxalic acid by copper oxide (Mugdan and Wimmer (19)), and dry distillation of calcium acetate, which decomposes to form acetone [Dulian (20)], cannot be used for the determination of acetic acid in urine, because other organic acids undergo similar conversions under these conditions. Hutchens and Kass (21) have recently proposed a photometric method, using the lanthanum reaction of Kruger and Tschirch (22), but propionic acid reacts quite similarly to acetic acid and therefore this process lacks the necessary specificity.

According to Caselli and Ciaranfi (23), acetic acid can be determined oxidimetrically in a mixture of organic products by means of the following principle: acetic acid is distilled quantitatively as its methyl ester into an alkali solution, while at the same time the other fatty acids present in solution are only partly distilled as methyl esters. After saponification of the fatty acid esters, the alkaline distillate is divided into 2 equal parts and evaporated. One part is completely oxidized with silver bichromate and 60% H_2SO_4, and the other part is oxidized with potassium bichromate and 50% H_2SO_4, i.e. under conditions in which acetic acid remains unchanged, whereas other acids are attacked in a definite percentage. This method was later developed somewhat by Caselli and Ciaranfi (24) for the determination of acetic acid in urine. In order to detect acetic acid in blood and tissues, the above method has recently been modified in part by Ciaranfi and Fonnesu (25). According to this method, methyl acetate can be better separated from the other fatty acid esters by repeated distillation as only hydrogen sulfide and sodium formate, along with sodium acetate, are left in the residue; this has the advantage that oxidation can be carried out in the same sample. The amount of reduced

bichromate is determined photometrically.

Within the framework of tests on the experimental loading of the organism with Na-acetate, it became necessary to determine the concentration of acetic acid in different small urine samples. Since the methods previously described were not suitable for this special problem, we have worked out the following procedure.

Reagents and Instruments

1. Copper sulfate, cryst., $CuSO_4$ $5H_2O$, analytical grade. 2. Sodium hydroxide, analytical grade, pellets. 3. 15% lime milk. 4. Saturated copper sulfate solution. 5. Methanol, analytical grade; to remove traces of volatile acids, methanol is distilled in a glass apparatus with 2 g solid NaOH/l. 6. Concentrated sulfuric acid (d. 1.84). 7. 1.25% NaOH solution. 8. 0.05 N potassium bichromate solution, analytical grade. 9. Saturated silver nitrate solution. Five standard micro Kjeldahl flasks, 100 cc and 50 cc, with ground necks carrying markings of the liquid contents at 12 cc; two 80 cm-long ground reflux condensers, a 50 cc Kjeldahl flask with ground neck. One Coleman spectrophotometer (S.14) with round cells (19 x 105 mm) or a similar instrument.

Procedure

Solid $CuSO_4$ $5H_2O$ (0.5 g) is added to 10 cc urine (as such or diluted when containing large amounts of acetic acid) made alkaline to litmus paper by addition of solid NaOH. After repeated shaking to dissolve the solid products, the liquid is placed into a refrigerator, allowed to stand at least 12 hours and then centrifuged. To a 5 cc portion of clear, centrifuged liquid 5 cc dist. H_2O, 2.5 cc saturated $CuSO_4$ solution and 4 cc of 15% lime milk solution is added. After strong shaking, the mixture

is centrifuged and filtered through paper into a 100 cc Kjeldahl flask, the precipitate and filter are washed several times with some water which was added to the first filtrate; then, 2 small glass beads are added and the flask is immersed into a water bath containing a concentrated boiling $CaCl_2$ solution, whereby the vapors are sucked out of the flask by means of a water jet pump. If the residue is well dried, the flask is cooled under running water and 15 cc methanol is added from the ground joint, followed by 3 cc conc. sulfuric acid. The flask is connected with the receiver, stirred gently to mix the solution and the mixture distilled over a small direct flame; the distillate is caught in a 50 cc Kjeldahl flask containing 4 cc methanol. The tip of the distillation tube must be submerged in the methanol. After distilling off 8 cc (i.e. down to the 12 cc marking), the distillation is stopped. The tip of the distillation tube is washed with 4 cc methanol and the washings combined with the distillate. During distillation, particularly when it is performed too quickly, light-breaking rings are formed, which should by no means reach the angle formed by the ascending and descending parts of the distillation tube. The flask containing the distillate is connected to another receiver, after first wetting the ground joint with H_2SO_4, and the contents are distilled as above. This process is repeated 3 times, and the product of the last distillation is caught in 4 cc of 1.25% NaOH solution instead of methanol. After adding a glass bead and stirring the liquid, it is left standing for 30 min. to allow saponification of the esters. Then, methanol and water are evaporated on a water bath, and the dry residue, after adding 7 cc of 0.05 N potassium bichromate solution and 7 cc conc. sulfuric acid, is boiled for 30 min, after connecting the flask with a ground reflux cooler. The ground

joint is wetted with conc. H_2SO_4, and the upper opening of the cooler is covered with an inverted beaker. After the solution has cooled, 4 cc are poured into a centrifuge tube and diluted with 6 cc distilled water (so-called unknown potassium). After adding 2 drops of a saturated silver nitrate solution and 3 cc conc. H_2SO_4, the remaining liquid is boiled for 1 hour and a second 4 cc sample is collected and diluted with 6 cc distilled water in another centrifuge tube (so-called unknown silver). Blank samples are prepared at the same time with the reagents used (potassium blank sample and silver blank sample). After separation of a light precipitate by centrifugation, the clear liquids are poured into a series of spectrophotometer cells and the amount of bichromate present in each cell is determined spectrophotometrically at 450 mu. The unknown potassium, which correspounds to a zero concentration of acetic acid, is set on an extinction of 0.4, and the extinction of the potassium blank sample is measured. This value serves as the initial (zero) point for the subsequent reading. The silver blank sample is set on this initial point and the extinction value of the unknown silver is recorded. The calibration curve is plotted as usual with a dilution series of a standard acetic acid solution, whereby the extinction of 0.4 corresponds to a sample without acetic acid. The calibration curve given in the study of Ciaranfi and Fonnesu (25) shows a linear relationship between the acetic acid concentration and the extinction.

Results

With the aid of the method described above, we have determined the amounts of acetic acid added to urine.

Table

	(1) Essigsäure bestimmt in 5 cm³ Harn* mg	(2) Essigsäure zugesetzt mg (3)	Essigsäure gefunden** mg (4)	Differenz mg	(5) Difference Fehler %
1	0.28	0.12	0.12	0.00	0.0
2	0.24	0.15	0.14	− 0.01	6.7
3	0.26	0.20	0.17	− 0.03	15.0
4	0.28	0.25	0.26	+ 0.01	4.0
5	0.24	0.30	0.32	+ 0.02	6.7
6	0.34	0.40	0.38	− 0.02	6.7
7	0.28	0.50	0.46	− 0.04	8.0
8	0.25	0.60	0.56	− 0.04	6.7
9	0.27	0.75	0.70	− 0.05	6.7
10	0.20	1.00	1.02	+ 0.02	2.0
(6) Durchschnitt	0.27	—	—	—	6.0

1. Acetic acid determined in 5 cc urine*
2. Acetic acid
3. Added
4. Found
5. Error
6. Average

* Human urine 8 hours after food intake
** The amounts found are calculated by substracting the amount of acetic acid present in urine samples to which no acetic acid was added.

Discussion

Determination of known amounts of acetic acid added to different urine samples shows that the values found are sufficiently accurate for biological purposes. As can be seen from the table, the probable error is generally smaller than ± 10% and seldom exceeds this value. The acetic acid concentration in the urine of normal people of both sexes after 8 hours of fasting probably depends on the type of food ingested and on the concentration ability of the kidneys, and agrees well with the values obtained volumetrically by Caselli and Ciaranfi (24).

The advantage of the photometric acetic acid determination, in contrast to the volumetric method, lies in the fact that this method requires

only small amounts of urine, thus allowing its use in metabolism tests on small laboratory animals, and also that repeated distillations allow a sharper separation of acetic acid from eventually present lower fatty acids, since the latter are already completely removed at the end of the process or only trace amounts are distilled. In addition, products such as formic acid and hydrogen sulfide are not measured photometrically at the same time; these products cannot be separated from acetic acid by distillation and are completely oxidized by potassium bichromate and 50% sulfuric acid.

Summary

A photometric micro method for the determination of acetic acid in urine is described. After clarification and sugar removal from the urine by precipitation with copper sulfate in an alkaline calcium hydroxide solution, the filtrate is evaporated, and the acetic acid present in the residue is esterified and repeatedly distilled as methyl acetate, in order to effect a good separation from other low fatty acid esters. After saponification of the methyl acetate, the alkaline distillate is evaporated. Formic acid, hydrogen sulfide and other products which cannot be removed by distillation are oxidized with potassium bichromate and 50% sulfuric acid, while acetic acid remains unchanged under these conditions. Complete oxidation of acetic acid is achieved by addition of silver nitrate and by increasing the concentration of sulfuric acid to 60%. Reduction of the bichromate is measured spectrophotometrically, and the amount of acetic acid is determined by difference of the extinction values with the aid of a calibration curve. The method makes it possible to determine 0.1-1.0 mg acetic acid in small amounts of urine and is specific and sufficiently accurate for biological purposes, since the probable error is $\pm$ 10%.

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Photometrische Anpassung für die Mikrobestimmung der Essigsäure im Harn.

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Die wichtige Stellung der Essigsäure im intermediären Stoffwechsel, wobei diese Substanz als Bruchstück des Kohlenhydrat-, Fett- und Proteinabbaus gebildet wird, und anderseits als Aufbaustein an mehreren biochemischen Synthesen teilnimmt, zeigt die Notwendigkeit, eine Methode auszuarbeiten, die es ermöglicht, die Essigsäure in organischen Flüssigkeiten genau zu bestimmen.

Die Kenntnis, daß die Essigsäure ein Bestandteil des normalen Harnes darstellt, verdanken wir den alten Arbeiten von PROUST [1] und LIEBIG [2], in welchen die Essigsäurebestimmung, nach vorangegangener Trennung durch Wasserdampfdestillation, titrimetrisch durchgeführt wurde. Bestimmungsmethoden der Essigsäure, begründet auf die physikalischen Eigenschaften dieses Stoffes, können wir einteilen in: Destillationsverfahren (PROUST [1]; LIEBIG [2]; DUCLAUX [3]; WELDE [4]; DYER [5]; OLMSTED, WHITAKER u. DUDEN [6]; JANKE u. KROPACSY [7]; McCLENDON [8]; BLACK [9]; BROUWER u. HIJKAMP [10]), Partitionsmethoden (BEHRENS [11]; WERKMAN [12]), Kombinationsmethoden der beiden obengenannten Verfahren (FRIEDEMANN [13]) und endlich in chromatographische Methoden (RAMSEY u. PATTERSON [14]; ELSDEN [15]; RAMSEY [16]; MOYLE, BALDWIN u. SCARISBRICK [17]; PETERSON u. JOHNSON [18]). Nur die letzten Methoden besitzen eine genügende Spezifität, aber sie sind zu unbequem, um Routinebestimmungen der Essigsäure im Harn durchzuführen. Andere Methoden sind auf die chemischen Eigenschaften der Essigsäure begründet. Unter solchen, die Oxydation der Essigsäure zu Oxalsäure durch Kupferoxyd (MUGDAN u. WIMMER [19]), die trockene Destillation des Ca-acetats, wobei dasselbe in Aceton zerfällt (DULIAN [20]), sind als Bestimmungsmethoden der Essigsäure im Harn nicht anwendbar, weil andere organische Säuren unter diesen Verhältnissen gleichartige Umbildungen durchmachen. HUTCHENS u. KASS [21] haben eine photometrische Methode, die die Lanthanreaktion von KRUGER u. TSCHIRCH [22] benützt, neuerdings vorgeschlagen, aber Propionsäure reagiert der Essigsäure ganz ähnlich und demnach fehlt einem solchen Verfahren die notwendige Spezifität.

Nach CASELLI u. CIARANFI [23] kann man die Essigsäure in einer Mischung organischer Stoffe oxydimetrisch bestimmen mit Hilfe des folgenden Prinzips: die Essigsäure wird quantitativ als Methylester in eine Alkalilösung überdestilliert, während gleichzeitig die übrigen in der Lösung anwesenden Fettsäuren nur zum Teil als Methylester destillieren. Nach Verseifung der Fettsäureester wird das alkalische Destillat in 2 Hälften geteilt und verdampft. Eine Hälfte wird durch Silberbichromat und 60%ige Schwefelsäure vollkommen oxydiert, die andere durch Kaliumbichromat und 50%ige Schwefelsäure oxydiert, d. h. unter Bedingungen, bei welchen die Essigsäure unverändert bleibt, während die anderen Säuren in einem bestimmten Prozentsatz angegriffen werden. Die Methode wurde später von CASELLI u. CIARANFI [24] für die Bestimmung der Essigsäure im Harn etwas ausgearbeitet. Um die Essigsäure im Blut und Gewebe zu erfassen, haben neuerdings CIARANFI u. FONNESU [25] die vorhergehende Methode zum Teil verändert. Nach diesem Verfahren wird das Methylacetat von den anderen Fettsäureester durch wiederholte Destillationen besser getrennt, indem in dem Rückstand, neben Na-acetat, nur Schwefelwasserstoff und Ameisensäure Natrium verbleiben, mit dem Vorteil, daß die Oxydation in demselben Probemuster durchgeführt wird. Die Menge des reduzierten Bichromats wird photometrisch bestimmt.

Im Rahmen von Versuchen über die experimentelle Belastung des Organismus mit Na-acetat ergab sich die Notwendigkeit, die Essigsäurekonzentration in verschiedenen kleinen Harnproben festzustellen. Da die beschriebenen Methoden sich für diese spezielle Frage nicht eigneten, kamen wir zu folgendem Verfahren.

Reagentien und Geräte.

1. Kupfersulfat krist., $CuSO_4 \cdot 5\,H_2O$, pro anal. 2. Natriumhydroxyd, pro anal. Plätzchenform. 3. 15%ige Kalkmilch. 4. Gesättigte Kupfersulfatlösung. 5. Methanol, pro anal.; um Spuren von flüchtigen Säuren zu entfernen, wird das Methanol unter Zusatz von 2 g festen Natriumhydroxyds pro Liter in einer Glasapparatur destilliert. 6. Konz. Schwefelsäure (D. 1,84). 7. 1,25%ige Natronlauge. 8. 0,05 N Kaliumbichromat, pro anal. 9. Gesättigte Silbernitratlösung. 5 gewöhnliche Mikro-KJELDAHL-Kolben, 100 cm³ und 50 cm³, mit geschliffenem Hals, letztere mit Markierung des Flüssigkeitsstandes bei 12 cm³. — 2 80 cm lange Schliffrückflußkühler, 50 cm³-KJELDAHL-Kolben mit geschliffenem Hals. — Ein COLEMAN-Spektrophotometer (S. 14) mit runden Cuvetten (19 × 105 mm) oder ähnliches Instrument.

Ausführung.

Zu 10 cm³ Harn (als solcher oder verdünnt, wenn er große Essigsäuremengen enthält) durch festes Natriumhydroxyd lakmusalkalisch gemacht, werden 0,5 g $CuSO_4 \cdot 5\,H_2O$ in Substanz hinzugefügt. Nach wiederholtem Schütteln, um die festen Stoffe in Lösung zu bringen, wird die Flüssigkeit in den Eisschrank gestellt, mindestens 12 Std stehen gelassen und danach zentrifugiert. Zu 5 cm³ klar zentrifugierter Flüssigkeit fügt man 5 cm³ dest. Wasser, 2,5 cm³ gesättigte Kupfersulfat-

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lösung und 4,0 cm³ 15%ige Kalkmilchlösung hinzu. Nach starkem Schütteln zentrifugiert und filtriert man durch Papier in einen 100 cm³-Kjeldahl-Kolben, wäscht wiederholt das Präzipitat und das Filter mit etwas Wasser, das dem ersten Filtrat zugegossen wird, gibt 2 kleine Glaskugeln hinein und taucht den Kolben in ein Wasserbad, welches eine konzentrierte, kochende $CaCl_2$-Lösung enthält, wobei die Dämpfe aus dem Kolben durch eine Wasserstrahlpumpe abgesaugt werden. Ist der Rückstand gut getrocknet, läßt man den Kolben unter fließendem Wasser erkalten und gießt 15 cm³ Methanol sowie darauffolgend 3 cm³ konz. Schwefelsäure vom Schliff an hinein. Man verbindet den Kolben mit der Vorlage, bewegt ihn sorgfältig, um die Lösung zu mischen und destilliert über kleiner direkter Flamme, wobei das Destillat in einen 50 cm³-Kjeldahl-Kolben, der 4 cm³ Methanol enthält, aufgefangen wird. Das Ende des Destillationsrohres muß in das Methanol eintauchen. Nach Abdestillieren von 8 cm³ (d. h. bis zur Marke 12 cm³) wird die Destillation beendigt. Man wäscht das Ende des Destillationsrohres mit 4 cm³ Methanol und vereinigt mit dem Destillat. Während der Destillation, besonders wenn sie zu schnell durchgeführt wird, treten lichtbrechende Ringe auf, die den Winkel zwischen aufsteigendem und absteigendem Teil des Destillationsrohres keineswegs erreichen sollen. Der das Destillat enthaltende Kolben wird mit einer anderen Vorlage verbunden, nachdem man vorher den Schliff mit Schwefelsäure befeuchtet hat, und destilliert wie oben. Das Verfahren wird 3mal wiederholt, wobei das Produkt der letzten Destillation in 4 cm³ 1,25%iger Natronlauge statt Methanol aufgefangen wird. Man gibt eine Glaskugel hinein, rührt die Flüssigkeit um und läßt eine halbe Stunde stehen, um die Ester verseifen zu lassen. Darauf werden Methanol und Wasser im Wasserbad verdampft und der trockene Rückstand unter Zusatz von 7 cm³ 0,05 n Kaliumbichromat und 7 cm³ konz. Schwefelsäure 30 min gekocht, nachdem der Kolben mit einem eingeschliffenen Rückflußkühler verbunden worden ist. Der Schliff wird mit konz. Schwefelsäure befeuchtet. Die obere Öffnung des Kühlers wird mit einem umgestülpten Becherglas bedeckt. Nach Abkühlen der Lösung werden 4 cm³ in ein Zentrifugenrohr gegossen und mit 6 cm³ destilliertem Wasser verdünnt (sogenanntes *unbekanntes Kalium*). Nach Zugabe von 2 Tropfen gesättigter Silbernitratlösung und 3 cm³ konzentrierter Schwefelsäure wird die übrige Flüssigkeit noch 1 Std lang gekocht und eine zweite 4 cm³-Probe abgenommen und mit 6 cm³ dest. Wasser in einem anderen Zentrifugenrohr verdünnt (sogenanntes *unbekanntes Silber*). Gleichzeitig mit den angewandten Reagentien werden Leerproben ausgeführt (*Kalium-Leerprobe* und bzw. *Silber-Leerprobe*). Nach Abtrennung eines leichten Niederschlages durch Zentrifugieren werden die klaren Flüssigkeiten in eine Reihe von Spektrophotometer-Cuvetten gegossen und die Menge des in jeder Cuvette anwesenden Bichromats wird bei 450 mμ spektrophotometrisch bestimmt. Das *unbekannte Kalium*, welches einer Konzentration gleich Null an Essigsäure entspricht, wird auf eine Extinktion von 0,4 gesetzt und die Extinktion der *Kalium-Leerprobe* gemessen. Dieser Wert dient als *Nullpunkt* für die darauffolgende Lesung. Man setzt die *Silber-Leerprobe* auf *diesen Nullpunkt* und liest den Extinktionswert des *unbekannten Silbers* ab. Die Eichkurve wird wie gewöhnlich mit einer Verdünnungsreihe einer Essigsäure-Standardlösung hergestellt, wobei die Extinktion von 0,4 einer Probe ohne Essigsäure entspricht. Die Eichkurve, die in der Arbeit von Ciaranfi und Fonnesu [25] wiedergegeben ist, zeigt eine lineare Beziehung zwischen Essigsäurekonzentration und Extinktion.

Ergebnisse.

Mit Hilfe der beschriebenen Methode haben wir zu Harn zugesetzte Essigsäure bestimmt. Die gefundenen Werte zeigt die folgende Tabelle.

Tabelle.

	Essigsäure bestimmt in 5 cm³ Harn* mg	Essigsäure zugesetzt mg	Essigsäure gefunden** mg	Differenz mg	Fehler %
1	0,28	0,12	0,12	0,00	0,0
2	0,24	0,15	0,14	0,01	6,7
3	0,26	0,20	0,17	0,03	15,0
4	0,28	0,25	0,26	− 0,01	4,0
5	0,24	0,30	0,32	− 0,02	6,7
6	0,34	0,40	0,38	0,02	6,7
7	0,28	0,50	0,46	0,04	8,0
8	0,25	0,60	0,56	0,04	6,7
9	0,27	0,75	0,70	0,05	6,7
10	0,29	1,00	1,02	− 0,02	2,0
Durchschnitt	0,27				6,0

* Harn von Menschen 8 Std nach Nahrungsaufnahme.

** Die gefundenen Mengen werden durch Subtrahieren der Essigsäuremenge berechnet, die in Harnproben ohne Essigsäurezusatz enthalten ist.

Diskussion.

Die Bestimmung von bekannten zu verschiedenen Harnproben zugesetzte Essigsäuremengen zeigt, daß die gefundenen Werte für biologische Zwecke hinlänglich genau sind. Wie man aus der Tabelle ersehen kann, ist der wahrscheinliche Fehler im allgemeinen kleiner als ± 10% und überschreitet selten diesen Wert. Die Essigsäurekonzentration im Harn von normalen Menschen beider Geschlechter nach 8stündigem Fasten hängt wahrscheinlich von der Nahrungsart und von dem Konzentrationsvermögen der Niere ab und stimmt gut mit den Werten, die Caselli u. Ciaranfi [24] volumetrisch erhalten haben, überein.

Der Vorteil der photometrischen Essigsäurebestimmung im Gegensatz zum volumetrischen Verfahren besteht darin, daß die Methode nur kleine Harnmengen braucht, was sie für Stoffwechselversuche an kleine Laboratoriumstiere anwendbar macht, und daß die wiederholten Destillationen eine schärfere Trennung der Essigsäure von eventuell begleitenden niedrigen Fettsäuren ermöglichen, indem diese Stoffe am Ende des Verfahrens schon vollkommen entfernt sind oder nur in Spuren überdestillieren. Außerdem werden Substanzen wie Ameisensäure und Schwefelwasserstoff photometrisch nicht miterfaßt, Substanzen, die durch Destillation von der Essigsäure nicht zu trennen sind, und welche mit Kaliumbichromat und 50%iger Schwefelsäure vollkommen oxydiert werden.

Zusammenfassung.

Es wird eine photometrische Mikromethode für die Bestimmung der Essigsäure im Harn beschrieben. Nach Klärung und Entzuckerung des

Harnes durch Fällung in calciumhydroxydalkalischer Lösung mit Kupfersulfat, wird das Filtrat verdampft und die im Rückstand vorhandene Essigsäure verestert und als Methylacetat wiederholt überdestilliert, um eine gute Trennung von den anderen niedrigen Fettsäureestern durchzuführen. Nach Verseifung des Methylacetats wird das alkalische Destillat verdampft. Ameisensäure, Schwefelwasserstoff und übrige Stoffe, die durch Destillieren nicht entfernt werden können, werden durch Kaliumbichromat und 50%ige Schwefelsäure oxydiert, während die Essigsäure unter solchen Bedingungen unverändert bleibt. Die vollkommene Oxydation der Essigsäure wird durch Zusatz von Silbernitrat und durch Vermehrung der Schwefelsäurekonzentration auf 60% ausgeführt. Die Reduktion des Bichromats wird spektrophotometrisch gemessen und die Menge der Essigsäure durch die Differenz der Extinktionswerte mit Hilfe einer Eichkurve bestimmt. Die Methode erlaubt 0,1—1,0 mg Essigsäure in kleinen Harnmengen zu bestimmen und ist spezifisch und genügend genau für biologische Zwecke, da der wahrscheinliche Fehler ±10% ist.

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NETHERLANDS

1714 Preservatives Decree of 22. XII. 67.

(Staatsblad 1967, Text 691, p. 1919.)

Basic text, implementing EEC Directives (see in particular 77 CFAL 1131).

In principle, only the following preservatives may be used in foodstuffs:

Sorbic acid and its Na, K and Ca salts
Benzoic acid and its Na, K and Ca salts
Ethyl ester of p-hydroxybenzoic acid and its Na salt
n-Propyl ester of p-hydroxybenzoic acid and its Na salt
Sulphur dioxide; sodium sulphite and bisulphite; sodium, potassium and calcium disulphites (pyrosulphites, metabisulphites)

The following substances, intended principally for other purposes but capable of having a secondary preservative effect, are also authorized:

Sodium nitrite; sodium and potassium nitrates
Acetic acid and its Na, K and Ca salts
Lactic acid
Propionic acid and its Na and Ca salts
Carbon dioxide

Sodium nitrite may be used only in mixtures with sodium chloride; sodium and potassium nitrates may be used alone or in mixtures with sodium chloride. These substances may be used only in products within the purview of the Cheese Decree (see 79 CFAL 1205), the Processed Cheese Decree (of 5. XI. 59; not abstracted in CFAL) and the Meat and Meat Products Decree (see 116 CFAL 1715 below), and observing the limitations established in those Decrees.

Until a date to be established, diphenyl, o-phenylphenol and sodium o-phenylphenate may be present in/on citrus fruits and in the products within the purview of the Jam and Lemonade Decree (see 116 CFAL 1715 below), observing the limitations established in that Decree.

Until 7. XI. 68, boric acid and its salts and its salts and hexamethylenetetramine may be present in the products within the purview of the Eggs Decree (see 116 CFAL 1715 below) and the Fish Decree (see 116 CFAL 1715 below), observing the limitations established in those Decrees.

General and specific purity criteria are included.

1715 Decree No 22. XII. 67, amending the Jam and Lemonade Decree, the Meat and Meat Products Decree, the Eggs Decree, the Fish Decree and the Spices Decree.

(Stbl. 1967, Text 692, p. 1925.)

Decree making amendments consequential on the Preservatives Decree of the same date (see 116 CFAL 1714 above). Its major innovations are to extend uses of benzoic acid to include sorbic acid and its salts, and to insert cut-off dates on the use of boric acid and hexamethylenetetramine.

MODIFICATION OF BLOOD KETONES AND GLUCOSE LEVELS BY INFUSION OF SODIUM ACETATE IN PANCREATECTOMIZED DOGS

Galli, del Castillo, Boldan and Rietti

Source: C.R. Soc. Biol. 162:1620-1621 (1968).

We have studied fluctuations of plasma glucose, free fatty acids (FFA), ketone bodies, total lipids, and cholesterol, in normal dogs and in dogs after pancreatectomy, or pancreatectomy with hypophysectomy, following infusions of sodium acetate, sodium chloride, or normal saline.

The site of infusion was the jugular vein, and the duration was 1 hour (0-1 hr for a constant volume of 50 ml of fluid). The following concentrations were given: Na-acetate 6.1 mM/kg/hr with Na concentration 6.1 mM/kg/hr; Na-chloride 6.1 mM/kg/hr; and normal saline was 0.9% NaCl. Experiments lasted 6 hours.

The normal control dogs infused with Na-acetate showed no fluctuations of plasma glucose level, FFA, ketone bodies, cholesterol, or total lipids. The pancreatectomized dogs (deprived of insulin 60 hrs before infusion) given Na-acetate showed a slow but significant drop in blood glucose between the 1st and 6th hour, with significant rises of FFA and ketone bodies (1st hour) but no change in cholesterol or total lipids.

The pancreatomized-hypophysectomized dogs given Na-acetate responded similarly to those merely pancreatectomized, indicating that under these conditions hypophysectomy was incapable of correcting the metabolic dysfunctions induced by Na-acetate in the absence of the pancreas.

The pancreatectomized dogs given Na-chloride equimolar with the Na-acetate showed a slow, significant decrease of blood glucose (from the 1st to the 6th hour) and a rise of FFA (6th hour). Hematocrit was unchanged during the experiment (6 hrs).

Infusions of Na-acetate and NaCl (equimolar) both diminished the blood glucose levels, and this could be attributed entirely to the Na ion.

The normal saline induced only a rise of FFA (at the 6th hour) in the pancreatectomized dogs.

In the pancreatectomized-hypophysectomized dogs, there was a significant rise of plasma ketones which was not seen at the end of the infusion; the return to initial level between the 3rd and 6th hours demonstrated that there was a mechanism for utilization that operated in the absence of insulin.

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Physiologie.

Modification de la cétonémie et de la glycémie par perfusion d'acétate de sodium chez le chien sans pancréas.

C.R. Soc. Biol. 162: 1620-21, (1968)

par Maria Elisa Galli, E. J. del Castillo, Alicia G. Roldán et C. T. Rietti.

On a étudié les variations de la glycémie, des acides gras libres (AGL), des corps cétoniques, des lipides totaux et du cholestérol dans le plasma de chiens normaux, privés de pancréas, privés de pancréas et d'hypophyse, après perfusion de solution d'acétate de sodium, de chlorure de sodium et de solution physiologique.

Les chiens ont été perfusés par voie endoveineuse jugulaire pendant 1 heure (de 0 à 1 heure avec un volume constant de 50 ml au total de liquide de perfusion). Les solutions ont été administrées aux concentrations suivantes : acétate de sodium 6,1 mM/kg de poids/heure, avec une concentration de sodium de 6,1 mM/kg de poids/heure ; chlorure de sodium, 6,1 mM/kg de poids/heure et une solution physiologique à 9 ‰. Les expériences ont duré 6 heures.

Les chiens témoins normaux, perfusés avec de l'acétate de sodium, n'ont pas présenté de variations de la valeur plasmatique de la glycémie, les AGL des corps cétoniques, du cholestérol et des lipides totaux. Chez les chiens sans pancréas (privés d'insuline 60 heures avant la perfusion), perfusés avec la solution d'acétate de sodium, la glycémie a diminué de façon lente mais significative (de la 1re à la 6e heure), les AGL et les corps cétoniques (1re heure) ont augmenté de façon significative, sans modification du taux du cholestérol et des lipides totaux.

Les chiens privés à la fois de pancréas et d'hypophyse et perfusés avec l'acétate de sodium ont répondu comme les chiens simplement privés de pancréas ; ceci signifie que l'hypophysectomie n'est pas capable, dans les conditions expérimentales envisagées, de corriger les troubles métaboliques provoqués par l'acétate de sodium en l'absence de pancréas.

Les chiens sans pancréas, perfusés avec du chlorure de sodium, à une concentration millimolaire de sodium semblable à la solution d'acétate de sodium, ont montré une diminution significative et lente de la glycémie (de la 1re à la 6e heure) et une augmentation de AGL (6e heure). La valeur de l'hématocrite ne s'est pas modifiée pendant la durée de l'expérimentation (6 heures).

Les perfusions d'acétate de sodium et de ClNa équimolaire ont toutes deux fait baisser la glycémie ; cette baisse peut être due à la seule action de l'ion sodium.

La solution physiologique produit seulement une augmentation de AGL (à la 6e heure) chez des chiens sans pancréas.

Chez les chiens sans pancréas et chez ceux sans pancréas et sans hypophyse, l'augmentation du taux des corps cétoniques est significative, mais n'est pas élevée à la fin de la perfusion ; le retour au taux initial entre la 3e et la 6e heure prouve qu'il existe des mécanismes d'utilisation qui fonctionnent sans la présence d'insuline.

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Biochimie.

Action *in vivo* d'œstrogènes artificiels sur le système $NADH_2$-oxydase d'organes de Rat.

par J. A. Brignone, Clara M. C. de Brignone et A. O. M. Stoppani.

Des rats normaux surrénalectomisés et castrés ont été traités pendant sept jours, par des injections sous-cutanées, avec 0,1 mg/100 g de diéthylstilbestrol (stilbestrol, DES) ou avec des quantités équimolécu-

Œstrogène injecté	Variation de l'activité de $NADH_2$-oxydase en relation avec les témoins					
	Rats normaux		Rats surrénalectomisés		Rats castrés	
	Activité spécifique	Activité totale	Activité spécifique	Activité totale	Activité spécifique	Activité totale
DES.....	20 ± 1 (*) (16)	49 ± 5,5 (*)	21 ± 1,1 (*) (10)	26 ± 6,2 (*)	30 ± 1,8 (*) (10)	44 ± 7,6 (*)
DES-DP...	10 ± 0,7 (16)	0	—1 ± 0,6 (11)	29 ± 9,0	15 ± 1,4 (10)	40 ± 5,6
DES-MME..	12 ± 0,8 (10)	29 ± 6,7	18 ± 1,3 (10)	57 ± 5,0	14 ± 1,9 (10)	20 ± 8,6
DES-DME.	9 ± 0,6 (10)	15 ± 5,7	—4 ± 0,5 (12)	0	10 ± 1,7 (10)	12 ± 6,9
HEX.....	—5 ± 0,3 (10)	7 ± 6,2	18 ± 1,2 (11)	26 ± 6,5	20 ± 1,5 (10)	20 ± 7,9
HEX-DME.	1 ± 0,6 (10)	19 ± 7,5	9 ± 1,0 (11)	12 ± 9,6	9 ± 1,4 (10)	—4 ± 1,2
DIEN.....	12 ± 0,8 (10)	11 ± 6,2	36 ± 2,1 (13)	65 ± 4,3	16 ± 1,3 (10)	32 ± 5,3

(*) Erreur standard de la moyenne $P < 0,05$.

Action d'œstrogènes artificiels sur le système $NADH_2$-oxydase de cœur. $NADH_2$-oxydase obtenue du myocarde ventriculaire par extraction avec tampon de phosphate 5 mM (*pH* = 7,3) et centrifugation différentielle entre 1000 et 1500 g à 4°C. Activité spécifique : $NADH_2$ oxydé/mn/mg d'oxydase. Activité totale : $NADH_2$ oxydé/mn/g de muscle. Entre parenthèses nombre de rats dans chaque expérience.

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Effect of Volatile Fatty Acids on *Salmonella typhimurium*[1]

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It has been established for many years that volatile fatty acids have an inhibitory or bactericidal effect on a number of bacterial species. D. J. Hentges (3) noted that shigellae were inhibited when grown in association with *Klebsiella* sp. He identified the compounds responsible for this inhibition as formic acid and acetic acid, products of the metabolism of *Klebsiella*. In a separate publication, Hentges (4) demonstrated that the effect of the fatty acids was *p*H-dependent. Brownlie and Grau (1) reported that both *Escherichia coli* I and *Salmonella* were rapidly eliminated from the rumen and were rarely detectable in the feces of cattle fed a normal daily ration of hay. They attributed the demise of the enteric microorganisms in part to the high volatile fatty acid and low *p*H values in the rumen of these cattle.

In order to define the conditions affecting the sensitivity of *S. typhimurium* to volatile fatty acids, the following experiments were performed. In all experiments the inoculum of *S. typhimurium* was prepared by diluting a 24-hr nutrient broth culture to provide an initial level of 5 × 10[illegible] to 5 × 10[illegible] cells per ml in the test solution. The test solutions consisted of a nutrient broth base to which the desired concentration of fatty acid was added. The *p*H was adjusted with HCl or KOH prior to autoclaving at 121 C for 15 min. Tubes containing the test solution were tempered to the appropriate temperature in a water bath prior to inoculation. Samples were withdrawn periodically and surviving salmonellae were enumerated by spread-plating on nutrient agar.

Table 1 shows the effect of *p*H on the bactericidal activity of formic, acetic, propionic, and butyric acids. The effectiveness of the acid diminished as the *p*H of the medium was increased.

TABLE 1. *Effect of pH on the bactericidal activity of volatile fatty acids*

Acid	*p*H	No. of survivors per ml				
		Incubation time (hr)[a]				
		0	8	12	24	48
Formic	5.0	1.4×10^4	<10			
	5.5	1.1×10^4	1.1×10^3	3.0×10	<10	
	6.0	1.0×10^4	7.6×10^3	4.5×10^3	2.6×10^3	6.4×10^2
Acetic	5.0	1.5×10^4	<10			
	5.5	1.2×10^4	4.8×10^3	1.9×10^3	8.2×10^2	6.0×10
	6.0	1.1×10^4	8.6×10^3	6.3×10^3	4.4×10^2	3.9×10^2
Propionic	5.0	1.8×10^4	<10			
	5.5	1.8×10^4	4.8×10^3	1.5×10^3	1.2×10^2	<10
	6.0	1.3×10^4	6.3×10^3	4.0×10^3	3.8×10^3	5.9×10^2
Butyric	5.0	1.7×10^4	$<10^2$	<10		
	5.5	1.9×10^4	5.3×10^3	9.0×10^2	$<10^2$	1.0×10
	6.0	1.3×10^4	1.8×10^4	1.7×10^4	1.1×10^4	1.9×10^3
Control[b]	5.0	1.7×10^4		6.9×10^7	1.9×10^3	
	5.5	6.3×10^3		5.2×10^3	$>10^5$	

[a] Incubations at 37 C. Acid concentration was 0.5% in nutrient broth.
[b] Control: *p*H adjusted with HCl.

[1] Published with the approval of the Director of the Wisconsin Experiment Station.

These results are in keeping with the premise that the undissociated acid molecule is the bactericidal moiety (5). Although not of a pronounced nature, a general trend of decreasing bactericidal effect with increasing chain length of the fatty acid was noted.

The rate of death of the cells in the presence of volatile fatty acids at a given *p*H and temperature was concentration-dependent (Fig. 1). An increase in temperature increased the killing rate at fixed *p*H and concentration (Fig. 2). Lowering the temperature resulted in a longer lag period before the maximal death rate. A similar lag, although of longer duration, was noted at 7.5 C and 13 C, as reported previously (2). The lag period preceding the maximal death rate is unexplained, but characterized many of the survivor-time plots, particularly at lower concentrations of acid or higher *p*H values.

Various quantities of peptone, glucose, and sucrose were added to the test medium to ascertain whether proteins or sugars would protect the salmonellae. Each of the added substances was protective; the degree of protection increased with the concentration of the substance. Table 2 shows that glucose was the most protective substance when each was tested at the 20% level. It is possible that this reflects the larger number of glucose molecules present at the 20% level. The mechanism of this protection has not been elucidated.

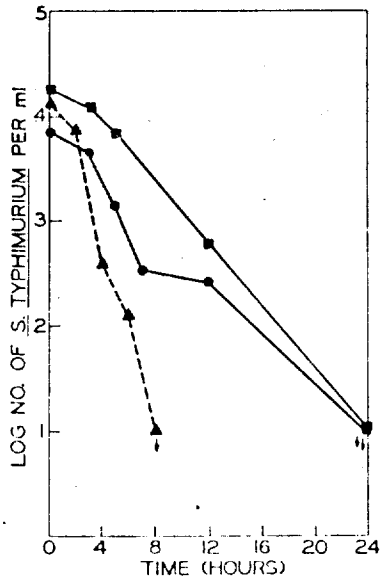

FIG. 1. *Effect of concentration of acetic acid on the rate of death of S. typhimurium in nutrient broth at pH 5 and 37 C. Symbols:* (■) *0.1% HAc,* (●) *0.2%,* (▲) *0.5%.*

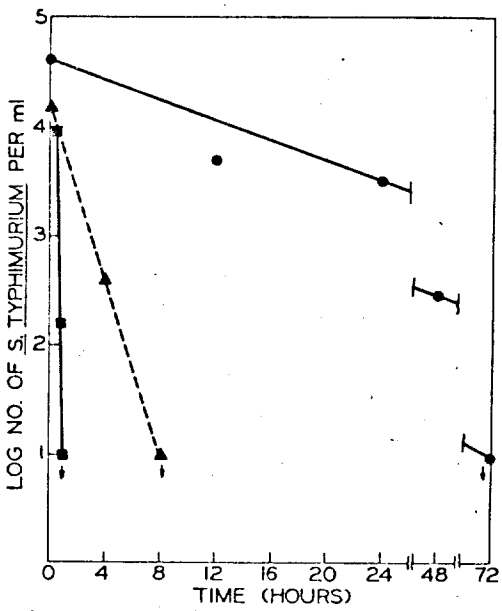

FIG. 2. *Survival of S. typhimurium in nutrient broth with 0.5% acetic acid at pH 5. Symbols:* (●) *25 C,* (▲) *37 C,* (■) *43 C.*

TABLE 2. *Effect of added glucose, sucrose, and peptone on the rate of death of S. typhimurium in 0.5% acetic acid at pH 5 and 37 C*

Incubation time (hr)	Number of survivors per ml			
	20% Glucose	20% Sucrose	20% Peptone	Control[a]
0	2.0×10^4	4.9×10^3	1.8×10^4	1.3×10^4
2	1.9×10^4	4.4×10^3	2.6×10^4	1.8×10^3
6	1.8×10^4	2.2×10^3	2.9×10^4	1.6×10^2
12	1.3×10^4	6.8×10^2	1.4×10^4	<10
24	3.5×10^3	<10	<10	
48	2.3×10^2			
72	<10			

[a] Control was nutrient broth plus 0.5% acetic acid at *p*H 5.0.

In summary, the sensitivity of *S. typhimurium* to volatile fatty acids depended not only on the *p*H of the medium but also on the chain length and concentration of the acid and the temperature and composition of the medium. Other data (Goepfert, *in preparation*) has indicated that available moisture plays a key role in the sensitivity of *Salmonella* to acetic acid.

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Russian Translation

Selection of a Method of Treatment in Poisoning with Vinegar Essence

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In spite of the fact that the clinical picture and pathogenesis of poisoning with vinegar essence have been studied to a considerable extent (M.R. Byrsan, A.G. Kissin, et al.), the number of fatal cases is still high.

On the basis of our own experience and literature data, we have attempted to determine the effectiveness of various methods of treatment depending upon the severity of poisoning.

Since 1966, a total of 93 patients suffering from vinegar essence poisoning, consisting of 30 men and 63 women, were treated in the intensive care section. The age distribution was as follows: 17 patients were over 20, 50 were 20 to 40 years old, 18 were 40 to 60 years old, 6 were 60 to 80 years old, and 2 were over 80 years old.

The degree of poisoning was determined from the extent of burns and shock due to burns, and the intensity of hemolysis, hemoglobinuria and intoxication. Light poisoning was noted in 33 patients, who exhibited slight burns of mucous membranes in the oral and pharyngeal cavity, accompanied by subfebrile temperature, moderate leucocytosis and accelerated erythrocyte sedimentation reaction, traces of protein in the urine with no symptoms of hemolysis and hemoglo-

binuria. Characteristic features of this type of poisoning were, along with clearly expressed local changes (burns of mucous membranes, esophagus and stomach), a burn shock of the 1st and 2nd degree, a rise in temperature up to 38°C and above, leucocytosis, a hemoglobinemia of up to 1000 mg%, hemoglobunuria, and a proteinuria of up to 10-20%. In severe cases of poisoning the following symptoms were observed: clearly expressed and extensive burns of mucous membranes, a flabby appearance of the face, vomiting with blood expectoration, a burn shock of the 2nd and 3rd degree, a high leucocytosis, hyperthermia, severe hemolysis (hemoglobinemia above 1000 mg%), hemoglobinuria, a proteinuria of up to 100% due mainly to hemoglobinuria, jaundice, liver enlargement, an increase of the potassium and residual nitrogen level, and acidosis in the blood serum. Cases of severe poisoning included 31 patients, and 4 patients were admitted in a terminal state (confused thinking, psychomotor excitation, cyanosis of the skin and mucous membranes, dyspnea, copious foamy sputum, thready pulse, arterial pressure down to 60-50 mm and lower, several drops of black urine in the bladder).

On admission of all patients, attempts were made to perform a gastric lavage with water by means of a gastric probe after administration of atropine and omnopon (pantopon); an urine analysis was performed to determine the degree of hemoglobinuria and severity of poisoning.

In cases of light poisoning the following treatment was used: milk, eggs, sour cream, alkaline inhalations, administration of novocaine and Anesthesin (benzocaine), coating compounds, antibiotics,

intravenous injection of a 5% glucose solution and 500 ml of a 4% sodium bicarbonate solution. All patients with light poisoning recovered. In cases of medium-degree poisoning, up to 100 mg hydrocortisone was also given to prevent collapse and cicatricial stenosis of the esophagus following burns, as well as large doses of vitamins B and C, analgetics, cardiac, antihistaminic and hemostatic drugs.

Special attention was given to alkalization therapy and stimulation of diuresis, in order to stop hemolysis and acidosis and to effect a rapid elimination of erythrocyte decomposition products; for this purpose, the patients were given up to 2 liter of a 4% sodium bicarbonate solution, 2 ml of a 2% papaverine solution 4-5 times per day (24 hrs), 2-3 intravenous injections of 10 ml euphyllin solution, and up to 1 liter of a glucose-novocaine mixture. Out of 25 patients one female patient died, in whom an autopsy revealed a hemorrhage into the cerebellum and under the pia mater as a result of a blunt head injury.

In the case of patients with severe poisoning or in a terminal state, the dosage of corticosteroids was increased, a paranephral novocaine block and a more intensive alkalization therapy (up to 4 liter of a 4% sodium bicarbonate solution) were carried out, and up to 2 liter/day of glucose-novocaine mixture was given. Simultaneously, when diuresis occurred, urea or mannitol was given in a dose of 30-60 g. Blood losses were substituted by intravenous or intra-arterial blood and plasma transfusion. Such a complex therapy was carried out on 26 patients, 14 of whom died; 11 patients died during the first 24 hours after admission, and the cause of their death was shock due to burns, intoxication, bleeding and pulmonary edema. Three patients died within 1 1/2 to 38 days:

two from growing renal and hepatic insufficiency and one from aspiration pneumonia and cachexia following a tracheal-esophageal fistula.

In 9 patients suffering from servere poisoning, a partial blood exchange transfusion of 1200 to 5500 ml was performed after preliminary intravenous injection of 1.5-2 liter of a 4% sodium bicarbonate solution and glucose-novocaine mixture. The blood was taken from the elbow vein and was introduced either into the elbow vein, or into the radial artery in case of hypotension. Out of 9 patients treated in this manner, 6 died, including 3 during the first 12 hours after admission from shock due to burns, intoxication, and pulmonary edema, and 3 died within 2 to 7 days from renal-hepatic insufficiency (2) and profuse hemorrhage from the esophagus (1). In 2 of the 3 surviving patients, a secondary hemolysis set in after partial blood exchange transfusion, and this hemolysis was cut (stopped) by alkalization therapy.

Out of 35 patients with severe poisoning 20 died, 13 of whom were over 40 years old (in 6 of these, the following concomitant diseases were noted: marked atherosclerosis, coronary-cardiosclerosis, nephrosclerosis, bilateral hydronephrosis, and hypertonic diease). Four of the patients who died were admitted in a terminal state, and in 7 patients who died a gastric lavage could not be performed in view of their serious condition, edema of the esophagus, or the categorical refusal to undergo such treatment on the part of the patients themselves.

Conclusions

1. Complex therapy, including urine alkalization and diuresis stimulation, is sufficiently effective to cut (stop) hemolysis, acidosis and hemoglo-

binuria in cases of medium and severe poisoning with vinegar essence.

2. Partial blood exchange transfusion does not improve the prognosis in cases of severe poisoning with vinegar essence.

3. In order to reduce lethality, removal of the poison from the organism should be done as early as possible by means of gastric lavage and intravenous infusion of sodium bicarbonate.

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УДК 615.917:547.292.08

К ВОПРОСУ О ВЫБОРЕ МЕТОДА ЛЕЧЕНИЯ ПРИ ОТРАВЛЕНИИ УКСУСНОЙ ЭССЕНЦИЕЙ

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Тер. Арх. - 43(8): 120-122

Несмотря на то что клиника и патогенез отравления уксусной эссенцией довольно хорошо изучен (М. Р. Бырсан; А. Г. Киссин, и др.), смертность продолжает оставаться высокой.

На основании собственного опыта и литературных данных мы попытались проанализировать эффективность различных методов лечения в зависимости от степени тяжести отравления.

С 1966 г. в отделении интенсивной терапии находились на лечении 93 больных с отравлением уксусной эссенцией: 30 мужчин и 63 женщины. В возрасте от 20 лет было 17 больных, от 20 до 40 лет —50, от 40 до 60 лет—18, от 60 до 80 лет — 6, старше 80 лет — 2.

Степень отравления определяли по выраженности ожогов и ожогового шока, интенсивности гемолиза и гемоглобинурии и интоксикации. Отравление легкой степени было у 33 больных. У них наблюдался небольшой ожог слизистых оболочек полости рта и зева, сопровождающийся субфебрильной температурой, умеренным лейкоцитозом и ускоренной РОЭ, следами белка в моче, без явлений гемолиза и гемоглобинурии. Отравление средней степени констатировано у 25 больных. Оно характеризовалось наряду с выраженными местными изменениями (ожоги слизистых оболочек, пищевода и желудка) ожоговым шоком I—II степени, повышением температуры до 38° и выше, лейкоцитозом, гемоглобинемией до 1000 *мг%*, гемоглобинурией, протеинурией до 10—20‰. При тяжелой степени отравления наблюдались выраженный и распространенный ожог слизистых оболочек, пастозность лица, рвота с кровью, ожоговый шок II—III степени, высокий лейкоцитоз, гипертермия, резкий гемолиз (гемоглобинемия выше 1000 *мг%*), гемоглобинурия, протеинурия до 100‰ за счет главным образом гемоглобинурии, желтуха, увеличение печени, повышение уровня калия и остаточного азота, в сыворотке крови ацидоз. С отравлением тяжелой степени был 31 больной, 4 больных поступили в терминальном состоянии (спутанное сознание, психомоторное возбуждение, цианоз кожи и слизистых оболочек, одышка, обильная пенистая мокрота, нитевидный пульс, артериальное давление до 60—50 *мм* и ниже, в мочевом пузыре несколько капель черной мочи).

Всем больным при поступлении старались промыть желудок через зонд водой после введения атропина и омнопона, исследовали мочу для выяснения степени гемоглобинурии и тяжести отравления.

При отравлении легкой степени проводилось следующее лечение: молоко, яйца, сметана, щелочные ингаляции, внутрь новокаин, анестезин, обволакивающие средства, антибиотики, внутривенно 5% раствор глюкозы и 4% раствор бикарбоната натрия по 500 *мл*. Все больные с легкой степенью отравления выздоровели. При отравлении средней степени дополнительно назначали гидрокортизон до 100 *мг* для борьбы с коллапсом и профилактики рубцовых сужений пищевода после ожогов, витамины группы В и С в больших дозах, анальгетики, сердечные, антигистаминные и гемостатические средства.

Особое внимание уделяли ощелачивающей терапии и стимуляции диуреза для прекращения гемолиза и ацидоза и быстрого выведения продуктов распада эритроцитов: давали 4% раствор бикарбоната натрия до 2 *л*, 2% раствор папаверина по 2 *мл* 4—5 раз в сутки, 2,4% раствор эуфиллина по 10 *мл* 2—3 раза внутривенно, глюкозо-новокаиновую смесь до 1 *л*. Из 25

больных умерла одна, у которой на секции выявлено кровоизлияние в мозжечок и под мягкую мозговую оболочку в результате тупой травмы головы. Таким образом, смерть больной не связана с отравлением уксусной эссенцией.

Больным в тяжелом и терминальном состоянии дозу кортикостероидов увеличивали, проводили паранефральную новокаиновую блокаду и более интенсивную отщелачивающую терапию (4% раствор бикарбоната натрия до 4 *л*), вводили до 2 *л* глюкозо-новокаиновой смеси в сутки. Одновременно при появлении диуреза вводили мочевину или маннитол в дозе 30—60 *г*. Кровопотерю возмещали внутривенным или внутриартериальным переливанием крови и плазмы. Подобная комплексная терапия проведена у 26 больных, из которых умерли 14. Одиннадцать больных умерли в течение первых 24 часов после поступления, причиной смерти их явились ожоговый шок, интоксикация, кровотечение, отек легких. Трое больных умерли в сроки от $1^1/_2$ до 38 суток: 2 — от нарастающей почечно-печеночной недостаточности, 1 — от аспирационной пневмонии и кахексии после трахео-пищеводного свища.

У 9 больных с отравлением тяжелой степени после предварительного внутривенного введения 1,5—2 *л* 4% раствора бикарбоната натрия и глюкозо-новокаиновой смеси производили частичное замещение крови в объеме от 1200 до 5500 *мл*. Кровь выводили из локтевой вены, а вводили либо в локтевую вену, либо в лучевую артерию при гипотензии. Из 9 больных, леченных таким методом, умерли 6, в том числе 3 в течение первых 12 часов после поступления от ожогового шока, интоксикации, отека легких, 3 — в сроки от 2 до 7 суток от почечно-печеночной недостаточности (2) и профузного кровотечения из пищевода (1). У 2 из 3 выживших больных после частичного замещения крови наступил вторичный гемолиз, купированный ощелачивающей терапией.

Всего из 35 больных с отравлением тяжелой степени умерли 20, из которых 13 были старше 40 лет (у 6 из них обнаружены сопутствующие заболевания: выраженный атеросклероз, коронаро-кардиосклероз, нефросклероз, двусторонний гидронефроз, гипертоническая болезнь). Четверо из умерших поступили в терминальном состоянии, у 7 не удалось промыть желудок из-за тяжести состояния, отека пищевода или категорического отказа самих больных.

Выводы

1. Комплексная терапия, включающая алкалинизацию мочи и стимуляцию диуреза, достаточно эффективна для купирования гемолиза, ацидоза и гемоглобинурии при отравлениях уксусной эссенцией средней и тяжелой степени.

2. Частичное обменное переливание крови не улучшает прогноза при тяжелых отравлениях уксусной эссенцией.

3. Для снижения летальности необходимо возможно раннее освобождение организма от яда с помощью промывания желудка и внутривенной инфузии бикарбоната натрия.

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Поступила 21/XII 1970 г.

CHOICE OF THE METHOD OF TREATMENT IN POISONING WITH VINEGAR ESSENCE

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Summary

The authors analysed the efficacy of treatment in poisoning with vinegar essence in 93 patients depending upon the severity of poisoning and the method of treatment. In poisoning of moderate and severe degree complex therapy proved to be sufficiently effective, first of all alkalinization therapy. Of 51 patients there were 15 lethal outcomes. Additional partially substituted blood transfusion did not lessen lethality; of 9 cases 6 patients died. Prognosis in severe poisoning with vinegar essence is worse in elderly persons with concomitant diseases, in untimely removal of poison, in late beginning and insufficient intensity of alkalinization.

Steroid Hormone Formation in the Human Ovary: I. Identification of Steroids Formed *in Vitro* from Acetate-1-^{14}C in the Corpus Luteum

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ABSTRACT. Slices of 6 human corpora lutea of the menstrual cycle and ectopic pregnancy were incubated *in vitro* in the presence of acetate-1-^{14}C. By means of carrier dilution and stringent purification measures, the biosynthesis of 7 radioactive steroids has been demonstrated in the 2 types of corpora lutea. These steroids include progesterone, 20α-hydroxy-Δ^4-pregnen-3-one, 17-hydroxyprogesterone, Δ^4-androstene-3,17-dione, estradiol-17β, estrone and pregnenolone (3β-hydroxy-Δ^5-pregnen-20-one). Quantitative measures of incorporation of radioactivity in these tissues indicate progesterone to be the major steroid formed *in vitro* in the human corpus luteum. (*J Clin Endocr* 24: 597, 1964)

GONADOTROPIN stimulation of progesterone synthesis *in vitro* has recently been demonstrated in the bovine corpus luteum (1-3). The same techniques have been used to explore the relationship of gonadotropins and steroidogenesis in the human corpus luteum, using the increment in formation of radioactive steroids from acetate-1-^{14}C as the measure of gonadotropin action. This report describes the characterization of seven radioactive steroids regularly formed from acetate-1-^{14}C during incubations of corpora lutea of the menstrual cycle and ectopic pregnancy. The accompanying paper (4) deals with the action of various gonadotropins *in vitro* on the formation of these same steroids.

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Materials and Methods

Chemicals. All solvents were commercial reagent grade and were redistilled prior to use. Sodium acetate-1-^{14}C (20 mc/mmole) was purchased from Nuclear Research Chemicals, Orlando, Florida, and was used without purification. The steroids used as carriers were obtained commercially and were found to be chromatographically homogeneous and had appropriate melting points.

Measurement of carrier steroids. Δ^4-3-Ketosteroids were measured in methanol by their absorption at 240 mμ. The estrogens were measured by their absorption in methanol at 281 mμ or, in the case of a small amount, by the modified Kober reaction (5). Δ^4-3-Ketosteroids were located on paper chromatograms using a short wave ultraviolet lamp (Mineralite). The estrogen zones were identified with an automatic recording spectrograph equipped to scan paper chromatograms at 280 mμ. The zone occupied by pregnenolone[4] acetate was visualized by means of an ethanolic sulfuric acid reagent (6). Zones of radioactivity on the paper chromatograms were detected on an automatic recording gas flow chromatogram strip counter.

[4] Abbreviations used are: HCG = human chorionic gonadotropin; pregnenolone = 3β-hydroxy-Δ^5-pregnen-3-one; androstenedione = Δ^4-androstene-3,17-dione; dehydroepiandrosterone = 3β-hydroxy-Δ^5-androsten-17-one; 17-hydroxyprogesterone = 17-hydroxy-Δ^4-pregnene-3,17-dione.

TABLE 1. Radiochemical purification of steroids biosynthesized from acetate-1-^{14}C by human corpora lutea

Compound	Corpus luteum		Total		Crystallization data					
					1st		2nd		3rd	
	No.	Source	Radioactivity, dpm	Steroid carrier, mg	Crystals	Mother liquor	Crystals	Mother liquor	Crystals	Mother liquor
					specific activity (dpm/mg)					
progesterone	H-44	ectopic pregnancy	52,350	10	4,670	6,180	4,540	4,560		
	H-45	ectopic pregnancy	476,000	10	45,100	53,600	43,700	45,300		
	H-46	ectopic pregnancy	84,200	10	7,450	8,000	7,440	7,870		
	H-47	ectopic pregnancy	38,150	11.1	3,530	3,780	3,200	4,750	3,070	3,090
	H-48	menstrual cycle	24,200	10	2,045	2,750	1,990	2,100		
	H-57	menstrual cycle	109,300	15.9	6,230	8,130	5,800	5,870		
20α-hydroxy-Δ^4-pregnen-3-one	H-44	ectopic pregnancy	4,140	13	129	785	125	133		
	H-45	ectopic pregnancy	28,700	5.8	3,760	5,250	3,500	3,690		
	H-48	menstrual cycle	1,170	6.7	115	876	118	441‡		
17-hydroxyprogesterone	H-45	ectopic pregnancy	166,500	10	14,300	19,250	14,870	14,000		
	H-46	ectopic pregnancy	33,400	10	3,140	3,250	3,100	3,200		
	H-47	ectopic pregnancy	50,000	11.1	4,190	4,180				
	H-48	menstrual cycle	6,710	11.2	598	640				
	H-57	menstrual cycle	12,500	16.4	732	1,020	699	665		
Δ^4-androstene-3,17-dione	H-45	ectopic pregnancy	75,000	10	1,480	12,100	836	2,130	842*	792*
	H-46†	ectopic pregnancy	7,610	10	734	824	789	720		
	H-47	ectopic pregnancy	31,400	10	3,040	3,630	2,920*	2,980*		
	H-48†	menstrual cycle	614	5.8	96	254	92	103‡		
	H-57†	menstrual cycle	1,190	25.6	42	206	35	57	37	—
estradiol-17β	H-45	ectopic pregnancy	16,100	10	1,340	1,770	1,360	1,370		
	H-46	ectopic pregnancy	14,400	10.4	1,210	1,500	1,220	1,280		
	H-47	ectopic pregnancy	35,200	10	3,200	3,700				
	H-48	menstrual cycle	990	5.9	107	298	132	127		
	H-57	menstrual cycle	2,860	15.5	128	343	125	146		
estrone	H-45	ectopic pregnancy	8,300	10	700	1,425	714	726		
	H-46	ectopic pregnancy	2,040	10.2	206	199	202	216		
	H-47	ectopic pregnancy	10,700	10	1,060	1,570				
	H-57	menstrual cycle	822	15.5	36	126	38	55‡		

* After further addition of crystalline carrier; values are adjusted for this second dilution.
† Crystallization after additional purification of Zone II by means of acetylation (see text).
‡ High error due to small quantities recovered.

Acetylations. The dry samples were treated with 1 ml of pyridine and 0.5 ml of acetic anhydride for 18 hr at room temperature. Methanol was added and the reagents were evaporated in a stream of nitrogen.

Measurement of radioactivity. Samples containing carbon-14 were measured in a Packard Tri-Carb liquid scintillation spectrometer, Model 314, in duplicate, at 50-57% counting efficiency. All measurements were corrected to 100% counting efficiency and were recorded as disintegrations per minute (dpm).

Tissues. Six human corpora lutea were used for the studies to be described in this communication. Four specimens (H-44, H-45, H-46, H-47) were obtained at laparotomy for ectopic pregnancy; 2 corpora lutea (H-48 and H-57) were removed from patients without obvious endocrine disorders on days 21 and 22 of the menstrual cycle.

Incubations. The corpora lutea were imr

ately chilled, transported to the laboratory and processed in the cold. As previously described (1), corpora lutea were sliced on a Stadie-Riggs hand microtome (approx 0.5 mm thick). The slices were weighed and incubated in 5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing sodium acetate-1-^{14}C and, where used, human chorionic gonadotropin, as given in detail in the tabulated data. Incubation for 3 hr at 37 C in an atmosphere of 95% oxygen and 5% carbon dioxide in a Dubnoff metabolic incubator was terminated by freezing. The material was stored in the frozen state until analyzed.

Extraction and separation of the radioactive steroid products. Medium and slices were homogenized, 1 mg of each of the carrier steroids listed in Table 1 was added, and the mixture was extracted with ether as previously described (1). The residue of the ether extract was partitioned between ligroin (boiling range 65 110 C) and 90% aqueous methanol. The aqueous methanolic phase containing the polar lipid fraction was evaporated and the steroids were extracted from the water phase with benzene. The phenolic steroid fraction was separated from the neutral fraction by shaking the benzene solution with 1N sodium hydroxide according to Engel and associates (7).

The *neutral fraction* was further purified on a silica gel column eluting the steroids with benzene containing increasing proportions of ethyl acetate. The neutral steroids were then separated by paper chromatography in the ligroin/propylene glycol system.

The *phenolic steroid fraction* was separated on paper using the toluene/propylene glycol system. In experiments H-47 and H-48, a separation of neutral and phenolic fractions was not performed prior to chromatography in the ligroin/propylene glycol system. The area of the chromatogram containing material less mobile than 17-hydroxyprogesterone (see Fig. 1) included the 2 estrogen carriers and associated radioactivity. This area was eluted and the phenolic fraction prepared as above, and chromatographed in the toluene/propylene glycol system.

Identification and quantitative measurement of the radioactive steroids. The identification of the radioactive steroids formed from acetate-1-^{14}C is based upon the coincidence of radioactivity with the various individual carrier steroids in the above procedure, followed by crystallization to constant specific activity (8). This latter step was carried out on several samples of the steroids from the various incubations of corpora lutea of ectopic pregnancy and the menstrual cycle (Table 1). Each radioactive steroid was eluted from the paper and measured, then diluted accurately with 5 15 mg additional carrier steroid. Crystallization was performed from appropriate solvents and the specific activity determined on the crystals and mother liquor residues at each step. The latter residues often revealed the presence of trace radioactive contaminants by a high specific activity. Usually 2 crystallizations were sufficient to achieve constant values. At times, first crystals and mother liquor residue had the same specific activity as that calculated from the dilution of the eluted radioactivity, denoting radiochemical homogeneity. In these instances, further crystallizations were not carried out. In the case of some steroids (pregnenolone, estrone, estradiol-17β), further steps for the verification of homogeneity were taken. These are described under Results or given in Table 2.

Results

Qualitative Results

Since the final isolation and purification steps differed from steroid to steroid, details are given for each substance separately. The data of the different crystallizations (specific activity), performed in this study, are compiled in Table 1.

Neutral steroid fraction. Paper chromatography in ligroin/propylene glycol for four to five hours uniformly showed three main radioactive areas, as illustrated in Fig. 1. Zone I proved to be progesterone alone. The other two areas, Zones II and III, contained more than one steroid. Zone II was found to contain androstenedione (IIa) and pregnenolone acetate (IIb) after acetylation. The slowest moving zone (III) was resolved into a 20α-hydroxy-Δ^4-pregnen-3-one (IIIa) and 17-hydroxyprogesterone (IIIb) by chromatography for 35 to 40

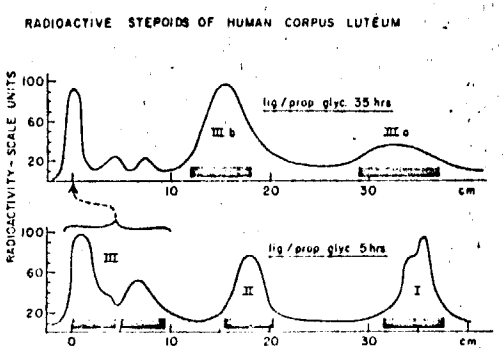

FIG. 1. Distribution of radioactivity on paper chromatograms, from slices of human corpus luteum of ectopic pregnancy (H-47) incubated in presence of 40 μc (2.0 μmoles) of acetate-1-^{14}C. Shaded areas indicate location of carrier steroids: Zone I = progesterone; Zone II = androstenedione and pregnenolone; Zone IIIa = 20α-hydroxy-Δ^4-pregnen-3-one; Zone IIIb = 17-hydroxyprogesterone; radioactive peaks between start line (0 cm) and Zone IIIb are associated with estrone and estradiol-17β. Chromatogram run initially for 5 hr; area III eluted and chromatography repeated for 35 hr.

hours. Two or three minor polar areas of radioactivity were seen but not further investigated.

Progesterone (Zone I). Radioactive progesterone was isolated from the incubations of the six corpora lutea investigated. In most cases, two crystallizations from ligroin were required to achieve constant specific activity.

20α-Hydroxy-Δ^4-pregnen-3-one (Zone IIIa). Radioactivity accompanied the carrier steroid in all six experiments, but was not always apparent as a peak as in Fig. 1. In two corpora lutea of pregnancy and one of the menstrual cycle the final identification by crystallizing to constant specific activity was carried out in acetone and/or methanol. Considerable amounts of radioactive contaminants were removed by this measure in samples from incubations of the two corpora lutea of pregnancy.

17-Hydroxyprogesterone (Zone IIIb). Crystallization from methanol to constant specific activity was carried out in five cases. There appeared to be little extraneous radioactivity associated with this steroid.

Δ^4-Androstenedione (Zone IIa). It was not recognized at first that Zone II contained more than one steroid. Consequently, in our first experiments (H-45 and H-47), Zone II was eluted, diluted with additional carrier androstenedione and crystallized from ethyl acetate or acetone-ligroin. In experiment H-47, constant specific activity was achieved after two crystallizations. In experiment H-45, three crystallization steps were necessary; however, a significant drop in the level of radioactivity occurred, indicating the presence of other radioactive material (Table 1). This latter proved to be pregnenolone. In all subsequent experiments, the components of Zone II were acetylated and rechromatographed. Androstenedione was obtained free of significant radioactive contamination on rechromatography (H-46, H-48, H-57, Table 1).

Pregnenolone (Zone IIb). After acetylating components of Zone II, a faster moving zone was separated from androstenedione by paper chromatography in ligroin/propylene glycol and had the same mobility as pregnenolone acetate. This procedure was carried out in experiments with three corpora lutea of pregnancy and two of the menstrual cycle. The proportion of this second component of Zone II varied considerably and ranged from 13 to 89% of the total radioactivity present in the original Zone II. The rigorous proof of the identity of this second substance as pregnenolone was carried out in experiment H-45. In this experiment, described in greater detail in the accompanying paper (4), several incubations with slices from corpus luteum H-45 were carried out. In the course of the isolation of a

TABLE 2. Demonstration of radiochemical homogeneity of estradiol-17β and estrone biosynthesized from acetate-1-^{14}C by human corpora lutea

	Experiment H-45			Experiment H-57		
	Ectopic pregnancy			Menstrual cycle		
	Radioactivity, dpm	Steroid carrier, mg	Specific activity, dpm/mg	Radioactivity, dpm	Steroid carrier, mg	Specific activity, dpm/mg
			estradiol-17β			
starting material	14,990	9.71	1,544	1,360	10.62	128
after digitonin precipitation	11,670	7.4	1,576	900	7.28	124
after acetylation and thin layer chromatography*	5,060	3.32	1,522	645	4.85	133
			estrone			
starting material	9,430	11.9	790	389	9.46	42
after acetylation and thin layer chromatography:						
estrone acetate*	3,340	4.19	793	265	5.415	49
estrone†	1,243	1.55	802	18	0.388	45

* Calculated as free steroid.
† For explanation see text.

drostenedione from these incubations, several mother liquors from the first crystallization of the latter were obtained and pooled; the total radioactivity was 83,900 dpm. Ten mg of free pregnenolone was added and two crystallizations from aqueous methanol were performed; the crystals had specific activities of 4,240 and 3,750 dpm/mg, respectively. The latter crystals and mother liquor were combined and contained 27,000 dpm. This material was acetylated, chromatographed on paper in ligroin/propylene glycol and the single radioactive zone further purified by chromatography on a silica gel column to give a total of 26,400 dpm. Two crystallizations from methanol resulted in crystals having constant specific activities of 3,345 and 3,410 dpm/mg, respectively (calculated as the free compound).

Due to the late recognition of pregnenolone in the course of this study, only the qualitative demonstration of its presence is presented here.

Phenolic steroid fraction. Paper chromatography of the phenolic extract in toluene/propylene glycol system revealed two distinct radioactive zones coincident with carrier estradiol-17β and estrone in all incubation studies. There were no indications of other radioactive substances in this fraction.

Estradiol-17β. Crystallization of estradiol-17β diluted with additional carrier was carried out in 80% methanol. Constant specific activity was reached in the five experiments where this steroid was examined (Table 1).

Because of the importance of this steroid, further purification steps were carried out on separately pooled samples of radioactive estradiol-17β of constant specific activity from one corpus luteum of ectopic pregnancy (H-45) and from one corpus luteum of the menstrual cycle (H-57). These two samples were treated with digitonin, the insoluble estradiol-digitonide complex was washed twice with aqueous methanol and decomposed according to Baggett and associates (9). The regenerated estradiol-17β was acetylated and chromatographed on thin layer plates of silica gel in cyclohexane: ethyl acetate (100:50) for 40 minutes.

TABLE 3. Quantitative aspects of incorporation of acetate-1-^{14}C into steroids by human corpus luteum slices *in vitro*[1]

Incubation						Results[2]					
Corpus luteum			Additions			Acetate-1-^{14}C incorporation (dpm/g tissue)					
Source	Age, days or wks	Wt, g	Gonadotropin IU	Acetate-1-^{14}C μc	Acetate-1-^{14}C μmole	Progesterone	20α-hydroxypregnen-3-one	17-hydroxyprogesterone	Androstenedione	Estradiol-17β	Estrone
H-45	8 wks[3]	.768	100[4]	50	2.5	1,190,000	37,100	231,500	15,190	31,900	23,900
H-46	8 wks[3]	.778	100[5]	50	2.5	119,000	present[6]	55,900	14,200	40,800	11,050
H-47	8 wks[3]	.446	1[4]	40	2.0	384,000	present[6]	192,000	84,000	116,000	51,600
H-48	21 days	1.115	175[5]	50	2.5	26,100	1,050	8,330	8,610	3,390	trace
H-57	22 days	1.347	350[6]	50	2.5	81,800	present[6]	11,690	716	3,920	2,570

[1] Incubation carried out in 5 ml Krebs-Ringer bicarbonate buffer medium, pH 7.4, for 3 hr in oxygen-carbon dioxide.
[2] Based on recovery of 1 mg of each steroid added as carrier, followed by further addition of carrier and crystallization to constant specific activity; total dpm accumulation in each steroid adjusted to 1 g tissue.
[3] Ectopic pregnancy.
[4] Human chorionic gonadotropin, API, Ayerst Laboratories, New York.
[5] Human chorionic gonadotropin, Schering A.G., Berlin.
[6] Not crystallized to constant specific activity.

As is shown in Table 2, there was no change in the specific activity of the two samples throughout these steps.

Estrone. Crystallization to constant specific activity in aqueous methanol was accomplished in experiments with three corpora lutea of ectopic pregnancy and one of the menstrual cycle (Table 1). In the case of the second corpus luteum of the menstrual cycle (H-48), only traces of radioactivity were associated with the carrier.

Pooled samples of estrone from experiments H-45 and H-57, previously crystallized to constant specific activity, were acetylated and submitted to thin layer chromatography as described for estradiol-17β. Two steroid areas were observed on the plates. The faster moving zone was estrone acetate, as indicated by the two absorption peaks at 275 and 268 mμ in methanol (10). The slower moving zone was free estrone. Its presence is considered to be due to decomposition of the acetate prior to thin layer chromatography. The specific activities of both substances were identical and did not differ from the value of the starting material (Table 2).

Quantitative Results

Individual steroids. The results of carrier dilution and crystallization of the samples of radioactive steroids eluted from the chromatograms show that the isolation procedure affords a considerable degree of purification of the radioactive steroid products. This is borne out by the relatively low accumulation of extraneous radioactivity in respective mother liquors, and particularly by comparison of the values of radioactivity at constant specific activity with the specific activities before crystallization (Table 1, columns 1 and 2) of each sample. The proportion of extraneous radioactivity in the steroid products as eluted from the chromatograms in general consisted of the following: 7 to 14% in the progesterone samples, 0 to 13% in 17-hydroxyprogesterone, 0 to 22% in androstenedione, 9 to 22% in estradiol-17β and 0 to 14% in estrone. Contamination, however, was higher in the minor product, 20α-hydroxy-Δ^4-pregnen-3-one in three samples studied and also in two steroids obtained in experiment H-57, where acetate-1-^{14}C incorporation was low. This evaluation of the extent

purification by the isolation method described is important to the results of the studies with gonadotropins described in the accompanying paper (4).

Over-all biosynthesis. Since the method of carrier dilution used consists of the addition of carrier steroids at the earliest step in the analysis (8, 11), the total radioactivity in each steroid product formed in any single incubation can be calculated from the respective specific activity determined at radiochemical homogeneity. Such calculations have been carried out for the products isolated and purified from each of the incubation studies described; the total radioactivity in each case was further adjusted to 1 g of tissue in order to compare the results of the various incubation studies. These quantitative data are compiled in Table 3. The results show that progesterone is the major product formed in the corpora lutea of the cycle and of ectopic pregnancy. The relative order of magnitude of incorporation of radioactivity into the other steroids in various tissues is very nearly always the same.

Discussion

The results of the present study demonstrate the regular formation *in vitro* of radioactive pregnenolone, progesterone, 20α-hydroxy-Δ^4-pregnen-3-one, 17-hydroxyprogesterone, androstenedione, estradiol-17β and estrone from acetate-1-^{14}C in corpora lutea both of the menstrual cycle and of ectopic pregnancy. Previous studies with a human corpus luteum of the menstrual cycle *in vitro* revealed the formation of radioactive progesterone and 20α-hydroxy-Δ^4-pregnen-20-one from radioactive acetate (12). Other studies with radioactive steroid substrates *in vitro* have shown the over-all biosynthetic process in the human corpus luteum to proceed along the pathway: pregnenolone→progesterone (→20α-hydroxy-Δ^4-pregnen-20-one)→17-hydroxyprogesterone→androstenedione→estradiol-17β and estrone (12–15). Indications of a similar pathway have been reported for the follicle of the human ovary (16). The data of the present study consequently serve to complete the evidence for the formation of all seven steroids in the above biosynthetic scheme by the human corpus luteum. By its formation of many steroids, the human corpus luteum differs from corpora lutea of other species, notably the mare (17, 18), the ewe (19) and the cow (13, and unpublished data from this laboratory), which appear to synthesize only progesterone and one of the epimers of 20-hydroxy-Δ^4-pregnen-3-one.

Studies of steroidogenesis *in vitro* in separate organelles of the ovary afford a means of determining the site of origin of various ovarian hormones. Reservations may be held as to the effectiveness of *in vitro* experiments in reflecting physiological processes, due to the possible exhaustion of substrates, co-factors, energy sources, and a lack of blood supply for removal of *de novo* formed steroids from their site of formation. Nonetheless, the results reported here are in excellent accord with the recent report of the isolation and measurement of progesterone, 20α-hydroxy-Δ^4-pregnen-3-one, 17-hydroxyprogesterone and androstenedione in ovarian venous blood collected from women in the luteal phase of the menstrual cycle (20). The results of the acetate-1-^{14}C incorporation studies agree remarkably well with the relative quantities measured in these isolation studies.

The demonstration of the biosynthesis *in vitro* from acetate-1-^{14}C of progesterone, 17-hydroxyprogesterone, androstenedione and 20α-hydroxy-Δ^4-pregnen-3-one thus establishes the corpus luteum as a site of their formation in the human ovary *in vivo*, already suggested by earlier chemical isolation studies (21, 22).

The incorporation of acetate-1-^{14}C into estradiol-17β and estrone, demonstrating the total biosynthesis *in vitro* of these two estrogens by the human corpus luteum, is of particular physiological significance and confirms earlier indications of their likely formation in this organelle which were based on direct chemical isolations (28). Estrogen synthesis was also indicated by the conversion *in vitro* of radioactive pregnenolone (15), progesterone (15) and androstenedione (12) into radioactive estrogens by various preparations of human corpora lutea. These results suggest that the corpus luteum may be a source of urinary estrogens excreted during the luteal phase of the menstrual cycle and in early pregnancy.

Of the various other steroids investigated in these studies, pregnenolone appears to be a consistent product formed by the human corpus luteum *in vitro*. This steroid has not been found previously by chemical isolation or incubation studies. The demonstration of its biosynthesis from acetate, however, is in accord with its key role in steroidogenesis, recently demonstrated in the human corpus luteum of pregnancy (15). Two other steroidal products, testosterone and dehydro*epi*androsterone, were sought but did not seem to have been formed from acetate-1-^{14}C in these studies. Testosterone normally appears in chromatograms in the area occupied by 17-hydroxyprogesterone (Fig. 1, Zone III) and can be separated from the latter after conversion to testosterone acetate. This manipulation, when carried out on the radioactive material (4800 dpm) in Zone III from experiment II-50, gave rise to a negligible amount of radioactivity associated with testosterone acetate. This confirms the impression of the absence of radioactive testosterone already indicated in all the incubations by the homogeneity of the 17-hydroxyprogesterone samples discussed above (Table 1). No conclusive evidence for the formation of radioactive dehydro*epi*androsterone in these incubations has been obtained; this agrees with the conclusion of Ryan (15) that it is not formed as an intermediate in the biosynthesis of estrogen in the human corpus luteum.

As a final consideration, it is evident (Table 3) that corpora lutea of the menstrual cycle synthesize the same pattern of steroidal products *in vitro* as corpora lutea of ectopic pregnancy in the same relative proportions, with progesterone as the major steroid. The very high formation of radioactive steroids in the corpora lutea of ectopic pregnancy, relative to those of the menstrual cycle, is attributable to the environment from which the tissues were derived and is discussed in greater detail in our subsequent paper (4).

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RELATIONSHIP OF ACIDS TO TASTE AND FLAVOR OF BUTTERMILK

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This paper presents the results of an investigation concerning the relationship of total acidity, pH, lactic and volatile acids to the acid taste and total flavor quality of commercial buttermilk.

The total flavor of fluid cultured products, such as cultured buttermilk, results from the combination of volatile and non-volatile compounds that make up the aroma and taste components of the flavor. Obviously the acids produced by the fermentation process are important to both taste and total flavor, but specific information is lacking as to the role of total acidity and specific individual acids in the development of the optimum flavor character of cultured products.

The lack of understanding of the nature of the acid flavor character of buttermilk is emphasized by the frequent reports in industry that titratable acidity is not a reliable index of the intensity of acid flavor in buttermilk. In earlier work on cottage cheese, the concentration of total lactic acid was found to relate directly to the intensity of acid taste in the cheese.[2]

Experimental

In the first phase of the study, commercial buttermilks were analyzed for total acidity (titratable acidity),[3] and concentration of volatile acids,[1] and the buttermilks were examined for flavor quality. Organoleptic evaluation was made by a panel of three judges to determine independently the intensity of acid taste and the characteristic aroma character of the buttermilk. Thirteen commercial buttermilks were examined and the results are presented in Table 1. The samples are arranged in order of increasing acid flavor intensity.

By separating the major taste and aroma components of flavor, the significance of the findings to these two aspects of flavor could be more accurately assessed. Intensity of acid taste ranges from 0, which is the normal desired acidity to a very high acid taste, or score of 3. Desired aroma ranged from 0 to 7, and overall flavor quality varies from poor to excellent. Intensity of acid taste and characteristic aroma varied independently. Total flavor quality was highest when acid taste values were between 1 to 2 with corresponding aroma intensity greater than 5.

The intensities of acid taste were unrelated to the level of titratable acidity. For example, three samples (Numbers 2, 5, and 10) had acid taste intensities of 0, 1 and 2 with identical titratable acidity values of 0.87%.

There was a general relationship between the intensity of acid taste and formic acid content with increased formic acid contents being associated with increased acid taste intensities, although several inconsistencies in this trend were noted. Formic acid values ranged from 16 to 744 micromoles per hundred grams. The average formic acid content was 50, 240, 280 and 450 micromoles per hundred grams for samples with acid intensities of 0, 1, 2 and 3, respectively.

Acetic acid values ranged from 383 to 701 micromoles per hundred grams. With the exception of sample Number 1, there was a general tendency for acetic acid values to be higher in those samples with higher characteristic aroma, but the acetic acid levels did not correlate to the intensity of acid flavor. The concentration of the volatile acids with carbon chain lengths from 3 to 8 (C3-C8 group) ranged from 0 to 274 micromoles per hundred grams. Concentration of this group of acids were unrelated to acid flavor but the concentration of the C3-C8 acid group was associated with aroma. The samples with aroma scores ranging from 0 to 3 had an average concentration of C3-C8 acids of approximately 22 micromoles per hundred grams, whereas those samples with aroma scores ranging from 5 to 7 contained an average of 179 micromoles per hundred grams of the C3-C8 acid group.

Intensity of Taste

In the next phase of the study, 37 commercial buttermilks were examined for intensity of acid taste, titratable acidity, lactic acid and pH. The data presented in Table 2 is in order of increasing concentrations of lactic acid.

About one-half of the 37 commercial samples were found to exhibit acid taste scores of less than 2, with one-third of these samples not being criticized for acid taste (acid taste of 0). The other half of the samples were considered to have excessive acid taste with acid taste ratings of greater than 2.0. About 25% of this latter group high acid had acid taste scores of >3.0.

There was a statistically significant correlation between the total lactic acid content of the samples and the intensity of acid taste. With lactic acid values of <0.8%, acid flavor criticism was lacking; with lactic acid values of 0.8-0.9% the acid taste intensity generally was between 1 and 2 lactic acid values greater than 0.9 were associated with acid taste intensities greater than 2.0.

There was no definite connection between: (a) titratable acidity and acid taste, (b) titratable acidity and pH, (c) titratable acidity and total lactic acid content, (d) pH and acid taste or (e) pH and titratable acidity. There was a general, but not specific relationship, between pH and total

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lactic acid content. Samples with lactic acid values of less than 0.7 has pH values of pH 4.6 or greater and samples with lactic acid values greater than 1.05 had pH levels of 4.6 or less.

The inconsistencies between lactic acid content and titratable acidity values merits special attention. Generally, the samples grouped into three categories; those with titratable acidity greater than the lactic acid content (samples 1 to 9), those with titratable acidity values essentially the same as the lactic acid content (samples 10 to 21) and those with titratable acidity values less than the lactic acid values (samples 22 to 37).

Significance of Starter

To determine the significance of the starter organism in the variations observed between titratable acidity and lactic acid contents, analyses were made for total lactic acid and titratable acidity and pH in milk cultures of a variety of single strain starter cultures. All cultures were inoculated into the same milk at the rate of 1% and incubated for 16 hours at 21° C. The cultures were also evaluated for acid taste. The ratio of lactic acid to developed titratable acidity (LA/TA ratio) was calculated to determine if differences in relative production titratable acidity and total lactic acid existed between cultures. Five cultures of *S. lactis*, 7 cultures of *S. cremoris* and 4 cultures of *S. diacetilactis* were investigated.

About one-third of the samples were found to be slow acid producers, with 0 acid taste scores, and lactic acid values of less than 0.45%, another third of the samples had acid taste intensities of 2 or greater and lactic acid values >0.84%.

Individual cultures varied in the production of titratable acidity and lactic acid as indicated by the lactic acid/titratable acidity ratios (LA/TA ratios) that varied from 0.53 to 1.13. The variation appeared to be species independent with ratios ranging from .73 to 1.01, 0.53 to 1.13 and 0.56 to 1.00 for *S. lactis*, *S. cremoris* and *S. diacetilactis* respectively. However, the differences in LA/TA ratio were related to individual differences in organisms within each of these species.

The LA/TA ratio was associated with acid taste intensity produced by the culture organism. For cultures with LA/TA ratios between .56 and 1.00, the acid flavor score was less than 2.0, and for those cultures with ratios of 1.0 or above the acid taste score was 2 or greater.

Summary

Acid flavor intensity in cultured buttermilk was correlated to the formic acid and total lactic acid content of the product with no direct relationship being observed between acid flavor intensity and titratable acidity or pH. Aroma intensity was related to the C2 to C8 group of fatty acids and unrelated to lactic acid, formic acid and acetic acid.

The concentration of total lactic acid, titratable acidity and pH in commercial buttermilks and in lactic acid starter cultures varied independently of one another. Wide differences in ratios of lactic acid and titratable acidity (0.53-1.13) were obtained with different single strain cultures of lactic streptococci grown in the same medium under the same conditions.

References

1. Hankinson, C. L., Harper, W. J. and Mikolajcik, E. M. A Gas-Liquid Chromatographic Method for Fatty Acids in Milk and Milk Products. J. Dairy Science, 41:1502. 1958.
2. Harper, W. J. and Randolph, H. E. Lactic Acid in Cottage Cheese. J. Am. Milk Rev., 22(6) 32. 1960.
3. Milk Industry Foundation Laboratory Manual: "Methods of Analysis of Milk and Its Products." pp. 316-318. Third Edition. Milk Industry Foundation. 1959.

Table 1
Relationship of Acid Taste and Characteristic Aroma to Titratable Acidity and Volatile Acids in Commercial Buttermilks

No.	Total Flavor Quality	Acid Taste[1]	Characteristic Aroma[2]				
1	Poor	0	0	.78	16	701	—
2	Poor	0	2	.87	87	418	4
3	Excellent	1	6	.83	85	501	131
4	Excellent	1	7	.90	285	563	122
5	Good	1	5	.87	352	535	94
6	Fair	2	3	.94	49	428	75
7	Poor	2	1	.81	416	383	8
8	Poor	2	2	.86	251	439	99
9	Fair	2	5	.91	276	816	161
10	Excellent	2	7	.87	387	588	176
11	Poor	3	3	.90	285	563	34
12	Fair	3	6	.90	744	551	214
13	Fair	3	5	.83	330	520	274

1. Acid Taste Intensity—0 = no excess acid flavor; 1 = slight/high acid flavor; 2 = high acid flavor; 3 = very high acid flavor; 4 = sour flavor.
2. Characteristic Aroma Intensity — range 0 to 10;. 0 = no aroma and 10 = maximum aroma.
3. Determined by Gas-Chromatographic method of Hankinson, et al. J. Dairy Sci., 41:1502. 1958

Table 2
Relationship of Acid Taste to Lactic Acid, Titratable Acidity and pH of Commercial Buttermilk

Sample No.	Acid Taste Character[1]	Lactic Acid %[2]	Titratable Acid %	pH
1	0	0.58	0.81	4.70
2	0	0.60	0.79	4.65
3	0	0.64	0.88	4.80
4	0	0.68	0.81	4.60
5	0	0.70	0.87	4.55
6	1.5	0.72	0.78	4.65
7	1	0.73	0.83	4.50
8	1	0.74	0.83	4.50
9	0	0.74	0.85	4.50
10	1	0.80	0.80	4.45
11	1	0.80	0.82	4.75
12	1.5	0.81	0.81	4.50
13	1.5	0.82	0.82	4.45
14	1	0.84	0.78	4.50
15	1	0.84	0.82	4.70
16	1	0.86	0.83	4.65
17	1.5	0.86	0.83	4.45
18	2	0.88	0.75	4.70
19	2	0.88	0.98	4.50
20	2	0.88	0.84	4.55
21	2	0.92	0.92	4.40
22	1.5	0.94	0.81	4.70
23	3	0.94	0.77	4.40
24	2	0.96	0.78	4.50
25	2	0.96	0.82	4.55
26	3	1.02	0.82	4.50
27	2	1.02	0.84	4.40
28	3	1.04	0.81	4.55
29	2	1.05	0.82	4.60
30	3	1.05	0.84	4.40
31	3	1.08	0.84	4.35
32	3	1.12	0.81	4.50
33	3	1.16	0.90	4.45
34	3.5	1.18	0.85	4.40
35	4	1.24	0.94	4.35
36	4	1.28	0.86	4.40
37	4	1.40	0.83	4.40

1. Acid Flavor — 0 = no excess acid flavor; + 1 = slight/high acid flavor; + 2 = high acid flavor; + 3 = very high acid flavor; + 4 = sour flavor.
2. Determined by Ling colorimetric method. Am. Milk Rev., 22 (6): 26. 1960.

Table 3
Lactic Acid, Titratable Acidity and pH Relationships of Single Strain Lactic Cultures

Culture No.	Culture Species	Acid Intensity	Titratable Acidity	Lactic Acid	LA/TA Ratio[1]	pH
192	S. lactis	0	.48	.35	.73	5.60
193	"	0	.45	.37	.82	5.65
C10W	"	1	.79	.65	.82	4.45
C9W	"	0	.58	.42	.72	4.90
C2W	"	1	.70	.66	.97	4.50
C6	"	2	.85	.86	1.01	4.80
ML1	S. cremoris	2	.82	.88	1.07	4.70
C1	"	2	.87	.87	1.00	4.55
C13	"	2	.85	.88	1.03	4.50
SC1	"	3	.83	.95	1.13	4.40
PLC1	"	0	.65	.34	0.53	4.65
C3W	"	1	.78	.70	0.89	4.40
DA1	"	1	.91	.71	0.78	4.70
18-16	S. Diacetilactis	0	.46	.26	0.56	5.95
26-2	"	0	.42	.33	0.75	5.75
KDIA	"	1	.66	.63	0.95	4.80
DRC2	"	2	.84	.84	1.00	4.70

1. $\frac{\text{Lactic acid}}{\text{Titratable acidity}}$ = LA/TA Ratio.

ACTION PROGRAMS

(Continued from page 46)

livelier concern and participation by membership and help to attract new members. One example is the new Dairy Merchandising Academy created by MIF-IAICM to help dairy processors better understand and deal with food store operations.

Among other things that need dedicated industry attention are:

1. The life and death issue of economic attrition.

2. A promotional war chest by milk dealers to supplement contributions of producers. Milk dealers have as big a stake in promotion as do the producers and do in fact actively promote their branded products. Somehow we give the impression though of being only interested observers when it comes to non-brand promotion.

3. Greater coordination so that the dairy industry can speak with a more authoritative voice. The Special Dairy Industry Advisory Board is a step in the right direction.

4. Legislation and moral suasion against price wars and unfair trade practices.

5. A confrontation with imitation products.

6. Understanding, complementing and competing with the growing role of supermarkets and private labeling.

Conventions and associations play a major role in disseminating information and educating their membership, and rightly so. But perhaps they do not often enough play the role of sympathetic participation and action.

It is not that leadership and ideas are lacking but there is perhaps a disinclination to rock the boat. A little boat rocking might be in order, however, beginning with some clear statements of 1965-66 goals set by the various associations — what they hope to accomplish, how they hope to do it and what they will need in funds, personnel and support to do this.

Let's get some positive action programs — not simply for the sake of action — but to get the dairy industry moving toward positive and clear-cut goals. •

German Translation

Effects and Fate of Acids in the Organism. I. Determination of the Hydrogen Ion Concentration in Circulating Rabbit Blood After Intravenous Injection of Free Acids

S. Hermann, R. Neiger and M. Zentner (From the Private Research Institute Prague II/1482 (Director: Dr. S. Hermann, Lecturer at the German University in Prague)]

Source: Arch. exptl. Path. Pharmakol. 188:526-32, 1938

In earlier studies (1) we have already established that the hydrogen ion concentration increases after intravenous injection of gluconic acid. From different effects observed after peroral administration of gluconic acid,we could also draw the conclusion that an increase of the hydrogen ion concentration takes place in the organism. Thus, calcification symptoms caused by Vitasterin (irradiated ergosterol-trade name) could be inhibited by Kombuchal (a bacterial yeast preparation containing gluconic acid), which contains 30% free gluconic acid, and even a regression of these symptoms could be achieved (2). A change of the calcium index (3), i.e. the increase of dissociated calcium in serum, can also be attributed to a change in the hydrogen ion concentration. Gluconic acid belongs to the group of difficultly combustible acids, and therefore it is easy to understand that this acid can shift the pH to the acid side in the organism. There are acids, however, which exhibit the opposite behavior, e.g. acetic acid, which is considered for different reasons as an easily "combustible" acid. Findings were noted in our studies, which could not always be brought into agreement

with previous concepts. For this reason, we decided to carry out a systematic study of a larger number of acids. The present study is concerned exclusively with changes in the hydrogen ion concentration of blood after administration of free acids (not salts!). In selecting a method of pH measurement, we decided to use the intravital method of V. Brehmer (4), since we got the impression, from the studies of Bucheler, Zucker and Capaldi (5) and Wunsche (6), as well as from our own comparative measurements, that this method yields very useful results for comparative determinations.

Method

Rabbits with an average weight of 2000 g were put on an oats and water diet prior to the start of tests. In carrying out the test, it is important that the animals do not come into contact with metal parts. When necessary, clamping devices should be wrapped with insulating tape. The rabbit is stretched out on a wooden board, and its right front paw is stuck through a hole in the board and fixed. After removing hair and fat, the paw is placed into a vessel filled with a potassium chloride solution. This vessel is conductively connected by means of a tube filled with KCl agar with the measuring vessel of the ionization gauge (Lautenschlager). The antimony blood vein needle is inserted into the ear helix vein of the rabbit and the circuit is thus closed. The usefulness (fitness) of the specific blood vein needle used is checked on solutions with a known pH and can be already determined from a check of the normal pH value of the rabbit. Occasionally, a sudden drop of the pH may take place; if this drop is due only to clot formation, perfect pH values can be again obtained after removal of the clot. Otherwise, replacement of the blood vein needle is necessary. The

rectal temperature of the rabbit was taken into account in correcting the pH values; those values were considered normal which remained constant for 30 minutes prior to injection. The acid was injected through a tap cannula (syringe) into the right jugular vein; 10 cc of the appropriate acid was injected in the course of 3 minutes, the pH of the circulating blood was then measured during 10 minutes, the acid was injected again, and so on. The results of our measurements after injection of various acids are given further in this article in the form of curves. The time of observation amounted to about 6-8 hours. Although 4-6 animals were used for each acid, for the sake of brevity only one of the curves obtained is given here, for the case in which they virtually coincide.

Results of pH Measurements in Circulating Blood After Intravenous Injection of Free Acids

1. Phosphoric acid

An n/2 phosphoric acid of pH 1.45 was injected; this acid is lipoid (fat)-soluble. The lethal dose was 62.30 cc of n/2 acid or 1 g H_3PO_4/kg body weight (BW). The average pH drop was 0.58. Death of the animals takes place suddenly with the appearance of dyspnea and cramps of extensor muscles. Attempts to save the animals by intravenous injection of sodium bicarbonate immediately after onset of the first poisoning symptoms failed. The effect of the acid on circulating blood in individual phases is illustrated by curve 1 in Figure 1.

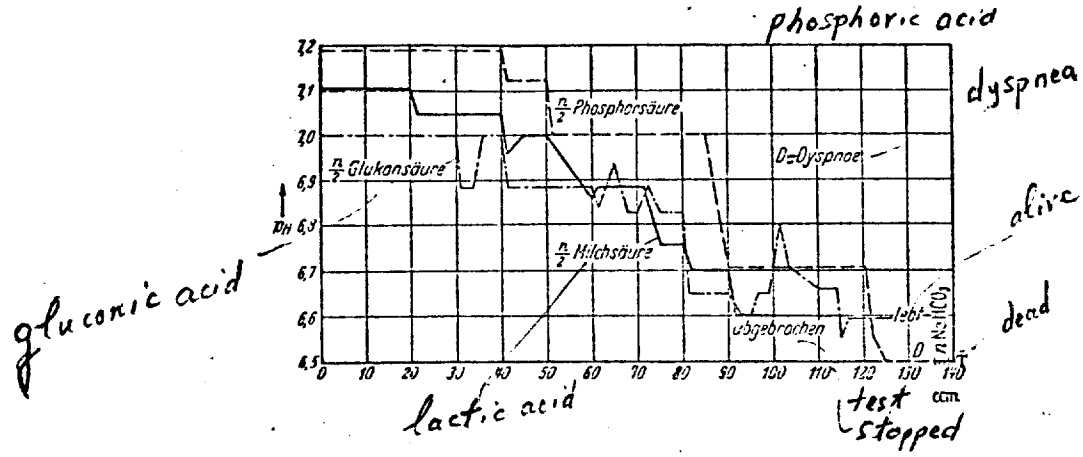

Fig. 1

2. Gluconic acid

An average pH decrease of 0.46 is obtained with n/2 gluconic acid; the pH of this acid is 2.05. Gluconic acid is insoluble in lipids (fats). Very large amounts of gluconic acid are tolerated, when the acid is given both perorally and parenterally. In our tests, a lethal dose could not be reached. The animals received intravenously 6 g or 61 cc n/2 gluconic acid per kg. At this dosage, the onset of dyspnea was noted, however, but it was suppressed by 2-3 injections of 10 cc of a normal sodium bicarbonate solution. The blood pH values also returned to normal. The animals lived for several days after removal of the cannula (Fig. 1, curve 2).

3. Lactic acid

A definite acidification of the blood is noted with n/2 lactic acid, which has a pH of 1.90. Relatively large amounts of this acid are tolerated by rabbits; lactic acid is fat-soluble. The toxic dose is 1.76 g/kg BW, and the lethal dose is 2.01 g or 80 cc of n/2 acid per kg BW (Fig. 1, curve 3).

4. Tartaric acid

An n/2 acid of pH 1.8 was injected. The average pH decrease after

intravenous injection was 0.4. Tartaric acid is fat-soluble. After oral ingestion, it is reported to be noncombustible (7). The lethal dose was 1.1 g or 30 cc n/2 tartaric acid per kg BW. Extension spasms are noted only prior to death (Fig. 2, curve 1).

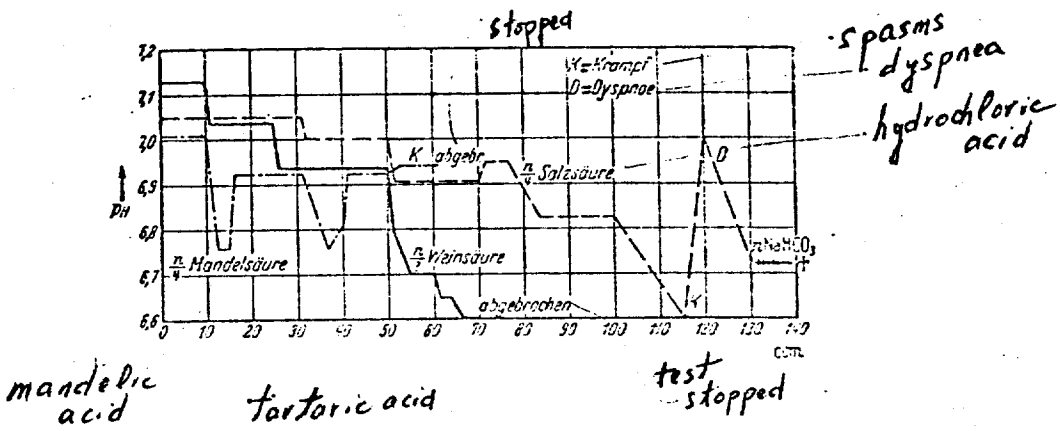

Fig. 2.

5. Hydrochloric acid

An n/4 hydrochloric acid was injected, and its pH was below 1.0. The acid is fat-soluble and noncombustible. The largest amount that can be injected into rabbits until the onset of death is 0.6 or 54.2 cc n/4 HCl per kg BW. The average pH drop is 0.35, and the hydrogen ion concentration is increased accordingly. Death occurs with the same symptoms as noted with phosphoric acid. Injections of sodium bicarbonate to counteract poisoning were unsuccessful (Fig. 2, curve 2).

6. Mandelic acid

n/4 Solutions with a pH of 2.05 were injected. The average pH drop was 0.25. Mandelic acid is fat-soluble and noncombustible in the organism; its strong toxicity must be stressed. The lethal dose was 1.65 g or 15.5 cc of n/4 acid per kg BW; in view of its high toxicity, n/2 solutions of the acid could not be injected. The effect of the acid on the pH is shown in

Fig. 2, curve 3.

7. Acetic acid

A definite alkalization of the blood is noted after intravenous injection of n/4 acetic acid. The blood pH increases on the average by 0.5, and this increase must be attributed to sodium acetate formation. The pH of an n/2 sodium acetate solution is 7.9. Injection of 50 cc of n/4 acetic acid resulted in a sharp drop of the blood pH, and the animals then died quickly with symptoms of dyspnea and spasms. By this time, acetic acid had bound available alkali reserves, so that excess acetic acid very rapidly leads to death. The spontaneously excreted urine contains blood. Measurements of the pH of the urine during the test showed a slight increase from 5.3 to 5.45. Acetic acid is fat-soluble, and the pH of the n/4 acid is 2.70. The lethal dose is 0.5 g or 33.5 cc of n/4 acid per kg BW (Fig. 3, curve 1).

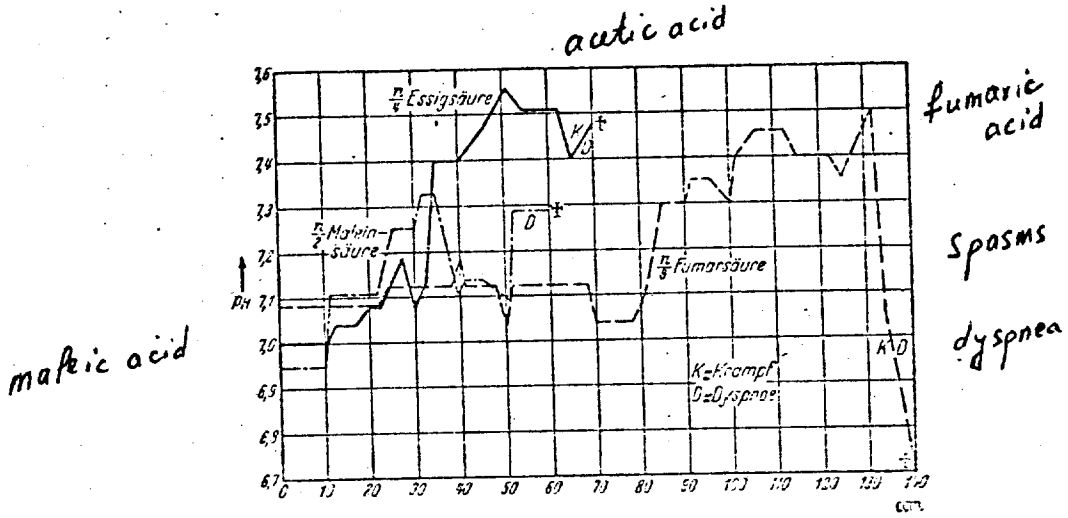

Fig. 3.

8. Maleic acid

Injections were carried out with n/2 solutions of pH 1.50, n/4 acid with a pH of 1.60, and n/8 acid with a pH of 1.70. The n/2 acid is extremely toxic. After injection of the n/2 acid, the pH drops rapidly and soon

increases again slightly; death sets in at this time. After injection of the n/4 acid, the pH rises slightly, then soon goes back to its initial value and again increases strongly. Only shortly before death does the blood pH drop down almost to its normal value, when dyspnea and spasms are noted. Alkalization is quite clearly expressed after injection of the n/8 acid. Maleic acid is fat-soluble. The lethal dose for the n/8 acid is 0.19 g maleic acid or 26 cc per kg BW (Fig. 3, curve 2).

9. Fumaric acid

Fumaric acid is a structural isomer of maleic acid. If we examine curve 3 in Fig. 3 during the course of injections with n/8 fumaric acid, we can notice the difference between the fumaric and maleic acid curves. It is possible to inject 70 cc fumaric acid without noting any change in pH whatsoever. Only later does the pH show a rather steep rise, and drops very rapidly below the norm before death. Fumaric acid is fat-soluble, and the n/8 acid has a pH of 2.25. The lethal dose of n/8 fumaric acid is 0.47 g/kg BW, and thus is more than twice as high as that of maleic acid.

10. Malic acid

In four different tests, curves of varying shape were obtained during injection of n/4 malic acid, which has a pH of 2.20. Two curves showed an increase of the pH, and two others a lowering of the pH. Perhaps this fact is related to the capacity of this acid to undergo a change in the organism. It is also possible that malic acid, as a dibasic acid, can form both acid and neutral salts in the blood, so that the reaction is determined by one or the other type of salt. Malic acid has a low solubility in lipids. The lethal dose of the n/4 acid is 2.40 g or 106 cc per kg BW (Fig. 4, curve 4).

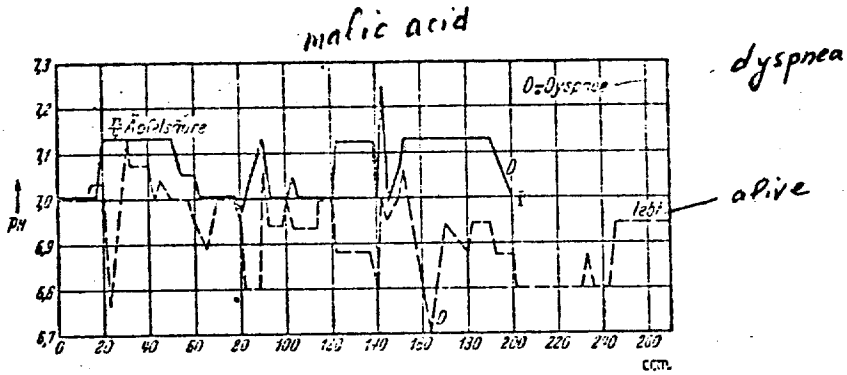

Fig. 4.

11. Succinic acid

The great difference in tolerance, according to whether an n/4 acid or an n/2 acid is injected, must be pointed out. Over 200 cc of the n/4 acid are tolerated, whereas a maximum of 50 cc of the n/2 acid can be injected. The shape of the curves is similar to the one observed with malic acid (the chemical relationship of the two acids is very close), and a zig-zag shape is also noted here. The pH values fluctuate around the initial value. The partial formation of both acid and neutral salts by succinic acid is possible, as in the case of malic acid, so that a clear-cut change in the pH cannot take place. Succinic acid is not soluble in lipids. The tolerated amounts are 0.54 g or 18.1 cc per kg BW for the n/2 acid, and 1.63 g or 88 cc per kg BW for the n/4 acid (Fig. 5, curve 1).

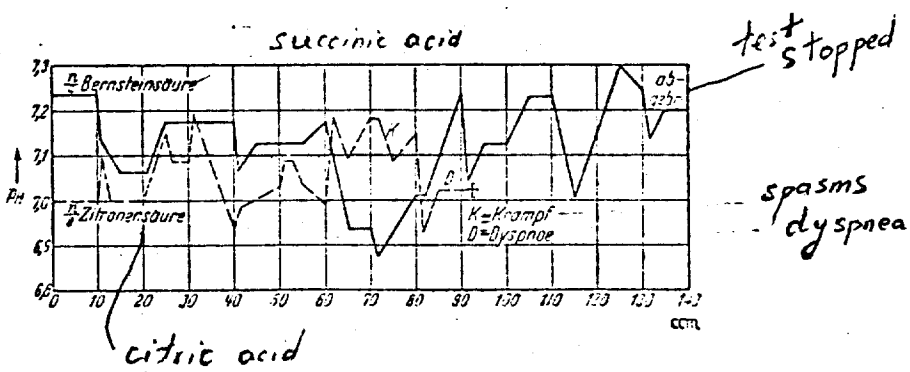

Fig. 5.

12. Citric acid

n/4 and n/8 solutions were injected. The pH of the n/4 acid is 2.15, that of the n/8 acid is 2.3. Citric acid has a low solubility in lipids. According to Langecker (8), it is combustible when given orally. The lethal dose for the n/4 acid is 0.28 g or 17.10 cc per kg BW, and 0.22 g or 39.90 cc per kg BW for the n/8 acid. The tendency to produce a temporary alkalization can be seen from Fig. 5, curve 2. The pH shows a temporary rise, and the hydrogen ion concentration is accordingly lowered.

Summary

1. Changes of the hydrogen ion concentration were determined in circulating venous blood after intravenous injection of various acids.

2. Three basic types of acids can be distinguished: acids which acidify the blood, acids which make the blood alkaline, and finally acids which can turn the blood into an acid and alkaline direction.

3. According to their blood acidifying ability, the acids can be arranged in the following series (in decreasing order): phosphoric > gluconic > lactic > tartaric > hydrochloric > mandelic.

4. According to the ability of the acids to make the blood alkaline, the following series is obtained (in decreasing order): acetic > maleic > fumaric.

5. Malic acid has both acidifying and alkalization properties; succinic acid shows temporary shifts to the acid side, while citric acid shows temporary shifts to the alkaline side.

Aus dem Privat-Forschungsinstitut Prag II/1482.
(Vorstand: Dr. Siegwart Hermann, Privat-Dozent der Deutschen Universität in Prag.)

Arch. exptl. Path. Pharmakol. 188: 526-32 (1938)

Säurewirkungen und Säureschicksal im Organismus.

I. Mitteilung: Bestimmung der Wasserstoffionenkonzentration im strömenden Kaninchenblut nach intravenöser Injektion freier Säuren.

Von

Siegwart Hermann, Richard Neiger und Margot Zentner.

Mit 5 Textabbildungen.

(Eingegangen am 15. Dezember 1937.)

In früheren Untersuchungen[1] haben wir bereits festgestellt, daß sich die Wasserstoffionenkonzentration nach intravenöser Zufuhr von Gluconsäure erhöht. Aus verschiedenen Wirkungen nach peroraler Zufuhr von Gluconsäure konnten wir auch den Schluß ziehen, daß eine Zunahme der Wasserstoffionenkonzentration im Organismus stattfindet. So konnten mit Kombuchal, welches 30% freie Gluconsäure enthält, Verkalkungserscheinungen nach Vitasterin verhindert, ja zum Teil sogar rückgängig gemacht werden[2]. Auch die Veränderung des Calciumquotienten[3], d. h. die Zunahme von dissoziiertem Calcium im Serum kann auf Änderung der Wasserstoffionenkonzentration zurückgeführt werden. Die Gluconsäure gehört zu den schwer verbrennbaren Säuren und es ist infolgedessen leicht einzusehen, daß sie im Organismus das p_H nach der sauren Seite verschieben kann. Es gibt aber auch Säuren, die ein entgegengesetztes Verhalten zeigen, z. B. die Essigsäure, die man aus verschiedenen Gründen zu den leichter „verbrennbaren" Säuren rechnet. Bei unseren Untersuchungen ergaben sich Befunde, die mit den bisherigen Vorstellungen nicht immer in Einklang zu bringen waren. Aus diesem Grunde haben wir uns entschlossen eine größere Zahl von Säuren systematisch zu untersuchen. Die vorliegende Arbeit soll sich ausschließlich mit den Änderungen der Wasserstoffionenkonzentration im Blute nach Zufuhr von freien Säuren (nicht Salzen) befassen. Bei der Auswahl der Methode zur Messung der Wasserstoffionenkonzentration haben wir uns für die intravitale Methode von v. Brehmer[4]

[1] Hermann, Siegwart: Naunyn-Schmiedebergs Arch. **154**. 144—192 (1930). — [2] Hermann, Siegwart: Klin. Wschr. **1929**, Nr. 38, S. 1752—57. — [3] Hermann, Siegwart u. Margot Zentner: Naunyn-Schmiedebergs Arch. **175**. 500 (1934). — [4] v. Brehmer: Sonderdruck aus dem Paracelsus Inst. Schriftenreihe des Vereins Deutsche Volksheilkunde, 2.

entschieden, da wir sowohl aus den Arbeiten von Bücheler, Zucker und Capaldi[5], Wünsche[6] als auch aus eigenen Vergleichsmessungen den Eindruck gewonnen haben, daß diese Methode für vergleichende Bestimmungen sehr brauchbare Resultate liefert.

Methodik.

Kaninchen im Gewichte von durchschnittlich 2000 g wurden vor Versuchsbeginn auf Hafer-Wasserkost gesetzt. Für die Durchführung des Versuchs ist es wichtig, daß die Tiere nicht mit Metallteilen in Berührung kommen. Erforderlichenfalls sind Klemmvorrichtungen mit Isolierband zu umwickeln. Das Kaninchen wird auf einem Holzbrett aufgespannt, die rechte Vorderpfote durch eine Öffnung im Brett durchgesteckt und fixiert. Die von Haaren und Fett befreite Pfote wird in ein Gefäß mit gesättigter Kaliumchloridlösung getaucht. Dieses Gefäß wird durch eine mit Kaliumchloridagar gefüllte Röhre mit dem Meßgefäß des Ionometers (Lautenschläger) leitend in Verbindung gebracht. Die Antimon-Hämovennadel wird in die Ohrrandvene des Kaninchens eingeführt und dadurch der Stromkreis geschlossen. Die Brauchbarkeit der jeweils verwendeten Hämovennadel wird an Lösungen mit bekanntem p_H geprüft und ergibt sich auch schon aus der Prüfung der normalen p_H-Werte des Kaninchens. Bisweilen kommt es zu einem plötzlichen Abfall des p_H. Ist nur eine Thrombenbildung daran schuld, so erhält man nach Entfernung des Thrombus wieder einwandfreie Werte. Anderenfalls ist die Hämovennadel durch eine andere zu ersetzen. Die rektale Temperatur des Kaninchens wurde bei der Korrektur der p_H-Werte berücksichtigt. Als normale p_H-Werte wurden diejenigen angesehen, die vor der Injektion ½ Stunde konstant blieben. Die Injektion der Säure erfolgte durch eine Hahnkanüle in die Vena jugularis dextra. Im Verlauf von 3 Minuten wurden 10 ccm der betreffenden Säure injiziert, hierauf durch 10 Minuten das p_H des strömenden Blutes gemessen, wieder injiziert usw. Im nachstehenden bringen wir die Ergebnisse unserer Messungen nach Injektion verschiedener Säuren an Hand von Kurven. Die Beobachtungsdauer betrug ungefähr 6—8 Stunden. Obwohl für jede Säure 4—6 Tiere verwendet wurden, veröffentlichen wir der Kürze halber nur je eine der erhaltenen Kurven, für den Fall, daß sie im wesentlichen übereinstimmen.

Ergebnisse der p_H-Messungen nach intravenöser Zufuhr freier Säuren im strömenden Blute.

1. Phosphorsäure.

Injiziert wurde n/2 Phosphorsäure von einem p_H 1,45. Sie ist lipoidlöslich. Die tödliche Dosis beträgt 62,30 ccm n/2 Säure bzw. 1 g H_3PO_4 pro kg Kaninchen. Die p_H-Erniedrigung beträgt im Mittel 0,58. Der Tod erfolgt plötzlich unter Auftreten von Dyspnoe und Krämpfen der Streckmuskulatur. Versuche, sofort nach Eintreten der ersten Vergiftungssymptome Tiere durch intravenöse Injektion von Natriumbicarbonat zu retten, schlugen fehl. Der Einfluß der Säure auf das strömende Blut ist in den einzelnen Phasen aus der Kurve 1, Abb. 1 zu ersehen.

[5] Zucker u. Capaldi: Radiologische Rundschau VI, 1937. — [6] Wünsche, Otto: Klin. Wschr. **1936**, Nr. 31, S. 1102.

2. Gluconsäure.

Durch n/2 Gluconsäure wird das p_H im Durchschnitt um 0,46 erniedrigt. Das p_H der n/2 Säure beträgt 2,05. Gluconsäure ist lipoidunlöslich. Von Gluconsäure werden sowohl oral als auch parenteral sehr

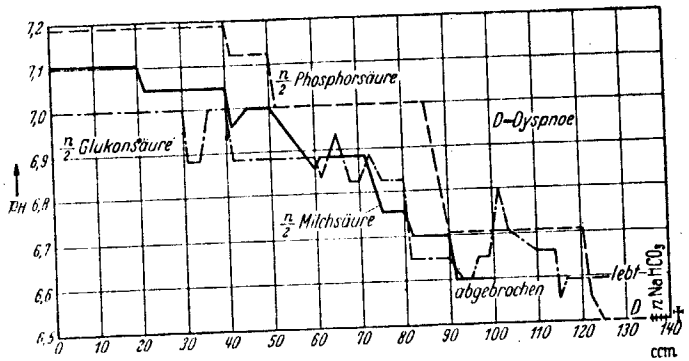

Abb. 1.

große Mengen vertragen. In unseren Versuchen wurde die letale Dosis überhaupt nicht erreicht. Die Tiere erhielten 6 g bzw. 61 ccm n/2 Gluconsäure pro kg intravenös. Bei diesen Mengen trat allerdings bereits Dyspnoe auf, die aber durch Injektion von zwei- bis dreimal 10 ccm einer n Natriumbicarbonatlösung aufgehoben wurde. Auch das Blut-p_H kehrte wieder zur Norm zurück. Die Tiere lebten nach Entfernung der Kanüle noch mehrere Tage (Abb. 1, Kurve 2).

3. Milchsäure.

n/2 Milchsäure, die ein p_H von 1,90 hat, säuert deutlich das Blut. Sie wird in verhältnismäßig großer Menge vom Kaninchen vertragen. Sie ist lipoidlöslich. Die toxische Dosis beträgt 1,76 g pro kg Tier, die letale Dosis 2,01 g bzw. 80 ccm n/2 Säure pro kg (Abb. 1, Kurve 3).

4. Weinsäure.

Injiziert wurde n/2 Säure von p_H 1,8. Die durchschnittliche Erniedrigung des p_H beträgt nach der intravenösen Injektion 0,4. Weinsäure ist lipoidlöslich. Nach oraler Aufnahme soll sie unverbrennbar sein[7]. Die tödliche Dosis betrug 1,1 g bzw. 30 ccm n/2 Weinsäure pro kg Kaninchen. Erst vor dem Tode kommt es zu Streckkrämpfen (Abb. 2, Kurve 1).

5. Salzsäure.

Injiziert wurde n/4 Salzsäure. Ihr p_H ist kleiner als 1,0. Sie ist lipoidlöslich und unverbrennbar. Die größte Menge, die pro kg Kaninchen bis

[7] Underhill, Gross u. Jalesky: J. of Pharm. 43, 359 (1931).

zum Eintritt des Todes injiziert werden kann, beträgt 0,6 g bzw. 54,2 ccm n/4 Salzsäure. Das p_H wird im Durchschnitt um 0,35 gesenkt, die Wasserstoffionenkonzentration dementsprechend erhöht. Der Tod erfolgt unter den gleichen Symptomen wie bei der Phosphorsäure. Injektionen von Natriumbicarbonat als Gegengift sind ohne Erfolg (Abb. 2, Kurve 2).

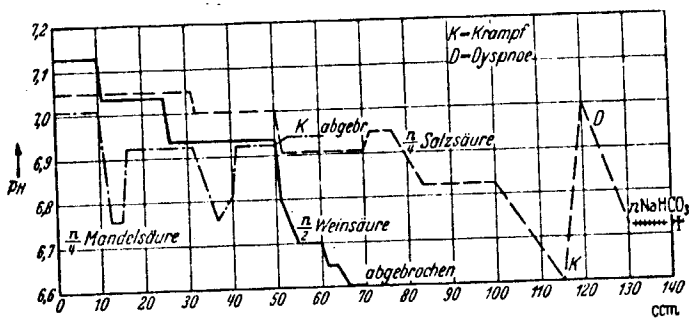

Abb. 2.

6. Mandelsäure.

Injiziert wurden n/4 Lösungen von einem p_H 2,05. Die Durchschnittserniedrigung des p_H betrug 0,25. Mandelsäure ist lipoidlöslich und im Organismus unverbrennbar. Hervorzuheben ist die starke Toxizität. Die tödliche Dosis beträgt 1,65 g bzw. 15,5 ccm einer n/4 Lösung pro kg Kaninchen. n/2 Lösungen konnten wegen der großen Giftigkeit überhaupt nicht mehr injiziert werden. Der Verlauf der p_H-Beeinflussung ist aus der Abb. 2, Kurve 3 zu ersehen.

7. Essigsäure.

Nach intravenöser Injektion von n/4 Essigsäure sieht man eine deutliche Alkalisierung des Blutes. Das Blut-p_H wird durchschnittlich um 0,5 erhöht. Diese Erhöhung ist auf Natriumacetatbildung zurückzuführen. Das p_H einer n/2 Natriumacetatlösung beträgt 7,9. Nach Injektion des 50. ccm von n/4 Essigsäure erfolgte ein steiler Abfall des Blut-p_H. Die Tiere gingen dann unter Auftreten von Dyspnoe und Krämpfen rasch zu Grunde. Die Essigsäure hat in diesem Zeitpunkt die verfügbare Alkalireserve abgebunden, so daß ein Überschuß der freien Essigsäure sehr rasch zum Tode führt. Der spontan entleerte Harn ist blutig. Die Messungen des Harn-p_H während des Versuchs ergaben einen leichten Anstieg von 5,3 auf 5,45. Die Essigsäure ist lipoidlöslich, das p_H der n/4 Säure beträgt 2,70. Die tödliche Dosis ist 0,5 g CH_3COOH bzw. 33,5 ccm einer n/4 Lösung pro kg Tier (Abb. 3, Kurve 1).

8. Maleinsäure.

Injiziert wurden n/2 Lösungen mit einem p_H 1,50; n/4 Säure mit einem p_H 1,60 und n/8 Säure mit einem p_H 1,70. Die n/2 Säure ist außer-

ordentlich giftig. Nach der Injektion einer n/2 Säure fällt das p_H rasch ab, um alsbald ein wenig wieder anzusteigen. In diesem Zeitpunkt tritt der Tod ein. Nach Injektion der n/4 Säure steigt das p_H ziemlich an, kehrt alsbald zum Ausgangspunkt zurück um wieder stark anzusteigen. Erst kurz vor dem Tode erfolgt unter Auftreten von Dyspnoe und Krämpfen ein Abfall des Blut-p_H bis fast zur Norm. Nach der Injektion der n/8 Säure ist die Alkalisierung ganz deutlich ausgeprägt. Maleinsäure ist lipoidlöslich. Die letale Dosis beträgt für die n/8 Lösung 0,19 g Maleinsäure bzw. 26 ccm pro kg Tier (Abb. 3, Kurve 2).

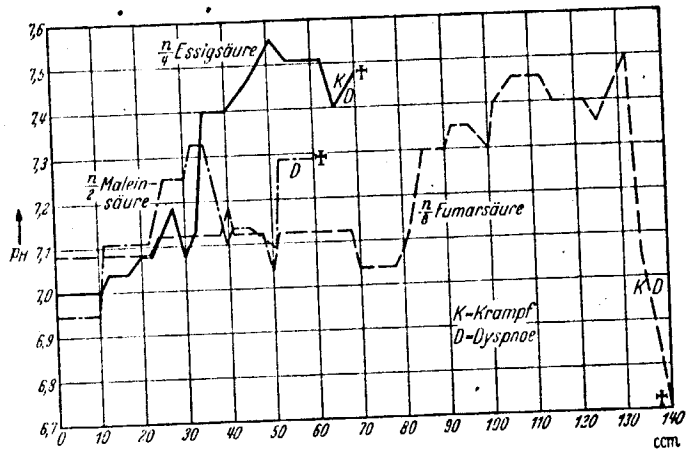

Abb. 3.

9. Fumarsäure.

Die Fumarsäure ist mit der Maleinsäure strukturisomer. Betrachtet man die Kurve 3 der Abb. 3 im Verlauf der Injektionen mit einer n/8 Säure, so fällt der Unterschied gegenüber der Maleinsäurekurve auf. Es können 70 ccm Fumarsäure injiziert werden, ohne daß überhaupt eine p_H-Änderung erfolgt. Erst später steigt das p_H ziemlich steil an, um vor dem Tode sehr rasch unter die Norm abzusinken. n/8 Fumarsäure hat ein p_H von 2,25. Sie ist lipoidlöslich. Die letale Dosis der n/8 Fumarsäure beträgt 0,47 g pro kg Tier, also mehr als das Doppelte der Maleinsäure.

10. Äpfelsäure.

n/4 Äpfelsäure, die ein p_H von 2,20 hat, zeigt in vier verschiedenen Versuchen während der Injektion einen wechselnden Verlauf der Kurven. Zwei Versuche zeigten eine Erhöhung, zwei eine Erniedrigung des p_H. Vielleicht hängt dies mit der Umwandlungsfähigkeit dieser Säure im Organismus zusammen. Es ist auch möglich, daß die Äpfelsäure als zweibasische Säure sowohl saure, als auch neutrale Salze im Blute bildet, so daß

die einen oder die anderen die Reaktion bestimmen. Äpfelsäure ist wenig lipoidlöslich. Die letale Dosis einer n/4 Säure beträgt 2,40 g bzw. 106 ccm pro kg Tier (Abb. 4).

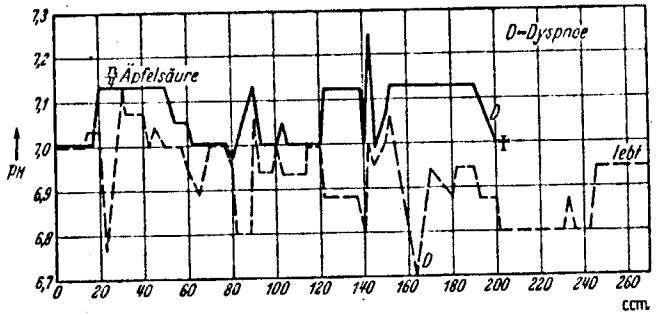

Abb. 4.

11. Bernsteinsäure.

Hervorzuheben ist der große Unterschied in der Verträglichkeit je nachdem ob n/4 oder n/2 Säure injiziert wurde. Von der n/4 Säure wurden über 200 ccm vertragen, während von der n/2 maximal 50 ccm injiziert werden konnten. Der Verlauf der Kurven erinnert insofern an den der Äpfelsäure (die chemische Verwandtschaft ist eine sehr nahe) als auch hier ein Zick-Zack-Verlauf vorliegt. Die p_H-Werte schwanken um den Anfangswert. Bei der Bernsteinsäure dürften sich vielleicht ebenso wie bei der Äpfelsäure teils das saure, teils das neutrale Salz bilden, so daß es zu keiner eindeutigen p_H-Änderung kommen kann. Die Bernsteinsäure ist lipoidunlöslich. Von der n/2 Säure wurden 0,54 g bzw. 18,1 ccm pro kg, von der n/4 1,63 g bzw. 88,0 ccm pro kg Tier vertragen (Abb. 5, Kurve 1).

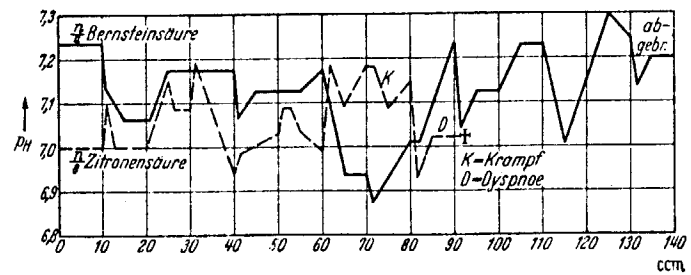

Abb. 5.

12. Citronensäure.

Es wurden n/4 und n/8 Lösungen injiziert. Das p_H der n/4 Säure beträgt 2,15, das der n/8 Säure 2,3. Sie ist wenig lipoidlöslich. Bei oraler

Zufuhr ist sie nach Langecker[8] verbrennbar. Die letale Dosis beträgt für die n/4 Säure 0,28 g bzw. 17,10 ccm pro kg, für die n/8 Säure 0,22 g bzw. 32,90 ccm pro kg Tier. Aus Abb. 5, Kurve 2 ersieht man die Tendenz vorübergehend zu alkalisieren. Das p_H wird vorübergehend erhöht, die Wasserstoffionenkonzentration dementsprechend erniedrigt.

Zusammenfassung.

1. Nach intravenöser Injektion verschiedener Säuren wurde die Änderung der Wasserstoffionenkonzentration im strömenden venösen Blute bestimmt.

2. Man kann drei Grundtypen von Säuren unterscheiden: Solche die das Blut säuern, solche die es alkalisieren und schließlich solche die Ausschläge nach der sauren und alkalischen Richtung geben.

3. Nach ihrem Blutsäuerungsvermögen ergibt sich folgende Reihe: Phosphorsäure > Gluconsäure > Milchsäure > Weinsäure > Salzsäure > Mandelsäure.

4. Nach dem Blutalkalisierungsvermögen geordnet ergibt sich folgende Reihe: Essigsäure > Maleinsäure > Fumarsäure.

5. Sowohl Säuerung als auch Alkalisierung zeigt Äpfelsäure; Bernsteinsäure zeigt nach der sauren, Citronensäure nach der alkalischen Seite vorübergehende Ausschläge.

[8] Langecker, Hedwig: Naunyn-Schmiedebergs Arch. **171**, 744 (1933).

THE IDENTIFICATION OF SOME VOLATILE CONSTITUENTS OF CONCORD GRAPE JUICE[a]

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In the classical procedure for the identification of the volatile constituents of fruits, a volatile oil is prepared from 100 to 1000 kg. or more of fruit. The volatile oil is fractionated by distillation or by chemical or chromatographic procedures, and the fractions are identified *(2, 3, 4, 5, 7, 8, 15, 16, 20)*. In the present article, a procedure is described which should prove useful for the identification of volatile constituents of fruits starting with as little as 10 kg. of material. In this new procedure a volatile oil is not isolated, but instead the volatile constituents are identified in aqueous solution, usually by preparation of water-insoluble derivatives which are identified chromatographically.

Two other features of this new procedure are noteworthy. A total carbon determination has been introduced in order to obtain a material balance and thus minimize the possibility of overlooking a major volatile constituent. Techniques have been devised for the identification of esters in an aqueous solution containing a large amount of free alcohols. Ordinarily in the identification of an ester, the ester is saponified and the acid and alcohol components are identified. This general approach is satisfactory for the identification of the acid components of esters in the presence of alcohols. However, free alcohols must be removed before saponification of esters if the alcohols formed by the saponification are to be detected. This has been accomplished by using platinum black-catalyzed oxidation to remove the free alcohols *(11)*. Once the free alcohols are removed, the esters are saponified and the alcohol components of the esters are determined in the aqueous solution.

The procedure is described below as it has been used in the identification of the volatile constituents of Concord grape juice (*Vitis labruscana* Bailey). Less than 10 liters of grape juice sufficed for the complete analysis, with all determinations run in duplicate.

EXPERIMENTAL

High quality commercial (1953) Concord grape juice produced by pressing after the grapes were heated to 150°F. (66°C.) was subjected to flash vacuum distillation followed by fractionation of the distillate *in vacuo* (15 in. vacuum), in an apparatus similar to that described by Milleville and Eskew *(12)*. The essence,[b] 1.2% of the original volume of the juice, had an odor very close to, but stronger than, that of the original juice. The essence was subjected to the various analytical procedures described below. It is anticipated that the procedure would be the same with other fruits, after

[a] Journal Paper No. 982, New York State Agricultural Experiment Station, Geneva, N. Y.

[b] We are greatly indebted to the Welch Grape Juice Company, Westfield, N. Y. for the gift of this essence.

preparation of a similar essence, using techniques designed to recover the natural aroma of the fruit.

Identification of alcohols. The alcohols in the essence were identified as 3,5-dinitrobenzoates, according to the method for the identification of alcohols in dilute aqueous solution *(6)*. Because of the high concentration of alcohols in the essence, 1.0 ml. of essence was used in each analysis. The results were made quantitative by comparison with the results obtained from solutions of known mixtures of ethanol and methanol in water.

Identification of esters. *Removal of Free Alcohols.* In a 3-liter glass-stoppered flask was placed 20 ml. of essence and 200 mg. of platinum black *(21)*, and the mixture was shaken mechanically at room temperature for 72 hours. (The large flask was required in order to furnish sufficient oxygen in the air space to complete the oxidation of the alcohols. The flask must be stoppered to prevent losses of esters to the atmosphere during the long oxidation process.) An additional 50 mg. of catalyst was added after 2 hours, and 75 mg. after 24 hours. Aliquots (1 ml.) were removed at 24, 48, and 72 hours, and were titrated with 0.1 *N* sodium hydroxide. (Primary alcohols are oxidized to acids.) The titration at 72 hours was the same as that at 48 hours, indicating that the oxidation was complete in 48 hours.

Identification of Alcohol Components of Esters. The catalyst was removed by filtration, and half of the solution was treated with 3,5-dinitrobenzoyl chloride according to the usual procedure for the determination of alcohols in aqueous solution *(6)*. Chromatography of the 3,5-dinitrobenzoates showed that only traces of ethanol and methanol remained in the solution after oxidation. The other half of the solution was mixed with 1.4 ml. of 5 *N* sodium hydroxide and was heated in a stoppered flask (stoppered to avoid loss of alcohols during the heating period) for 2 hours in a boiling water bath. Excess sodium hydroxide was neutralized with 1 *N* hydrochloric acid, and the solution was treated with 3,5-dinitrobenzoyl chloride. Chromatography of the 3,5-dinitrobenzoates showed that after saponification the solution contained considerable ethanol and some methanol, in the ratio of approximately 20:1. (Controls indicated that during the oxidation procedure there was not a significant amount of esterification or transesterification.)

Identification of Acid Components of Esters. A mixture of 5 ml. of essence and 8.0 ml. of 0.120 *N* sodium hydroxide was refluxed 2 hours. The solution was titrated, to a weak phenolphthalein end-point, with 0.75 ml. of 1.03 *N* hydrochloric acid. The solution was evaporated *in vacuo* to approximately 1 ml., acidified with 0.05 ml. of 1:1 sulfuric acid:water and diluted with water to 2.5 ml. A mixture of 1.7 ml. of this solution, 3.0 g. of silicic acid *(22)*, and 20 ml. of chloroform was made into a slurry, placed at the top of a silicic acid column and the material was chromatographed *(11, 17)*. The only acid found was acetic acid, and within experimental error the amount of acetic acid found accounted for all the ester in the essence, as determined by the saponification equivalent.

Since acetic acid was the only acid component of the esters in the essence, the ethanol and methanol, found as the alcohol components of the esters, must have been present as ethyl acetate and methyl acetate respectively.

Identification of carbonyl compounds. The carbonyl compounds were precipitated from the essence as 2,4-dinitrophenylhydrazones, and the 2,4-dinitrophenylhydrazones were determined chromatographically by the method of White *(19)*. In each experiment, 5.0 ml. of essence was mixed with 5 ml. of an 0.4% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. The solution was allowed to stand overnight in the refrigerator. The mixed 2,4-dinitrophenylhydrazones, weight 5 mg., were chromatographed on bentonite-celite *(19)*. For comparison, mixtures of acetaldehyde and acetone 2,4-dinitrophenylhydrazones were also chromatographed.

Methyl anthranilate. Methyl anthranilate was determined by the standard method *(1)*. Its concentration in the essence was too low for the anthranilic acid to be detected as an acid component of the esters. [To establish the identification of the compound, a derivative, 1-(2′-carbomethoxyphenylazo)-2-naphthol, was prepared from methyl anthranilate isolated from Concord grape juice, and was found to have the same melting point and mixed melting point as that of authentic derivative prepared from methyl anthranilate.]

Identification of acids. The essence did not contain a significant amount of acid. However, the original flash distillate (not fractionated) did contain acid. This acid was isolated as the sodium salt and chromatographed on silicic acid *(11, 17, 23)*. The only acid found was acetic acid. Its absence from the essence is understandable since acetic acid does not form an azeotropic mixture with water and would have been removed from the essence as high boiling material during fractional distillation.

Analysis for total carbon. Aliquots of approximately 0.10 and 0.20 ml. of essence were subjected to the Van Slyke-Folch *(18)* wet combustion procedure. The carbon dioxide was absorbed in dilute sodium hydroxide in a special absorption tube, and was determined gravimetrically as barium carbonate.

Specific gravity. The specific gravity of the essence was 0.9929 at 20°C. (68°F.). This is the specific gravity of an aqueous ethanol solution containing 39 mg./ml.

Ultraviolet absorption spectrum. The ultraviolet absorption spectrum of the essence is shown in Figure 1 (1 cm. cell).

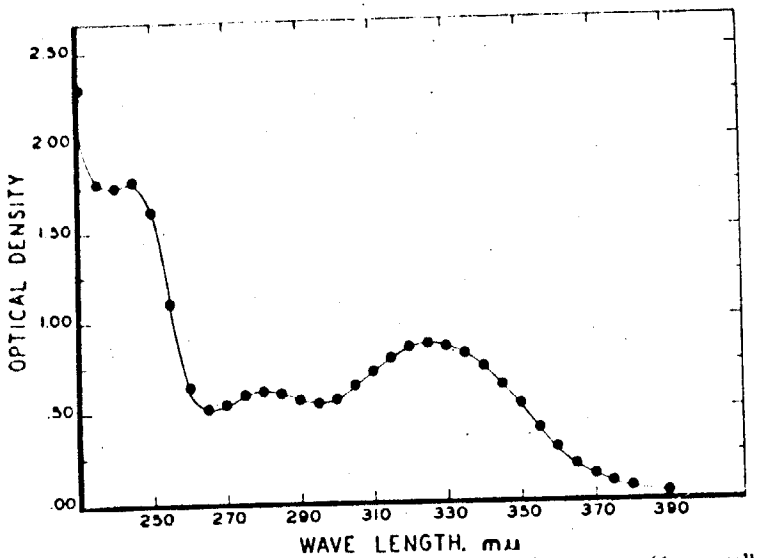

Figure 1. Ultraviolet absorption of Concord grape juice essence (1 cm. cell).

Synthetic essence. A synthetic essence was prepared with the composition shown in Table 1. Its specific gravity was 0.9932 at 20°C. (68°F.). The ultraviolet absorption spectrum was the same as that of the essence except that the weak maximum at 280 mμ was missing in the synthetic essence, and was replaced by a minimum at 270 mμ, optical density 0.14. The odor of the synthetic essence is discussed below.

RESULTS AND DISCUSSION

The results are summarized in Table 1.[c] As shown in this table the constituents which have been identified in Concord grape juice essence account for the total carbon content of the essence within experimental error. This indicates that no constituent present in sizeable amount has been overlooked. However, it would be entirely possible for the volatile constituents present in the greatest amount, and hence accounting for the

[c] The same volatile organic constituents were found in an essence prepared in the laboratory from 1950 Concord grape juice which had been prepared in a small tomato juice processing line *(13)* under conditions designed to minimize the possibility of chemical or microbiological changes during preparation.

major fraction of the carbon, to fail to account for the odor. This is true in the present instance since the odor of a synthetic essence prepared with the composition shown in Table 1 only slightly resembles the odor of the natural Concord grape juice essence. The identification of the volatile constituents is therefore incomplete.

It might be mentioned, however, that rarely has it been reported that the odor of a fruit is accounted for by the compounds which have been identified among its volatile constituents. In most instances no mention has been made of the odor of a mixture of the identified compounds, and it is likely that most identifications have been incomplete in this sense. In the present instance, it is known that the missing volatile constituents are present in the essence only in traces. Some information about this material has been obtained.

TABLE 1

Volatile constituents of Concord grape juice essence

Compound	Concentration, mg./ml.	Calculated millimoles carbon/ml.
Ethanol	35	1.52
Methanol	1.5	.05
Ethyl acetate	3.5	.16
Methyl acetate	0.15	.01
Acetone	0.3	.02
Acetaldehyde	0.03	....
Methyl anthranilate	0.033	....
Acetic acid	....*	....
Total		1.76[1]

[1] Total carbon found by combustion: 1.8₁ millimoles/ml.

* Acetic acid was absent from the essence but is present to the extent of approximately 0.03 mg./ml. in the vacuum distillate from grape juice.

If the Concord grape juice essence or the flash vacuum distillate from Concord grape juice is extracted with chloroform, and if the chloroform solution—after it has been washed with acid to remove methyl anthranilate—is evaporated, a small amount of oil is obtained. This oil is obtained to the extent of approximately 0.02 mg./ml., from the essence, and 0.004 mg./ml. from the vacuum distillate. If this oil is added at 0.02 mg./ml. to the synthetic essence, the odor of the mixture closely approximates the odor of the natural Concord grape juice essence. The composition of this oil is under investigation.

Data in Table 1 also lead to conclusions of interest in quality control work concerned with Concord grape juice. Approximately 90% of the volatile organic material in Concord grape juice is ethanol. Therefore, procedures for the quantitative determination of volatile matter by means of dichromate or permanganate oxidation *(9, 10)* if applied to Concord grape juice do little more than determine the amount of ethanol in the juice. Similarly, ethyl acetate and methyl acetate account for well over 95% of the volatile esters, and quantitative determinations of volatile esters in Concord grape juice do little more than determine ethyl and methyl acetate. In spite of their limitations, however, these procedures,

which determine the low-boiling volatile constituents, combined with the determination of a high boiling constituent such as methyl anthranilate probably furnish the best quality control procedure for volatile constituents that can be devised at present.

SUMMARY

A procedure is described for the identification of volatile constituents of fruits starting with as little as 10 kg. of material. Using this procedure, ethanol, methanol, ethyl acetate, methyl acetate, acetone, acetaldehyde, methyl anthranilate, and acetic acid have been identified as volatile constituents of Concord grape juice (*Vitis labruscana* Bailey). In addition to these compounds, the presence of at least one unidentified, chloroform-extractable constituent has been established. Conclusions of interest in quality control work are mentioned.

Acknowledgment

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Lipogenesis from glucose-2-^{14}C and acetate-1-^{14}C in aorta

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ABSTRACT Lipogenesis was measured with glucose-2-^{14}C and acetate-1-^{14}C in the everted aortas of normal and atherosclerotic rabbits. More glucose-2-^{14}C than acetate-1-^{14}C was incorporated into lipids in both the normal and the atherosclerotic aorta.

Radiocarbon from glucose-2-^{14}C appeared mainly in triglycerides and phospholipids with a small amount in cholesteryl esters. Incorporation increased almost threefold with atherosclerosis, most of the radioactivity being in the glycerol moiety; radioactivity was predominantly in carbon 2 of glycerol.

About 70% of the acetate-1-^{14}C incorporated into phospholipids and triglycerides was in the fatty acids, and the remainder was in glyceride-glycerol; 98% of the radioactivity in cholesteryl esters was in the fatty acid moiety. Incorporation into cholesteryl esters was increased most during the development of atherosclerosis.

Fatty acid synthesis was similar from both acetate-1-^{14}C and the 2 carbon unit derived from glucose-2-^{14}C, viz., predominantly de novo synthesis of fatty acids with 14 and 16 carbon atoms, and elongation for those of 18 carbons and longer.

SUPPLEMENTARY KEY WORDS aortic lipogenesis · lipid degradation

LIPOGENESIS[1] has been studied with various radioactive substrates in normal and atherosclerotic aortas. Several investigators have used glucose-^{14}C to study lipogenesis in intact aortic tissue preparations of various species (1–7). Glucose-^{14}C incorporation into fatty acids (1), phospholipids (4), and total lipids (2, 3, 5, 7) has been measured, and the total lipids have been separated into the individual lipid classes (5). The high degree of incorporation of ^{14}C from labeled glucose into the glycerol fraction of total glycerides compared with the low amount of label in fatty acids has also been noted (1, 4, 6).

In this paper I report studies on lipogenesis in normal and atherosclerotic rabbit aorta with glucose-2-^{14}C and with acetate-1-^{14}C. Data on the incorporation of these two substrates into total lipids, into different lipid classes, and particularly into the glycerol vs. fatty acid moieties of the lipids are compared. Since acetate-1-^{14}C arises from glucose-2-^{14}C the mode of synthesis of the fatty acids can be assessed and compared with results obtained when acetate-1-^{14}C is used as substrate.

MATERIALS AND METHODS

Animals, Diets, and Aorta Preparation

Female New Zealand rabbits were maintained 3–4 months on Purina rabbit chow pellets. The control group received pellets coated with corn oil (Mazola); this diet contained 1.4 g of oil/kg. The atherogenic group received pellets coated with corn oil (1.4 g/kg of diet) containing cholesterol (8) (U.S.P., Nutritional Biochemicals Co.) at a level of 0.8 g/kg of diet.

The rabbits were killed by cervical dislocation; the aorta from the descending arch to the diaphragm was removed and flushed with 0.9% saline solution, and the adherent material was stripped away. The

[1] "Lipogenesis" is defined here as incorporation of radioactive substrate into any portion of the total lipids, viz., glycerol, fatty acids, cholesterol, choline, etc., by any enzymic mechanism. "Fatty acid synthesis" is used to indicate radioactive substrate incorporation only into fatty acids, either by elongation or by de novo synthesis.

Abbreviations: TLC, thin-layer chromatography; PL, phospholipid; TG, triglyceride; CE, cholesteryl ester.

aorta was everted over a glass rod and tied, and the ends were wrapped with Parafilm to exclude access of the medium to the adventitial portion. The aorta was placed in 2.5 ml of Krebs-Ringer salts medium with either 2.8 mM glucose-2-^{14}C (5.4 μCi/μmole) or 2.8 mM acetate-1-^{14}C (10.4 μCi/μmole) (New England Nuclear, Inc., Boston). During the incubation at 39°C for 3 hr, oxygen–carbon dioxide (95%:5%) was bubbled from the bottom of the tube; this ensured adequate movement of the medium over the entire surface.

Analyses

After incubation, the aorta was cut from the glass rod and inspected for the extent of atherosclerosis. The control groups had intimal surfaces that were smooth and lesion free; the rabbits maintained on cholesterol had raised, white lesions which covered 70–95% of the intimal surface. The weights of the intima and most of the media (stripped away from the adventitia) averaged 168 mg and 542 mg for control and atherosclerotic aortas, respectively. The intima plus media was weighed and homogenized in $CHCl_3$–CH_3OH 2:1, and the lipid was extracted (9). The lipid extract was passed through a Sephadex G-25 column (10) to further reduce the amount of nonlipid material. Lipid was separated into classes on TLC according to the method of Skipski et al. (11) with heptane–ethyl ether–acetic acid–methanol 85:15:3.5:2.5. Areas that contained lipids were visualized after the plates were sprayed with 2,6-dichlorofluorescein; the silica gel was scraped into pipets, the lipid was eluted, and aliquots of the eluate were assayed for radioactivity.

To determine if there was any contamination of the lipids by radioactive substrate, nonincubated tissue was homogenized in chloroform–methanol, substrate was added, and the lipids were isolated. The results indicated that no more than 1.4% of the radioactivity in the phospholipids could have been due to contamination from glucose-2-^{14}C and no more than 2.6% from acetate-1-^{14}C. Triglycerides would not have been contaminated by radioactive substrate. When a sufficient amount of radioactivity was present, the lipid was hydrolyzed in ethanolic KOH, excess ethanol was evaporated with nitrogen, water was added, and the aqueous solution was extracted with petroleum ether (bp 40–60°C) before and after acidification of the aqueous portion. Aliquots from the aqueous and petroleum ether fractions were assayed for radioactivity. Under these conditions, the aqueous fraction contains glycerol or glycerophosphate, the petroleum ether extract of the alkaline aqueous layer contains nonsaponifiable compounds, and the petroleum ether extract of the acidified aqueous layer contains the fatty acids.

Substrate Characterization

After the aortas had been incubated with glucose-2-^{14}C, the water-wash of the chloroform-extracted aorta was analyzed to determine the distribution of ^{14}C in acetate. The acetate was isolated, converted to phenylacetophenone, and degraded to iodoform and phenylbenzoic acid according to the procedure of D'Adamo and D'Adamo (12). The distilled acetate-^{14}C was diluted with carrier sodium acetate, converted to acetyl-^{14}C chloride with phosphorus oxychloride (13), and the acetyl-^{14}C chloride was distilled off. Phenylacetophenone-^{14}C was prepared by refluxing the acetyl-^{14}C chloride, biphenyl, and 10% molar excess of anhydrous aluminum chloride in carbon disulfide. Portions of the phenylacetophenone-^{14}C, mp 119–120°C (unc; Ref. 12, 120–121°C), were cleaved with KI–I_2. Judging by the slurred melting point of 110–125°C (Ref. 13, 120°C), the iodoform (methyl carbon of acetate-^{14}C) was impure even after several recrystallizations. The phenylbenzoic acid (carboxyl carbon of acetate-^{14}C) was recrystallized and it melted at 223.5–224°C (unc; Ref. 14, 224°C).

Glycerol-^{14}C from triglyceride-glycerol and from phospholipid-α-glycerophosphate was degraded. The α-glycerophosphate was first purified by column chromatography (15) and then treated with acid phosphatase. The glycerol-^{14}C from each fraction was diluted with carrier glycerol and passed through a mixed bed resin (Bio-Rad AG 501-X8 [D]) to remove ionic contaminants. Periodate degradation (16) of the glycerol-^{14}C yielded carbons 1 and 3 as formaldehyde and carbon 2 as formic acid. The formaldehyde was precipitated as the dimedone derivative (17) (mp 189.5–190°C, unc; Ref. 18, 189–190°C). Formic acid was collected, reduced to formaldehyde (19), and converted to the dimedone derivative. Degradation of commercial glycerol-1,3-^{14}C and glycerol-2-^{14}C after passage through mixed bed resin showed that <0.3% of carbons 1 + 3 contaminated the formic acid from carbon 2 that was reduced to formaldehyde, and <0.1% of carbon 2 contaminated the formaldehyde from carbons 1 + 3.

Fatty acids were separated by gas–liquid chromatography (HI-EFF 2BP on Chromosorb W [AW], Applied Science Laboratories Inc., State College, Pa.). Certain fatty acids were decarboxylated [illegible] synthesis was de novo or by elongation.

RESULTS

Incorporation and Distribution of Radiosubstrates

The incorporation of ^{14}C into the aortic lipids is listed in Table 1. The amount of radioactivity incorporated into the lipids of control aortas was often low; extensive

TABLE 1 INCORPORATION OF ^{14}C INTO LIPIDS OF NORMAL AND ATHEROSCLEROTIC RABBIT AORTAS

	Glucose-2-^{14}C	Acetate-1-^{14}C
	*pmoles substrate incorporated/mg wet wt/3 hr**	
Normal	8.6 ± 1.2 (6)	0.22 ± 0.10 (5)
	$P = 0.04$	$P = 0.02$
Atherosclerotic	23.5 ± 6.0 (6)	15.6 ± 5.3 (6)

* Values are means ± SEM, with the number of animals given in parentheses; P values are for differences between normal and atherosclerotic aorta incubated with the same labeled substrate.

analyses were not possible. The production of atherosclerosis caused a significant increase in the amount of both substrates incorporated into lipids.

The distribution of radioactivity among the various lipid classes is shown in Table 2. In each type of aorta, about two-thirds of the label from glucose-2-^{14}C appeared in phospholipids. Atherosclerosis induced no significant changes in the distribution of ^{14}C from glucose-2-^{14}C. Vost (6) found approximately 66% of ^{14}C from glucose-^{14}C in phospholipids and 10–19% in the triglycerides of normal rabbit abdominal aorta after 2 hr of perfusion.

In control aortas, acetate-1-^{14}C was incorporated mostly into phospholipids; the induction of atherosclerosis increased incorporation into triglycerides and cholesteryl esters ($P < 0.001$ for each). The cholesteryl esters were rechromatographed in heptane–ethyl ether 19:1, and the radioactivity moved again to the cholesteryl ester spot; none cochromatographed with squalene. Rao and Rao (21) reported that 14% of acetate-^{14}C was incorporated into normal rabbit aortic triglycerides and 45% into phospholipids; Day and Wilkinson (22) found 10% in triglycerides, 56% in phospholipids, and 29% in cholesteryl esters of atherosclerotic rabbit aortic lipids.

Table 3 lists the amounts of radioactive substrate acetate and glucose incorporated into phospholipids and triglycerides of normal and atherosclerotic aortas. The ratio Atherosclerotic/Normal indicates the increase caused by atherosclerosis. Even though the incorporation of glucose-2-^{14}C into lipids was about the same in normal and atherosclerotic aortas, there was about a threefold increase in substrate incorporation on a per milligram basis into both triglycerides and phospholipids. The increase of acetate-1-^{14}C into both triglycerides and phospholipids was greater than the increase of glucose-2-^{14}C caused by atherosclerosis, but even the maximum incorporation of acetate-1-^{14}C into each of these lipids was not much greater than the amounts of glucose-2-^{14}C incorporated into control aortas.

Distribution of Radioactivity in Lipids

The two radioactive substrates differed markedly with regard to their incorporation into locations within the lipids (Table 4). ^{14}C from glucose-2-^{14}C was present in triglycerides and phospholipids almost exclusively in the glycerol moiety; the incorporation of the radioactive glucose into fatty acids was significantly different ($P = 0.004$) between phospholipids and triglycerides. Acetate-1-^{14}C was incorporated predominantly into fatty acids, with about one-third present in the glycerol moiety of triglycerides and one-fourth in the glycerophosphate of phospholipids. The radioactivity in

TABLE 2 DISTRIBUTION OF SUBSTRATE RADIOACTIVITY INTO LIPIDS OF NORMAL AND ATHEROSCLEROTIC RABBIT AORTA

Lipid Class	Glucose-2-^{14}C Normal	Glucose-2-^{14}C Atherosclerotic	Acetate-1-^{14}C Normal	Acetate-1-^{14}C Atherosclerotic
		% *		
Cholesteryl ester	0	1.7 ± 0.3	5.2 ± 1.6	26.9 ± 3.1
Triglyceride	22.0 ± 2.6	26.4 ± 1.3	4.3 ± 1.8	18.3 ± 2.1
Fatty acid	0.3 ± 0.1	0.5 ± 0.1	7.1 ± 1.3	5.8 ± 0.9
Cholesterol + diglyceride	10.4 ± 0.5	5.1 ± 0.2	24.9 ± 8.1	4.1 ± 0.7
Monoglyceride	3.2 ± 0.6	1.7 ± 0.3	9.9 ± 1.9	3.7 ± 0.3
Phospholipid	64.0 ± 2.2	64.6 ± 1.5	48.7 ± 8.0	41.2 ± 1.6

* Percentage of total radioactivity removed from the TLC plates.

TABLE 3 INCORPORATION OF ^{14}C INTO PHOSPHOLIPIDS AND TRIGLYCERIDES OF NORMAL AND ATHEROSCLEROTIC AORTAS

	Substrate Incorporated					
	Glucose-2-^{14}C			Acetate-1-^{14}C		
Lipid Class	Normal	Atherosclerotic	Atherosclerotic/Normal	Normal	Atherosclerotic	Atherosclerotic/Normal
	pmoles/mg wet wt/3 hr					
Triglyceride	1.8	6.2	3.4	0.094	2.9	31
Phospholipid	5.48	15.2	2.8	0.107	6.4	59

* Values are products of the percentage incorporation for each lipid class (Table 2) and the total incorporation into lipid (Table 1).

TABLE 4 Percentage Distribution of Radioactivity in Lipids of Atherosclerotic Rabbit Aorta

Lipid Class	Glucose-2-^{14}C			Acetate-1-^{14}C		
	Glycerol	Fatty Acid	Non-saponified	Glycerol	Fatty Acid	Non-saponified
			%*			
Triglyceride	96.5	3.3	0.2	31.6	68.3	0.1
Phospholipid	94.0	6.0	0.0	26.1	73.2	0.7
Cholesteryl ester	1.2	98.0	0.8	2.1	97.4	0.5

* Percentage of the sum of the ^{14}C in the three hydrolysis fractions.

cholesteryl esters was present predominantly in fatty acids. Rechromatography of the nonsaponifiable fraction showed slight radioactivity cochromatographing with cholesterol, but this was not characterized further. Yost (6) found 3.3% of the glucose-^{14}C radioactivity in the fatty acids of total lipid, while Day and Wilkinson (22) reported that over 90% of acetate-^{14}C appeared in the phospholipid fatty acids of rabbit aorta.

Presumably, the ^{14}C from either acetate or glucose is present in the aqueous fraction as glycerol or glycerophosphate. Contamination of phospholipid by substrate acetate-1-^{14}C was ruled out by acidifying the water hydrolysate and evaporating it to dryness; over 90% of the radioactivity remained after this treatment, contrary to the loss of added acetate-1-^{14}C in control experiments.

Cholesterol cochromatographs with 1,3-diglycerides on TLC, and since this compound was sometimes incompletely separated from 1,2-diglycerides in aortic lipids from cholesterol-fed animals, these three lipids were considered together (Table 2). With glucose-2-^{14}C as substrate, over 90% of the radioactivity was in the glycerol fraction, indicating predominantly diglycerides. Over half of the ^{14}C from acetate-1-^{14}C was in the fatty acids contained in that spot. After hydrolysis and rechromatography, the amount of radioactivity that cochromatographed with cholesterol would have accounted for <0.6% of the total acetate-1-^{14}C incorporated into aortic lipids.

Table 5 is designed to place in perspective the relative amounts of the two substrates incorporated in the glycerol and fatty acid moieties of phospholipids, triglycerides, and cholesteryl esters by atherosclerotic aortas. The ^{14}C of glucose-2-^{14}C would yield the triose dihydroxyacetone-2-^{14}C phosphate for conversion to glycerol-2-^{14}C. A portion of the triose phosphate isomerizes to glyceraldehyde-2-^{14}C-3-phosphate and provides precursor for acetyl-1-^{14}C CoA used in fatty acid synthesis. Since the specific activity of this acetyl-1-^{14}C CoA is diluted by half of the original glucose-2-^{14}C molecule, the values for fatty acids synthesized from this substrate are doubled in Table 5. No corrections are made for additional dilution by endogenous metabolites, nor are glycerol-^{14}C incorporation values altered because of possible dilution by nonradioactive glyceraldehyde-3-phosphate arising from glucose-2-^{14}C. The activity for glycerol-^{14}C arising from substrate acetate-1-^{14}C is also doubled because of the randomization and loss of ^{14}C that would occur during the conversion of acetate-^{14}C to glycerol-^{14}C. The amount of ^{14}C from glucose-2-^{14}C in the glycerol moiety was 8–15 times more than in fatty acids, whereas acetate-1-^{14}C was distributed almost equally between the glycerol and fatty acid moieties.

Glycerol-^{14}C from the triglycerides and phospholipids was degraded as outlined in the Materials and Methods section. When acetate-1-^{14}C was the substrate, an average of 98.5% of the ^{14}C was in carbons 1 + 3, as would be expected. With glucose-2-^{14}C, 95% of the ^{14}C was in carbon 2 and 5% was randomized into carbons 1 + 3: this is in contrast to 25% or more randomization found in adipose tissue (23) and probably indicates a lower activity of the hexosemonophosphate shunt in aortic tissue than in adipose tissue. Acetate-1-^{14}C arising from glucose-2-^{14}C had >95% of the radioactivity in carbon 1: this establishes the validity of using glucose-2-^{14}C for fatty acid and lipid synthesis.

TABLE 5 Comparison of Amounts of Radioactive Substrate Incorporated into Different Moieties of Lipids of Atherosclerotic Rabbit Aortas

	Total Substrate Incorporation* (Table 1)	Lipid Class	Percentage of Total Lipid† (Table 2)	Total (Table 3)	Glycerol‡	Fatty Acids‡
					pmoles of substrate incorporated/mg wet wt of aorta/3 hr	
Glucose-2-^{14}C	23.5	TG	26.4	0.2	0.0	0.2 × 2 = 0.4
		PL	64.6	15.2	14.5	0.9 × 2 = 1.8
Acetate-1-^{14}C	15.6	TG	15.5	2.9	0.90 × 2 = 1.8	2.0
		PL	41.2	6.4	1.7 × 2 = 3.4	4.7

* pmoles of substrate incorporated/mg wet wt of aorta/3 hr.
† Percentage of substrate incorporated into all lipids.
‡ Values are the products of the percentage of each fraction, glycerol or fatty acid, from Table 2 and the absolute amount from Table 3.

TABLE 6 DECARBOXYLATION RATIO* FOR PHOSPHOLIPID FATTY ACIDS OF ATHEROSCLEROTIC RABBIT AORTA

Fatty Acid†	Glucose-2-^{14}C	Acetate-1-^{14}C
14:0	7.0	5.3
16:0	7.0	6.2
18:0	1.1	1.2
18:1	1.2	1.2

* The decarboxylation ratio is total fatty acid radioactivity/carboxyl radioactivity. A ratio of 1.0 indicates that elongation was the only reaction, while a higher ratio indicates increased de novo synthesis, e.g., 7.0 for 14:0 and 8.0 for 16:0.

† Fatty acid = number of carbon atoms:number of double bonds.

Table 6 presents decarboxylation ratios for some of the phospholipid fatty acids synthesized in atherosclerotic aortas. The fatty acids with 14 and 16 carbons arise mainly by de novo synthesis, whereas those with 18 carbons have radioactivity incorporated by elongation, similar to the results of St. Clair, Lofland, and Clarkson (24). Both glucose-2-^{14}C and acetate-1-^{14}C gave similar results.

DISCUSSION

These studies demonstrate the feasibility of using glucose-2-^{14}C to study lipogenesis in normal and atherosclerotic aorta. This substrate is metabolized in a manner consistent with known biochemical pathways. There is little randomization of label: ^{14}C appears predominantly where predicted in glycerol-2-^{14}C and acetate-1-^{14}C. Substrate acetate-1-^{14}C used concomitantly showed that fatty acid synthesis was similar from both labeled substrates. Any estimates on substrate incorporation are minimum values, since the degree of dilution of labeled substrates by endogenous metabolites is unknown. Considering that the radioactive glucose passes through more enzymic steps with more possibilities of endogenous, nonradioactive substrate dilution than the radioactive acetate, the amount of ^{14}C incorporated from glucose-2-^{14}C into fatty acids is quite substantial. This may be physiologically more important due to the greater availability of glucose for in vivo metabolism. In general, there was good agreement between the data obtained here and comparable data from other work (6, 21, 22, 24) in which whole aortic tissue was incubated under similar conditions.

Approximations based on the rates of glyceride-glycerol synthesis from glucose-2-^{14}C and on the amounts of lipid that accrue during the development of atherosclerosis in rabbits fed cholesterol (25–27) indicate that glucose could furnish virtually all of the glycerol in triglycerides and glycerophospholipids synthesized de novo in the aorta. In normal and atherosclerotic rabbit aorta, but not in human aorta (28, 29), the concentration of triglycerides is greater than that of phospholipids; however, the rates of increase due to atherosclerosis are similar. The aortic preparation used here excluded periaortic fat tissues that could have caused contamination with triglyceride-synthesizing enzymes. Numerous workers (5, 6, 22, 30, 31) have noted this problem and have likewise taken care to prevent contamination. The active triglyceride synthesis observed here and elsewhere probably represents metabolism of the aortic intima plus media. Acetate-1-^{14}C incorporation into glyceride-glycerol results from the equilibration of that substrate with endogenous metabolites, but no net glycerol synthesis results. Fatty acids synthesized from either substrate could result in net accumulation.

The incorporation of glucose-^{14}C into cholesteryl esters has not been reported to this extent before, although the substantial incorporation of acetate-1-^{14}C into cholesteryl esters, primarily in the fatty acid moiety, has been reported in this work and that of others (22, 24, 32). The rate of incorporation of acetate-1-^{14}C into the fatty acids that are esterified to cholesterol (Tables 1 and 2) could account for 20% (4) to > 90% (25) of the cholesteryl ester fatty acid that accumulates during the development of atherosclerosis. These data do not differentiate between the net accumulation of cholesteryl esters by the esterification of nascent fatty acids with endogenous cholesterol and the turnover of fatty acids from cholesteryl esters already present in the aorta (32); they merely indicate a potential for net accumulation.

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Volatile Ester Equilibria in Plant Tissue*

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SUMMARY

Variations in the apparent dissociation constants of ethyl acetate for several different samples of banana pulp are noted. It is hypothesized that the variations might be due to the involvement of coupled reactions that supply or delete one or more of the reacting species at a rate faster than that at which the tissue esterases function to establish equilibrium. In the same samples, the constants for methyl acetate and iso-amyl acetate remained relatively constant.

Enzymes responsible for the hydrolysis of carboxylic ester linkages have been found in a wide variety of materials. The aliesterases, which act on aliphatic esters of low molecular weight, are widely distributed in the animal body and in the higher and lower plants. Although it is often assumed that the aliesterases catalyze both the synthesis and hydrolysis of the simple esters, evidence has been presented that ethyl acetate is produced in fermentations by the yeast *Hansenula anomala* by a different mechanism (Baba, 1956; Cantarelli, 1955; Peel, 1951; Tabachnick and Joslyn, 1953). Baba (1956) observed that the amount of ethyl acetate formed varied in no consistent pattern with the accumulation of ethanol and acetate, indicating that something more than a simple condensation of these substances is involved in the metabolic production of ethyl acetate. It has been postulated that ethyl acetate is produced by means of the formation of an active two-carbon group such as acetyl-coenzyme A, probably from ethanol, followed by a condensation reaction with ethanol to the acetate ester (Cantarelli, 1955; Peel, 1951). Calcium pantothenate, a precursor of coenzyme A, has been shown to enhance the ethyl acetate synthesis by yeast (Cantarelli, 1955). There is no evidence that the acid moiety of the ester is derived from acetate (Peel, 1951). The ethyl acetate synthesis in yeast requires oxygen; is not inhibited by low pH's (as are most esterase-catalyzed reactions) or by the general esterase inhibitor diisopropylfluorophosphate; and gives much higher yields than would be expected if the ester were formed by a simple condensation of ethanol and acetate by an esterase.

A quantitative analysis of some of the volatile compounds produced in banana pulp at different ripeness stages and under various conditions likely to affect the metabolism of the banana, such as the effect of various gases on the fruit during ripening and storage, has been recently reported (Hultin and Proctor, 1961). Table 1 shows data relating to the volatile acids, alcohols, and esters expressed in moles per kg of tissue.

The green, half-ripe, ripe, and overripe samples represent different ripeness stages of the banana. Ripening of the ethylene-ripened sample was hastened with ethylene stimulation. The scalded sample was also treated with ethylene, and in addition the carbon dioxide content of the storage atmosphere was allowed to increase above 10%. The coated sample was dipped in a mixture of acetylated monoglycerides and stored at a low temperature (13.3°C) for 4 days. The coated control was stored in the same manner but without the dip treatment. These samples and the methods used in the analyses were described in detail by Hultin and Proctor (1961).

Table 1. Some volatile acids, alcohols, and esters of banana pulp (moles per kg banana pulp).

	Acetic acid ($\times 10^5$)	Ethyl alcohol ($\times 10^6$)	Ethyl acetate ($\times 10^6$)	Methyl alcohol ($\times 10^6$)	Methyl acetate ($\times 10^6$)	Iso-amyl alcohol ($\times 10^6$)	Iso-amyl acetate ($\times 10^6$)
Green	4.3	2.4	25.4	6.9	6.1	0.3	1.5
Half-ripe	9.3	0.9	10.8	57.8	12.8	0.5	7.7
Ripe	8.0	1.1	*	4.7	1.8	0.1	1.5
Ethylene-ripened	8.3	2.8	18.0	12.5	5.4	0.5	7.7
Scalded	7.0	2.4	12.5	17.2	14.9	0.5	53.9
Overripe	26.2	49.1	19.2	17.5	6.8	6.4	92.4
Coated	10.2	162	13.5	9.4	8.1	0.8	15.4
Coated control	17.7	194	8.4	9.4	12.0	1.7	15.4

* No detectable ethyl ester in the ripe sample.

Apparent equilibrium constants (K) for the dissociation of the esters were calculated from the data in Table 1 as follows:

$$K = \frac{(ROH)\ (HOAc)}{(ROAc)\ (HOH)}$$

where ( ) indicates the concentration of each molecular species in moles/kg of banana pulp. The apparent equilibrium constants are presented in Table 2. In calculating these constants, it was assumed that the water content of each of the banana samples was 75%.

The calculated apparent equilibrium constants for the methyl and iso-amyl acetate esters did not vary greatly, indicating that in all samples there was a relatively constant relation between the concentrations of the ester and its component parts. This may indicate that an esterase-catalyzed reaction is possibly important in synthesis and degradation of these esters *in vivo*.

Table 2. Apparent dissociation constants (K) of volatile esters in various samples of banana pulp.

Sample	Ethyl acetate ($K \times 10^5$)	Methyl acetate ($K \times 10^6$)	Iso-amyl acetate ($K \times 10^5$)
Green	1.0	1.2	2.0
Half-ripe	1.9	10.1	1.4
Ripe	>1800*	5.0	1.3
Ethylene ripened	3.1	4.6	1.3
Scalded	3.2	1.9	0.2
Overripe	162	16.2	4.3
Coated	292	2.9	1.3
Coated control	427	1.5	2.2

* No detectable ethyl ester was present in the ripe banana pulp. On the basis of the sensitivity of the analytical methods employed, the equilibrium constant was calculated as being greater than 1800×10^{-5}.

On the other hand, the extremely large variations in the ethyl acetate dissociation constants among some of the samples led to the tentative conclusion that the formation of ethyl acetate does not necessarily depend only on the concentrations of ethanol and acetate in the tissue under all circumstances. There are three explanations that could account for this fact. The first is that equilibrium is not reached, because of an unfavorable reaction rate caused by lack of a catalyst, i.e., esterase. This is considered unlikely in view of the evidence cited above pertaining to the methyl acetate and iso-amyl acetate equilibrium data. Owing to the low specificity of the esterases (Ammon and Jaarma, 1951), it would be expected that if an esterase is present that can catalyze the formation and degradation of these esters from their respective alcohols and acetate, then it could also catalyze the formation and degradation of ethyl acetate from ethanol and acetate.

A second explanation is that the over-all concentrations of the reacting species are unrelated to the activities, or effective concentrations, in the tissue due to adsorption on cell particulate matter, concentration at interfaces or other physical phenomena that would tend to concentrate one or more of the reacting substances in one area in the cell. Again, however, the relative constancy of the equilibrium values of the other esters leads to the conclusion that this would probably not be the major factor for the large variations in the ethyl ester constant, i.e., if the methyl and iso-amyl alcohols and esters are not concentrated in one or more particular areas of the cell, there is no reason

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Table 2. The dissociation constants (K)* of methyl, ethyl, and iso-amyl acetate.

Compound	K	Reference
Ethyl acetate	0.26	Taussig and Petreanu, 1956
	0.26	Glasstone, 1946
	0.23	Parks and Huffman, 1932
Methyl acetate	0.17	Taussig and Petreanu, 1956
	0.12	Friedman and Elmore, 1941
Iso-amyl acetate	2.7	Mardashev and Kuznetzoy, 1935

$$* K = \frac{(\text{acid})\ (\text{alcohol})}{(\text{ester})\ (\text{water})}$$

assume that ethanol or its acetate ester would be.

The other explanation is that, in some cases at least, a coupling reaction(s) is involved that is supplying or deleting one or more of the reactants or products of the hydrolysis at a rate that is considerably faster than the rate at which the esterases function to establish equilibrium. This would allow for the presence of at least one component of the reaction in excess of its equilibrium value, which would make the apparent equilibrium constants differ from the true dissociation equilibrium constant and might cause the variations noted among the several banana tissue samples. This seems the most probable explanation for the variability of the apparent ethyl acetate equilibrium constants. Similar anomalies for the yeast *Hansenula anomala*, as noted above, have been ascribed to an oxidative synthesis of ethyl acetate from ethanol that does not involve acetate.

One other fact that must be considered is that the apparent equilibrium constants obtained in this study showed very large variations from the true thermodynamic equilibrium constants, as calculated from approximately equimolar concentrations of all reacting species and reported by other investigators. Some of these latter constants are given in Table 3.

The major implication to be derived from the differences between the apparent equilibrium constants calculated from the data in Table 1 and the true thermodynamic equilibrium constants as expressed in Table 3 is that the calculated values may not represent a true equilibrium state. It is surprising, however, that the constants for the methyl and iso-amyl esters should be so constant under such varying conditions if a true equilibrium state is not represented. A possible explanation for these discrepancies is that in calculating the constants for the banana esters it was assumed that all the water in the tissue was free to take part in the reaction, which of course is not the actual situation, since much, if not most, of the water is bound to cellular protein or polysaccharides or is made unavailable at the reaction site by physical barriers.

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The Acetic Acid Content of Sour French Bread and Dough as Determined by Gas Chromatography[1]

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ABSTRACT

The total acidity of sour French bread, dough, and starter was determined by extraction of these materials with acetone followed by titration of the extracts with standard alkali. The results were compared with extracts of conventional bread and dough. There was ten times more acid in the sour bread than in the conventional bread. Percentage of acetic acid present in the acid fractions was determined by gas chromatography. Approximately half of the total acidity of sour French bread and three-fourths of the total acidity of conventional bread was acetic acid.

Sour-dough French bread, made only from flour, water, salt, and the all-important starter sponge, appears to be unique in being produced only in northern California, although its popularity is widespread. The starter sponge provides both the leavening and the souring action and is maintained commercially by rebuilding approximately every 6 to 8 hr. Although this process has been perpetuated in this manner for over 100 years, nothing has been reported, to our knowledge, either on the identity of the microorganisms involved or on the nature

[1]Contribution from Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Albany, Calif. 94710.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

of the acids and other fermentation products produced by them. This study is part of a general study on the nature of this process and deals specifically with the acetic acid content of sour doughs and breads. The acetic acid content of conventional breads and doughs is also reported for comparative purposes.

MATERIALS AND METHODS

Sour French Bread Preparation

The "starter" or mother sponge used was one which had been carried through many transfers from the original sponge obtained from a local sour-dough bakery. Transfers were made about four times a week with the following formulation:

Previous sponge	100 parts
Hi-gluten flour (14% protein)	100 parts
Water	53 parts

The freshly prepared mother sponge was incubated 7 to 8 hr. at 80°F., during which time the pH value dropped from 4.5 to 3.9. Sponges were held at 50° to 55°F. between transfers.

The bread was prepared with the following formulation:

Bread patent flour (12% protein)	100 parts
Water	60 parts
Salt	2 parts
Mother sponge	18 parts

The bread dough was rounded and allowed approximately 1 hr. floor time before scaling and moulding. It was allowed to proof either directly on canvas or in perforated metal pans. Proofing was for 7 to 8 hr. at 86°F., during which time the pH value of the bread dough dropped from 5.3 to between 3.9 and 4.0. The dough was baked in a rotary hearth oven for 45 min. at 375°F. with low-pressure steam bled in for the first half of the baking time.

Conventional Bread Preparation

Conventional white bread was prepared by the straight-dough procedure as described by Kline and Sugihara (1).

Extraction of Organic Acids and Preparation for Analysis

From doughs and starters: Fifty grams of frozen dough and 100 ml. of acetone were placed in a 1-pint Osterizer jar whose blade assembly was modified with an "O" ring and Teflon seal to prevent leakage. Samples were ground for approximately 5 min. at low speeds to prevent the temperature of the mixture from rising. Jar and contents were then placed in a refrigerator for approximately 30 min. The cold mixture was then filtered on a Buechner funnel through Celite. The precipitate was washed several times with acetone, and washings and filtrate were combined and made up to a volume of 200 ml. with acetone. A 50-ml. aliquot of the acetone solution was placed in an Erlenmeyer flask and 50 ml. of water was added. The mixture was then titrated to pH 8 with 0.1N sodium hydroxide, and total acidity calculated as meq. of acetic acid. The remainder of the stock solution was neutralized with 0.1N sodium hydroxide, and then evaporated to

extracted twice with equal volumes of dichloromethane to remove any nonacidic, nonvolatile components that might interfere with subsequent chromatography; the dichloromethane extract was in turn extracted once with water, and the aqueous extract thus obtained was added back to the original extracted alkaline solution. The latter was then evaporated to a volume of 1 to 2 ml. and the residue transferred to a 12-ml. centrifuge tube with about 4 ml. of water, to which was added 1 ml. of dilute HCl (1:3 acid:water); the mixture was shaken well, centrifuged, and then made up to a volume of 7 ml. with water, reshaken and recentrifuged; a portion of the supernatant was poured into a 5-ml. volumetric flask which was then capped with a serum bottle rubber septum.

From baked bread: The procedure for extracting organic acids from baked bread was similar to that used for extracting acids from dough except for the following modifications. The baked loaf, usually weighing a little under 500 g., was cut into four quarters. A quarter was then cut into 1/4-in. pieces which were placed in an Osterizer jar with 150 ml. of acetone, ground to a slurry, cooled in the refrigerator, and then filtered on a Buechner funnel through Celite. The precipitate of crumbs was washed with acetone; the washings were combined with the filtrate and made up to a volume of 350 to 400 ml. with acetone. An aliquot of the total solution was placed in an Erlenmeyer flask, and an equal volume of water was added. The titer was determined as for the doughs in the previous section and then the total acidity and acidity per g. were calculated. The stock solution was made alkaline, reduced in volume, extracted, further reduced in volume, made acid, centrifuged, and transferred to a 5-ml. volumetric flask that was capped with a rubber serum bottle closure.

Determination of the Acetic Acid Content of Extracts of Bread and Dough by Gas Chromatography

The gas chromatography apparatus used in this study consisted of a heated Barber-Colman flame ionization detector, a Beckman Thermotrac oven, a Jarrel-Ash electronic amplifier, and a 5-mv. recording potentiometer. Separations were made on a stainless-steel column (10 ft. X 1/8 in. o.d.) packed with 10% LAC-1-R (296) on Gas-Chrom Q (80 to 100 mesh). Column temperature was 100°C. Nitrogen for the column (50 ml. per min. at 38 psig. monitored by a gage at the head of the column) was passed through a toggle valve connected to a self-storing nylon hose, which in turn was connected to the head of the column by a quick-connect tube fitting with double end shut-off.

Samples for analysis were taken from the septum-covered 5-ml. volumetric flasks containing the free organic acids. As the needle was withdrawn from the flask its tip was wiped clean by the rubber septum. Prior to injection of the sample, the nitrogen supply to the column was cut off with the toggle valve, the quick connects leading to the column were separated, and the pressure at the head of the column slowly dropped to zero. The other end of the quick-connect tube was emptied of nitrogen and the quick connects were reconnected. The sample was injected on the column through an on-column injection port heated to 150°C. The toggle valve was flipped on and the inlet checked for leaks by means of the device described by Hunter and Walden[2]. Retention time for the acetic acid peak under the conditions

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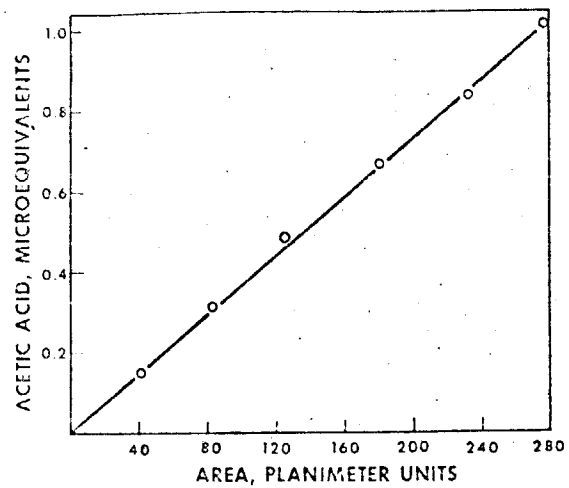

Fig. 1. Calibration curve of acetic acid in microequivalents vs. area in planimeter units.

described was 5.5 min. Peak areas were determined by planimeter. A linear response (see Fig. 1) for acetic acid was obtained with the gas chromatograph when 2 to 5 μliters of a standard solution in water, containing 10.45 γ of acetic acid per μliter, was injected. Initial injections of the acid mixtures gave a slightly smaller response than did subsequent injections. After two or three injections the response remained constant. Only those data obtained after the response stabilized were used.

Recovery values for acetic acid by gas-chromatographic analysis of the

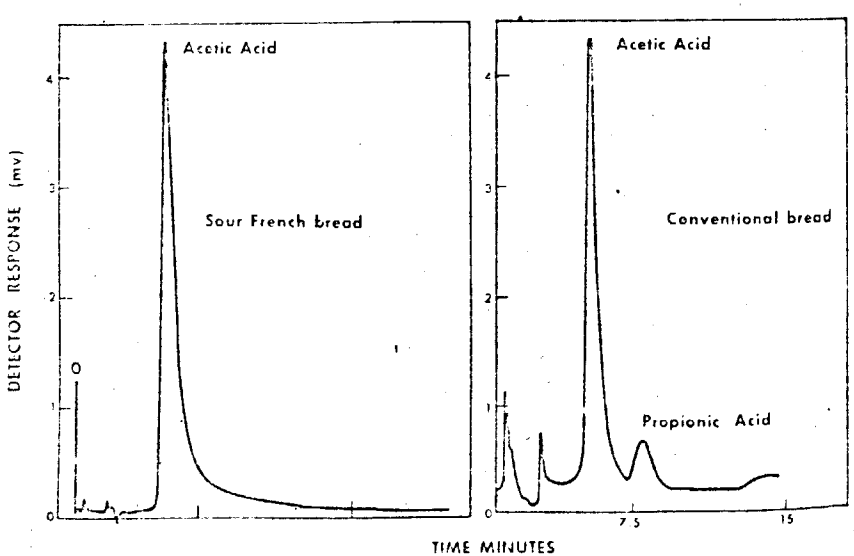

Fig. 2. Typical chromatograms of extracts of sour French bread and conventional bread.

concentrated extracts of bread and dough were determined by adding to a dough or bread sample known amounts of acetic acid. After grinding and extracting, the final concentrate was assayed for acetic acid. Average recovery ranged between 98 and 102%.

Chromatograms prepared from extracts of sour bread and dough showed only acetic acid peaks, whereas chromatograms of extracts of conventional breads and doughs indicated the presence of substantial amounts of propionic acid as well as acetic acid. Typical chromatograms are shown in Fig. 2.

Percent acetic acid present in the total acids extracted was determined by the following formula:

$$\text{Percent acetic} = \frac{\text{microequiv. acetic acid}}{\text{microequiv. total acidity}} \times 100$$

RESULTS AND DISCUSSION

The results of the assays for total acidity and acetic acid content of sour and conventional breads and doughs are shown in Table I. Acetic acid represented from

TABLE I. ACETIC ACID CONTENT OF BREAD AND DOUGH[a]

Type	Total Acidity A[b]	Total Acidity B[b]	Acetic Acid A	Acetic Acid B
	meq./g.		% of total acidity	
Sour mother sponge	0.0472	0.0496	48.9	50.1
Sour French bread dough	0.0384	0.0384	53.5	51.4
Baked sour French bread	0.0476	0.0468	49.4	51.1
Conventional bread dough	0.00982	0.00780	57.0	56.1
Baked conventional bread	0.00527	0.00504	75.8	75.8

[a]All values are an average of two or more determinations.
[b]Sample designation.

56 to 76% of the total acidity in conventional breads and doughs and about 50% of the acidity in the sour breads and doughs. However, the total acidity of the sour bread samples was, not surprisingly, 5 to 10 times that in the conventional breads. The predominance of acetic acid suggests that acetic acid may contribute in an important way to the flavor of sour-dough French bread.

Acknowledgment

The authors thank Linda Bele for preparing the bread and dough samples used in this study.

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ACIDITY CONTROLLING ANTISEPSIS BY WEAK ACIDS

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Ever since antiseptics have been used in food preservation, it has been known that weak acids are more efficient in acid solution. Vermast (1921) calculated the amount of undissociated benzoic acid necessary to prevent growth and found the same amount necessary in neutral as in acid media, and so concluded that the undissociated acid was the effective antiseptic. This paper was apparently overlooked by all later workers in the field. Several other acids were found to be far more efficient in acid media, and the authors suggested that this could be explained by assuming that the inhibitory substance was the undissociated fraction of the acid, but none of these authors proved this suggestion by demonstrating that the inhibitory dose at all acidities represents a constant amount of undissociated acid. This proof was furnished by Rahn and Conn (1944) for salicylic and benzoic acids. With H_2SO_3, these authors found *Escherichia coli* to be inhibited by the HSO_3 ion, whereas yeast was affected only by the undissociated H_2SO_3 fraction.

This investigation was undertaken to determine whether or not this conclusion can be generalized to include all weak acids which are antiseptics. The majority of the weak acids tested had dissociation constants between 10^{-3} and 10^{-5}. Therefore, if the undissociated acid were the antiseptic, the efficiency should increase greatly between pH 5 and 3. This required a test organism capable of growing at pH values between 2.5 and 6.

A burgundy wine yeast was chosen for this purpose. The medium contained yeast extract, tryptone, glucose, and phosphate, and was adjusted to approximately pH 6, 5, 4, 3, and 2.5 with solid citric acid, using a glass electrode. The media were inoculated with about one drop of a 24-hour yeast culture per 100 ml, and then enough antiseptic, adjusted to similar acidities, was added to give the desired concentrations. The total in each tube was 10 ml. All cultures were incubated at room temperature.

Growth was measured by the turbidity of the culture after 1, 2, 4, 7, and 10 days. Sterile test tubes were used and the percentage of transmittance was read in a Coleman universal spectrophotometer using the uninoculated medium as a standard transparency of 100. These readings were converted into turbidity by subtracting the value from the zero reading. A wave length of 620 millimicrons was used. The final pH of each tube was determined.

Table 1 shows the type of data thus obtained. The inhibiting dose of acetic acid at pH 4.9 was between 1.4 and 1.2 per cent. The lower concentrations show the rate and type of retardation, which is of value for an analysis of the mode of action of the antiseptic. This will be discussed later.

Similar sets of data were obtained for each of the acids tested at five different acidities. Table 2 shows only the inhibiting concentrations. It is plainly seen that the undissociated acid was the bacteriostatic agent with acetic, propionic, butyric, monochloracetic, bromopropionic, and nitrous acids. In the case of oxalic and selenious acids the undissociated fractions are efficient antiseptics, but the $HC_2O_4^-$ and the $HSeO_3^-$ ions are also bacteriostatic.

With monobasic acids, the agreement at all acidities was so good that it was possible to calculate the dissociation constant K from the antiseptic concentrations, using the formula

$$K = \frac{H_1 H_2 (C_1 - C_2)}{H_2 C_2 - H_1 C_1}$$

where H is the hydrogen ion concentration, and C the concentration of the antiseptic; the subscript *1* referring to the higher concentration, and *2* to the lower concentration of the antiseptic. It can be seen from table 2 that fairly good

TABLE 1

Inhibition of growth of Saccharomyces ellipsoideus by acetic acid at pH 4.9 measured by turbidities

% ACETIC	1 DAY	2 DAYS	4 DAYS	7 DAYS	10 DAYS	FINAL pH
1.4	0	−1	−1	−1	−1	4.9
1.2	−2	−2	−2	61	84	4.9
1.0	0	17	77	87	86	4.9
0.9	3	58	87		90	4.9
0.8	22	75	90		91	4.9
0.7	40	80	95		100	4.9
0.6	54	90			94	4.9

values for the dissociation constants can be obtained by this method. It was found that the greater the difference in the concentrations necessary to kill or prevent growth over a narrow pH range, the better the value for the dissociation constant. The only unexplained deviation was the value for K with bromopropionic acid between pH 4.8 and 4.5.

Figure 1 shows the effect of acetic acid at pH 4.9 and bromopropionic acid at pH 3 on the growth of *Saccharomyces ellipsoideus*. These curves show that acetic acid affects chiefly the length of the lag phase, as do the dyes, whereas the bromopropionic acid, as shown by the fan-shaped curve typical for most antiseptics, affects chiefly the multiplication rate.

Phthalic, nicotinic, sulfanilic, and tellurous acids were too weak within the investigated range of pH 2.5 to 6.0 to permit any conclusions. Hydrozoic acid and aniline were equally efficient in the dissociated and in the undissociated forms. The dissociation constant of aniline is 4.6×10^{-10}. If the undissociated base were the effective agent, there should be a great decrease in efficiency between pH 5 and 4. This did not occur.

Sodium azide or hydrozoic acid is a very efficient antiseptic but the pH of the

medium does not affect the amount of azide necessary to prevent growth (figure 2).

TABLE 2

Inhibition of growth of Saccharomyces ellipsoideus at various acidities by different weak acids

ACID	pH	INHIBITING CONCENTRATIONS, MG PER 100 ML	DISSOCIATION CONSTANT	UNDISSOCIATED ACID		DISSOCIATION CONSTANT COMPUTED FROM INHIBITORY CONCENTRATIONS
				Fraction	mg per 100 ml	
Acetic	6.0	>2,000	1.86×10^{-5}	.051	>102	
	4.9	1,200-1,400		.411	493-575	1.8 to 3.7×10^{-5}
	4.0	500- 600		.813	421-506	
	3.1	400- 500		.977	390-488	
Propionic	5.8	>1,500	1.4×10^{-5}	.103	>155	
	4.9	700-800		.481	337-385	1.2 to 2.9×10^{-5}
	3.9	300-400		.903	271-361	
	3.1	300-400		.983	295-393	
Butyric	5.8	>2,000	1.48×10^{-5}	.0976	>195	
	4.9	400-500		.468	187-234	.56 to 3.3×10^{-5}
	4.1	200-300		.842	168-253	
	3.1	100-200		.982	98-196	
Monochloracetic	5.0	>1,000	1.55×10^{-3}	.0064	>6.4	
	4.0	300-400		.0606	18.2-24.2	2.6 to 36×10^{-3}
	3.0	40-50		.39	15.6-195	.57 to 2.6×10^{-3}
	2.5	20-30		.67	13.4-20.1	
Bromopropionic	4.8	400-500	1.08×10^{-3}	.0146	5.8-7.3	1.6 to 6.4×10^{-5}
	4.5	~300		.0288	~8.6	Impossible to calculate
	4.0	60-70		.0847	5.1-5.9	2.1 to 44×10^{-3}
	3.1	9-10		.422	3.7-4.2	1.5 to 5.6×10^{-3}
	2.6	3-5		.698	2.1-3.5	
Oxalic	3.0	1,200-1,400	$K_1 = 3.8 \times 10^{-2}$	.026	31-36	
	2.5	~600	$K_2 = 4.9 \times 10^{-5}$	.075	~45	
Selenious	6.1	150-225	$K_1 = 3 \times 10^{-3}$	.000263	.04- .06	
	5.4	150-225	$K_2 = 5 \times 10^{-5}$	.00133	.2- .3	
	4.0	>75		.0323	>2.4	
	3.1	30-45		.208	6.2-9.4	
Nitrous	6.1	180-600	4×10^{-4}	.00198	.36-1.19	
	5.3	18-60		.0123	.22- .74	

At pH 6, at which the azide ion predominates, the fan-shaped growth curves indicate that the antiseptic retards the rate of reproduction. However, at pH 4, at which the undissociated hydrozoic acid predominates, an entirely different type of curve is obtained. Hydrozoic acid causes an increase in the lag phase,

but after the organism once starts to multiply there is no effect on the rate of reproduction. Thus, although the azide ion and the undissociated hydrozoic acid are about equally efficient in preventing growth, they do so by different mechanisms. Lichstein and Soule (1944) found that, in inhibitory concentra-

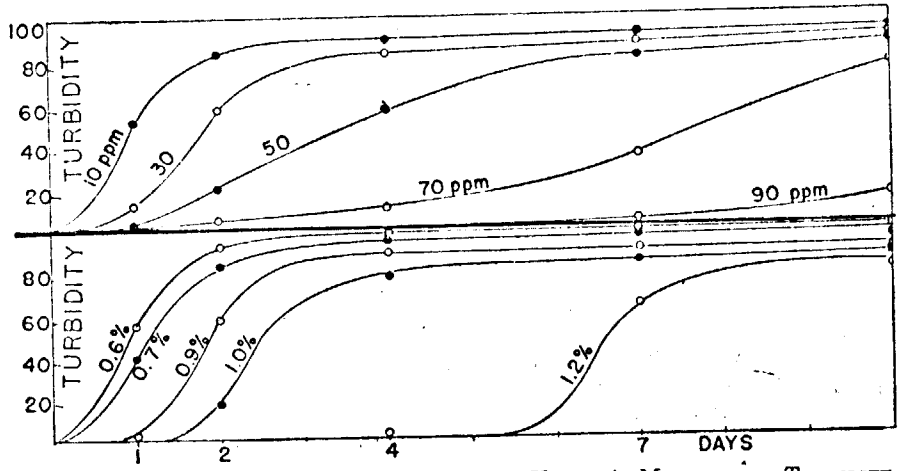

FIG. 1. EFFECT OF ANTISEPTICS ON THE GROWTH OF YEAST, AS MEASURED BY TURBIDITY
Above: α bromopropionic acid at pH 6. Below: acetic acid at pH 4.9.

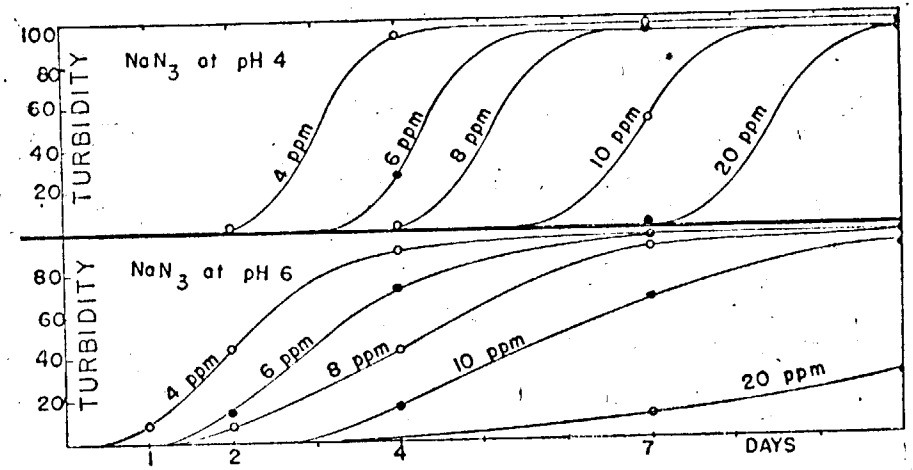

FIG. 2. EFFECT OF SODIUM AZIDE ON THE GROWTH OF YEAST, AS MEASURED BY TURBIDITY
Above: at pH 4. Below: at pH 6.

tions of azide, the respiratory quotients suggest that the respiration is almost entirely anaerobic. These experiments were conducted at pH 7.2 and therefore describe antisepsis by the azide ion.

SUMMARY

The increased antiseptic efficiency by increased acidity of weak acids has been proved in the majority of cases to be due to the increase of undissociated

acid molecules. The undissociated fraction is the chief, and in many cases the only, compound with antiseptic properties. This has been proved by demonstrating that, with any one antiseptic, the inhibiting concentrations contain at all acidities the same amount of undissociated acid. The experiments included acetic, propionic, butyric, chloracetic, bromopropionic, oxalic, selenious, and nitrous acids. Previous investigators have shown the same to be true for benzoic, salicylic, sulfurous, and hypochlorous acids, and also for phenol. It is possible to compute the approximate dissociation constant of weak acids from the inhibitory doses at different acidities.

Some antiseptics are equally efficient in the dissociated and in the undissociated forms. Hydrozoic acid and aniline are examples of this. It could be shown for hydrozoic acid that the undissociated acid inhibits by a different mechanism than does the azide ion.

Phthalic, nicotinic, sulfanilic, and tellurous acids as antiseptics were too weak within the investigated range of pH 2.5 to 6.0 to permit any conclusions.

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Skand. Vet. Tidksr. 33(11): 659-677

Poisoning of Horse in Connection with Lavage with a Solution of Acetic Acid.

By

A. Johannsen.

Summary.

The author gives an account of four cases of poisoning of horse occasioned by lavage with a solution of acetic acid. Six horses in a drove had been treated, for an oxyuris invasion, with, as was stated, a 2,5 % solution. All the horses sickened with symptoms of colic. Four of the animals displayed more serious tokens of disease, and two died, one first and then the second after an interval of a week.

Clinically, there existed general symptoms of an intoxication. The most important pathological-anatomical find consisted of highly developed alterations in the terminal part of the colon, in the form of diffuse, brownish-gray discoloration of the mucous membrane, together with extensive necrotic or ulcerous processes in the mucous membrane. Similar alterations existed in the rectum, together with highly developed inflammatory edema, chiefly in the submucosa. In one of the horses there was also found a fresh aspiration pneumonia.

For the sake of comparison, the author carried out experiments on rabbits in order to determine the concentration

which would be so sufficiently small that it would not give rise to alterations in the intestinal mucous membrane on rectal application. On lavage with acetic acid solutions with concentrations from 5 % down to as low as 0,5 %, the animals died after courses of 1—8 days. The animal which had been given a 5 % solution displayed inconsiderable local alterations, while the others showed more or less highly developed and extensive necrotic and inflammatory processes in the walls of the little colon and of the rectum. The animals which had been given 0,25 and 0,1 % solutions recovered. The rabbit which had received a 5 % solution probably died in consequence of a general action of the acetic acid, while the cause of death of the other rabbits was probably connected with intoxication caused by alterations in the mucous membrane, occasioned by corrossive action and disturbances of the circulation in the walls of the intestine.

Judging by the above-mentioned cases and the experiments, the value of a therapy employing acetic-acid lavage in cases of oxyuris invasion is probably little or nothing.

In connection with the description of these experiments, the author gives an orientation -- based on studies of the literature -- respecting the general effect of acetic acid on the organism when administered in various ways and doses, etc.

Meddelande från Statens veterinärmedicinska anstalt.
(Chef: Professor A. HJÄRRE.)

Skand. Vet. Tid Skr. 33(11): 659-677

Förgiftning hos häst i anslutning till lavemang med ättiksyralösning.

Av

K+A. Johannsen.

Förgiftningsfall med ättiksyra synes av litteraturen att döma vara ovanligt hos husdjuren, och då sådana förekommit har det oftast rört sig om terapevtisk överdosering. Någon gång har också utfodring med förjäst drank föranlett ättiksyraförgiftning (ECKHARDT, STOCKFLETH).

Inom humanmedicinen förekomma ättiksyraförgiftningar väsentligt oftare bl. a. genom av misstag intagen ättiksyra eller vid självmord och mord. Bland i litteraturen beskrivna fall utgöra självmorden övervägande antalet. Omkring sekelskiftet och fram till början av förra världskriget, d. v. s. tiden då mera koncentrerade ättiksyrapreparat började komma i handeln förekommo talrika fall framför allt i Tyskland och Ryssland. I dessa länder utfördes i samband härmed utförliga utredningar med ingående djurförsök för närmare kännedom om ättiksyrans toxiska verkningar, dosering m. m. Sådana arbeten ha utförts av bl. a. FRANZ, MARX, SCHÄFFER, SCHEPPEL, SCHIBKOW, KAZNELSON m. fl.

I litteraturen beskrives fall med ytterligt snabbt förlopp med *mors* inom några timmar, men också sådana, vilka gått till hälsa trots ganska stora intagna mängder. Detta förhållande visar att ättiksyran är ett synnerligen nyckfullt gift, som för sin verkan är beroende av ett flertal faktorer.

De allmänna kliniska symtomen vid förgiftning med svagare ättiksyra uppgivas vara nedsatt hjärtverksamhet, sänkning av kroppstemperaturen, relativ anemi till centrala nervsystemet och be-

nägenhet för blödning. Resorberad från blodet åstadkommer ättiksyran rubbning av bas-syrakvoten i hjärnan med medvetslöshet som följd. Vid förgiftning med starkare syra förhåller sig allmänsymtomen på likartat sätt, men här tillkomma dessutom symtom och komplikationer från lokala processer till följd av ättiksyrans etsande verkan, varför bilden närmast kommer att likna den, man finner vid förgiftning med vanliga oorganiska syror. Vid sådana fall får man således symtom på en akut korrosiv förgiftning med blodiga kräkningar och starka smärtyttringar. Ofta ses etsverkan på huden i ansiktet, på halsen och läpparna samt på slemhinnan i svalg och övre luftvägar. Kommer syran ned i tarmen inställer sig diarré med meteorism och stark törst. Döden inträder vanligen inom 24 timmar under koma och fortskridande nedsättning av hjärtverksamheten med påskyndad slapp och liten puls *(pulsus insensibilis)*. Någon gång kan komplikationer till övre luftvägarna dominera bilden med cyanos och död genom kvävning.

Vid sektion finner man bilder av varierande utseende allt efter den intagna ättiksyrans mängd och koncentration, tiden för dess inverkan, tarmkanalens fyllnadsgrad m. m. Efter inverkan av starkare syra föreligger oftast etsning och uppmjukning med vitgrå färg av huden och av slemhinnor i övre delarna av digestionskanalen och eventuellt även i larynx. I magsäcken och främre delen av tarmen finner man en ringa mängd innehåll med ett smutsigt gråbrunt utseende. Ibland har det lukt av ättiksyra och tarminnehållet kan ge sur reaktion mot lackmus. Slemhinnorna i magsäck och tarm visa ödem, blödningar, nekrotiska förändringar, defekter med vulstartade kanter och ibland förekommer perforationer. Kärlen stå fyllda med segt koagulerat blod.

Njurarna anges vara svällda, ha spänd kapsel och ett fläckigt ödematöst utseende. Barken förefaller svullen och de hyperemiska märgstrålarna framträda tydligt. Urinen har ofta alkalisk reaktion.

Många gånger förefinnas mer eller mindre utbredda och höggradiga, lokala inflammatoriska processer å bukhinnan med fibrinösa adherenser.

Mjälten är svullen, har spänd kapsel samt fast konsistens. Snittytan är blodrik och till färgen mörkbrun eller blåröd.

Vid mikroskopisk undersökning av blodet finner man ofta skrumpna eller uppblåsta och delvis avfärgade röda blodkroppar. Blodserum kan vara rödfärgat, och spektroskopiskt kan hemoglobin påvisas i såväl blodserum som i urin.

Ett påpekat observandum är, att urinen ofta ger alkalisk reaktion, ett förhållande som anses specifikt för förgiftning med organiska syror.

De histologiska njurförändringarna beskrivas som en hårdformigt bristande färgbarhet av kärnorna i såväl glomeruli som i njurkanalernas epitel. I kanalerna förekomma rikligt med hemoglobin och cylindrar med tillplattning av epitelet.

Inom veterinärmedicinen användes ättiksyra ofta som lavemang vid behandling av oxyurisinvasion hos häst. BERGSTRANDS vademekum upptager i korthet denna behandlingsmetod utan närmare angivande av doser eller koncentration. Vanligtvis bereder veterinären lösningen omedelbart före användandet genom att till 1 lit. vatten sätta c:a 1 matsked ättiksyra. Den koncentrerade ättiksyran, isättikan, farmakopens *Acid. aceticum concentratum*, innehåller minst 96 % ättiksyra och *Acidum aceticum* omkring 29 %. Lösningar beredda på ovan angivet sätt erhålla således en ungefärlig styrka av 1,5 resp. 0,4 %. Sistnämnda lösning har använts otaliga gånger utan olägenheter. Metoden är emellertid långt ifrån ofarlig, om den utövas av personer som icke närmare känna dess risker. Gränsområdet mellan terapevtisk dos och sådan som framkallar toxiska symtom och anmärkningsvärda patologisk-anatomiska förändringar är nämligen synnerligen liten. Som ett ytterligare riskmoment vid användandet av ättiksyrelavemang får man räkna med den växlande koncentrationen hos de i handeln förekommande olika preparaten, vilket framgår av följande sammanställning:

Acidum aceticum concentratum	96 %
Acidum aceticum dilutum	30 %
Matättika	6 %
Bordsättika	2— 4 %
Ättiksprit	10—12 %
Ättikessens	50—80 %
Träättika	5— 7 %.

Denna senare innehåller dessutom metylalkohol, fenoler och naftalin.

I början av 1943 behandlades i ett stall i Stockholmstrakten 6 st. hästar med ättiksyralavemang mot oxyuris. Enligt uppgift användes härvid c:a 15 liter lösning med 2,5 %:s

styrka till varje djur. Inom kort insjuknade samtliga hästar i koliksymtom med tydliga buksmärtor, starka krystningar och skummande uttömningar från anus.

Fyra av de värst angripna infördes till Veterinärhögskolans medicinska klinik, varifrån följande sjukdomsberättelser erhållits (prof. CARLSTRÖM).

Fall n:r 1. Med. klin. J. nr 18/43. Arbetshäst, vallack, 12 år. I anamnesen meddelades endast, att hästen förutom ovannämnda symtom visat tilltagande oro. Status vid ankomsten den $^{20}/_{1}$: Hästen, som har medelgott hull, är påfallande slö och ligger gärna. Temp. 39,0°. Conjunctivalslemhinnorna äro diffust rodnade och kärlinjicerade samt ha ett något grådaskigt, ikteriskt utseende. Hjärtfrekvens 70/min. Andningsfrekvens 24/min. Andningtypen är blandad costal abdominell. Hästen synes ogärna vilja arbeta med bukmuskulaturen, antagligen beroende på smärtförnimmelser från peritoneum. Vid rektalundersökning kännes tarmen fylld av en ganska lös, ej formad träck. Väggen i rectum kännes ödematös och slemhinnan synes starkt diffust rodnad. I rectum inlagt lackmuspapper visar neutral reaktion. Faecesundersökning: Lös konsistens, grågul färg, neutral reaktion, katalas: 0, urobilin 5+, blod 0, digestion: god, mask: strongylider.

Klinisk diagnos: Ättiksyraförgiftning.

Hästen behandlades med oljelavemang och linfröslem *per os*. Utskrevs frisk den $^{22}/_{1}$.

Fall nr 2. Med. klin. J. nr 19/43. Arbetshäst, vallack, 9 år. Samma anamnes som i föregående fall. Status vid ankomsten $^{21}/_{1}$: Hästen har slapp hållning, slött temperament och ruggig hårrem. Hullet är dåligt. Synliga slemhinnor äro diffust rodnade och kärlinjicerade samt ha ett grådaskigt och ikteriskt utseende. Hjärtfrekvens 60/min. Andningsfrekvens 20/min. Aptiten är dålig och buken uppdragen. Temp. 39,4°. Rectums vägg är ödematös och slemhinnan starkt rodnad. Innehållet är illaluktande.

Urin: Albumin +. Blod: 12,0 milj. röda blodkroppar per kbmm. SAHLI 68 %, MEULENGRACHT 24.

Klinisk diagnos: Ättiksyraförgiftning.

Den $^{23}/_{1}$ är allmäntillståndet betydligt försämrat. Puls 120/min: Andningsfrekvens 30/min. Vid punktion av bukhålan erhålles hemorrhagiskt transsudat. Vid sondering av magsäcken märkes vidrig lukt. Äggvitehalten i blodserum bestämdes enligt ESSBACH till 6 %. (Blodserum från frisk häst visade enligt samma metod

8 %). Vid bestämning av de röda blodkropparnas resistens gentemot hypotoniska koksaltlösningar erhölls begynnande hemolys vid 0,76 % och fullständig sådan vid 0,42 %. Allmäntillståndet försämrades än ytterligare och hästen dog samma dag.

Samtliga organ undersöktes å Veterinärhögskolans patologisk-anatomiska avdelning (P. 110/43).

I œsophagus föreligger en idiopatisk muscularishypertrofi. Tunntarmen visar framför allt i bakre partierna rikligt med i regel små subserösa blödningar och liknande förekomma i tunntarmskröset nära dess fäste på tarmen. Kröslymfkörtlarna äro tydligt hyperemiska. Cœcum och stora colon visa frånsett en del smärre subserösa blödningar inga anmärkningsvärda förändringar. De äro väl fyllda med tarminnehåll av normalt utseende.

I lilla colon är innehållet tämligen sparsamt, icke format till bollar, samt i de bakre 2,5 m. starkt blodfärgat. Tarmlumen är i detta avsnitt något dilaterat. Tarmväggen är särskilt i mellersta tredjedelen starkt ödematöst förtjockad (upp till över 1 cm) och säte för utbredda difteroida-nekrotiserande processer av brungrå färg. På en del ställen äro pseudomembranerna ganska lätt avskrapbara. Kärlen i tarmväggen äro i stor utsträckning tromboserade. Serosan är starkt hyperemisk och visar rikligt med punkt- till 2-öringstora blödningar. Förändringarna i slemhinnan sluta med en ganska skarp gräns c:a 2,5 m. framför anus. Rectum saknar innehåll och den väsentligaste förändringen är här ett företrädesvis submuköst ödem, varigenom väggen blivit upp till 3 cm tjock.

I övriga organ har inga anmärkningsvärda förändringar kunnat iakttagas.

Vid histologisk undersökning påvisades i lilla colon en akut difteroid-nekrotiserande, eller ibland ulcerös enterit. I den ödematösa submukosan finns tämligen rikligt med fibrin och ett flertal kapillära kärl äro tromboserade. Venerna i kröset äro fyllda med koagulerat blod. Artärerna visa däremot inga förändringar.

Vid bakteriologisk undersökning av mjälte och kröslymfkörtlar kunde någon bakteriell infektion icke påvisas.

Fall nr 3. Med. klin. J. nr 20/43. Arbetshäst, vallack, 9 år.

Status vid ankomsten den $^{22}/_{1}$: Hästen har slapp hållning, slött temperament, dåligt hull och ruggig hårrem. Slemhinnorna äro diffust rodnade, samt något ikteriska. Hjärtfrekvens 66/min. Andningsfrekvens 18/min. Dålig aptit, uppdragen buk. Rektalslemhinnan är ödematös och kraftigt rodnad. Faeces har lös konsistens, brungul färg, neutral reaktion. Katalas +, urobilin +, blod och mask: 0. Digestion: god. Urinen har alkalisk reaktion och

spec. vikt 1,032. Sediment avsättes i mycket liten mängd och består av kalciumkarbonat och detritus. Blod: 10,6 milj. röda blodkroppar, SAHLI 87 %, MEULENGRACHT 14.

Klinisk diagnos: Ättiksyraförgiftning.

Den $^{28}/_{1}$ är allmäntillståndet tydligt försämrat. Temp. 40,1°. Puls 60/min. Hästen äter obetydligt och ligger mest. Den $^{1}/_{2}$: puls 90, andningsfrekvens 36/min. Hästen ligger gärna, men kan resa sig själv. Den $^{2}/_{2}$ är allmäntillståndet ytterligare försämrat och djuret förmår ej resa sig utan ligger utsträckt på sidan. Lindriga krampanfall förekomma. Äggvitehalten i blodserum enligt ESSBACH 6 %. Hästen dog samma dag.

I likhet med föregående fall undersöktes samtliga organ på Veterinärhögskolans patologisk-anatomiska avdelning. Härvid påvisades förändringar i colon av i stort sett samma karaktär som i föregående fall.

Innehållet i stora colon är av normal mängd och konsistens. Vid bäckenflexuren är slemhinnan i andra colonläget på ett några dm:s långt område måttligt hyperemisk. I lilla colon är innehållet betydligt lösare än normalt och slemhinnan med undantag för den närmaste metern intill fjärde colonläget säte för utbredda gärna längsgående ulcerationer med gråsvart botten, i vilka finnas mindre epiteliserade öar. Förändringarna äro starkast utpräglade vid tænierna, medan slemhinnan i haustrabildningarna på relativt stora områden är tämligen oskadd. Rectum visar i stort sett samma förändringar som lilla colon. I *ampulla recti* är slemhinnan mindre skadad än i övriga avsnitt.

Lungorna äro i spetsarna och i främre, nedre delarna av diafragmaflikarna säte för härdformiga, nekrotiserande pneumbnier med på vissa ställen kaverner med gråbrunt innehåll.

Mjälten visar bilden av en akut infektionsmjälte,

Histologisk undersökning av lilla colon visar en ulcerös colit. I periferien av ovannämnda slemhinneöar synas tydliga regenerationsbilder. Botten av ulcera utgöres av en subakut granulationsvävnad utan epitelbeklädnad. *Muscularis mucosae* synes vara helt förstörd.

I myokardiet föreligger måttlig findroppig förfettning av muskeltrådarna jämte en härdformig akut myokardit med trådsönderfall och rundcellsinfiltration.

Vid bakteriologisk undersökning påvisades infektion med *Streptococcus schütz* i mjälte, kröslymfkörtlar och lunga.

Fall nr 4. Med. klin. J. nr 21/43. Arbetshäst, sto, c:a 18 år.

Status den $^{22}/_{1}$: Slapp hållning, slött temperament och relativt dåligt hull. Slemhinnorna äro starkt rodnade och något ikteriskt

missfärgade. Hjärtfrekvens 60/min. Andningsfrekvens 20/min. Hästen äter med relativt god aptit. Vid rektalundersökning kännes tarmväggen förtjockad och atonisk. Slemhinnan i rectum är starkt, diffust rodnad. Urinen har neutral reaktion. spec. vikt 1,027 och albumin +. Blod: 10,6 milj. röda blodkroppar, SAHLI 60 %, MEULENGRACHT 13. Äggvitehalt i serum enligt ESSBACH 6 %.

Klinisk diagnos: Ättiksyraförgiftning.

Efter behandling med oljelavemang samt linfröslem *per os* förbättrades allmäntillståndet dag för dag och hästen utskrevs som frisk ²/₂.

Kliniskt ha de ovan relaterade fyra fallen visat tämligen enhetliga sjukdomsbilder. Sannolikt torde symtomen få tillskrivas såväl en allmän inverkan på organismen från resorberad ättiksyra, som av lavemangen framkallade lokala förändringar i tarmen och återverkningar från dessa. Genomgående för samtliga är det slöa temperamentet och den slappa hållningen liksom de kärlinjicerade och ikteriska slemhinnorna. Symtombilden tyder på en intoxikation. I de fall fullständig urinundersökning skett har förekomsten av albumin påvisats. Blodbilden visar intet anmärkningsvärt med undantag av något ökat bilirubinvärde och sänkt resistens hos de röda blodkropparna gentemot hypotoniska koksaltlösningar (begynnande hemolys vid 0,76 % i ett fall).

I de två fall, som undersökts patolog-anatomiskt, utgjordes de huvudsakligaste förändringarna av en akut — subakut nekrotiserande eller ulcerös colit, lokaliserad till lilla colon. Denna var förbunden med höggradigt inflammatoriskt ödem i submukosan med trombosering av slemhinnans finare kärl. I lilla colons krös förelåg stark fyllnad i venerna med anmärkningsvärt väl koagulerat blod. Den i sista fallet beskrivna aspirationspneumonin får uppfattas som sekundär komplikation.

Med anledning av förgiftningsfall hos människa har åtskilliga forskare gjort närmare undersökningar över experimentella ättiksyreförgiftningar hos olika försöksdjur. Vid dessa har applikationen i regel skett *per os*.

Redan 1831 fann ORFILA att c:a 150 gr vanlig vinättika (konc. ej angiven) för en hund medförde döden på 12—15 timmar.

KOBERT ansåg den sannolika dödliga dosen för köttätare vara väsentligt större än för växtätare. (I förh. 15—0,49 syra per kg kroppsvikt.) SCHEPPEL anställde en del försök på hund och för att hindra djuren att kräkas upp ättiksyran gjordes efter ingivningen oesophagotomi med avbindning av nedre oesophagusdelen. Det visade sig att 1 ml. isättika per kg kroppsvikt ledde till döden inom relativt kort tid och den tredubbla dosen t. o. m. inom 24 timmar. SCHIBKOW och WERTEGRADOW visade vid försök på kaniner och hundar att de tidigare som letala angivna doserna vid tillförsel *per os* voro alldeles för höga, och att en många gånger mindre dos hade dödlig verkan. För att framkalla toxisk verkan på slemhinnor anger SCHÄFFER ättiksyra med en koncentration av minst 12 % vara erforderlig. Konjunktivalslemhinnan hos marsvin uppges giva uttryck för toxisk reaktion av en 9 % lösning, under det att, för att åstadkomma verkan på huden, erfordras minst 30 % sådan.

BOKAI anger att ½—1 % lösning av ättiksyra insprutat i tarmen på försöksdjur framkallar en kraftigt ökad peristaltik i hela tunntarmen och långvarig muskelkramp i grovtarmen. Vid större doser av samma koncentration uppkommer diarré och inflammatoriska processer. Detta i samband med mina här nedan relaterade försök synes mig tala för att åtminstone tarmslemhinnan reagerar för betydligt svagare koncentrationer av ättiksyra.

De patologisk-anatomiska förändringarna vid experimentell ättiksyraförgiftning hos försöksdjur ha beskrivits av ORFILA, HERTWIG, MITCHERLICH, HEINE, SCHEPPEL, SCHIBKOW, WERTEGRADOW m. fl.

Vid syratillförsel *per os* äro förändringarna synnerligen variabla beroende bl. a. på syrans koncentration och mängd. I allmänhet har man funnit mer eller mindre höggradig etsverkan i övre delarna av digestionskanalen, mörkröd—brunröd slemmig beläggning på magslemhinnan, vilken ofta ses förtjockad och ödematös med histologiskt påvisbar nekros i epitelet och uppsvällda förtjockade bindvävstrådar. I inre organ har man vanligtvis funnit stashyperemi eller stasödem.

Vid försök utförda av WERTEGRADOW genom att droppvis införa syra *per os* kunde blott obetydliga lokala förändringar iakttagas i oesophagus och ytterst lindriga sådana eller inga alls i magsäcken. Däremot fann han till följd av aspiration av ättiksyraångor diffus bronkopneumoni och ibland larynxstenos.

SCHIBKOW har vid försök med möss visat att inandning av ättiksyraångor leder till döden under intoxikationssymtom på tider

varierande mellan 38 minuter och 8½ dagar. Härvid erfordras icke nödvändigtvis koncentrerad syra utan mera utspädd sådan har samma effekt. Makroskopiskt voro lungorna starkt hyperemiska och blodet i lungkärlen hade ett koagulerat och segt utseende. Samma var förhållandet i magsäckens kärl vid resorption från magslemhinnan. Den histopatologiska undersökningen visade tydligt utpräglad hyperemi med diapedesisblödning och ödem i lungalveolerna samt lindrigare perivaskulära inflammatoriska infiltrat, d. v. s. initialstadium till en bronkopneumoni. I dessa försök får man räkna med att ättiksyran genom att ångorna, som uppblandats med luft, haft en relativt låg koncentration vid sin inverkan å vävnaderna.

Genom försök med intravenös injektion av utspädd ättiksyra studerade HEINE de röda blodkropparnas reaktion mot syran. Han påvisade därvid vissa formförändringar hos de röda blodkropparna jämte sönderfall och utträde av hemoglobin. Vid sektionen iakttogs hyperemi och ödem i lungorna, staslever samt i den injicerade venen fasta blodkoagel, vilka sträckte sig ända till hjärtat.

Experimentella ättiksyraförgiftningar efter rektal applikation av syran har jag icke funnit omnämnda i litteraturen. Med tanke på de ovan relaterade förgiftningsfallen hos häst har jag anställt en del sådana försök med kaniner. Avsikten var närmast att utröna vilken syrakoncentration i lavemangen, som var erforderlig för att undvika lokala förändringar å tarmslemhinnan samt allmänverkan från ättiksyran.

Kanin nr 1 (vikt 2 kg) erhöll den $^{18}/_{3}$ c:a 30 ml 5 % ättiksyralösning rektalt med rekordspruta. Sjukdomssymtom inställde sig tämligen snart, i det att kaninen upphörde att äta och satt hopkrupen i ett hörn av buren. Den dog den $^{20}/_{3}$.

Sektion: Slemhinnan i främre delen av rectum har ett torrt, brungult utseende på ett område av c:a 5 cm. Innehållet i hela colon och cœcums tjocka del har anmärkningsvärt fast consistens. Serosan å rectum och bakre delen av colon visar sparsamt med smärre blödningar och lindriga fibrinbeläggningar. Histologisk undersökning av rectum visar nekrotisering av slemhinneepitelet, härdformigt inflammatoriskt ödem i submukosan samt akut fibrinös peritonit. I skelettmuskulaturen förekom en lindrig härdformig vaxartad degeneration.

Kanin nr 2 (vikt 1,73 kg) erhöll den $^{18}/_{3}$ c:a 30 ml 2,5 % ättiksyralösning rektalt med rekordspruta. Den visade liknande sjukdomssymtom som föregående och dog den $^{26}/_{3}$.

Sektion: I colon omkring 5 cm från anus finns å ett c:a 5 cm långt område en höggradig, nekrotiserande inflammation i tarmväggen samt omedelbart framför detta parti mindre fläckformiga liknande processer. I framförliggande del av colon och cœcum föreligger höggradig koprostas.

Histologisk undersökning av colon visar en höggradig akut, difteroid-nekrotiserande colit med tämligen starkt inflammatoriskt ödem i submukosan. Fibrinfärgning enligt WEIGERT ger negativt resultat.

Kanin nr 3 (vikt 1,36 kg) erhöll den $^{18}/_{3}$ c:a 30 ml 1,25 % ättiksyralösning med rekordspruta. Liknande sjukdomssymtom som hos de föregående djuren. Dog den $^{26}/_{3}$.

Sektion: I rectum och så gott som hela colon finnas fläckformiga, difteroida nekrotiserande processer i slemhinnan, vilka äro mest höggradiga i de tarmdelar, som ligga invid kröslymfkörtlarna, där hela tarmväggen förefaller nekrotiserad och där inflammationen gripit över på dessa.

Histologisk undersökning av colon och rectum visar en höggradig difteroid-nekrotiserande inflammation med stark leukocytinfiltration. Fibrinfärgning ger negativt resultat. På andra ställen förlöper processen som akut ulcererande enterit. Tarmserosan visar en akut varig peritonit. I myokardiet föreligger härdformig akut myokardit.

Kanin nr 4 (vikt 3,1 kg) erhöll den $^{7}/_{1}$ lavemang med 0,75 % ättiksyralösning. Efter likartade sjukdomssymtom som hos de föregående dog djuret den $^{10}/_{4}$.

Sektion: Cœcum och colon, som har ett fast innehåll, visa multipla subserösa punkt- och fläckformiga blödningar. På övergången mellan stora och lilla colon är slemhinnan hyperemisk och säte för strimformiga nekrotiska processer. Vid ungefär lilla colons mitt är tarmen starkare dilaterad och visar diffusa nekrotiska processer i slemhinnan. I bakre delen av det nekrotiska partiet är slemhinnan kraftigt veckad och gråfärgad samt skarpt avgränsad från den hyperemiska rektalslemhinnan. På peritoneum finnes utbredda akuta inflammatoriska processer med fibrinösa adherenser mellan olika tarmavsnitt.

Histologisk undersökning av colons främre del visar nekrotiska processer i slemhinnans ytligare skikt samt höggradigt inflammatoriskt ödem i submukosan. I colons mellersta och bakre

delar är praktiskt taget hela tarmväggen fullständigt nekrotiserad. Förändringarna äro starkast i de anala partierna. I skelettmuskulaturen finnes en måttlig vaxartad degeneration med skolligt-skivigt sönderfall av en del muskeltrådar samt härdformig findroppig förfettning. Njurarna visa en måttlig findroppig enkel fettinfiltration i samlingsrörens epitel.

Kanin nr 5 (vikt 3,4 kg) erhöll den 7/4 lavemang med 0,5 % ättiksyrelösning. Sjukdomssymtom som hos de föregående djuren och djuret dog den 12/4.

Sektion: Vid lilla colons mitt finnes en c:a 10 cm lång, cirkulärt utbredd nekrotisk härd i slemhinnan. Analt härom äro slemhinnenekroserna mera strimformiga eller fläckformiga och upphöra c:a 10 à 15 cm framför anus.

Histologisk undersökning av colons mellersta del visar fullständig nekros i hela tarmväggen. I de bakre delarna finnas härdformiga nekroser i slemhinnan med demarkerade cellinfiltrationer och ödem i submukosan. Njurarna visa lindrig enkel fettinfiltration i samlingsrörens epitel. I myokardiet förekommer en måttlig härdformig subakut myokardit samt härdformig findroppig förfettning. Levern visar en lindrig akut stas jämte lindrig diffus stordroppig, enkel fettinfiltration.

Kaninerna nr 6 och 7 (vikt 2,4 resp. 2,7 kg) erhöllo den 8/5 lavemang med 0,25 % ättiksyralösning. Båda djuren visade de närmaste dagarna efter behandlingen slöhet och minskad foderlust, men tillfrisknade senare fullständigt.

Som synes äro de vid försöken påvisade tarmförändringarna i stort sett likartade och yttra sig i form av difteroida-nekrotiserande enteriter av växlande grad och utbredning. Anmärkningsvärt är de med hänsyn till koncentrationen av ingiven syra tämligen obetydliga förändringarna hos kanin nr 1. I detta fall torde man till stor del få räkna med en allvarligare allmänverkan av ättiksyran än hos de övriga djuren, där lokal etsverkan och cirkulationsrubbningar i tarmväggen synbarligen dominerat bilden. I tre fall har koprostas förelegat, troligen beroende på en av ättiksyran framkallad atoni i colon. Akut peritonit har påvisats i tre fall, vilken får anses ha kommit sekundärt till tarmförändringarna. Kaninerna nr 3 och 5 visade härdformiga akuta myokarditer och nr 1 och 4 vaxartad degeneration i skelettmuskulaturen. Dessa senare

förändringar torde knappast ha något mera direkt samband med ättiksyreförgiftningen.

Tabellarisk översikt över försöken.

Sektionsfynd m. m.

Nr	Konc.	Död inom antal dygn	Nekrotiserande colit (grad och utbredning) + ++ +++	Koprostas	Peritonit	Myokardit	Vaxdeg.
1	5 %	2	(+)	+	+	—	+
2	2,5 %	8	++	+	—	—	—
3	1,2 %	8	+++	—	+	+	—
4	0,75 %	3	+++	+	+	—	+
5	0,5 %	5	++(+)	—	—	+	—
6	0,25 %	Frisk					
7	0,1 %	Frisk					

Av försöken att döma synas de svagare syrakoncentrationerna ha givit mera utpräglade förändringar än de starkare. Försöken äro emellertid allt för få för att tillåta någon säker sådan slutsats och i övrigt får man räkna med att åtskilliga faktorer förutom syrans koncentration måste ha influerat på graden och utbredningen av tarmförändringarna, så t. ex. bl. a. tarmens fyllnadsgrad och tiden för syrans inverkan. Genom krystningar hos djuren kan såväl mängden av den insprutade syran som tiden för dennas inverkan på slemhinnan ha växlat. Att exakt fixera mängden ingiven syra hos de olika försöksdjuren är överhuvudtaget icke möjligt. Enbart en irritation av sprutspetsen var tillräckligt för att djuret skulle avlämna en större eller mindre mängd av lösningen. En svagare syra retar givetvis mindre, varför den ingivna syramängden i dessa fall kan ha utövat sin verkan under en relativt längre tid med åtföljande höggradigare förändringar i tarmväggen genom lokala cirkulationsrubbningar. En mera koncentrerad syra torde genom starkare irritation av slemhinnan endast haft

tillfälle att inverka under kortare tid och därvid endast framkallat ytligare etsverkan.

Förändringarna uppkomna i samband med ättiksyrans inverkan på rektalslemhinna hos mina försöksdjur synas i princip vara analoga med dem, andra forskare funnit i övre delarna av digestionskanalen hos människa och djur efter peroral applikation.

Ättiksyra betraktas i allmänhet som ett blodgift eller protoplasmatiskt cellgift, som förändrar eller förstör den levande protoplasmans struktur.

Alla syror ha en hemolyserande förmåga varvid H-jonerna anses ha en katalytisk verkan. Hemolysens grad är beroende av syrans natur, koncentration, tiden för dess inverkan, temp. m. m. Trots att ättiksyran är relativt obetydligt dissocierad har den dock en tämligen intensiv hemolytisk verkan, vilket skulle bero på att erytrocyternas lipoider äro lättlösliga i ättiksyra. Härigenom anses ättiksyrans toxiska verkan i jämförelse med oorganiska dissocierade syror höjas c:a 4 ggr. Man kan således skilja mellan en kemisk och en osmotisk tolerans hos de röda blodkropparna gentemot hemolyserande ämnen. Enligt OVERTON är den protoplasmatiska cellmembranen genomtränglig för icke dissocierade syror och enligt LOEB diffundera dessa syror snabbare än oorganiska.

Vid vissa inre sjukdomar såsom kancer, leversjukdomar m. fl. anges blodkropparnas resistens mot ättiksyra vara stegrad under det att den vid diabetes, rubbningar i fettomsättningen, uremi, helminthiasis m. fl. har ett lägre värde.

Enligt ARRHENIUS har ättiksyra en dubbelverkan på blodet. Tillförseln av en stor syramängd verkar hastigt koagulerande på erytrocyternas äggviteämnen under bildning av flockor. Vid tillförsel av mindre mängd svagare syra blir resultatet i stället en agglutination. Ju starkare denna agglutination är, desto mindre blir hemolysen beroende på att den koagulerade ytan av de agglutinerade blodkropparna hindrar utträde av hemoglobin och vidare påverkan av syran. Nedsättes agglu-

tinationen under ett visst värde, ökas åter hemolysen. Under ett visst koncentrationsvärde upphör densamma.

Närvaron av koagulations- och agglutinationsbefordrande ämnen i blodbanorna ger givetvis ökade möjligheter för uppkomsten av tromboser med lokala cirkulationsrubbningar och infarceringar som resultat. Åtskilliga forskare ha iakttagit kapillärtromboser särskilt i magsäckens kärl vid ättiksyraförgiftning hos människa. De bildade tromberna hindra i viss mån vidare resorption av syra till blodet.

Genom direkt etsverkan och trombosbildning få de lokala slemhinneförändringarna sin naturliga förklaring. Vid relativt svaga koncentrationer synes den toxiska verkan till stor del vara beroende av syrans löslighet i lipoider, varigenom den får växlande möjligheter att tränga på djupet och framkalla vävnadsskador.

Den största mängden ättiksyra anses resorberas via lymfkärlen och här äro betingelserna för uppkomsten av trombos betydligt ogynnsammare, då lymfan jämfört med blodet är fattigare på äggvita och formade beståndsdelar. Den innehåller dessutom rikligt med från tarmen resorberade ämnen av lipoid karaktär, vilket ger den möjlighet att binda större mängder syra.

I många fall särskilt vid intagande av en större mängd syra med starkare koncentration är förloppet mycket hastigt (döden inträder redan inom några timmar) utan att lokala processer hinna utvecklas. I sådana fall är det allmänpåverkan, som dominerar bilden. Sedan syran upptagits i blodbanorna framträder tecken på acidos. En akut ättiksyraförgiftning ger sig tillkänna på likartat sätt som diabetiskt koma, vilket ju också erhåller sin förklaring genom ökad syrahalt i blodet eller sänkt blodalkalescens. Utdrages förloppet utan att blodets alkalireserv helt förbrukas, domineras bilden i stället av sekundära processer under det att symtomen på acidos försvinna.

Nedsatt blodalkalescens för med sig sänkning av oxidationsprocesserna inom organismen. Till följd härav inträder en nedsatt pulsfrekvens och mindre sänkning av kroppstem-

peraturen. Hjärtverksamheten minskas, varför relativ anemi till centrala nervsystemet och benägenhet för blödningar inställer sig. Centrala nervsystemet är ju ytterligt känsligt för rubbningar i blodets syra-bas kvot. När denna rubbning blir tillräckligt stor inträder medvetslöshet med påskyndad slapp puls *(pulsus insensibilis)*, koma och död.

Den närmaste dödsorsaken vid okomplicerad ättiksyraförgiftning anser man vara hjärtförlamning, vilket bl. a. bevisats vid försök, då ättiksyra införts i magsäcken på djur under morfinnarkos. Man har då iakttagit att andningsverksamheten fortsatt en stund efter hjärtstillestånd. Samma resultat har man kommit till vid intravenös injektion av starkt utspädd ättiksyra. Vidare har man genom försök (BOBRIK) med avskärning av båda *Nerv. vagii* på ättiksyraförgiftade djur ävenledes visat att hjärtpåverkan sker direkt genom blodet och icke är av nervös natur.

I alla de fall där i tarmen påvisats utbredda, nekrotiska processer, vilka för sin utveckling fordrar en viss tid, torde dödsorsaken icke kunna förklaras som en ren ättiksyraförgiftning utan snarare som en sådan komplicerad med en intoxikation från tarmen genom de där förefintliga förändringarna.

Som synes är ättiksyran ett synnerligen nyckfullt gift med komplicerad inverkan på organismen, vilket inbjuder till försiktighet vid dess användning inom terapin. Då såväl de ovan relaterade förgiftningsfallen hos häst som mina experimentella undersökningar på kanin visat att ättiksyra applicerad på rektalslemhinnan endast är ofarlig i mycket svag koncentration, bör en terapi med ättiksyrelavemang vid behandling av oxyurisinvasion hos häst, om icke helt undvikas så under alla förhållanden utföras med vederbörlig försiktighet. Frågan är för övrigt, om inte en sådan terapi får anses vara mera riskabel än nyttig, enär den svaga koncentrationen av ättiksyra som bör användas, kan uppväcka tvivelsmål om syran överhuvudtaget har någon inverkan på parasitinvasionen.

Zusammenfassung.

Verf. berichtet über vier Fälle von Vergiftung bei Pferden infolge von Essigsäurelösungsklystieren. In einem Bestande wurden 6 Pferde gegen Oxyurisinvasion angeblich mit 2,5-proz. Lösung behandelt. Alle Pferde erkrankten unter Koliksymptomen. Vier Tiere zeigten ernsthaftere Krankheitssymptome und zwei starben mit einen Zeitinterwall von 7 Tagen.

Klinisch lagen allgemeine Anzeichen von Intoxikation vor. Der hauptsächlichste patholog-anatomische Befund bestand in hochgradigen Veränderungen im kleinen Colon in Form diffuser, braungrauer Verfärbung der Schleimhaut mit ausgebreiteten nekrotischen oder ulcerösen Prozessen in derselben. In Rectum lagen ähnliche Veränderungen sowie ausserdem ein vorzugsweise submucös gelegenes, entzündliches Ödem vor. Bei einem der Tiere fand sich zudem eine frische Aspirationspneumonie vor.

Zwecks Vergleichs stellte Verf. Versuche mit Kaninchen an, um die Konzentration festzustellen, welche genügend gering war, um bei rektaler Applikation keine Veränderungen in der Darmschleimhaut hervorzurufen. Rektale Infusion von Essigsäurelösungen von Konzentrationen von 5 % bis zu 0,5 % verursachten den Tod der Tiere im Laufe von 1—8 Tagen. Dasjenige Tier, welches eine 5-proz. Lösung erhalten hatte, zeigte unbedeutende lokale Veränderungen, während bei den übrigen mehr oder weniger hochgradige und ausgebreitete nekrotische und entzündliche Prozesse in der Wand des kleinen Colons und Rectums vorhanden waren. Die mit 0,25- und 0,1-proz. Lösung behandelten Tiere genasen. Das Kaninchen, welches 5-proz. Lözung erhalten hatte, dürfte infolge allgemeiner Wirkung seitens der Essigsäure gestorben sein, während die Todesursache bei den übrigen auf eine Intoxikation infolge der durch Ätzwirkung und Zirkulationsstörungen in der Darmwand zustande gekommenen Schleimhautveränderungen zurückszuführen sein dürfte.

Mit Rücksicht auf die genannten Fälle muss der Wert

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einer Therapie mit Essigsäureklystieren gegen Oxyurisinvasion als sehr zweifelhaft beurteilt werden.

Im Anschluss an die Beschreibung dieser Versuche gibt Verf. eine auf Literaturstudien gestützte Orientierung über den allgemeinen Einfluss der Essigsäure auf den Organismus bei verschiedener Applikationsweise, Dosierung u. a.

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RELEASE OF HISTAMINE INTO GASTRIC VENOUS BLOOD FOLLOWING INJURY BY ACETIC OR SALICYLIC ACID

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It has frequently been suggested[1, 2] that histamine is liberated within the gastric mucosa during stressful or damaging situations. Davenport has shown that, when the mucosa is injured by salicylic or acetic acid, a number of changes occur,[3-5] some of which could be caused by an intramucosal liberation of histamine. The flow of fluid from the damaged mucosa exceeds that of control experiments, indicating increased capillary permeability as well as increased mucosal permeability. In addition, plasma proteins appear in the gastric contents in excess of erythrocytes, which is evidence that the volume changes are not primarily caused by bleeding into the lumen. Whether acid secretion increases as well as volume flow has not been determined, since hydrogen ions move through the damaged mucosa toward the serosal side and are neutralized by HCO_3 ions in the mucosal extracellular fluid which enters the lumen. Topical irrigation of the damaged mucosa with histamine produces a model of the effects of histamine release. There is a large increment in volume, most but not all of which is plasma. If acid secretion is trapped with glycine buffer and if the parietal cells secrete acid at a concentration of 150 mM, Davenport has calculated that secretion accounts for approximately 11% of the volume increment during histamine irrigation and for 65% of the increment during the next 30 min. Histamine applied to the normal mucosa has no effect.

Tentative evidence for the intramucosal liberation of histamine during damage was provided by the observation that histamine appeared in Heidenhain pouch contents during and following salicylate damage more often than in control experiments.[5] Further evidence comes from work with the pylorus-ligated rat. We have shown that, when the rat gastric mucosa is irrigated with 100 mM acetic acid or 20 mM salicylic acid in 100 mM HCl, histamine appears in the gastric contents and the histamine content of the oxyntic glandular area simultaneously decreases.[7] These changes do not occur when the stomachs are filled with 154 mM NaCl or with 100 mM HCl. We inferred from this that, during injury, histamine is present in the mucosal interstitial fluid where it can exert its characteristic effects.

Unequivocal evidence for histamine liberation within the mucosa would be the finding of increased levels of histamine in the interstitial fluid during injury. Appearance of histamine in gastric venous blood or lymph would be presumptive evidence of its presence in the interstitial fluid. The present report concerns an investigation of the histamine content of gastric venous blood during injury.

Materials and Methods

Fifteen dogs weighing 15 to 22 kg were fasted overnight and then anesthetized with sodium pentobarbital, administered intravenously. The

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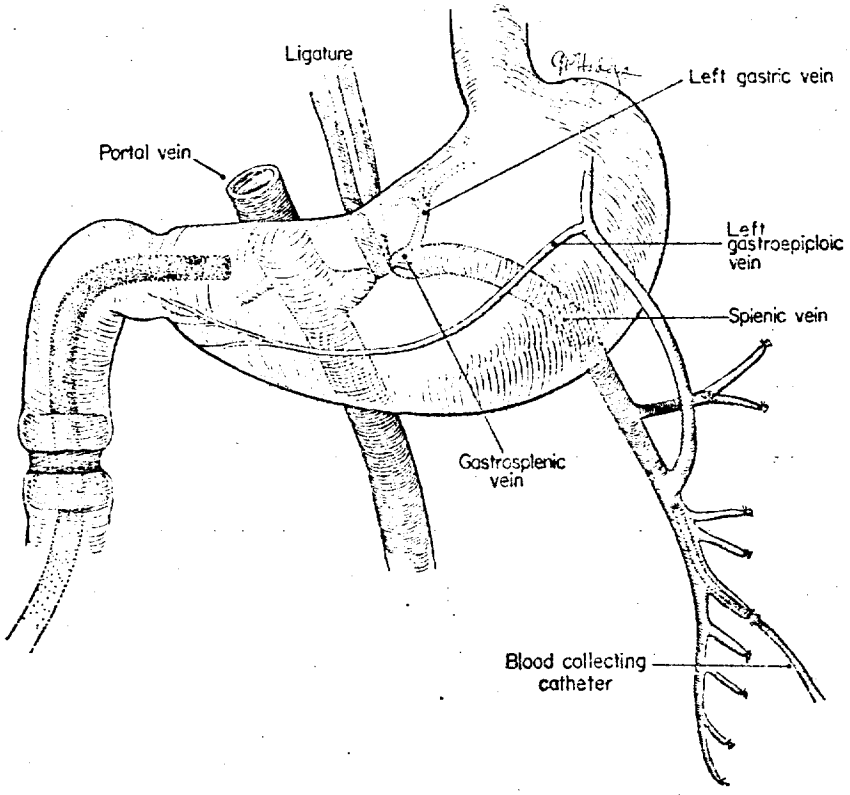

FIG. 1. Diagram of method used to isolate and collect gastric venous blood.

spleen was delivered through a midline abdominal incision, and a catheter was inserted into one of the splenic veins (fig. 1). Following splenectomy, a length of umbilical tape was passed under the gastrosplenic vein between its junctions with the portal and left gastric veins. During collection of venous blood, the tape was pulled, occluding the gastrosplenic vein and causing a retrograde flow of gastric venous blood from the catheter. Normal blood flow resumed when the umbilical tape was released.

A tube was then passed into the stomach through an incision in the duodenum and tied in place, as illustrated in figure 1. The stomach was filled with 300 ml of one of four solutions: (1) 154 mM NaCl, (2) 100 mM acetic acid, 15 mM NaCl, 78 mM mannitol, (3) 100 mM HCl, 15 mM NaCl, 78 mM mannitol, 20 mM salicylic acid, (4) 100 mM HCl, 15 mM NaCl, 78 mM mannitol.

Just prior to irrigation and at various times thereafter, 5-ml gastric venous and femoral arterial blood samples were collected. Blood samples were immediately combined with 10 ml of 10% w/v trichloroacetic acid, mixed, and filtered as described by Code.[5] Samples of the irrigation solutions were also removed from the stomachs for histamine assay. The trichloroacetic acid filtrates of the blood and the samples of irrigation fluid were combined with 5 ml of concentrated HCl to begin the histamine extraction procedure described by Code and McIntire.[9] Histamine content of the final extracts of both blood and fluid was determined by assay using an isolated terminal segment of guinea pig ileum suspended in atropinized Tyrode's solution.[10] Potassium concentrations of the final extracts were measured by internal standard flame photometry and found to be well below the level which would affect the isolated ileum.[11] Contractions attributed to histamine were abolished by 1.0 mg per liter of pyrilamine maleate. All results are expressed as micrograms of histamine base. The minimal amount of detectable histamine was 1.2 μg per liter of blood or gastric contents, and quantities below this are recorded as zero.

This method recovers total histamine present in the blood. In each of the 15 dogs, two arterial and two gastric venous blood samples were assayed for histamine before filling the stomachs. The four values were always essentially the same in each dog and ranged from 0.0 μg per liter to

56.8 μg per liter in the 15 dogs, with a mean of 6.4 μg per liter. The second highest concentration was 20.3 μg per liter. Recoveries of histamine added to blood samples varied from 62 to 100%. The errors of replication in duplicate determinations ranged from 2 to 17%.

Results

Histamine Levels in Gastric Venous Blood

NaCl and HCl Instillations. In five stomachs filled for 120 min with the NaCl solution and three filled with the 100 mM HCl solution, there was no increase in arterial or gastric venous histamine over preinstillation levels.

Acetic acid instillations. During exposure to acetic acid, histamine appeared in the gastric venous blood within 5 min, and the increase over preinstillation levels was 6.8 to 9.0 μg per liter (fig. 2). In the four acetic acid experiments, histamine concentration dropped within 10 or 15 min, returning to control levels by 45 min. Arterial samples taken at the same time as the venous samples containing the peak concentrations of histamine had only a fraction of the venous histamine or none at all. Before filling, arteriovenous differences were not discernible in any dog having detectable blood histamine at that time, and, at the end of a 60-min irrigation, there were again no detectable arteriovenous differences.

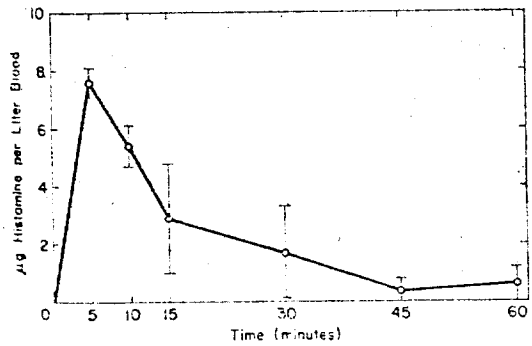

FIG. 2. Change in the histamine concentration of gastric venous blood during acetic acid irrigations. The amount of histamine present during the control period of an experiment was subtracted from that present at each time interval following instillation of acetic acid at zero time. These values were averaged for each time interval. *Vertical lines* indicate standard errors of the means. The increase over the control level is significant with $P < 0.01$.

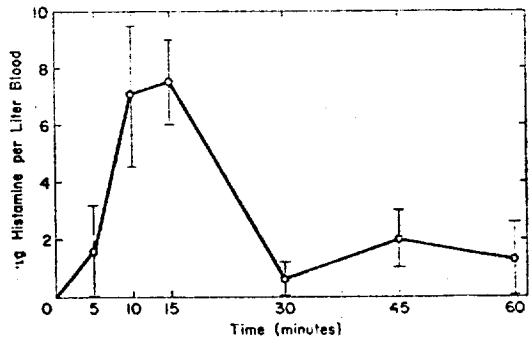

FIG. 3. Change in the histamine concentration of gastric venous blood during salicylic acid irrigations. The amount of histamine present during the control period of an experiment was subtracted from that present at each time interval following instillation of salicylic acid at zero time. These values were averaged for each time interval. *Vertical lines* indicate standard errors of the means. The increase over the control level is significant with $P < 0.05$.

Salicylic acid instillations. In the three salicylic acid experiments (fig. 3), histamine appeared in the gastric venous blood more slowly; the maximum postinstillation difference occurred at 15 min, the range being 4.5 to 9.8 μg per liter. As in the acetic acid experiments, blood histamine fell rapidly after 15 min and reached near control levels by 60 min. Again there were no arteriovenous differences at the beginnings and ends of the irrigations and no or only slight increases in arterial histamine during the increases in venous levels.

Histamine Levels in Gastric Contents

NaCl and HCl Instillations. In four NaCl and three HCl experiments, histamine was absent from samples of the solution removed from the stomach until 60 min had elapsed. In one experiment, low amounts of histamine were found at the beginning. The highest concentration found was only 3.1 μg per liter.

Acetic and salicylic acid instillations. Histamine, however, appeared in all acetic acid solutions (fig. 4) within 5 min. The range at 5 min was 2.5 to 9.0 μg per liter. All salicylic acid solutions also contained

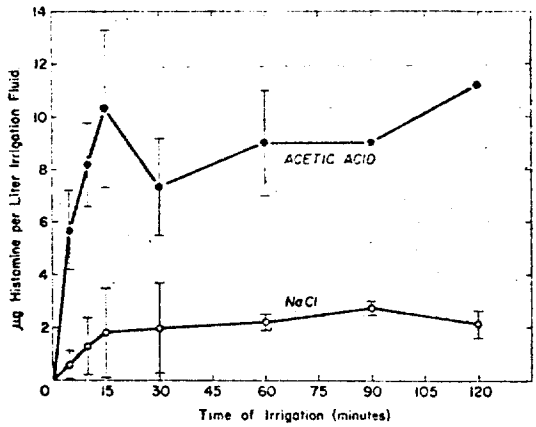

FIG. 4. Comparison of amounts of histamine liberated into the lumens of stomachs filled with NaCl versus those filled with acetic acid. Means and standard errors of the means at various time intervals are shown.

histamine within 5 min, the range being 5.4 to 39.1 μg per liter.

Discussion

A summary of our concept of the role of histamine in explaining a portion of the results observed during damage to the gastric mucosa has been presented by Davenport.[6] The acetic and salicylic acid are present in the lumen as almost 100% undissociated and lipid soluble molecules. As such they diffuse across the mucosa and ionize at the higher intracellular *p*H. The released H^+ damages the tissue, and acid diffuses through the mucosa, made much more permeable by the injury. The acid causes a liberation of histamine into the interstitial fluid. If the mucosa is permeable enough, some histamine appears in the gastric contents. Some histamine stimulates secretion and some increases mucosal capillary permeability, resulting in the presence of an abnormal amount of plasma proteins in the gastric contents.

It is difficult to assess the amounts of histamine needed to cause these changes, especially since all of the histamine is released from the gastric mucosa and can be expected to have a greater effect on the mucosa than the same amount originating somewhere else. The increase of approximately 9 μg per liter observed in the gastric venous blood in these experiments would seem to be more than sufficient to account for the hypothesized changes. In a group of experiments (*unpublished data*), we have found that intravenous infusion of a low concentration of histamine always produces a significant secretion of hydrogen ion by the oxyntic cells before gastric venous histamine concentrations have increased 5 μg per liter.

The time course of the histamine release into the gastric venous blood supports the general concept of the role histamine plays in damage to the gastric mucosa. In figure 2, following acetic acid injury the venous blood histamine appears quickly and peaks sharply at 5 min. With salicylic acid (fig. 3), there is little histamine present at 5 min, and the peak concentration does not occur until 10 to 15 min. This was predicted from the model, for the diffusion gradient for acetic acid is much greater than for salicylic acid (100 mM versus 20 mM). Therefore, it should take longer for the salicylic acid to diffuse through the mucosal barrier and injure the tissue.

There are, however, several interesting problems raised by the time course of release. The release occurs quickly and is of short duration. Our experimental setup does not allow us to differentiate between liberation of preformed histamine and synthesis and release of histamine. Therefore, the pulse of liberated histamine could represent a brief release of histamine from mast cells or a short stimulation of the histamine-forming capacity of the tissue. On the other hand, there is the less likely possibility that histamine is liberated throughout the experiment, but its rate of destruction increases to such an extent that after the first 15 min little escapes into the gastric venous blood.

Summary

The stomachs of anesthetized dogs were filled with 154 mM NaCl, 100 mM HCl, 100 mM acetic acid, or 20 mM salicylic acid in 100 mM HCl. Gastric venous and femoral arterial blood samples were taken at various intervals for the next 120 min, and their histamine content was determined. Within 5 min there was a significant increase in

the histamine concentration of the blood draining the stomachs filled with acetic acid. A similar increase occurred within 10 min in the blood from the salicylic acid-treated stomachs. Femoral arterial samples taken concurrently with the gastric venous samples containing the peak amounts of histamine contained little or no histamine. In no experiment was an increase in histamine detected in blood from a stomach containing NaCl or HCl. Histamine always appeared early in the salicylic and acetic acid irrigation solutions and reached peak concentrations at 15 min. Histamine was usually absent from the NaCl solutions until 1 hr of irrigation had elapsed, and then the concentrations reached were low. We conclude that the increased amount of histamine in the gastric venous blood during injury is proof that it is released into the mucosal interstitial fluid, where it can exert its characteristic physiological actions.

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THE EFFECTS OF THE ADMINISTRATION OF ACETIC, PROPIONIC, AND *n*-BUTYRIC ACIDS UPON THE BLOOD GLUCOSE AND KETONE BODY LEVELS OF GOATS

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WITHIN the last decade it has become increasingly evident that virtually all the carbohydrate ingested by the ruminant is degraded by anaerobic microorganisms to the volatile organic acids—acetic acid, propionic acid, and *n*-butyric acid—and that ruminants are dependent primarily upon these acids for their supply of energy.[10,11] The degree of the relative contribution of the three acids to energy production has, however, been the subject of considerable controversy.[10,11]

Apparently contradictory results, obtained by various methods of investigation used in the study of this difficult problem, have finally been largely resolved by the efforts of Phillipson and his coworkers. In their latest paper[10] they report mean absorption rates for sheep of 32 milliequivalents per hour of acetic acid, 18 milliequivalents per hour of propionic acid, and 23 milliequivalents per hour of *n*-butyric acid. They calculate that of the total energy of 25.3 kilocalories per hour represented by these amounts of acids, acetic acid accounts for 6.7 kilocalories, propionic acid 6.6 kilocalories, and *n*-butyric acid 12.0 kilocalories.

Since the advent of the use of isotopic tracers, the major pathways by which metabolites ultimately converge in the common energy-generating device known as Krebs' citric acid

cycle have been reasonably well defined in the usual laboratory animals.[11,15,16] Limited tracer studies with ruminants have yielded results consistent with the same pathways.[3,4,12,13,14]

Although these pathways indicate that propionic acid is the only one of the three acids giving rise to significant quantities of glucose, and that acetic acid and *n*-butyric acid are precursors of ketone bodies, numerous reports at variance with this conception have appeared in the literature. In view of the major energy contribution made by these acids and the obvious relationship of their metabolism to the problem of ketosis, a study was undertaken to determine whether the administration of the individual volatile fatty acids, and combinations thereof, causes changes in the blood glucose and ketone body levels consistent with the generally accepted mechanisms of metabolism. This study is the subject of this report.

EXPERIMENTAL PROCEDURE

Figures 1 to 10 summarize the experimental data. Grade female goats were employed. For the oral administration a constant-rate pump was used, the solution passing into the rumen via a tube passed through a nostril. Intravenous administration was made with a constantly supervised drip device through a small plastic tube inserted into the jugular vein.

Blood samples were collected from the jugular vein into test tubes containing sodium fluoride. Plasma glucose was determined by the method of Somogyi[27] using Benedict's[2] chromogenic reagent; the total plasma ketone bodies were determined by the method of Behre[1] modified for use with a Coleman spectrophotometer. As it was not possible to maintain a normal acid-base balance in these experiments, data on the CO_2 capacity, determined by the method of Van Slyke,[7] are included in order to indicate the degree of departure from normal conditions.

RESULTS AND DISCUSSION

CHANGES IN BLOOD KETONE BODY LEVEL

A. Effect of n-butyric acid. According to presently held

synthesis of fat and substances such as cholesterol, may be partially oxidized to acetoacetate and its derivatives, or may be oxidized via the citric acid cycle.[11] It thus tends to be ketogenic. That this is the case in ruminants has been shown by numerous investigators.[9,17,18,25,26] The experiments summarized in figures 1, 4, 5, 6, 7, and 8 likewise confirm this fact.

B. Effect of acetic acid. Acetic acid, in the usual experimental animals, may be used for the synthesis of fat and other compounds, may condense with itself to form acteoacetic acid and its derivatives, or may be oxidized via the citric acid cycle.[11] The question whether it is actually ketogenic or not in ruminants has been the subject of some disagreement. Schultz, Smith, and Lardy[26] in 1949, after administering acetic acid orally to goats, found no increase in blood ketones. In 1951 Schultz and Smith[25] concluded that a large excess of acetic acid causes a slight ketosis. Pennington[17] has observed a slight increase in ketone bodies in the venous blood from the rumen after acetate was placed in the rumen. On the other hand, investigators administering the acid intravenously as its sodium salt have concluded that it is ketogenic.[8,9,22,28]

Figure 3 indicates that orally administered acetic acid has a slight ketogenic effect, although only those experiments which show a drop in ketone bodies after the cessation of administration of acid rule out the possibility that the increase is due to starvation. The data of figure 9 show little ketogenic effect from orally administered acetic acid, while those of figure 10 indicate an increase in ketone bodies. When we consider that two moles of acetate must combine to form one mole of acetoacetate and that the rate of absorption of acetate is less than that of *n*-butyrate, the difference observed between the ketogenic action of the two acids is not surprising. If acetic acid is administered by both the oral and intravenous routes, as was done in the experiments summarized in figures 9 and 10, the ketogenic effect of acetic acid is apparent ($P<0.001$).*

*Probability was calculated by use of the *t* test.[6]

F–D C–A

's of fig. 9 + 's of fig. 10, t = 0.83, n-1 = 5

390

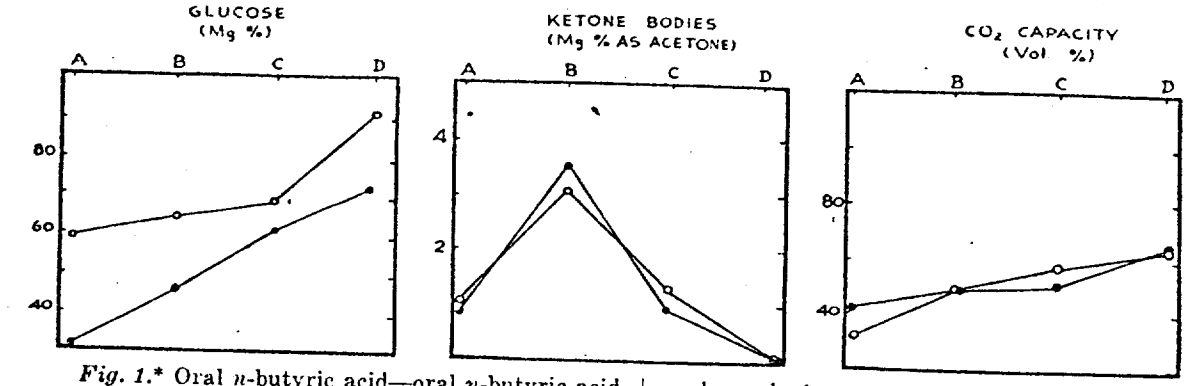


*Fig. 1.** Oral *n*-butyric acid—oral *n*-butyric acid + oral propionic acid—oral propionic acid

Hours of administration

53	24	24	24
A	B	C	D
<—Starved—>	<—0.5 mole *n*-butyric acid, 3.5 g KOH, 0.5 g NaOH/L (oral)—>	<—0.5 mole *n*-butyric acid, 0.25 mole propionic acid, 3.5 g KOH, 0.5 g NaOH/L (oral)—>	<—0.25 mole propionic acid, 1.75 g KOH, 0.25 g NaOH/L (oral)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration: A-B $\frac{}{B_o}$	B-C B_o	B-C P_o	C-D $\frac{}{P_o}$
○ 1	381	25	D	1.52	1.55	0.78	0.78
● 2	382	28	D	1.30	1.30	0.65	0.59

* The following abbreviations are used in the data accompanying figures:

Hours of administration — Capital letters indicate times at which blood samples were taken and correspond to the letters on the graphs.

Stage of lactation or pregnancy — D = dry and not more than 10 weeks pregnant, ( ) = weeks of lactation, [ ] = weeks before parturition.

Rate of administration — B, P, A, G, Na = administration of *n*-butyric acid, propionic acid, acetic acid, glucose, and $NaHCO_3$ respectively in millimoles per kilogram per hour. A subscript "o" indicates that administration was oral; a subscript "v" indicates intravenous administration.

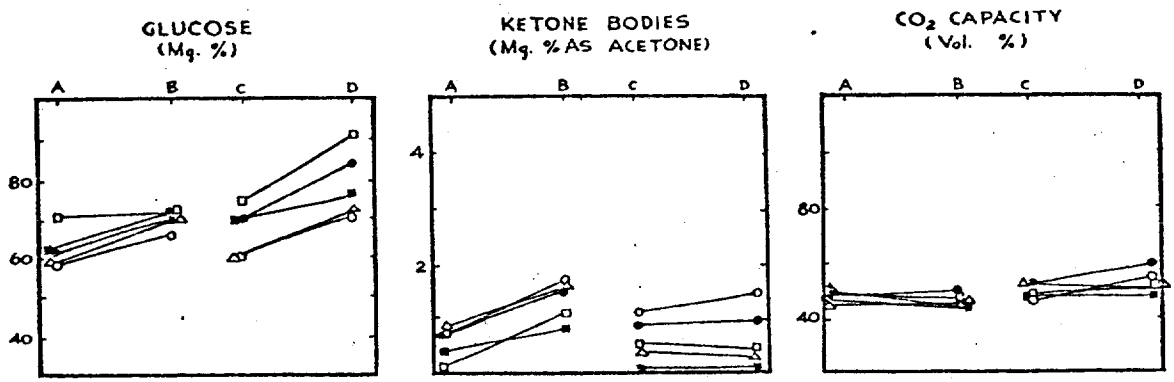


Fig. 2. Oral *n*-butyric acid + oral propionic acid — oral propionic acid

Hours of administration

55	23	25	23
A	B	C	D
<—Starved—>	<—0.5 mole *n*-butyric acid, 0.25 mole propionic acid, 3.5 g KOH, 0.5 g NaOH/L (oral)—>	<—Starved—>	<—0.25 mole propionic acid, 3.5 g KOH, 0.5 g NaOH/L (oral)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration: A-B B_o	A-B P_o	C-D P_o
○ 1	347	31	D	1.27	0.69	0.69
● 2	385	24	D	1.67	0.89	0.81
□ 3	316	38	D	0.87	0.44	0.43
■ 4	377	57	D	0.43	0.22	0.22
△ 5	378	59	[10]	0.44	0.22	0.22

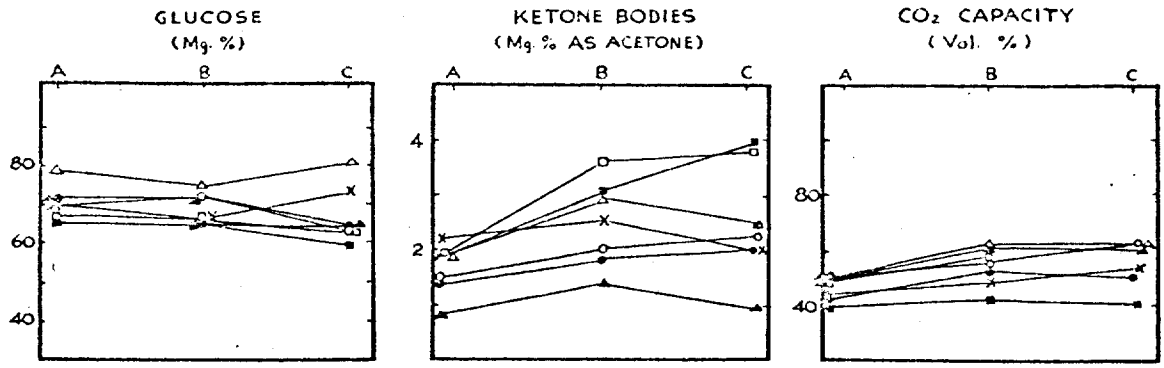


Fig. 3. Oral acetic acid

Hours of administration

53	24	24
A	B	C
<—Starved—>	<—0.67 mole acetic acid, 5.34 g KOH, 0.67 g NaOH/L (oral)—>	<—Starved—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration $\frac{A\text{-}B}{A_0}$
○ 1	438	59	D	0.48
● 2	377	57	D	1.17
□ 3	441	48	D	1.41
■ 4	440	42	D	1.42
Δ 5	442	30	D	1.93
▲ 6	443	39	D	1.50
X 7	385	34	D	1.61

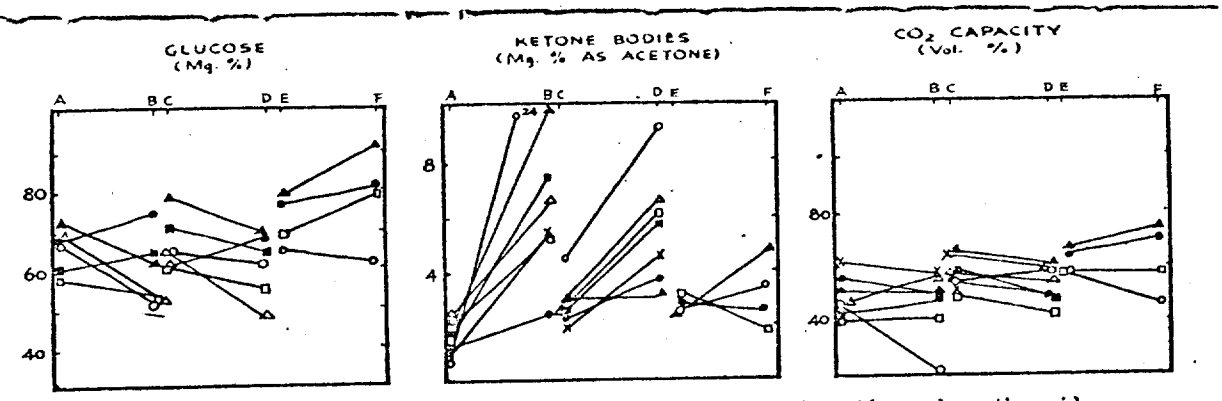


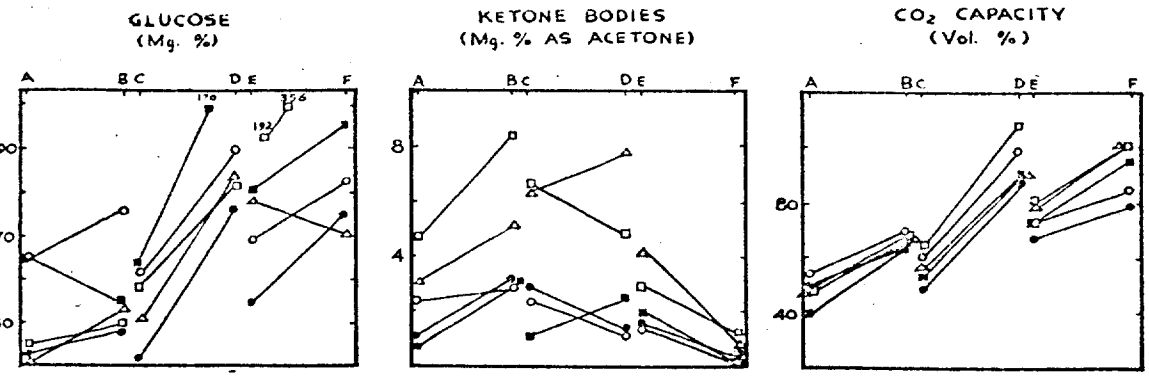
Fig. 4. Oral acetic acid + oral *n*-butyric acid—oral *n*-butyric acid—oral acetic acid

Hours of administration

53	24	24	24	24	24
A	B	C	D	E	F
<—Starved—>	<—0.67 mole acetic acid, 0.33 mole *n*-butyric acid, 8 g KOH, 1 g NaOH/L (oral)—>	<—Starved—>	<—0.33 mole *n*-butyric acid, 2.67 g KOH, 0.33 g NaOH/L (oral)—>	<—Starved—>	<—0.67 mole acetic acid, 5.34 g KOH, 0.67 g NaOH/L (oral)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B A_0	A-B B_0	C-D B_0	E-F A_0
○ 1*	377	57	D	1.96	0.98	0.99	1.96
● 2*	438	59	D	0.68	0.34	0.34	0.68
□ 3*	363	41	D	1.57	0.79	0.79	1.57
■ 4	440	42	D	1.31	0.66	0.66	
Δ 5	441	48	D	1.25	0.63	0.63	
▲ 6	442	30	D	1.92	0.96	0.96	2.80
X 7	443	39	D	1.95	0.98	0.99	

* During these runs, in which no base was added to the acid, the goats developed a very severe [illegible]a.

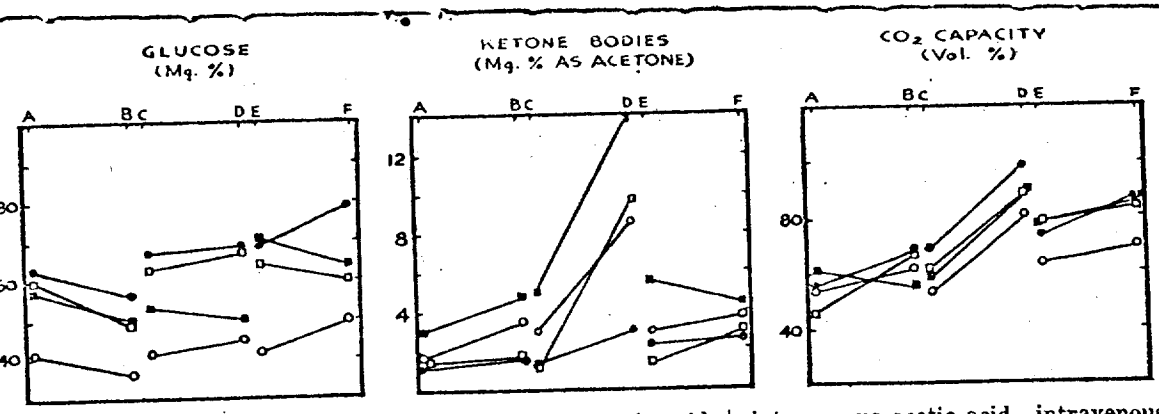


ig. 5. Intravenous *n*-butyric acid—intravenous *n*-butyric acid + intravenous propionic acid—intravenous propionic acid

Hours of administration

24	6	18	6	18	6
A	B	C	D	E	F
—Starved—>	<—0.375 mole—>	<—Starved—>	<—0.375 mole—>	<—Starved—>	<—0.375 mole—>
	n-butyric acid/L adjusted to pH 6.0 with NaOH (IV)		*n*-butyric acid, 0.375 mole propionic acid/L adjusted to pH 6.0 with NaOH (IV)		propionic acid/L adjusted to pH 6.0 with NaOH (IV)

Run	Goat no.	Weight kg	Stage of lactation or pregnancy	Rate of administration A-B Bv	C-D Bv	C-D Pv	E-F Pv
○ 1	344	30	(1)	2.36	2.50	2.50	2.50
● 2	142	63	[5]	1.19	1.19	1.19	1.19
□ 3	220	40	(2)	1.87	1.87	1.87	1.87
■ 4	316	37	(6)	2.03	2.03	2.03	2.03
△ 5	347	31	(21)	1.61	1.61	1.61	1.61



ig. 6. Intravenous *n*-butyric acid—intravenous *n*-butyric acid + intravenous acetic acid—intravenous acetic acid

Hours of administration

24	6	18	6	18	6
A	B	C	D	E	F
<—Starved—>	<—0.375 mole—>	<—Starved—>	<—0.375 mole—>	<—Starved—>	<—0.375 mole—>
	n-butyric acid/L adjusted to pH 6.0 with NaOH (IV)		*n*-butyric acid, 0.375 mole acetic acid/L adjusted to pH 6.0 with NaOH (IV)		acetic acid/L adjusted to pH 6.0 with NaOH (IV)

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B Bv	C-D Bv	C-D Av	E-F Av
○ 1	142	63	[1]	1.19	1.19	1.19	1.19
● 2	344	30	D	2.50	2.50	2.50	2.50
	316	37	(6)	2.03	2.03	2.03	2.03
	347	31	(14)	1.61	1.61	1.61	1.61

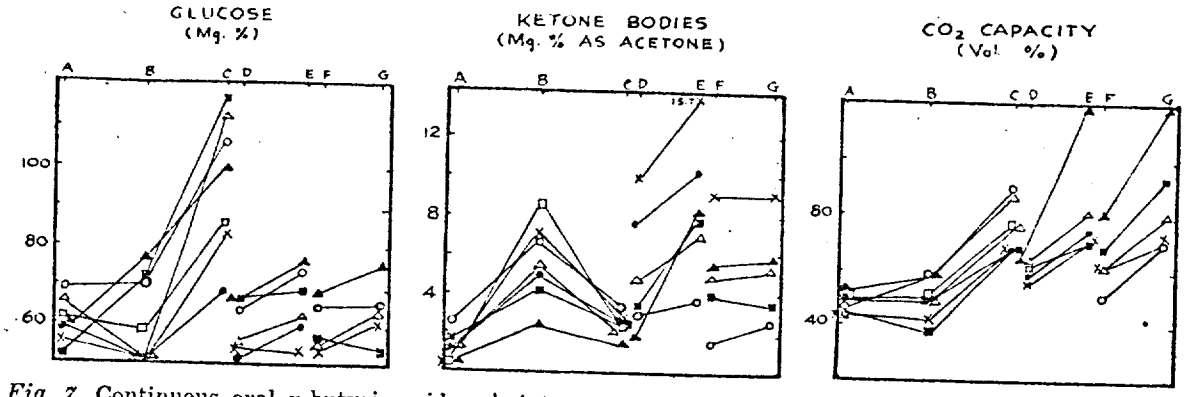


Fig. 7. Continuous oral *n*-butyric acid— + intravenous propionic acid— + intravenous acetic acid— + $NaHCO_3$

Hours of administration

48 | 22 | 6 | 18 | 6 | 18 | 6

A B C D E F G

<—Starved—><————— 0.5 mole *n*-butyric acid, 3.5 g KOH, 0.5 g NaOH/L (oral) ————>

<—0.375 mole—> propionic acid/L adjusted to pH 6.0 with NaOH (IV)

<—0.375 mole—> acetic acid/L adjusted to pH 6.0 with NaOH (IV)

<—0.375 mole—> $NaHCO_3$/L (IV)

	Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B B_0	B-C B_0	B-C Pv	C-D B_0	D-E B_0	D-E Av	E-F B_0	F-G B_0	F-G N_{AV}
○	1	220	40	(17)	1.39	1.25	1.87	1.23	1.23	1.87	1.23	1.23	1.87
●	2	142	63	D	1.15	1.21	1.19	1.27	1.15	1.19			
□	3	363	41	D	1.63	1.65	1.83						
■	4	316	37	(13)	1.47	1.46	2.03	1.15	1.32	2.03	1.46	1.55	2.03
△	5	347	31	D	1.78	1.82	2.42	1.23	1.29	2.42	1.24	1.29	2.42
▲	6	377	57	D	1.17	1.17	1.33	1.09	1.09	1.33	1.12	1.12	1.33
X	7	378	59	D	1.24	1.25	1.01	1.13	1.13	1.01	1.33	1.34	1.01

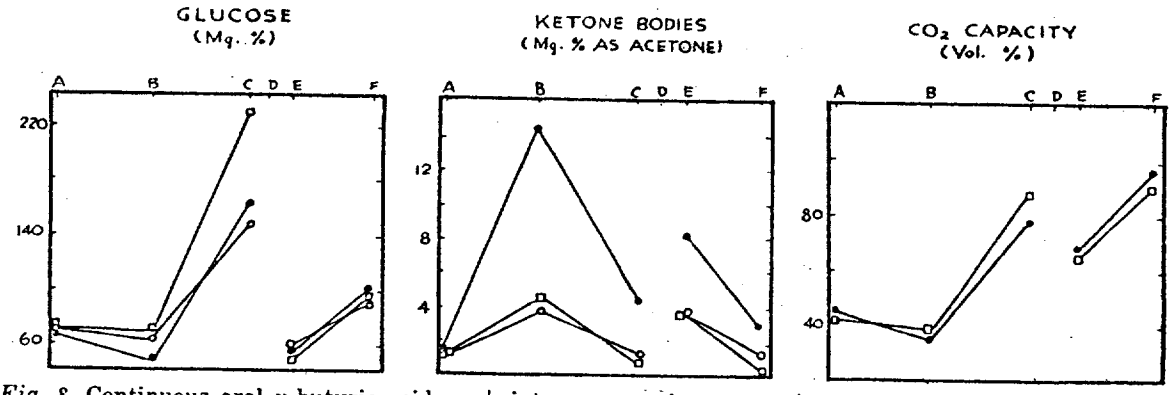


Fig. 8. Continuous oral *n*-butyric acid — + intravenous glucose — + intravenous propionic acid

Hours of administration

29 | 17 | 6 | 25 | 17 | 6

A B C D E F

<—Starved—><————— 0.5 mole *n*-butyric acid/L (oral) —><—Starved—><————— 0.5 mole *n*-butyric acid/L (oral) —>

<—0.1875 mole glucose, 0.375 mole $NaHCO_3$/L (IV) —>

<—0.375 mole propionic acid/L adjusted to pH 6.0 with NaOH (IV) —>

	Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B B_0	B-C B_0	B-C Gv	D-E B_0	E-F B_0	E-F Pv
○	1*	142	63	D	1.16	1.16	0.60	1.13	1.19	1.20
●	2	378	59	(1)	1.25	1.25	0.64	1.22	1.27	1.28
□	3	316	37	[5]	1.29	1.24	0.96	1.07	1.33	1.92

* No bicarbonate added to glucose.

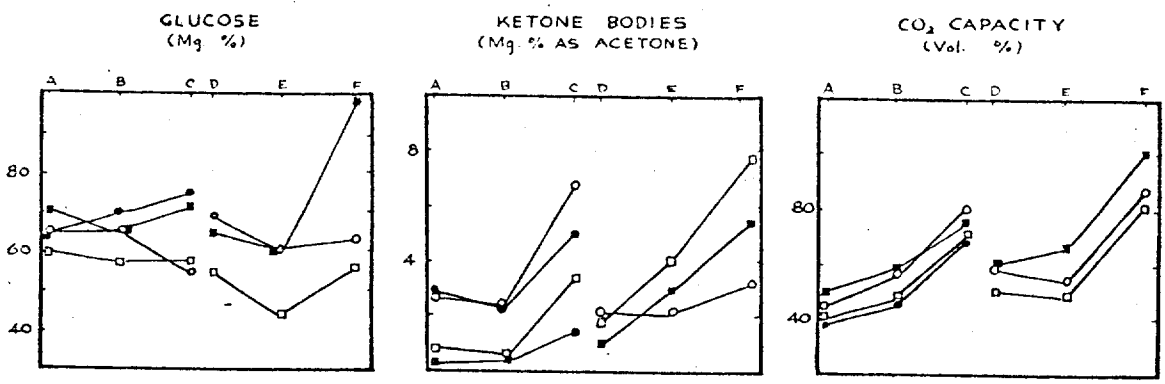


Fig. 9. Continuous oral acetic acid — + intravenous *n*-butyric acid — + intravenous acetic acid

Hours of administration

52 | 20 | 6 | 22 | 20 | 6

A B C D E F

<—Starved—><————1 mole acetic acid, 8 g KOH, 1 g NaOH/L (oral)><—Starved—><————1 mole acetic acid, 8 g KOH, 1 g NaOH/L (oral)>

<—0.4 mole *n*-butyric acid/L adjusted to pH 6.0 with NaOH (IV)—>

<—0.8 mole acetic acid/L adjusted to pH 6.0 with NaOH (IV)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B A_0	B-C A_0	B-C B_V	D-E A_0	E-F A_0	E-F A_V
1	441	48	D	2.74	2.32	1.66	2.60	2.50	3.22
2	440	42	D	2.40	2.26	1.90			
3	377	57	D	2.56	2.10	1.41	2.27	2.20	2.82
4	438	59	D	1.88	1.35	1.35	2.31	2.22	2.70

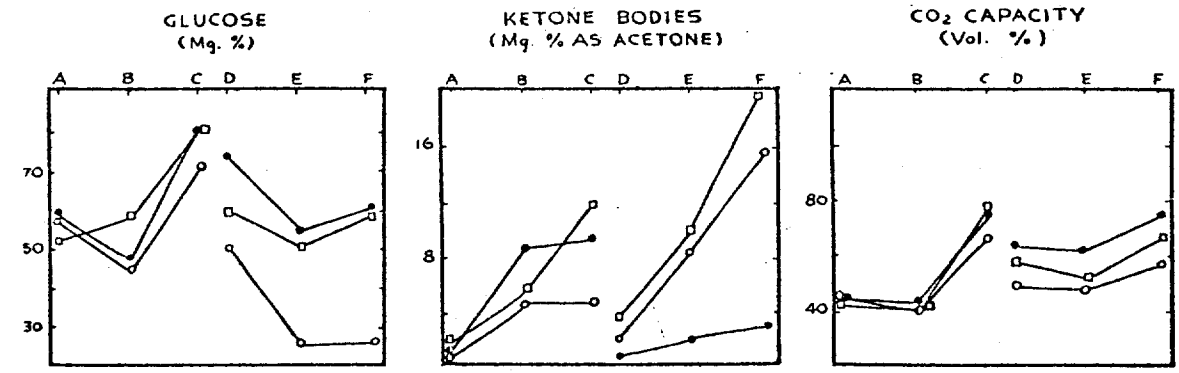


Fig. 10. Continuous oral acetic acid — + intravenous acetic acid — + intravenous *n*-butyric acid

Hours of administration

52 | 17 | 6 | 25 | 17 | 6

A B C D E F

<—Starved—><————0.75 mole acetic acid/L (oral)><—Starved—><————0.75 mole acetic acid/L (oral)>

<—0.4 mole acetic acid/L adjusted to pH 6.0 with NaOH (IV)—>

<—0.2 mole *n*-butyric acid/L adjusted to pH 6.0 with NaOH (IV)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B A_0	B-C A_0	B-C A_V	D-E A_0	E-F A_0	E-F B_V
1	377	57	[6]	1.54	1.54	1.41	1.62	1.46	0.70
2	385	24	[8]	2.51	2.51	1.64	2.02	2.51	0.82
3*	363	41	D	2.60	2.60	3.91	2.59	2.59	1.95

* Intravenous solutions were 0.8 molar and 0.4 molar.

As both acetic and *n*-butyric acids may form acetyl CoA fragments for oxidation via the citric acid cycle and as they may both form ketone bodies if this cycle is blocked or overloaded, the ketogenic effects of the individual acids should reinforce one another. Figures 4 ($P<0.05$,* 6 ($P<0.002$,† 7 ($P<0.002$,‡ 9, and 10 clearly demonstrate this reinforcement. The experiments summarized in figures 9 and 10 suggest that when the citric acid cycle is overloaded, two molecules of acetate may not be significantly inferior to one molecule of *n*-butyrate in ability to produce ketone bodies.

C. Effect of propionic acid. In other animals propionic acid does not form ketone bodies, since it may be converted to glucose or glycogen or it may be catabolized via the citric acid cycle, for which it can supply the necessary oxaloacetic acid as well as the acetyl CoA.[11] This is also true for ruminants.[9,17,18,25,26] Figures 2 and 5 summarize confirming experiments. Schultz[21] has shown sodium propionate to be useful in the treatment of clinical ketosis.

Because of its ability to supply the oxaloacetate necessary for the operation of the citric acid cycle, propionic acid should facilitate the complete oxidation of acetic and *n*-butyric acids. Jarrett *et al.*[9] demonstrated the ketolytic effect of propionic acid given concurrently with acetic acid but failed to observe a similar effect with *n*-butyric acid. That propionic acid inhibits the ketogenic action of *n*-butyric acid is demonstrated by figures 1, 5 ($P<0.06$,§ 7 ($P<0.001$),# and 8.

If *n*-butyric and propionic acids are given to the animal in sufficiently large quantities, there will be a rise in blood

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*$\frac{(B-A) - (D-C)}{(B-A)}$, $t = 2.67$, $n-1 = 6$.

†$\frac{(D-C) - (B-A)}{(D-C)}$, $t = 12.8$, $n-1 = 3$.

‡$\frac{E-F}{E}$, $t = 7.18$, $n-1 = 4$.

§$\frac{(B-A) - (D-C)}{(B-A)}$, $t = 2.70$, $n-1 = 4$.

#$\frac{B-C}{B-A}$, $t = 11.2$, $n-1 = 6$.

ketone bodies despite the presence of the propionate, as the amount of *n*-butyrate exceeds that which can be oxidized via the citric acid cycle or stored as fat under even the best conditions. This is shown in figure 2.

CHANGES IN BLOOD GLUCOSE LEVEL

A. Effect of propionic acid. It is commonly recognized that propionic acid is an efficient source of glucose.[11] This is true in ruminants as well as other animals.[9,20,21,23,25] Figures 1, 2 and 5, summarizing experiments in which propionic acid was given alone, and figures 5, 7, and 8, summarizing experiments in which propionic acid was given in combination with other acids, likewise confirm this effect.

B. Effect of n-butyric acid. Although in nonruminants *n*-butyric acid is not considered to be glucogenic, there is a known mechanism (*n*-butyrate —>acetoacetate—>acetone —>pyruvate) by which glucose may be formed.[11] The work which has been done on ruminants is contradictory. Schultz and Smith[25] found that when *n-butyric* acid was injected into the rumen of goats there was a brief initial increase in blood glucose level followed by a depression to less than one-half that of the initial level. They also found that caproic, caprylic, and capric acids caused similar decreases in blood glucose, the degree of change being inversely related to the rise in ketone bodies. Acetone, sodium and ethyl acetoacetate, or beta-hydroxybutyric acid did not have a comparable effect.

On the other hand, Jarrett, Potter, and Filsell[9] found that when 5 millimoles of sodium *n*-butyrate were administered intravenously to sheep over a period of 5 to 10 minutes, a marked rise in blood glucose occurred. They noted, however, that the animals responded with marked symptoms of distress and suggested that the rise may be due to glycogenolysis brought about by the sympatho-adrenal system. In a later paper Potter[21] reported that intravenous sodium *n*-butyrate relieved insulin convulsions in lambs and caused a rise in blood glucose greater than that produced by propionate, while acetate neither gave relief from the convulsions nor caused a rise in blood glucose. Convulsions, occurring in two adult ewes whose splanchnic nerves had been severed, responded [illegible]

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n-butyrate and propionate but not to acetate. He concluded that *n*-butyric acid may be a precursor of glucose. In interpreting experiments in which the quantity of radioactive carbon was determined in the lactose, casein, and fat of the milk secreted by cows which had received labeled *n*-butyrate, Kleiber *et al.*[12] concluded that a net synthesis of lactose had occurred. This very interesting work does not, however, indicate the mechanism by which this could take place, and other interpretations of the results are possible.

In the experiments here reported, the slow, continuous administration avoided any apparent alarm reaction except when anoxia, as evidenced by an extremely high CO_2 capacity, appeared to be intervening. The experiments involving oral administration of *n*-butyric acid are summarized in figures 1, 4, 7, and 8. The ketone values obtained in these experiments are lower than those attained by Schultz and Smith. Although there are exceptions, the results tend to confirm the finding that the drop in glucose level varies inversely with the degree of ketonemia. The experiments involving intravenous sodium *n*-butyrate (figures 5 and 6) reveal no consistent changes in blood glucose level.

C. Effect of acetic acid. The consensus of opinion is that in ruminants,[9,21,25] as in other animals,[11] acetic acid is not a precursor of glucose. The detection by Kleiber *et al.*[11] of radioactive carbon in the lactose and casein of the milk of cows given labeled acetate is readily explained as resulting from participation in Krebs' citric acid cycle rather than as net synthesis. The experiments involving the oral administration of acetic acid (figures 3, 4, 9, and 10) show no consistent influence upon blood glucose level. Those involving the intravenous injection of sodium acetate (figure 6) likewise show no meaningful changes. The experiments summarized in figures 9 and 10 indicate that the sodium ion necessarily introduced in intravenous experiments has a marked effect upon the blood glucose level.

As the blood glucose level is sensitive to many factors, these experiments are not sufficiently well designed to reveal whether *n*-butyric or acetic acid has a minor effect upon blood

glucose level, but they do indicate that these two acids are in no way comparable to propionic acid as precursors of glucose.

SUMMARY

The effects of the administration of acetic, propionic, and *n*-butyric acids upon the blood glucose and ketone body levels of goats are consistent with the generally accepted mechanisms of metabolism.

1. Acetic acid and *n*-butyric acid form ketone bodies.
2. Propionic acid counteracts the formation of ketone bodies.
3. Propionic acid causes a marked increase in blood glucose level.
4. The experiments were not suitable for the observation of slight effects upon the blood glucose level, but they clearly demonstrate that acetic acid and *n*-butyric acid do not cause an elevation of the blood glucose level comparable to that caused by propionic acid.

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FORMIC AND ACETIC ACIDS AS POISONS*

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Historical

THE appearance of the volatile fatty acids, formic and acetic, in toxicology is very patchy. According to Autenrieth (1928) formic acid has 'hardly ever come up as poison'. Five cases have however been reported from the Federated Malay States (*Analyst*, 1940, p. 459), 4 from the Straits Settlements (*Analyst*, 1937, p. 742; 1939, p. 744), and 2 from Ceylon (*Analyst*, 1932, p. 575) during the period of 1931–42.

Although acetic acid has been used for suicide, and accidental poisonings have also been fairly common in certain countries of Europe, notably Russia and Germany from the beginning of the present century, Autenrieth (1928) and Owens (1935) have not mentioned any instance of poisoning with this acid. This might be because, as Hunt and Gettler say, 'in the United States and England, poisoning by acetic acid seems to be rare'. There were according to these authors 4 fatal cases in England and Wales in 1912, while in Germany there were 256 cases of acetic acid poisoning in 1909 and in St. Petersburgh in 1908 it accounted for 34 per cent of all suicides and 72 per cent of all cases of poisoning. Blyth and Blyth (1920) refer to 12 cases as having occurred in England and Wales during the five years ending in 1916. Two instances of cases of acetic acid poisoning have been mentioned in the Annual Report of the Medical Officer of the Ministry of Health, 1937. Fifteen cases have been reported from Ceylon during 1931–42 (*Analyst*, 1932, p. 575; 1937, p. 469; 1938, p. 501; 1939, p. 512 and 1940, p. 652).

Owens (1935), while stating that 'a few fatal cases of poisoning by this have been reported as having occurred in Europe', does not mention a single case anywhere in India. It has been referred to as poison only once during the last decade by the Chemical Examiner to the Government of Madras (Annual Report of the Chemical Examiner's Department, 1940, p. 15).

Cases in Travancore

The number of cases from which we have been able to isolate formic and acetic acids during the last seven years is significant and interesting. Although toxicological analysis was introduced in Travancore 51 years ago, these acids had not figured in our toxicological history prior to 1936. Since then we have had 71 cases out of a total of 428 cases of poisoning distributed as follows :—

	1935–36	1936–37	1937–38	1938–39	1939–40	1940–41	1941–42	1942–43	Total
Formic acid ..	..	6	8	13	5	2	..	..	34
Acetic acid ..	7	1	3	1	5	11	6	3	37
TOTAL ..	60	54	47	55	53	64	51	44	428

The regional distribution of these cases is significant. Administratively the State is divided into three districts, and in one respect the districts are strikingly different from each other.

Travancore is the biggest rubber producing area in India. Of the 136,605,671 acres under rubber in India, 10,446,517 acres are in Travancore. But the distribution of rubber among the districts is unequal. Trivandrum division has little rubber. Although both Quilon and

*A paper presented before the Indian Science Congress, 1944.

Kottayam have extensive acreage under rubber, most of the small holdings are in the Kottayam district. Of the 71 cases under review, Kottayam claimed 62, Quilon 9 and Trivandrum none.

The close association of rubber with poisoning by the two acids is remarkable. Indeed, it is the development of this industry that has brought these acids into common use in this country and led to their deliberate or accidental use as poisons. The high incidence of such cases in Ceylon and Malaya, two of the leading rubber producing countries of the world, corroborates this view.

During the last two years, there has been no case of poisoning by formic acid because the outbreak of war has made it unavailable. Acetic acid has also become increasingly costly and more difficult to obtain, and it is to a large extent replaced by a sulphuric acid preparation called 'Sulpho'ex'. Because these acids have become more precious, the owners are naturally taking greater care of their stocks than they did before.

The 71 cases come under three heads : (*a*) suicidal, (*b*) accidental, (*c*) overdose—when deliberately taken as a medicine.

	Suicidal	Accidental	Over-dose	Total
Formic acid ..	27	6	1	34
Acetic acid ..	28	7	2	37

No case of homicidal poisoning has been reported, obviously because they do not satisfy the primary properties which criminals desire in their poisons—*i.e.* smallness of dosage and absence of unpleasant smell and taste. The choice of a poison for suicide is governed by its easy availability as well as by the level of intelligence of the victim. An intelligent person's suicide is seldom a momentary impulse. It is generally the result of long thought and brooding, and a quick-acting poison not likely to cause much agony, such as the cyanides, opium or arsenic will be preferred. In the case of an illiterate labourer, the thought of suicide is often a sudden impulse, following, say, a domestic quarrel, discharge from employment, etc. He has neither the knowledge of the more powerful poisons nor the cunning or means to procure them. He uses what he can easily get and is not deterred by fear of suffering. This explains the large use of these acids for suicidal purposes in this country. Their extensive use in the rubber industry made them available to anyone, and the victims invariably belonged to the lower strata of society.

A parallel to this can be found in the use of acetic acid for suicide in Russia and Germany during the early part of this century, referred to above. Essence of vinegar containing 80 per cent of acetic acid had just come into use as a food preservative, and was freely available to all, and the poor people, probably not more advanced in intelligence than the labourers engaged in the rubber industry, used it for suicide.

Accidental consumption of any poison is due to its domestic storage along with receptacles containing medicine or alcoholic drinks. Accidental poisoning by carbolic acid and other disinfectants has often occurred in Great Britain in this manner, and by acetic acid in Germany and Russia. In Travancore, while no domestic storage of these acids is done in the large rubber estates, the small holders keep small quantities at home in bottles similar in size and appearance to those used for keeping various indigenous medicines or alcoholic drinks. The smell of some of these indigenous preparations is not very different from that of these acids, and this leads to accidental poisoning.

Although the conditions under which acetic and formic acids have been used for suicide or consumed accidentally are more or less similar everywhere, there is another group of cases which, although small, is of a unique type. In these cases the victims were reported to have been using small doses of acetic acid for the relief of their stomach pain. It may be noted here that the incidence of gastro-duodenal disease in Travancore is estimated to be 3 to 4 times that of other parts of India (Orr, 1936) and occasionally larger doses of acids than usual have been consumed either accidentally or deliberately with the hope of getting the relief which smaller doses had failed to give. In a fair proportion of suicides also, the desire to escape for ever from their incurable gastric pain appears to have been the motive for the crime.

Signs and symptoms

These are in general similar to those caused by mineral acids. The post-mortem appearances are also of the same nature. Vomiting, salivation and a burning sensation of mouth are present invariably. The vomit contains bloody fluid. The victim usually rolls about in agonizing pain. The pulse becomes rapid first and then slows down. Respiration also follows the same course of rise and fall. The skin is often cold and the face cyanosed, and there is rattling of the throat and irritation of the respiratory passages, although in the majority of cases the larynx and trachea were found normal. If concentrated acid is taken, the whole of the mouth and tongue may be corroded, the skin over the chin, front of the neck and on the upper part of the chest showing brownish desquamation if the acid has flowed down the mouth. Sometimes dark-stained streaks run down from the angles of the mouth and the middle of lower lip just under the chin, through the trickling down of the acid.

Fatal period

Death in acetic acid poisoning is stated to occur in 48 hours by Reid Hunt and Gettler.

We have reliable information about this point in 14 cases of formic acid and 21 of acetic acid poisoning, and this is given below :—

	Deaths in less than 2 hours	2–4 hours	4–6 hours	6–12 hours	12–24 hours	24–48 hours	More than 48 hours
Formic acid ..	2	2	1	3	4	2	..
Acetic acid ..	1	2	4	4	6	1	3

Of the three victims of acetic acid who survived for more than 48 hours, one survived for 3 days, another for 7 days and the last for 14 days. The second was a young adult male (24) who had swallowed the acid with his stomach loaded with toddy.

Post-mortem appearances

They included the presence of blood-stained froth in the mouth and nostrils. The pupils were usually dilated. The right side of the heart was full with blood, and the left empty. The lungs were often congested and crepitant; in some cases they were normal. The liver, kidney and spleen were generally congested. If the concentrated acid was swallowed on an empty stomach, the latter was often distended and dark in appearance and contained dark thick fluid matter. With large doses the coats of the stomach had a dark and burnt appearance. This dark appearance was not noticed when the acid was taken on a loaded stomach. The coats might be normal, although a congested appearance was sometimes seen in such cases. If the dose was heavy enough, the intestines might also be dark in colour and distended in appearance. The bladder in formic acid poisoning cases was empty. This being so, examination of the urine which would have thrown light on the rate of absorption of the acid into the system and excretion could not be carried out. The membranes of the brain, the brain substance and ventricles were often congested, the latter containing sometimes cerebro-spinal fluid. Death appeared to result from asphyxia.

Quantities detected

In formic acid poisoning the quantities detected have ranged from 0.04, 0.17, and 0.55 to 31.8 grammes of anhydrous acid, and in acetic acid cases from a trace to 2.20 and 2.40 grammes of anhydrous acid.

Toxicity and fatal dose

The two acids, being corrosive liquids, destroy the epithelial tissues, formic acid being more irritant than acetic acid. It may be noted here that in the use of the two acids for the coagulation of rubber, 1 ounce of formic acid is enough for 18 lb. of latex whereas 1 ounce of acetic acid coagulates only 11 lb. of latex. In [illegible] of the cases of poisoning by formic acid did the victim survive; even 4 ounces of a 50 per cent diluted acid proved fatal. In two of the cases of acetic acid poisoning in which 2 ounces of undiluted acid were swallowed, recovery was seen. Death was however reported within 9 hours in a case in which the victim, a man of 35, took 2 ounces of the concentrated acid.

Separation, detection and estimation

For quantitative estimation of acetic acid, a known weight of the well-comminuted viscus (100 grammes) is taken in a litre flask with a long neck. 30 c.cm. of 25 per cent phosphoric acid is added, together with 200 c.cm. of water to make the volume up to 300 c.cm. A few glass beads are introduced into the flask. A still head is used and the distillation is conducted with vigorous boiling. The volume of the liquid is kept constant by adding distilled water drop by drop from a separating funnel, distillation being carried on until about 1,500 c.cm. of the distillate have passed over, when usually the point of neutrality to litmus is reached. Stir an excess of calcium carbonate with water and add to the distillate. Bring at once to the boil, and then evaporate in a flat dish on the water bath to dryness. Dry the whole residue for an hour at 100°C. Add 50 c.cm. of hot water, keep for a few minutes and filter, washing the dish several times with warm water and passing the liquid each time through the filter. Cool. Make up to 500 c.cm. A portion of this solution is evaporated to dryness on the water bath, dried at 100°C. and the residue is heated in a hard-glass test tube with arsenious acid. The presence of acetic acid is inferred from the smell of cacodyl produced. The ethyl acetate test can be applied to a portion of the residue; but often the presence of organic matter prevents the smell being noted. In such cases, the cacodyl test answers the purpose very well.

Take 25 c.cm. of the above solution in a long-necked flask of 300 c.cm. capacity and proceed as follows: Add 30 c.cm. of water free from CO_2 and 10 c.cm. of phosphoric acid (sp. gr. 1.7). The volume of the liquid in the flask is kept constant by adding CO_2 free distilled water. The distillation is carried on for 1½ to 1¾ hours, the distillate being collected in a flask containing excess of a known volume of standard NaOH, to which access of CO_2 is prevented by providing a bent tube containing soda lime. Ninety-five per cent of the acid is evolved during the first hour. The excess of NaOH is titrated with standard H_2SO_4, using phenolphthalein as indicator. Each c.cm. of N/10 NaOH represents 0.006 grammes acetic acid.

For the quantitative estimation of formic acid, the method suggested by Autenrieth (1928) may be followed. The presence of organic matter interferes with the usual reduction test (with silver nitrate). This can be avoided if the residue is treated with magnesium and dilute sulphuric acid in a distilling flask, the contents distilled, and the distillate tested for formaldehyde by the usual reagents namely phloroglucinol and resorcinol (Henry Leffmann in Allen's *Commercial Organic Analysis*, Vol. I, 1924).

Summary

Formic and acetic acids have figured in the toxicology of Travancore in a remarkable manner during the last 8 years. Prior to 1936 there were no recorded cases of poisoning due to these acids, but since then 71 cases have occurred out of a total of 428 cases in which poison was detected. Formic acid does not appear to have found a place in toxicology elsewhere in India, while acetic acid has been mentioned only once in the last decade by the Chemical Examiner to the Government of Madras.

The regional incidence of these cases shows their close association with the rubber industry, in particular with the distribution of the small rubber holders whose store room is a part of the house they live in. The scarcity of the acids resulting from the outbreak of the war has considerably reduced the incidence of poisoning by these acids.

In 77 per cent of cases the acids were swallowed for suicide; in 18 per cent it was accidental. There were no instances of their use for homicide, but in 3 cases death followed deliberate use for relief of stomach pain.

Death may occur within a few hours or up to 14 days after swallowing. While 2 ounces of the concentrated acid have been found generally fatal, in a few cases of acetic acid poisoning, that dose did not cause death. All cases of formic acid poisoning were fatal.

The signs and symptoms are those of irritant poisoning, and post-mortem appearances are suggestive of death from asphyxia.

The technique adopted by the authors for the qualitative and quantitative estimation of the acids is described.

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CANNING AND STORAGE EFFECTS

Volatile Water-Soluble and Oil Constituents of Valencia Orange Juice

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The nature of the chemical changes in the water and oil constituents of the volatile flavoring material of fresh, freshly canned, and stored canned California Valencia orange juices has been investigated. Acetic, propionic, isovaleric, and traces of butyric acid were found in the stored juice. There was a decreased acetaldehyde content and traces of diacetyl. A $C_6H_8O_2$ unsaturated acid previously found in grapefruit juice was present in all three orange juices. Twenty-nine compounds from the oil fraction were identified and their amounts determined in each of the three juices. Analyses were made by chromatographic techniques when possible. The major changes in the oil fraction of orange juice on canning and storage were loss in total volatile oil, conversion of hydrocarbons to alcohols, and loss in esters, aldehydes, and terpene aliphatic alcohols. The predominant off-flavor of stored canned orange juices appears to arise from the non-volatile precursors.

THE VOLATILE FLAVORING CONSTITUENTS of orange juice have been considered in this study to be materials that can be removed from the juice by distillation methods. These include low-boiling organic constituents and high-boiling oils, the latter being removed as aqueous azeotropes.

If these volatile constituents are removed from the juice by distillation at atmospheric pressure, the recovered fraction has an odor strongly suggesting that its composition has been considerably altered in the process. If they are removed at reduced pressures and correspondingly lower temperatures, the aroma of the recovered fraction resembles more closely the aroma of the original juice. The lower the temperature of distillation the more closely the volatiles resemble the original fruity aroma.

A citrus juice from which a portion of the water has been removed by distillation shows a definite change in flavor, and it was early recognized that the aroma of orange juice was associated with the volatile fraction (*10*). It was also shown that the flavor of orange juice is largely lost by evaporation, but that this loss may be partly overcome by the addition of fresh juice to the concentrate to restore flavor and aroma (*22*).

The flavor of a concentrated orange juice which has been restored to its original strength by the addition of pure water tastes insipid and is lacking in orange character (*27*). It is generally recognized that the flavor and aroma of fresh orange juice, as well as certain off-flavors of the processed juice, are associated with the volatile fractions (*2, 4, 11*). These fractions consist of constituents soluble and insoluble in water; whereas some of the aroma resides in the water-soluble fraction (*10, 16, 23*), most of the characteristic flavor and aroma of citrus juices is considered to be in the volatile, sparingly water-soluble, oil fractions (*14, 16, 23, 27*).

The method of extraction of the juice from the fruit results in the incorporation of some peel oil into the juice, but the juice itself contains some oil present in the juice sacs. In experiments in which fruit was carefully peeled and washed, appreciable amounts of oil were recovered from the juice sacs (*2, 4, 23, 27*). Globules of oil have been demonstrated by histological studies on the juice sacs of various citrus fruits (*6, 26*).

Orange juice has been investigated extensively, particularly as regards changes in the composition of the volatile fractions resulting from heating and storage. Hall and Wilson (*10*) investigated the volatile materials obtained from 10,000 gallons of fresh California Valencia orange juice.

They reported the following constituents: acetone, acetaldehyde, ethyl alcohol (0.018% on separate sample), formic acid, citronellal, caprylic acid as ester, acetic acid as ester, formic acid as ester, isoamyl alcohol, phenyl ethyl alcohol, possibly geraniol, α-terpineol, and a $C_{10}H_{18}O$ alcohol similar to linaloöl.

Hydrogen sulfide has been reported in green oranges and in orange juice (*1, 18*). Furfural has been detected in orange juice stored in the presence of oxygen (*24*), and seasonal acetaldehyde and alcohol contents of orange juice have been determined (*32*).

This paper reports the isolation and identification of the constituents in the volatile water-soluble and oil fractions from freshly reamed, canned, and stored canned California Valencia orange juices and represents a continuation of long term studies on citrus flavoring (*14, 16*).

Water-Soluble Constituents

Experimental

In the preparation of the canned single strength orange juice used in this study processing methods followed standard commercial practices. Arrangements were made to purchase the orange juice from a commercial processor, where the oranges were carefully selected to prevent the inclusion of soft and rotten fruit and thoroughly washed. Total soluble solids in the juice was 13.8%, and total acid (as citric acid) was 1.26%.

The juice, obtained from an automatic citrus juice extractor, was screened and run into a large stainless steel mixing tank. One third of the juice was quick frozen for delivery to the laboratory for immediate analysis, while the remaining juice was pasteurized at 92° C. (198° F.) and filled directly into 46-ounce citrus enamel-lined cans. After being filled and sealed, the cans were cooled to approximately 38° C. (100.4° F.) by passage through a cold water tank before being packed in cartons. The following volumes of orange juice were distilled and studied individually: 3000 gallons of freshly reamed juice, 2500 gallons of freshly canned juice, and 1520 gallons

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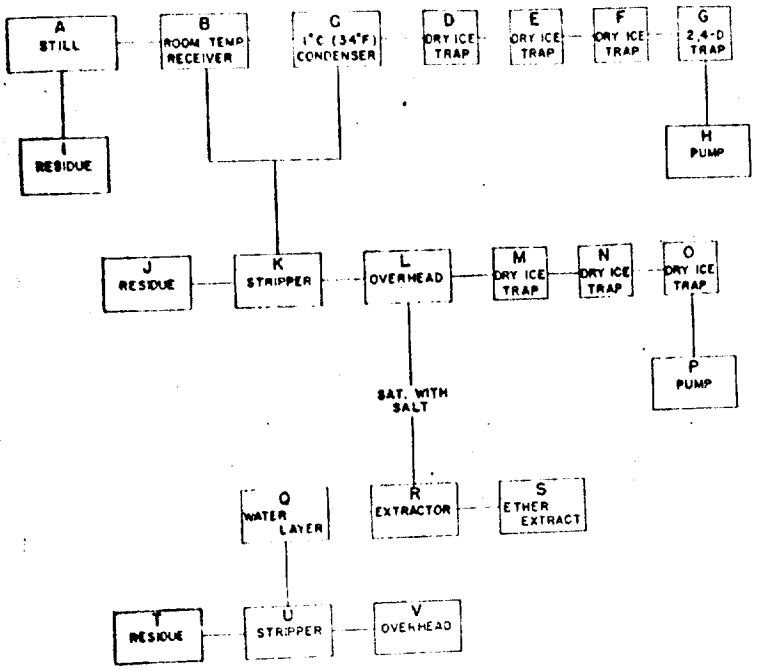

Figure 1. Flowsheet for separation of volatile constituents from orange juice

of stored canned juice held at room temperature for 3 years.

A flowsheet describing separation of the volatile material from the juice appears in Figure 1. The frozen, fresh Valencia orange juice (3000 gallons) was thawed by running through a Seprosieve into a warm water-jacketed kettle equipped with a stirrer, and then fed directly into the evaporator, *A*, while still cold. Distillation was carried out at 40 mm. of mercury absolute, with the evaporator contents maintained below 45° C. (113° F.) at all times, until 75% of the juice had distilled. The evaporator was heated by steam at 8-pound gage pressure (112° C., 233.6° F.). [It was found by test runs that orange juice lost its volatile oil less readily than grapefruit juice (*16*), so it was necessary to distill off 75% of the juice before essentially all of the oil was removed.] The still was a long vertical-tube, natural-circulation, vacuum evaporator with an external vertical-liquid separator. The vapors passed from the top of the evaporator through two water-cooled stainless steel condensers into flask, *B*, at room temperature. This flask was connected to the vacuum pump, *H*, through another condenser, *C*, refrigerated with a 1° C. (34° F) brine system, and a series of traps, *D,E,F*, maintained at −78° C. (−108° F.) with dry ice and alcohol. A trap, *G*, containing 2,4-dinitrophenylhydrazine in sulfuric acid was inserted just ahead of the pump to trap acetaldehyde. The condensate from the room temperature, *B*, and refrigerated receivers, *C*, was pumped to a stainless steel stripping column, *K*, in order to concentrate the volatile water-soluble materials. The stripping column was also operated under vacuum with a 1° C. (34° F.) condenser and refrigerated receiver, *L*, connected through a series of dry ice traps, *M,N,O*, to the vacuum pump, *P*, in order to collect the volatiles. By this means the evaporator distillate was reduced to 150 gallons. The distillate was saturated with salt and extracted with diethyl ether in a continuous extractor, *R*, under a pressure of 360.7 mm. of mercury absolute. Ether used in this work was anhydrous analytical grade containing not more than 0.01% alcohol. Prior to use it was checked to make sure no peroxides were present. Approximately 5 gallons of ether were used to each 1000 gallons of juice. The water layers in the various cold traps were separated from the oils, saturated with salt, and extracted with ether. All of the ether-extracted water fractions were then combined, freed of excess salt and ether, and concentrated in the stripping column to 5 gallons in order to recover the volatile water-soluble material, *V*. This concentrate was then carefully fractionated on a Podbielniak Hyper-Cal column, and the resulting fractions were systematically examined by microtests for carbonyl compounds, alcohols, esters, acids, and sulfur and nitrogen compounds.

The combined ether extracts of the water-layer condensates from the evaporation of the juice were freed of ether by distillation on the Hyper-Cal column. Low-boiling alcohols present in this fraction were fractionally distilled on this column. The combined oil layers from the condensers, *B,C*, the cold traps, *D,E,F,M,N,O*, and the oil remaining after removal of the low-boiling alcohols from the ether extract, were analyzed.

The freshly canned juice and the juice that had been stored for 3 years were distilled in a similar manner.

As the residue, *M*, from stripping the evaporator distillate from stored juice was acidic, a portion was worked up to determine which acids were present. Thirty gallons of the residue were neutralized with sodium hydroxide and evaporated to dryness, and 4.76 grams of sodium salts were recovered. From a similar treatment of 58 gallons of residues from fresh juice, and 14 gallons from freshly canned juice there were obtained 1.28 and 0.7 grams of sodium salts. This material was converted to the *p*-phenylphenacyl esters (*7*) for chromatographic separation and identification of the acids (*17*).

Table I gives the approximate amounts of the compounds found in the volatile water-soluble fractions of the three juices.

Results and Discussion

Carbonyls. A crystalline 2,4-dinitrophenylhydrazone (melting point 167.5° C.) was identified as that of acetaldehyde by a mixed melting point with a known sample. It was also identified by the dimedone derivative (melting point 143° C.), and by the preparation of ethylidene di-2-naphthyloxide (melting point 173° C.).

Table I. Volatile Water-Soluble Constituents of Fresh, Freshly Canned, and Stored Canned Valencia Orange Juices

	Mg. per Kg. of Juice		
	Fresh	Freshly canned	Stored canned
Acetaldehyde	3.0	3.0	0.8
Furfural	Trace	Trace	5.1
Acetone	Trace	Trace	Trace
Diacetyl	...	...	Trace
Ethyl alcohol	380	550	484
Methanol	0.8	Present	0.2
Hydrogen sulfide	Trace	Trace	None
Acetic acid	2.8	5.8	18.6
Propionic acid	...	...	0.1
Butyric acid	...	...	Trace
Isovaleric acid	...	...	0.4
C_6H_5COOH	0.1	0.1	0.7
Other acids	0.1	...	...

Fresh juice contained 3.0 mg. of acetaldehyde per kg. based upon analysis with 2,4-dinitrophenylhydrazine. Values for the freshly canned and stored canned juices were 3.0 and 0.8 mg., respectively.

Furfural was detected in trace amounts in both the fresh and the freshly canned juices by means of the *p*-toluidine acetate test. In the stored canned juice this aldehyde was found to the extent of 5.1 mg. per kg. It was positively identified by means of a semicarbazone derivative melting at 190–192° C., which showed no melting point depression when mixed with an authentic derivative. The 2,4-dinitrophenylhydrazone melted at 225–227° C. This furfural probably arises from the carbohydrate fraction of the juice.

Traces of acetone were found in all three juices. The 2,4-dinitrophenylhydrazones were mixtures which could not be resolved by recrystallization. They were resolved chromatographically (*28*) and were a mixture of acetone and acetaldehyde 2,4-dinitrophenylhydrazones. The presence of acetone had been demonstrated by Hall and Wilson (*10*) by means of a potassium nitroprusside test for methyl ketones and an iodoform test.

A trace of diacetyl was found in the stored canned juice. It was identified by preparing the semicarbazone which melted at 279° C.

Alcohols. Ethyl alcohol was determined semiquantitatively by specific gravity and refractive index measurements. It was positively identified by means of its 3,5-dinitrobenzoate derivative melting at 92–93° C., and was present in the fresh juice to the extent of 406 mg. per kg. (0.04%). Hall and Wilson (*10*) reported a value of 0.018%, but did not indicate the method of determination. The freshly canned juice contained 554 mg. per kg., and the stored canned juice 484 mg. per kg.

A small quantity of methanol (0.8 mg. per kg.) was found in the fresh orange juice as determined by its oxidation to formaldehyde and analysis for formaldehyde by the chromotropic acid test (*3*). The results were checked against the actual quantity of methanol isolated by fractionation. Qualitatively it was identified by the isolation of the 3,5-dinitrobenzoate (melting point 108–109° C.). It gave no melting point depression with an authentic sample of methyl 3,5-dinitrobenzoate. Unfortunately, the freshly canned juice fraction containing the methanol was lost before a quantitative determination could be made. It was, however, shown to be present qualitatively. The stored canned juice was found to contain 62 mg. per kg. of juice. The absolute amounts in the various alcohol fractions were determined by the refractometer method (*33*). The increase in methanol in the stored juice probably results from the breakdown of pectins.

Acids. The *p*-phenylphenacyl esters obtained from the acids in the stripper residues were separated by chromatographic adsorption on silicic acid (*17*). The major portion of the derivatives in all three juices was found to be the derivative of acetic acid and represented 2.8, 5.8, and 18.7 mg. per kg., respectively, in the fresh, freshly canned, and stored canned juices. The melting point of the ester was 109–111° C., and a mixed melting point with *p*-phenylphenacyl acetate gave no depression.

Other acids were found in the stored canned juice. Propionic acid was present to the extent of 0.1 mg. per kg. of juice. The *p*-phenylphenacyl ester of this acid melted at 102–103° C. and showed no depression with *p*-phenylphenacyl propionate. Isovaleric acid (0.4 mg. per kg. of juice) was identified as the *p*-phenylphenacyl ester melting at 75–77° C., and a trace of butyric acid was found with *p*-phenylphenacyl ester melting at 82° C. Traces of other acids were found in the fresh juice but in amounts too small to be identified.

The *p*-phenylphenacyl ester of one of the unsaturated acids found in grapefruit juice (*16*) was also found in all three orange juices. The *p*-phenylphenacyl ester melts at 120° to 126° C. and analyzes for the derivative of an acid with the formula $C_6H_8O_2$. The amounts of this acid in the fresh, freshly canned, and stored canned juice are respectively, 0.1, 0.1, and 0.7 mg. per kg. of juice.

Miscellaneous. During distillation of the juice small quantities of hydrogen sulfide were detected (*1, 18*) in both the fresh juice and the freshly canned juice, but not in the stored canned juice. Passage of nitrogen through unheated freshly reamed juice showed hydrogen sulfide to be present as such and not as an artifact produced by heating.

Oil-Soluble Constituents

Experimental

The oil layers obtained from the low-temperature distillation of the three lots of juice were separated into zones of similar R_f values by chromatographic processes. The individual components were obtained by further chromatographic separations, by distillation, or by chemical means.

The chromatographic methods used, establishment of the purity of isolated compounds, and determination of the physical properties of pure compounds have been described (*13–15, 20, 21*). In the identification of the compounds, mixed chromatograms with known compounds were run when these were available for comparison. The adsorbent used for preparing chromatostrips and adsorbent columns was reagent grade silicic acid.

An example of the separation of one of the oil layers serves to illustrate the procedures used. From the low-temperature distillation of the fresh lot of 3000 gallons of orange juice, the combined oil layers collected in the cold traps consisted of 1010 grams of a nearly colorless oil of n_D^{25} 1.468, α_D^{20} + 100° after drying over anhydrous sodium sulfate. (The oil obtained by ether extraction of the water layers was treated separately.) This oil was adsorbed on a column packed with 200 grams of silicic acid as a slurry of the adsorbent with 1200 ml. of benzene-free hexane. The column was washed with hexane until tests on chromatostrips showed all hydrocarbons had been removed. Development was continued using successively stronger solutions of ethyl acetate in hexane until chromatostrip tests showed all of the material had been washed into the eluate, which was collected in 10-ml. portions.

The hydrocarbon portion was kept as one fraction and separated by vacuum distillation on a Podbielniak Hyper-Cal column as a material having a lower boiling point than limonene, as limonene, and as a material having a higher boiling point than limonene. The low-boiling material was adsorbed on silicic acid with hexane as developer and separated into α-thujene (?) and β-myrcene. The high-boiling material was adsorbed on silicic acid with hexane as the developer and separated into two fractions, sesquiterpene I and sesquiterpene II.

The nonhydrocarbon eluate fractions from the original column were systematically tested on chromatostrips with 15% ethyl acetate in hexane as the developer, with fluorescein-bromine (*15*) and 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid used as indicators. Fractions of similar R_f value were combined and the solvent evaporated at reduced pressures. In this way the nonhydrocarbon fractions were divided into eight fractions. After examination on chromatostrips for the number of compounds present and the best solvent for separations, each fraction was adsorbed on columns of silicic acid and separated into components. For less strongly adsorbed compounds chloroform or benzene was most often used as a developer. For compounds of intermediate or strong adsorption 5% ethyl acetate in benzene, 15% diisopropyl ether in hexane, 5% ethyl acetate in chloroform, 5% ethyl acetate in benzene, or 5% ethyl acetate in hexane was used. For maximum separation of components of close R_f values, 1 to 1.5 grams of the oil mixture was used with 100 grams of silicic acid of 200 mesh and approximately 5% moisture content. It was often necessary to perform more than one separation on chromatographic columns to

effect separation of sufficient quantities of material for identification purposes.

Methods of testing the purity of compounds with one- and two-dimensional chromatograms and crude distillations of 50- to 100-mg. samples were the same as reported in the work on grapefruit volatile oil constituents (*14*). Spots on the chromatograms were detected by spraying with fluorescein-bromine, 2,4-dinitrophenylhydrazine, *o*-dianisidine, or concentrated sulfuric-nitric acid.

In contrast to finding a majority of the components of the volatile oil from grapefruit juice separable by chromatography, the orange oil constituents were most often separated into chromatographically homogeneous fractions which consisted of two or more compounds. It was often necessary to resort to distillation or chemical methods to separate these mixtures. These methods are described for the individual compounds in the summary of identification work for that compound.

Results and Discussion

The following compounds were isolated from the three juices.

Hydrocarbons

d-Limonene. α_D^{25} + 103.8°, n_D^{25} 1.4708.

β-Myrcene. Analysis. Calculated for $C_{10}H_{16}$, C 88.16; H 11.84. Found, C 87.69; H 11.94, α_D 0, n_D^{25} 1.4648, boiling point at 18 mm. 68.5° C., at 36 mm. 82.2° C.

Derivatives. Maleic anhydride adduct melting point 33–35° C. (from methanol) (lit. 34–35° C.).

α-Thujene (?). Analysis. Calculated for $C_{10}H_{16}$, C 88.16; H 11.84. Found, C 87.95, H 12.21, α_D^{25} + 31.14, n_D^{25} 1.4552, boiling point at 30 mm. 71° C.

Derivatives. An authentic sample of *α*-thujene could not be found for comparison with this unknown. This compound can be separated chromatographically from both limonene and *β*-myrcene, but is indistinguishable chromatographically from *α*-pinene. The only derivative which could distinguish between *α*-pinene and *α*-thujene was the hydrochloride. Repeated attempts to form the hydrochloride of *α*-pinene resulted in a solid compound melting at 98° to 118° C. after four recrystallizations from methanol (lit. 132° C.). It was not possible to isolate a crystalline hydrochloride from the unknown. On the basis of physical properties this compound is tentatively identified as *α*-thujene.

$C_{15}H_{24}$ (I). Analysis. Calculated for $C_{15}H_{24}$, C 88.16; H 11.84. Found, C 87.41; H 11.55, α_D^{25} + 86.18, n_D^{25} 1.5012, d_4^{25} 0.9053, boiling point at 16 mm. 140.5° C.

Derivatives. Bromide, melting point 140–142.5° C. (from ether-alcohol). Analysis. Calculated for $C_{15}H_{24}Br_4$, C 34.36; H 4.70. Found, C 34.38; H 4.62. This compound absorbs 2 moles of hydrogen per mole and, therefore, contains two double bonds. Repeated attempts to form a crystalline hydrochloride by saturating an ether or an acetic acid solution of the hydrocarbon with dry hydrogen chloride, or by adding the equivalent amount of hydrogen chloride in ether to the hydrocarbon in ether, were unsuccessful.

The properties of this compound do not agree with those of any of the well-characterized sesquiterpenes found in the literature.

$C_{15}H_{24}$ (II). Analysis. Calculated for $C_{15}H_{24}$, C 88.16; H 11.84. Found, C 86.10, H 11.52. α_D^{25} −6.56, n_D^{25} 1.5008, boiling point at 10 mm. 115° C., at 21 mm. 132.5° C.

Nonhydrocarbons

Esters. Ethyl Isovalerate. The ester was hydrolyzed with potassium hydroxide in diethylene glycol. The 3,5-dinitrobenzoate of the alcohol portion melted at 92–93° C. A mixed melting point with ethyl 3,5-dinitrobenzoate was not depressed. The *p*-phenylphenacyl ester of the acid portion melted at 77–79° C. A mixed melting point with *p*-phenylphenacyl isovalerate was not depressed.

This ester, isolated chromatographically, was found mixed with hexanal, octanal, and ethyl $C_6H_8O_2$. It was separated from the mixture by distillation at 30 mm. in a microdistillation column.

Ethyl $C_6H_8O_2$. The ester was hydrolyzed with potassium hydroxide in diethylene glycol. The 3,5-dinitrobenzoate of the alcohol melted at 93° C. The *p*-phenylphenacyl ester of the acid melted at 124–126° C. By comparison on chromatostrips, and by the melting point of the acid derivative, the acid portion of this ester was determined to be the same as the $C_6H_8O_2$ acid isolated from the distillation water of the juice.

This ester, isolated by adsorption from silicic acid, was not separable by chromatography from hexanal, octanal, and ethyl isovalerate. It was separated from these compounds by distillation at 30 mm. in a microdistillation column.

Methyl *α*-Ethyl *n*-Caproate. Analysis. Calculated for $C_9H_{18}O_2$, C 68.31; H 11.46. Found, C 67.57; H 11.35. Boiling point at 30 mm. 80° C., n_D^{25} 1.4101.

Derivatives. The compound was hydrolyzed with potassium hydroxide in diethylene glycol. The *p*-phenylphenacyl derivative of the acid melted at 54.5–55.5° C. (from 95% alcohol) (lit. 53–54° C.). Analysis. Calculated for $C_{22}H_{26}O_3$, C 78.07; H 7.74. Found, C 78.06; H 7.80.

The chromatographically homogeneous fraction in which this compound was found contained citronellyl acetate and citronellal. The aldehyde was removed by reaction with semicarbazide hydrochloride and separated from the esters by adsorption on silicic acid and development with ethyl acetate. The two esters were then separated by distillation in a microdistillation column.

Citronellyl Acetate. Boiling point at 8 mm. 110.5° C., n_D^{25} 1.4422.

Derivatives. The ester was saponified with potassium hydroxide in diethylene glycol. The alcohol portion compared with citronellol on chromatostrips showed no differences. The *p*-phenylphenacyl ester of the acid melted at 107–108° C. (from 95% alcohol) (lit. 111° C.).

Terpinyl Acetate. Analysis. Calculated for $C_{12}H_{20}O_2$, C 73.42; H 10.27. Molecular weight 196. Found C 71.79; H 10.34. Molecular weight 194.

Derivatives. The ester was saponified with potassium hydroxide in diethylene glycol. The alcohol portion compared with *α*-terpineol on chromatostrips showed no differences.

This compound was found as a mixture with $C_{15}H_{24}O$ (II) and was separated from it by reacting the aldehyde with semicarbazide hydrochloride and separating the two on a silicic acid column with ethyl acetate as developer.

Carbonyls. Hexanal. Boiling point 113° C., n_D^{25} 1.4175.

Derivatives. 2,4-Dinitrophenylhydrazone melting point 105–106° C. (from 95% alcohol) (lit. 106–107° C.). Analysis. Calculated for $C_{12}H_{16}N_4O_4$, C 51.42; H 5.75; N 19.99. Found, C 51.79; H 5.93; N 19.87. Semicarbazone melting point 106–108° C. (from benzene) (lit. 106° C.).

Octanal. Derivatives. 2,4-Dinitrophenylhydrazone melting point 109° C. (from 95% alcohol). Analysis. Calculated for $C_{14}H_{20}N_4O_4$, C 54.33; H 6.54; N 18.17. Found, C 55.16; H 7.01; N 18.19. Mixed melting point with octanal 2,4-dinitrophenylhydrazone showed no depression.

Decanal. Derivatives. 2,4-Dinitrophenylhydrazone melting point 104–106° C. (lit. 104° C.). The derivative melting point was not depressed when mixed with the 2,4-dinitrophenylhydrazone of decanal.

2-Dodecenal (?). Boiling point at 10 mm. 117° C., n_D^{25} 1.4585.

Derivatives. The 2,4-dinitrophenylhydrazone was formed and separated from reagent by adsorption on silicic acid and development with benzene. The resulting hydrazone melted at 74–77° C. (from 95% alcohol), but the product was amorphous. The semicarbazone was formed and separated from reagent and unreacted compound by adsorption on silicic acid and development with ethyl acetate. (Semicarbazones could be detected on developed chromatostrips by spraying with a solution of 50 mg. of bromophenol blue and 200 mg. of citric acid in 100 ml. of water. The semicarbazone was a blue spot on a yellow background.) The semicarbazone was waxy, even after precipitation from methanol and water, hexane, or ethyl acetate. A characteristic melting point could not be obtained.

CITRONELLAL. This compound was removed from a mixture with citronellyl acetate and methyl α-ethyl *n*-caproate by reacting with semicarbazide hydrochloride. The mixture was resolved by adsorption on silicic acid and developing with ethyl acetate. Semicarbazone melted at 85–86° C. from 50% methanol (lit. 86° C.). The 2,4-dinitrophenylhydrazone prepared by adding reagent to the mixture with the esters and recrystallizing from 95% alcohol melted at 76–77° C. (lit. 76.5–78° C.).

$C_{15}H_{24}O$ (I). Analysis. Calculated for $C_{15}H_{24}O$, C 81.76; H 10.98. Molecular weight 220. Found, C 81.93; H 11.01. Molecular weight 237. Boiling point at 12 mm. 125.5° C., n_D^{25} 1.5019.

Derivatives. The 2,4-dinitrophenylhydrazone was amorphous and melted at 183–186° C. (from 95% alcohol). The semicarbazone melted at 215–217° C. from methanol. The compound reacts with *o*-dianisidine and is probably an aldehyde.

$C_{15}H_{24}O$ (II). Analysis. Calculated for $C_{15}H_{24}O$, C 81.76; H 10.98. Found, C 81.85; H 10.39. Boiling point at 6 mm. 141° C., n_D^{25} 1.5038.

Derivatives. Semicarbazone melting point 126–129° C. from 50% methanol. Analysis. Calculated for $C_{16}H_{27}N_3O$, C 69.27; H 9.81; N 15.15. Found, C 69.32; H 9.36; N 14.95. 2,4-Dinitrophenylhydrazone melts at 60–62° C. from 95% alcohol (amorphous). When chromatographed on chromatostrips this compound appeared as an ultraviolet absorbing spot which reacted with 2,4-dinitrophenylhydrazine and with *o*-dianisidine. It was concluded to be a sesquiterpene aldehyde.

$C_{15}H_{22}O$. Boiling point at 3 mm. 146.5° C., n_D^{25} 1.5210.

Derivatives. Semicarbazone, melting point 192–194° C. (from 50% methanol). This compound was judged to be the same as the $C_{15}H_{22}O$ compound found in grapefruit juice by comparison on chromatostrips and by odor.

CARVONE. Derivatives. 2,4 - Dinitrophenylhydrazone melting point 185–188° C. The melting point of the derivative was not depressed when mixed with an authentic sample of carvone 2,4-dinitrophenylhydrazone. The sample has a strong odor of caraway.

Alcohols. $C_{15}H_{26}O$ (I). Analysis. Calculated for $C_{15}H_{26}O$, C 81.02; H 11.79. Molecular weight 222. Found, C 80.24; H 12.08. Molecular weight 232. Melting point 94–96° C. (from 80% methanol).

Derivatives. The allophanate was formed by adding the compound in benzene to cyanic acid, extracting the reaction mixture with hot 90% ethyl acetate in hexane, evaporating the solvent, and recrystallizing the product from 90% methanol. After 24-hour standing at 5° C. well-formed crystals were present (melting point 153–153.5° C.). The compound was mixed with benzene, pyridine, and 3,5-dinitrobenzoyl chloride, but did not form the benzoate derivative, as shown by chromatography of the reaction mixture, and by recovery of the unchanged compound. The compound failed to give the alkyl xanthate test for primary or secondary alcohols (*8*). It was concluded to be a tertiary sesquiterpene alcohol. Its properties did not agree with those of any of the known sesquiterpene tertiary alcohols.

$C_{15}H_{26}O$ (II). Analysis. Calculated for $C_{15}H_{26}O$, C 81.02, H 11.79. Molecular weight 222. Found, C 80.61; H 12.03. Molecular weight 242. Boiling point at 4 mm. 151° C., n_D^{25} 1.5013.

Derivatives. The compound reacted with cyanic acid and the formation of an allophanate was shown by chromatographing the reaction mixture on chromatostrips. A low R_f compound which reacted with fluorescein-bromine spray was formed. A crystalline derivative could not be obtained from hexane, dilute methanol or hexane ethyl acetate mixtures. The formation of an allophanate indicates this compound is an alcohol.

LINALOÖL. Analysis. Calculated for $C_{10}H_{18}O$, C 78.39; H 11.19. Molecular weight 154. Found, C 77.89; H 11.13. Molecular weight 183. Boiling point at 32 mm. 115° C., α_D^{25} + 16.47, n_D^{25} 1.4645.

Derivatives. Phenylurethane melting point 61–64° C. (from hexane) (lit. 65–66° C.). The compound was oxidized with chromic anhydride in glacial acetic acid, diluted with water, and extracted from the mixture with hexane. The product had the odor of citral, and when chromatographed on chromatostrips with several solvents, it was indistinguishable from citral, and gave colored spots when sprayed with *o*-dianisidine and 2,4-dinitrophenylhydrazine.

CARVEOL. Boiling point at 6 mm. 100° C., α_D^{25} + 9.30, n_D^{25} 1.4932.

Derivatives. 3,5 - Dinitrobenzoate melting point 119° C. (from alcohol–ethyl acetate, 3 to 1) (lit. *dl-trans*-carveol, 119° C.). The compound was oxidized with chromic anhydride in glacial acetic acid, extracted with hexane from the aqueous reaction mixture, compared to carvone chromatographically, and reacted with 2,4-dinitrophenylhydrazine. The 2,4-dinitrophenylhydrazone melted at 191° C. (from 10% ethyl acetate in alcohol) (lit. 189° C.). It was not depressed when mixed with carvone 2,4-dinitrophenylhydrazone.

1-DECANOL. This alcohol was found mixed with carveol. Fifty milligrams of the mixture were oxidized with 1 ml. of a saturated solution of chromic anhydride in glacial acetic acid. After dilution with water and extraction with hexane, the mixture was chromatographed on chromatostrips. The reaction products of decanal and carvone were identified by comparison to known samples. The 2,4 - dinitrophenylhydrazones formed from the mixture were adsorbed on silicic acid and developed with benzene. After removal of the solvent, the single band formed was dissolved in 95% alcohol and carvone 2,4-dinitrophenylhydrazone filtered from the cooled solution. After removal of as much red carvone derivative as possible the supernatant liquors were evaporated and yellow crystals obtained by crystallization from 95% alcohol. After several recrystallizations the melting point was 92–94° C. Mixed with octanal 2,4-dinitrophenylhydrazone of melting point 109° C., the melting point of the mixture was depressed to 86–95° C. Mixed with decanal 2,4-dinitrophenylhydrazone of melting point 104° C., the melting point of the mixture was 96–100° C. The amount of 1-decanol present in the mixtures was calculated from the refractive index of the original fractions.

1-OCTANOL. This alcohol was always found in the α-terpineol fractions. The mixture was oxidized with chromic anhydride, compared to octanal on chromatostrips, the 2,4-dinitrophenylhydrazone formed, and separated by adsorption on silicic acid with benzene as the developer. Melting point 105–107° C. (from 95% alcohol) (lit. 106° C.). The melting point was depressed with the 2,4-dinitrophenylhydrazones of decanal, hexanal, and dodecanal, but not with octanal. The amount of 1-octanol present in the mixture with α-terpineol was calculated from the refractive index of the original fractions.

α-TERPINEOL. Melting point 37–38° C. (from hexane) (lit. 38–40° C.), α_D^{25} + 86, n_D^{25} 1.4815.

Derivatives. Nitrosochloride melting point 105–108° C. from ethyl acetate (lit., 107–108° C.).

1-HEXANOL. Analysis. Calculated for $C_6H_{14}O$, C 70.53; H 13.81. Found, C 70.72; H 13.82. Boiling point at 58 mm. 90.5° C., n_D^{25} 1.4182.

Derivatives. 3,5-Dinitrobenzoate melting point 59–59.5° C. from 95% alcohol (lit. 60–61° C.). Allophanate melting point 163–164° C. from 10 to 1 hexane–ethyl acetate (lit. 165° C.). A portion oxidized with chromic anhydride in glacial acetic acid and compared on chromatostrips to hexanal showed no differences. The 2,4-dinitrophenylhydrazone of the oxidized sample melted at 99–101° C. from 95% alcohol, and its melting point was not depressed when mixed with a known sample of hexanal 2,4-dinitrophenylhydrazone.

3-HEXEN-1-OL. This compound was difficult to separate chromatographically from 1-hexanol. It could not be separated by distillation. The best fraction obtained was shown to contain some 1-hexanol.

Analysis. Calculated for $C_6H_{12}O$, C 71.87; H 12.07. Molecular weight 100. Found C 72.51; H 13.79. Molecular weight 126. Boiling point at 50 mm. 90° C., n_D^{25} 1.4275.

Derivatives. 3,5-Dinitrobenzoate melting point 47° C. from 95% alcohol (lit. 49° C.). The allophanate prepared by reaction of the best fraction with cyanic acid was separated by recrystallization from ethyl acetate into a derivative melting at 164° C., and shown by mixed melting point to be identical with the allophanate of 1-hexanol. Another derivative was obtained from this preparation by separating from an ethyl acetate solution all crystals melting above 153° C., and recrystallizing the supernatant liquors after evaporation of the solvent from 90% ethyl acetate in hexane to a constant melting point of 146–148° C. (lit. 146° C.). The original fraction was oxidized with chromic anhydride solution and the 2,4-dinitrophenylhydrazones formed from the reaction products were separated by adsorption on silicic acid and development with benzene into two hydrazones. The higher R_f derivative melted at 99–100° C., and its melting point was not depressed when mixed with known hexanal 2,4-dinitrophenylhydrazone. The other derivative melted at 150–151° C. from 95% alcohol (lit. 144° C.).

$C_7H_{16}O_2$. Boiling point at 38 mm. 95.5° C., n_D^{25} 1.4272. Derivatives. 3,5-Dinitrobenzoate melting point 63–64° C. (from 95% alcohol). Analysis. Calculated for $C_{14}H_{18}N_2O_7$. C 51.53; H 5.56; N 8.59. Found. C 51.16; H 5.16; N 8.53.

Polyoxygenated Compounds. Material which appeared in the volatile fractions of very low R_f values was isolated from the adsorption columns. These fractions were mostly insoluble in hexane, soluble in ethyl acetate, and of a viscous nature. The fractions gave an acid reaction with pH paper. By analogy to similar fractions obtained from grapefruit juice, these fractions were concluded to be a mixture of compounds with more than one oxygen in the molecule.

Other Compounds. Three other chromatographically homogeneous fractions were isolated from the canned stored orange juice. These fractions were set aside at 38° F. for several months before final identification was undertaken. During this period two of the fractions had badly decomposed as shown by chromatostrip tests and identification was impossible. By comparison on chromatostrips before the sample decomposed it was possible to determine that these compounds were not present in either the fresh or freshly canned juices. The fractions reacted with fluorescein-bromine spray and were of high R_f value, indicating an unsaturated ester, carbonyl, oxide, or high-molecular-weight type compound. One of the three fractions had not decomposed as much as the other two. It was adsorbed on a column and about one fourth of the original fraction was obtained in an unchanged form. When a distillation test showed it to be a mixture, an amount too small for identification remained. The sample was of high R_f value and gave a fluorescein-bromine test for unsaturation. Its boiling point was about 100° C. at 20 mm., and n_D^{25} 1.472. When compared to 1,8-cineol it had the same R_f values but would not give the resorcinol addition product. It appeared to react slowly with 2,4-dinitrophenylhydrazine. From the evidence it did not appear to contain either 1,8-cineol or 1,4-cineol.

Table II contains a summary of the compounds found with an estimation of the amounts obtained in the three orange juices. The amounts were estimated by weighing the oil before separation procedures, weighing the separated fractions obtained, and correcting the weight of the separated fractions for the over-all yield. As pointed out in the earlier work on grapefruit juices (*14*), the losses experienced were almost entirely confined to the process of removing developing solvent from the fractions. Corrections could not be made for differences in volatility of the components of a mixture because of its complexity. Where fractions were left as mixtures the components were estimated from refractive index data and observations of chromatographed portions on chromatostrips. Therefore, the quantitative figures in Table II must be regarded as estimations.

Other compounds have been reported present in orange peel or juice (Table III) which were not found in the orange juice samples examined in this study. For example, citral was isolated by chromatography from commercial orange peel oil, but was not found in the oil fractions of any of the juices tested. Isopulegol has been reported as a constituent of citrus oils (*25*), which also contain citronellal. Isopulegol was prepared by refluxing citronellal with acetic anhydride and hydrolyzing the mixture with potassium hydroxide (*31*). The purified compound was obtained by adsorbing the steam-distilled mixture on silicic acid with 50% isopropyl ether

Table II. Volatile Oil Constituents of Orange Juices

	Mg. per Kg. of Juice		
	Fresh	Freshly canned	Canned stored
Oil present by Clevenger analysis	105.00	88.06	62.36
Total oil obtained[a]	91.58	76.36	54.44
Hydrocarbons	88.39	70.96	45.96
Nonhydrocarbons	3.17	5.40	8.48
Hydrocarbons	80.10	63.60	43.0
Limonene	1.98	1.14	0.69
β-Myrcene	0.30	0.30	0.03
α-Thujene (?)	5.80	5.78	2.04
$C_{15}H_{24}$(I)	0.20	0.18	0.21
$C_{15}H_{24}$(II)			
Nonhydrocarbons			
Esters			
Ethyl isovalerate	0.01	0.01	0
Ethyl $C_6H_{12}O_2$	0.03	0.03	0
Methyl α-ethyl *n*-caproate	0.06	0.10	0.02
Citronellyl acetate	0.10	0.04	0.02
Terpinyl acetate	0.08	0.01	0
Carbonyls			
Hexanal	0.04	0.03	0
Octanal	0.06	0.06	0
Decanal	0.05	0.04	0.02
2-Dodecenal (?)	0.06	0.06	0
Citronellal	0.04	0.04	0.02
$C_{15}H_{24}O$(I)	0.14	0.10	0.15
$C_{15}H_{24}O$(II)	0.07	0.12	0
$C_{15}H_{22}O$	0.09	0.09	0.09
Carvone	0	0	0.08
Alcohols			
$C_{15}H_{26}O$ (I)	0.07	0.24	0.23
$C_{15}H_{26}O$ (II)	0.14	0.16	0
Linaloöl	0.93	1.10	0.12
Carveol	0.06	0.17	0.86
1-Decanol	0.10	0.09	0.07
1-Octanol	0.21	0.23	0.19
α-Terpineol	0.32	1.72	4.08
1-Hexanol	0.10	0.14	0.08
3-Hexen-1-ol	0.10	0.18	0.06
$C_7H_{16}O_2$	0.07	0.08	0.09
Polyoxygenated compounds	0.12	0.15	0.75
Other compounds			0.53

[a] Total oil obtained from 3000 gallons of fresh juice, 2500 gallons of freshly canned juice, and 1520 gallons of canned stored juice.

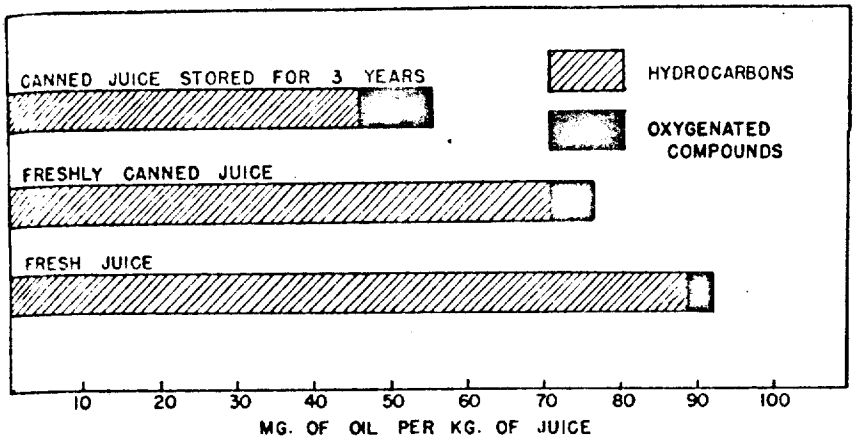

Figure 2. Volatile oils from orange juice

in hexane as the developer. The product. [n_D^{25} 1.4696, allophanate melting point 219° C. (lit. 219° C.)], was chromatographed on chromatostrips in various solvents and compared to fractions of similar R_f values from the juices. Isopulegol was not present in the isolated oil fractions. It was felt that citronellol should be a constituent of the volatile oils from these juices, particularly since citronellyl acetate and citronellal were shown to be present. None of the fractions contained citronellol by comparison on chromatostrips. Citronellal 2,4-dinitrophenylhydrazone was prepared from citronellol after oxidation with chromic anhydride. It was separable by chromatography from the 2,4-dinitrophenylhydrazones of octanal, decanal, and carvone which are the oxidation products of the compounds with R_f values similar to citronellol. By oxidation of a synthetic mixture of geraniol, 1-octanol, α-terpineol, 1-hexanol, and conversion of the oxidized products to their 2,4-dinitrophenylhydrazones it was possible to isolate the 2,4-dinitrophenylhydrazone of the citral (oxidation product of geraniol) in pure form by chromatography on silicic acid with benzene as developer. Since these alcohols have R_f values close to geraniol, and no such derivative was found by similar treatment of orange juice fractions, it was further concluded that geraniol was not present in the oil fractions. Nerol would react in the same way as geraniol in these tests.

The data presented in Figures 2, 3, and 4 show that the volatile oil portion of orange juice is greatly changed during pasteurization and subsequent storage. The total volatile oil decreases markedly, hydrocarbons are lost, and the nonhydrocarbons increase. There is a general decrease in the volatile esters, aldehydes, linaloöl, and the $C_{15}H_{26}O$ (II) compound (which may be farnesol). Other aliphatic alcohols remain essentially unchanged, and there is an increase in carveol, α-terpineol, $C_{15}H_{26}O$ (I), and polyoxygenated compounds. These findings agree in general, but with important differences, with the work of Blair and coworkers (*2*) and Henry and Clifcorn (*11*). Blair and coworkers concluded from infrared studies of oil isolated from heated and stored orange juices, that changes in flavor were caused by hydrolysis of esters and the production of 1,4-cineol, α-terpineol, and terpinolene from the hydrocarbons. Their experiments showed a decrease in recoverable oil and the production of oil-soluble volatile acids during storage. The work of Henry and Clifcorn presents evidence that the products responsible for some of the off-flavor of canned orange juice are volatile unsaturated alcohols and hydrocarbons. Results of the analysis of the volatile oils from the various orange juices are in contrast to the findings from a similar analysis of grapefruit juice (*14*). In grapefruit juice the recoverable oil does not decrease on canning and storage and the hydrocarbons are apparently changed to volatile alcohols without as great a conversion to α-terpineol. The nature of the changes in the volatile oil fractions of grapefruit juice reflect the greater stability of this juice over orange juice.

An attempt was made to evaluate the significant changes in the composition of the volatile oils from the three juices in relation to their effect on the flavor of orange juice. A mixture of 5 mg. of α-terpineol, 1 mg. of carveol, 0.1 mg. of carvone, 1 mg. of polyoxygenated compounds, 5 mg. of furfural, 62 mg. of methanol, and 16 mg. of acetic acid was added to 1 liter of freshly squeezed orange juice and compared to an aliquot

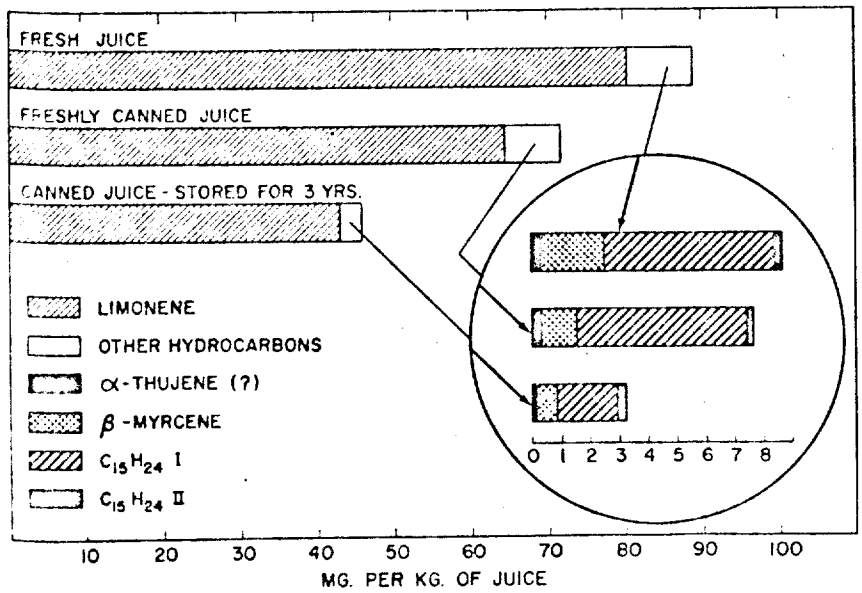

Figure 3. Composition of hydrocarbons from orange juice

Table III. Volatile Oil Constituents Previously Reported in Peel and Juice of Sweet Orange

Hydrocarbons	Aldehydes
Limonene	Citral
Myrcene	Decanal
Ocimine(?)	Nonanal
Terpinolene(?)	Octanal
α-Terpinene	2-Decenal
Terpinene	2-Dodecenal
Carene	Citronellal
Cadinene	
Alcohols	**Esters**
Linaloöl	Methyl anthranilate
1-Octanol (as ester)	Methyl *N*-methyl anthranilate
1-Nonanol	Nonyl caprylate
d-Terpineol	$C_{10}H_{17}OOC_8H_{15}$
l-Terpineol	
Nerol	
1-Decanol(?)	**Esters (acid portion)**
Geraniol(?)	
Farnesol (or nerolidol)	Formic acid
Ceryl alcohol	Acetic acid
$C_{15}H_{15}OH$	Capric acid
2-Phenylethanol	Caprylic acid
	Butyric acid

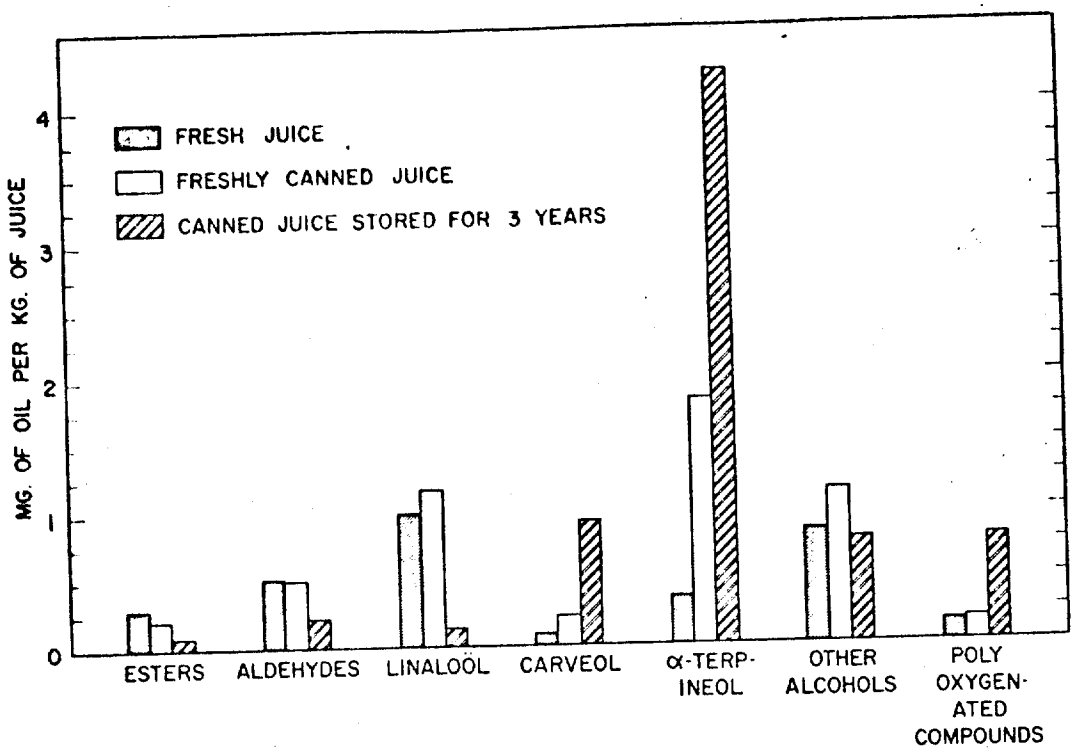

Figure 4. Changes in oxygenated constituents of orange juice

of the same juice without added oils. The flavor of the treated juice was easily distinguishable from that of the untreated juice. The flavor was described as stable and musty, but it did not resemble the flavor and odor of canned stored orange juice. As one of the findings of the work on the volatile oils in orange juice appears to be that the flavorful components are destroyed by canning and storage, a concentrate was prepared by low-temperature evaporation which was substantially free of any volatile oil (less than 0.001%). The reconstituted juice had little orange flavor but it did not have a canned or pasteurized taste. Addition to this juice of α-terpineol, carveol, carvone, polyoxygenated compounds, furfural, methanol, and acetic acid in the amounts found in stored canned orange juice produced a decided stale flavor which could be identified as characteristic of aged canned orange juice but which was not identical with canned juice ordinarily encountered. The $C_6H_8O_2$ acid found in the water-soluble volatile fractions was isolated only as its *p*-phenylphenacyl ester and its flavor contribution could not be studied.

Experiments were conducted to determine the effect of volatile oil decomposition on the flavor of orange juice and to determine, if possible, the extent of the contribution of the decomposition of the volatile oils to the flavor of canned orange juice. To accomplish this, freshly reamed juice was evaporated at low temperature to remove oil. After diluting to the original Brix, part of the lot was canned with added peel oil (same lot of oil as described below) and part without oil. All samples were pasteurized by agitating

Table IV. Triangular Taste Tests on Canned Orange Juice

Sample No. and Lot	Oil Level during Storage, %	Storage Temp., ° F.	Oil Added for Flavor Evaluation[a]	Storage Temp. of Oil Added, ° F.	Compared to Sample No.	Sample Preferred	Correct Judgments Out of 18, %	Statistical Significance[b]
1	0.002	40	Terpeneless	70	2	1	69	++
2	0.002	40	Whole oil	70				
3	0.002	40	Whole oil	0	4	3	83	+++
4	0.002	40	Whole oil	70				
5	0.002	40	Terpeneless	0	6	5	83	+++
6	0.024	40	None	..				
7	0.002	40	Terpeneless	0	8	..	23	−
8	0.002	40	Terpeneless	70				
9	0.002	70	Terpeneless	70	10	9	55	+
10	0.024	70	None	..				
11	0.002	70	Whole oil	70	10	11	60	+
12	0.024	40	None	..	10	12	73	+++
1A	0.001	40	Terpeneless	0	2A	2A	44	−
2A	0.001	40	Whole oil	0				
3A	0.016	40	None	..	2A	2A	67	++
4A	0.016	70	None	..	5A	..	55	+
5A	0.001	70	Whole oil	70				
6A	0.016	70	None	..	3A	3A	89	+++

[a] Oil added just before tasting to contain equivalent of nonhydrocarbon constituents as found in 0.024% whole oil in first lot and 0.016% in lot A.

[b] − no significance, + significant at 5% level, ++ significant at 1% level, +++ significant at 0.1% level.

the cans in a 200° F. water bath for 2.25 minutes and stored for 4 months at 40° and 70° F. A second lot of juice was treated in a similar fashion. A single lot of commercial orange oil of good fresh odor was deterpened by a chromatographic process (*13*). Part of this oil was stored at 0° F. and part at 70° F. for 4 months. A portion of the same lot of whole peel oil was stored at similar temperatures. Juices stored without oil were reconstituted with these oil samples to the same oil level (calculated on the basis of nonhydrocarbon content) as juices stored with oil before taste tests were made. The triangular taste test method was used to rate the samples. The results of these experiments are shown in Table IV.

The flavor of both lots of juice without any volatile oil initially present was distinctly bad. This is a strong indication that part of the unacceptable flavor of pasteurized orange juice does not arise from the volatile flavor fraction. The findings of Guyer and Boyd (*9*) substantiate this observation. The results of the taste tests shown in Table IV can be summarized as follows:

The storage temperature of either the juice or the flavoring oil had a great effect on the taste of the juice.

Terpeneless oil as added flavor was preferred over whole oil if the storage temperature was high.

Terpeneless oil as added flavor was not affected by storage.

Juice stored without oil and reconstituted with oil prior to tasting was preferred to juice stored with oil.

At high storage temperatures the off-flavor contributed by the nonvolatile fraction of the juice was the dominant flavor.

These conclusions and the results of the analysis of the volatile fractions show that contributions to the off-flavors in orange juice are produced by alterations of the hydrocarbon portion of the oil present in the juice, but that the off-flavor produced by the volatile oil decomposition is only part of the off-flavor of canned and stored canned juices.

The flavor of orange juice (*10, 23, 27*) and of heated and stored orange juice (*2, 4, 5, 9, 11, 12, 19, 29, 30*) has been the subject of numerous investigations. Hall and Wilson (*10*) investigated the volatile material from a large volume of fresh orange juice but largely ignored the contribution of the volatile oils to the flavor and aroma. Boyd and Peterson (*4*) recognized that the presence of volatile oils in fresh juice gave it the pleasant aroma and flavor and showed certain effects of storage, heating, and oxygen removal on the flavor quality of the juice. Henry and Clifcorn (*11*) distinguished between cooked flavor and off-flavor of canned orange juice. The off-flavor was claimed to be volatile, whereas the cooked flavor was nonvolatile and water-soluble. Theories of flavor change in orange juice have been reviewed by Swift (*30*). In addition, Swift (*30*) and Huskins, Swift, and Veldhuis (*12*) investigated the changes in the lipides on heating and storage and concluded that these compounds were not responsible for flavor change. Recently Guyer and Boyd (*9*) have shown that orange juice packed with low volatile oil deteriorated as fast as juice packed with a normal oil content.

Results of the analysis of the volatile fractions from fresh, freshly canned, and stored canned orange juice and the taste texts of variously treated juices allow tentative conclusions to be made about the nature of the flavor of orange juice. Most of the characteristic flavor of fresh orange juice is due to volatile oils. Low-temperature removal of volatile oils from fresh juice leaves a liquid which can still be distinguished as being of orange juice origin but which is insipid and flat. The flavorful components of fresh juice are largely destroyed by heating and storage. The volatile hydrocarbons appear to be degraded by heating and storage to alcohols, polyoxygenated compounds, and probably nonvolatile components. Furfural, acetic acid, and methanol are produced by heating and storage. These compounds probably arise from the nonvolatile portion of the juice. An unidentified acid ($C_6H_8O_2$) was found present in fairly large amounts in canned and stored juices, but its flavor effect was not evaluated. The addition to fresh orange juice of the major volatile products found in heated orange juice produces a definite off-flavor. The off-flavor produced by this addition still lacks some of the characteristics of canned and stored canned orange juices. These juices are dominated by an off-flavor derived from the nonvolatile portion of the juice. This off-flavor is produced by heating and its nonvolatile origin can be demonstrated by heating juices with the volatile flavors removed.

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INVESTIGATION OF THE BACTERICIDAL EFFECT OF ACETIC ACID ON *PASTEURELLA PESTIS* AND *VIBRIO COMMA**

(Author's Abstract)

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In a search for disinfectants harmless to human beings and suitable for disinfecting foodstuffs not submitted to heat processing the bactericidal effect of solutions of acetic acid on *Vibrio comma* and *Pasteurella pestis* was investigated. The work was carried out by the methods proposed by Kaganova (1945).

We introduced a loop of agar culture in the form of a chick smear (1–2 days' growth) on to sterile coverslips; after light drying in air we transferred the coverslips with the culture to a Petri dish with the solution of acetic acid under investigation, kept them in this for a given time (30 sec, 1, 3, 5, 7, 10, 15 and 20 min), washed off with sterile distilled water and put into tubes of meat peptone broth (5 ml). At the same time a control was carried out with a culture of the organism used in the experiment. Solutions of 2·5 per cent, 5, 10, 15, 20 and 25 per cent acetic acid were investigated. As control disinfectants mercuric chloride (1 : 1000) and also 2, 3 and 5 per cent solutions of lysol were taken. The results of the tests were read after 24–48 hr and finally after 7 days.

With acid of each concentration and at each exposure 10 tests were carried out with *V. comma* (strain No. 267) and 11 tests with *P. pestis* (8 tests with a culture of strain EV and 3 tests with the virulent strain No. 261).

In 2·5 per cent concentration acetic acid acted bactericidally on *V. comma* with 7–10 min exposure; commencing with 5 per cent concentration the bactericidal effect ensued within 5 min, i.e. with the same rapidity as the test solutions of mercuric chloride and lysol. A 2·5 per cent solution of acetic acid exerted a bactericidal effect on *P. pestis* in 15–20 min; commencing with a 5 per cent concentration solutions of it killed *P. pestis* within 5–7 min, whereas solutions of mercuric chloride and lysol exerted a bactericidal action within 3–5 min.

It was interesting to find out whether solutions of acetic acid would exert a bactericidal action on *V. comma* and *P. pestis* if the latter were present in the depths of an object which could, by virtue of its physical properties, be soaked in an aqueous solution. Tests were made with the liver and spleen of guinea-pigs which had died of cholera or plague septicemia. Small pieces of the organs 1·5 × 1·5 cm in size were placed in solutions of acetic acid in various concentrations and investigated at given intervals of time. Pieces of the organs of the guinea-pigs placed in saline and lysol served as control.

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The tests showed that in these conditions solutions of acetic acid exerted a marked bactericidal effect on *P. pestis* and *V. comma*.

Thus, being a food product, acetic acid can be widely used for the sterilization of foodstuffs which are not submitted to heat processing.

Translated by D. BURTON

PERORAL IMMUNIZATION WITH LIVE BRUCELLOSIS VACCINE*

(Author's Abstract)

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TAKING into account that in natural conditions the infection of persons and animals with brucellosis occurs mainly by way of the alimentary tract we immunized experimental animals orally with live brucellosis vaccine for the purpose of working out a simpler and more effective method of vaccination. We administered orally to white mice and guinea-pigs live brucellosis vaccine (of the Gamaleya Institute of Epidemiology and Microbiology, U.S.S.R. Academy of Medical Sciences) which was diluted in milk in accordance with our findings that this to a certain extent protects *Brucella* from the bactericidal effect of the gastric juice. An immunizing dose of 1,000 million bacilli was administered in 0·5 ml of milk. To the control animals the same vaccine was injected subcutaneously in the inguinal region in a dose of 100 million bacilli. In all there were 20 white mice and 36 guinea-pigs under test.

In the course of 6 months both the test and the control animals were examined at various times—after 1, 4, 7, 15 and then every 30 days; the appearance of the Wright and Burnet reactions was established in the animals, they were submitted to autopsy and cultures of the various organs were made on agar D.

The results of this experiment showed that in white mice and the guinea-pigs inoculated both orally and subcutaneously the Wright test became positive from the 6th–10th day and was maintained up to 2 months; at the same time agglutination was marked (++ and +++) but the titres were relatively low—from 20 to 160. The Burnet reaction was also identical in the guinea-pigs inoculated orally and subcutaneously, making its appearance on the 40th–45th day and remaining up to 5 months, the diameter of the skin infiltrate in the majority of the animals being 1·5 × 2 cm.

On autopsy no essential differences were noted in the white mice and guinea-pigs of the test and control groups. The extent of invasion of the body by the vaccine strain of *Brucella* was likewise identical in both groups (it was found only in 8 test and control animals).

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