SUMMARY

1. Dimethylthetin chloride incubated with rat-liver or -kidney slices in either bicarbonate or phosphate salines was not oxidized to sulphate, but the compound incubated with homocysteine gave rise to a larger amount of sulphate than was obtained with homocysteine alone.

2. Methylthioacetic acid was oxidized to sulphate by liver slices and to a lesser extent by kidney slices. Ethylthioacetic, mercaptoacetic and dithiodiglycollic acids were also oxidized by liver slices, whereas *S*-phenylthioacetic acid was not.

3. Suspensions of fresh liver or acetone-dried liver powders were unable to oxidize methylthioacetic acid. Addition of the following reagents singly, in groups, or altogether did not restore the oxidative ability of the tissue: $MgCl_2$, $MnCl_2$, glucose, diphosphopyridine nucleotide, cytochrome *c* and adenosine 5'-phosphate with sodium fumarate.

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REFERENCES

- Borsook, H. & Dubnoff, J. W. (1947). *J. biol. Chem.* **169**, 247.
- Cope, C. L. (1931). *Biochem. J.* **25**, 1183.
- Cuthbertson, D. P. & Tompsett, S. L. (1931). *Biochem. J.* **25**, 1237.
- Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 436.
- Dubnoff, J. W. (1948). *Arch. Biochem.* **17**, 327.
- Dubnoff, J. W. (1949). *Arch. Biochem.* **22**, 478.
- Dubnoff, J. W. & Borsook, H. (1948). *J. biol. Chem.* **176**, 789.
- Du Vigneaud, V., Loring, H. S. & Craft, H. A. (1934). *J. biol. Chem.* **105**, 481.
- Du Vigneaud, V., Moyer, A. W. & Chandler, J. P. (1948). *J. biol. Chem.* **174**, 477.
- Fromageot, C. (1947). *Advanc. Enzymol.* **7**, 369.
- Heimberg, M., Fridovich, I. & Handler, P. (1953). *J. biol. Chem.* **204**, 913.
- Hill, R. M. & Lewis, H. B. (1924). *J. biol. Chem.* **59**, 557, 569.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Maw, G. A. (1953). *Biochem. J.* **55**, 42.
- Maw, G. A. & du Vigneaud, V. (1948). *J. biol. Chem.* **176**, 1037.
- Medes, G. (1939). *Biochem. J.* **33**, 1559.
- Medes, G. & Floyd, N. (1942). *Biochem. J.* **36**, 259.
- Pirie, N. W. (1934*a*). *Biochem. J.* **28**, 305.
- Pirie, N. W. (1934*b*). *Biochem. J.* **28**, 1063.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Shen, C.-W. & Lewis, H. B. (1946). *J. biol. Chem.* **165**, 115.
- Smythe, C. V. (1943). *Arch. Biochem.* **2**, 259.
- Westerman, B. D. & Rose, W. C. (1927). *J. biol. Chem.* **75**, 533.
- Westerman, B. D. & Rose, W. C. (1928). *J. biol. Chem.* **79**, 423.

Studies on the Volatile Fatty Acids of Sheep Blood with Special Reference to Formic Acid

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The production of volatile fatty acids (VFA's) in the rumen, and their subsequent absorption is well established. The recognition of the nutritional significance of these acids to the ruminant (cf. Phillipson, 1948) has led to many investigations of the VFA's in the blood of these animals. Reid (1950) made a careful study of the uptake of VFA from the peripheral circulation by the tissues of the sheep, and VFA levels in the blood of cattle were reported by McClymont (1951*a*). In earlier work values were reported for the concentration of circulating VFA in men and dogs (McClendon, 1944), and in the sheep, pig, pony and rabbit (Barcroft, McAnally & Phillipson, 1944). Dog blood was subsequently investigated by Phillipson (1947).

* This work was started while the author was seconded to the Rowett Research Institute, Bucksburn, Aberdeenshire.

Investigation of the nature of the blood VFA's, however, had to await the development of reliable methods of analysis, based on partition chromatography (Elsden, 1946; Moyle, Baldwin & Scarisbrick, 1948). Reid (1950), using chromatographic methods, found that about 90% of the VFA of sheep blood was acetic acid, and similar findings with respect to cattle blood were reported by McClymont (1951*a*). The VFA's of goat blood have recently been examined chromatographically by Craine & Hansen (1952).

In each of these chromatographic investigations of blood VFA's the acids were first distilled from mercuric oxide by the method of Friedemann (1938) to remove acidic substances which might interfere with the subsequent analysis. Formic acid is destroyed by this procedure. Furthermore, the

liquid-gel partition methods used in these chromatographic analyses would not readily distinguish formic acid from acetic acid. The recently developed gas-liquid partition chromatographic method (James & Martin, 1952) separates these and the other lower VFA's, and the application of this method to the analysis of sheep blood during the course of another investigation (Annison & Pennington, 1954) gave results which suggested that formic acid comprised 10-30% of the VFA's of the venous blood of sheep under normal conditions. The further investigation of this finding is the subject of the present paper.

The studies were extended to include an examination of the VFA content of urine, saliva, gastric juice and cerebrospinal fluid. The possibility that formate was produced by bacterial fermentation in some part of the alimentary tract and subsequently absorbed into the blood stream was also investigated.

MATERIALS AND METHODS

Experimental animals. Three Clun Forest sheep were fed once daily on rations of hay (1 kg./day), casein (1 kg. hay and 150 g. casein/day) and dried grass (400 g. dried grass and 300 g. hay/day) respectively. The rations were usually consumed within 2 hr. The sheep maintained on dried grass had a permanently exteriorized carotid artery. Additional animals kept for other purposes were available when required.

Sampling procedures. Venous blood was obtained by withdrawal from the jugular vein, and arterial samples were taken from the sheep with an exteriorized carotid artery. Heparin was used as an anticoagulant throughout the investigation.

Urine samples were obtained from ewes by inserting a polythene catheter into the bladder and allowing free flow under gravity into a collection vessel. The animals were restrained during the collection period.

Small quantities of saliva were obtained from sheep by washing the mouth with water and then applying gentle suction to a rubber tube held in the mouth of the animal. In addition, samples were obtained from sheep anaesthetized with Nembutal (sodium pentobarbitone). The mouth and teeth were washed and saliva was allowed to drain from the mouth which was held slightly open with retractors. The first 50 ml. of saliva was rejected.

Gastric juice was obtained from a 10-week-old lamb which had an innervated gastric pouch; this was prepared by Mr K. Hill for another purpose.

Cerebrospinal fluid was taken from sheep by suboccipital puncture under light anaesthesia (Nembutal).

Determination of VFA's in body fluids

Blood. Most published methods for determining total blood VFA's involve deproteinization of the blood and subsequent steam distillation of the strongly acid filtrate (McClendon, 1944; Phillipson, 1947). A suitable volume of distillate is collected and titrated with alkali under CO_2 -free conditions and a second and equal volume of distillate is treated similarly to provide a correction figure (cf. Phillipson, 1947). In no case has the validity of this type of procedure

been checked by subsequent VFA analysis of the distillates, since suitable methods for the small quantities involved were not available. The complete recovery of added VFA's from blood examined by these methods is a valuable check on possible losses associated with the deproteinization procedures but gives no information on the nature of the acidic substances estimated.

A method for determining total blood VFA levels has been developed which yields sufficient acid for subsequent chromatographic analysis by the procedure of James & Martin (1952). It was obviously desirable that the volume of blood used for each analysis should be as small as possible. The limiting factor was the amount of VFA required for chromatographic analysis. In order to scale down the method, the strength of the alkali in the recording burette (for details see James & Martin, 1952) was reduced from 0.04 to 0.01N. However, the solutions of the sodium salts of the VFA's to be analysed were more dilute than those recommended by James & Martin (1952), and the extraction of VFA's from such solutions by the method of these authors gave low and irregular recoveries (50-70%, w/w). These low recoveries were attributed to adsorption of VFA on the Na_2SO_4 used in the extraction procedure. Results were obtained indicating that, under standard conditions, the losses from this cause increased with the chain length of the VFA examined. Reduction of the quantity of anhydrous Na_2SO_4 employed gave increased recoveries and the procedure outlined below, which allows the analysis of mixtures containing 5-20 μmoles of VFA, was finally adopted.

Blood samples (20 ml.) were laked with 70 ml. water and deproteinized with 10 ml. of freshly prepared 25% (w/v) metaphosphoric acid. After standing 20 min. to allow complete coagulation of the precipitated protein, the suspension was filtered at the pump. The flask and precipitate were washed with 25 ml. of water. The filtrate and washings were neutralized to pH 8.0-8.5 with 5N-KOH and concentrated *in vacuo* to a volume of 3-4 ml. at 30-40° using a water pump and a combined magnetic stirrer-hotplate (Baird and Tatlock, London Ltd., Chadwell Heath, Essex). This solution was carefully washed into a 10 ml. volumetric flask, adjusted to pH 2.8-3.0 with 10N- H_2SO_4 (bromophenol blue indicator) and made up to 10 ml. Duplicate samples (4 ml.) were distilled in a Markham still and two portions of distillate (100 ml. each) were collected and titrated with 0.01N-NaOH under CO_2 -free conditions using phenolphthalein as indicator. The titration of the second 100 ml. of distillate served as a blank. The distillates were concentrated and the sodium salts of the VFA's were dried in a small test tube (6 x 0.6 cm.) and retained for chromatographic analysis. In order to transfer the VFA's to the gas-liquid partition columns, 40 $\mu\text{l.}$ of 30% (w/v) aqueous H_3PO_4 were added from a micropipette to the sodium salts. After thorough mixing the solution was transferred to the top of a micro-column similar to that described by James & Martin (1952), but containing a smaller quantity of Na_2SO_4 and Celite. A 0.6 cm. column of anhydrous Na_2SO_4 (0.08-0.09 g.) above which was placed a 0.2-0.3 cm. column of Celite in a tube of 0.3 cm. internal diameter was employed. The test tube was rinsed with 0.4 ml. of VFA-free ether, which was transferred to the micro-column and passed through under slight pressure. This procedure was repeated with a further 0.3 ml. of ether, the same pipette being used throughout. The VFA's were transferred from the ethereal solution to the gas-liquid partition column as described by James & Martin (1952).

Improved separations of VFA's were obtained by using partition columns of modified composition. The amount of stearic acid in the liquid phase (DC 550 silicone, Albright and Wilson Ltd., Oldbury, Birmingham) was increased from 10 to 15% (w/w). Under these conditions 'tailing' was considerably reduced and good separations of formic acid and acetic acid were obtained. An operating temperature of 135° and a N_2 flow rate of 15 ml./min. were employed throughout. All percentages of VFA's are expressed on a molecular basis.

Several reagents for deproteinizing blood which had been laked with water were examined, including $ZnSO_4$ - $Ba(OH)_2$ (Weichselbaum & Somogyi, 1941), $ZnSO_4$ - $NaOH$ (Somogyi, 1930), perchloric acid (Grauer, Mendl, Strauss & Neuberg, 1950) and metaphosphoric acid (Phillipson, 1947). The $ZnSO_4$ - $Ba(OH)_2$ method is theoretically the most suitable, since the reagent is completely eliminated with the precipitated protein, but the recoveries from sheep blood of added VFA were always low (70-80%). Similar results were obtained with the Somogyi reagent (Somogyi, 1930), and the metaphosphoric acid method of Phillipson (1947) was found to be the most satisfactory. Recoveries from blood of added VFA were invariably in the range 95-100%.

The deproteinized blood filtrates were distilled at pH 2.8-3.0 to minimize the interfering effects of other substances normally present, such as pyruvic and lactic acids and chloride, which are partially steam volatile under acid conditions. Control experiments established that at pH 3.0 only 5-8% of pyruvic acid steam distilled under the conditions used for the blood filtrates, but at pH 1.5 about 30% steam distilled. Similarly, at pH 3.0 the influence of chloride, lactic acid and β -hydroxybutyric acid, when present at concentrations similar to those encountered in blood, is negligible. The utility of steam-distilling VFA's under conditions of minimum acidity when interfering substances are present was recognized by Scarisbrick (1952), who estimated blood VFA's by steam distillation at pH 4.0. At this pH, formic acid cannot be steam distilled quantitatively, but at pH 3.0 complete recoveries of all of the lower VFA's were obtained.

The reliability of the method described above for blood VFA analysis was checked by performing ten analyses on a large pooled sample of sheep blood (Table 1). Good agreement was obtained between the total VFA level found by titration and the VFA accounted for by subsequent chromatographic analysis of the distillates.

Urine. Total VFA levels were determined by steam distillation. Samples of urine (5 ml.) were adjusted to

pH 2.8-3.0 (bromophenol blue indicator) by the addition of 1.5 ml. of phosphate buffer (saturated aq. KH_2PO_4 adjusted to pH 2.8-3.0 with 88% (w/w) H_3PO_4) and 2 g. of $MgSO_4 \cdot 7H_2O$ were added before the samples were steam distilled in a Markham apparatus. The distillate (150 ml.) was collected, neutralized with alkali and concentrated to a small volume (4-5 ml.). The acids were then redistilled in the Markham apparatus at pH 3.0, and two 100 ml. portions of distillate were collected and titrated with 0.01N- $NaOH$ under CO_2 -free conditions, the titre of the second portion serving as a correction figure. The distillates were concentrated and retained for chromatographic analysis. Double steam distillation was found necessary, since a considerable amount of acidic substance other than VFA steam distilled at pH 3.0 in the first distillation, but after the second distillation the titratable acidity was all accounted for as VFA by the subsequent chromatographic analysis.

Saliva. Samples of saliva (5 ml.) were adjusted to pH 3.0 with 5N- H_2SO_4 saturated with KH_2PO_4 (bromophenol blue indicator) and steam distilled in the Markham apparatus after the addition of 2 g. $MgSO_4 \cdot 7H_2O$. Two 100 ml. portions of distillate were collected and titrated with alkali as described for urine VFA analysis and retained for chromatographic analysis.

Gastric juice. Duplicate samples (5 ml.) were adjusted to pH 2.8-3.0 (bromophenol blue indicator) by the addition of $m-K_2HPO_4$ and steam distilled in the Markham still after the addition of 2 g. $MgSO_4 \cdot 7H_2O$. Two 100 ml. portions of distillate were collected and titrated with alkali as described for urine VFA analysis and retained for chromatographic analysis.

Cerebrospinal fluid. Samples (5 ml.) were analysed for VFA's as described for saliva.

Total nitrogen. This was determined by the Kjeldahl method as modified by Chibnall, Rees & Williams (1943) and the ammonia, which was steam distilled in the Markham apparatus, was trapped in the boric acid reagent of Conway & O'Malley (1942) and titrated with standard acid.

RESULTS

Formate in sheep blood

Identification of formic acid in sheep blood. Irrespective of the methods employed to isolate the VFA's from sheep blood, subsequent chromatographic analysis invariably gave rise to an acid zone on the gas-liquid chromatogram which, from its retention volume under the conditions employed, was provisionally identified as formic acid. The problem at this stage was twofold. It was necessary not only to confirm the identity of this acid, but also to establish whether it is a normal constituent of sheep blood, or whether it is produced subsequent to the withdrawal of the sample either as a result of standing or by the procedures employed to isolate the blood VFA's.

The latter point was investigated first and, in preliminary experiments, it was shown that the VFA content of a sample of venous blood did not change appreciably on standing for several hours at room temperature. To investigate whether or not

Table 1. *Results of ten VFA analyses on the same sample of sheep blood (venous)*

Sample	Total VFA (m-moles/l.)	Molecular percentages of VFA's		
		Formic	Acetic	Propionic
1	1.37	12	87	1
2	1.70	12	87	1
3	1.36	13	86	1
4	1.24	13	86	1
5	1.36	12	87	1
6	1.37	11	88	1
7	1.36	12	87	1
8	1.37	13	86	1
9	1.32	14	85	1
10	1.40	13	86	1

the formic acid observed in sheep blood was produced during the isolation procedures, a large sample of blood (200 ml.) was heparinized and examined for VFA's by the following methods. (a) Deproteinization with metaphosphoric acid (procedure described above). (b) Deproteinization with $\text{ZnSO}_4\text{-NaOH}$ (Somogyi, 1930); the filtrate was concentrated *in vacuo* and steam distilled at pH 3.0 as in (a). (c) A portion of the blood (60 ml.) was lightly centrifuged to remove red cells and the heparinized plasma dialysed at 0° against successive changes of distilled water for 4 days. The diffusible VFA was obtained by steam distilling the concentrated solution at pH 2–3. (d) Plasma obtained from a further portion of blood (100 ml.) was dialysed against an equal volume of distilled water at 0° for 2 days. The diffusate was acidified and exhaustively extracted with ether previously shaken with alkali to remove any VFA contaminants. The ether was then extracted with a slight excess of alkali (0.1N-NaOH) and this aqueous solution was divided into two equal portions, one of which was acidified and steam distilled to measure the VFA content. The other portion was evaporated to dryness and, in spite of a large amount of contaminating solid, examined directly by gas-liquid chromatography. The VFA extraction procedure described earlier was somewhat modified: the solid material was dissolved in 100 $\mu\text{l.}$ of 40% (w/v) aq. H_3PO_4 and twice the normal amount of anhydrous sodium sulphate was used.

The results of these analytical procedures are shown in Table 2. The small proportions of formate observed in procedures (c) and (d) are probably due to incomplete recoveries of VFA, but the presence in blood of an acid which behaves like formic acid revealed by these mild methods of extraction suggests that the acid is normally present in sheep blood.

The VFA's from a large blood sample (200 ml.) were isolated and examined chromatographically and a zone tentatively identified from its retention volume as formic acid was observed. Addition of formic acid to the sample before analysis did not give rise to a new zone but only enlarged the original zone. Distillation of the VFA mixture from HgO by the method of Friedemann (1938), before chromatographic analysis, either greatly reduced or eliminated the apparent formic acid. A further portion of the VFA mixture was examined chromatographically and the apparent formic acid was collected by removing the titration cell contents after the emergence from the column of the first acid zone. This acid was isolated by steam distillation and examined chromatographically alone and with added formic acid. In each case only one acid zone was observed, with a retention volume characteristic of formic acid. In a further experi-

ment the apparent formic acid was isolated from two samples of sheep blood by the procedure described above. These acid samples were examined for formic acid by the colorimetric method of Grant (1948), which is based on the reduction of the acid to formaldehyde and measurement of the colour produced with chromotropic acid. The colours produced were similar to those given by formic acid standards examined at similar concentrations. The method of Grant (1948) is not specific for formic acid. In subsequent experiments the VFA's from four samples of sheep blood were isolated by the standard procedure described earlier and analysed for formic acid by this colorimetric method and by gas-liquid partition chromatography (Table 3). The poor agreement is attributed to the difficulty experienced in obtaining reproducible results by the chromotropic acid method of Grant (1948).

Conclusive proof of the identity of the apparent formic acid was obtained by the isolation of the acid from sheep blood.

Isolation of formic acid from sheep blood. Samples of venous blood (290 ml.) were withdrawn from two sheep 5 hr. after feeding and pooled. The blood was laked with 1 l. of water and deproteinized with 250 ml. of 25% metaphosphoric acid. The filtrate was neutralized with 5N-KOH, concentrated *in vacuo* to about 100 ml. and steam distilled after acidifying to pH 2–3 with 10N- H_2SO_4 . The distillate

Table 2. *Results of the chromatographic analysis of the VFA's of a sheep blood sample from which the VFA's were isolated by the methods indicated*

Method of isolation (see text)	Composition of VFA fraction (molecular proportions)		
	Formic	Acetic	Propionic
(a) Standard procedure	17	82	1
(b) Deproteinization of blood by $\text{ZnSO}_4\text{-Ba(OH)}_2$ technique	16	83	2
(c) Dialysis and steam distillation	14	84	2
(d) Dialysis and ether extraction	12	85	3

Table 3. *Results of analyses of blood samples for formic acid by the chromatographic and chromotropic acid methods*

Sample	Total VFA (m-moles/l.)	Formate content of isolated VFA's (moles/100 moles VFA)	
		Chromato- graphic analysis	Chromotropic acid method
1	0.9	12	10
2	1.0	18	13
3	0.8	15	12
4	0.7	21	20

was neutralized with alkali, concentrated to about 10 ml. and steam distilled from acid solution (pH 2-3) in the presence of added $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (6 g.). The distillate (200 ml.) was titrated with 0.1N-NaOH, which indicated that the total VFA present was 0.80 m-moles. Chromatographic analysis showed that the sample contained 28 % formic acid or 0.22 m-moles (10.1 mg.). This formic acid was isolated from the other VFA's by gas-liquid partition chromatography. The sample was divided into 10 approx. equal portions which were run separately on partition columns. After the emergence of the formic acid zone the cell contents were collected, and the remainder of the VFA's were quickly eluted from the column using a high rate of nitrogen flow. The formic acid from the pooled cell contents was recovered by steam distillation (0.19 m-moles). Chromatographic analysis of this sample showed that formate (94 %) and acetate (6 %) were present.

The identity of the acid was confirmed by the analysis of its barium salt which was precipitated from aqueous solution with five volumes of ethanol. This procedure was repeated once. Yield of barium salt, 23 mg.: chromatographic analysis indicated that formate (96 %) and acetate (4 %) were present. (Found: C, 10.6; H, 1.2. Calc. for mixture of barium formate, $\text{Ba}(\text{O}_2\text{CH})_2$, (96 %) and barium acetate, $\text{Ba}(\text{O}_2\text{C}_2\text{H}_5)_2$, (4 %), C, 10.88; H, 0.94 %.) The elementary analyses were made by Drs Weiler and Strauss, Oxford.

Further confirmatory evidence of the identity of this acid was obtained by utilizing the formic hydrogenylase enzyme system of *Escherichia coli* (Woods, 1936). The isolated formate (sodium salt) was incubated at 37° with a suspension of *Esch. coli* (0.39 mg. of bacterial-N/ml.) in Warburg manometric flasks (one side bulb), with KOH in the centre wells and under a gas phase of N_2 . The bacterial suspension (2 ml.) was placed in the main compartment and the isolated formate (14.8 μmoles) in the side arm. Control flasks containing 15 μmoles of authentic formate were examined in a similar manner. The volume of hydrogen liberated from the test samples was almost identical with that expected on the basis of the formate content.

VFA's of sheep blood

Daily variation in sheep blood VFA's. Blood samples for VFA analysis (20 ml.) were withdrawn at 2 hr. intervals throughout the course of a day from two sheep maintained on the hay and casein diets respectively. The results are shown graphically in Fig. 1. The increased level of blood VFA's after feeding is due almost entirely to a rise in the acetate content of the blood, the formate levels remaining fairly steady.

Blood VFA's of fasted sheep. Food was withheld from the two sheep maintained on diets of hay and casein respectively, and blood samples were withdrawn for VFA analysis at 4 hr. intervals throughout a period of 48 hr. The results shown in Fig. 2 indicate that, although the level of acetate in blood fell markedly, there was only a slight decrease in the formate concentration.

Higher VFA's in sheep blood. Small amounts of butyric acid (< 1 %) were frequently observed when sheep blood was analysed for VFA's and traces of the C_6 acids were occasionally seen. When larger amounts of blood were examined, traces of all the C_4 and C_5 VFA's which normally occur in the sheep's

Fig. 1. Daily variation in blood VFA concentration (m-moles/l.) of sheep maintained on hay (total VFA's, □—□; formate, ■—■) and hay with added casein (total VFA, ○—○; formate, ●—●). Sheep fed during 0-2 hr. period.

Fig. 2. Changes in blood VFA concentration (m-moles/l.) of fasted sheep normally maintained on hay (total VFA, □—□; formate, ■—■) and hay with added casein (total VFA, ○—○; formate, ●—●).

rumen (Annison, 1954) were observed but, under normal conditions, these acids account for less than 1 % of the total VFA level.

The distribution of blood VFA's between cells and plasma. The distribution of the component VFA's in sheep blood between cells and plasma was examined. Venous blood (100 ml.) was carefully withdrawn to avoid haemolysis and 20 ml. were examined for VFA's. The haematocrit of the blood was determined by the procedure of Meyerstein (1942) by centrifuging at 2500 g. for 25 min., and 70 ml. of blood were centrifuged simultaneously to separate cells and plasma. The VFA's of the cells and plasma were determined by the procedure described for whole blood (Table 4). It was assumed that the cells contained 5 % (v/v) entrained plasma and corrections to the haematocrit (30.9 %), cell volume and cell VFA value were applied. Considerably more formate was present in cells than in plasma. Similar results were obtained with ox blood (Table 4). In further experiments, cells were repeatedly washed with 0.9 % saline and then dialysed against 20 vol. of saline at 2° for 24 hr. The VFA content of the cells was compared with that of the original blood. A significant amount of VFA was present in the washed cells, and the proportion of formate was considerably higher than that of whole blood (Table 4).

VFA content of other body fluids

VFA content of sheep saliva. Samples were obtained from sheep under normal conditions and also

when anaesthetized with Nembutal. Blood samples were withdrawn for VFA analysis during the period in which saliva was collected. The results shown in Table 5 indicated the absence of formate from saliva, although formate was present in the blood samples obtained at the same time. The samples were obtained 2-4 hr. after feeding, i.e. in the period usually associated with high blood VFA levels.

VFA's of cerebrospinal fluid. Samples were obtained from two sheep on two separate occasions. In the second experiment jugular blood samples were taken at the same time and also examined for VFA's. The results shown in Table 6 indicated that the VFA level was considerably lower than in blood, but both formate and acetate were present in similar proportions to those normally found in blood.

VFA's of urine. The analysis of six samples from different sheep showed that only small amounts (0.2-0.9 m-moles/l.) were normally present. The relative amounts of formate and acetate present were similar to those usually observed in blood and the total VFA levels of both body fluids were of the same order. Traces (<1 % of the total VFA's) of propionate were occasionally observed in sheep urine. In further experiments, urine samples were taken for analysis at intervals throughout 24 hr. periods and the results of a typical experiment are shown in Table 7. The daily output of VFA in the urine of sheep was found to be within the range 0.15-1.5 m-moles.

Table 4. *Distribution of blood VFA's between cells and plasma*

Animal	Sample	Total VFA (m-moles/l.)	Molecular proportions of VFA's		
			Formate	Acetate	Propionate
Sheep	Cells	0.77	13	84	3
	Plasma	1.03	4	94	2
	Whole blood	0.96	7	90	3
Ox	Cells	0.54	11	89	0
	Plasma	0.80	9	91	0
	Whole blood	0.74	5	95	0
Sheep	Washed cells	0.24	52	48	0
	Whole blood	1.04	24	75	1

Table 5. *VFA content of blood and saliva samples taken simultaneously from sheep anaesthetized with sodium pentobarbitone (Expts. 1 and 2), and under normal conditions (3 and 4)*

Expt.	Sample	Total VFA (m-moles/l.)	Molecular proportions of VFA's		
			Formic	Acetic	Propionic
1	Saliva	2.1	0	98	2
	Carotid blood	1.7	7	91	2
2	Saliva	0.8	2	95	3
	Carotid blood	1.4	7	92	1
3	Saliva	0.6	0	98	2
	Venous blood	1.2	12	86	2
4	Saliva	0.8	0	100	0
	Venous blood	0.9	14	84	2

Bastrup (1947) has reported the occurrence of relatively large amounts (0.43–4.8 m-moles/l.) of formate in dog urine and this finding was investigated using the methods described for the analysis of sheep urine. Six samples were obtained from different dogs (mixed breeds) at various times after feeding. The animals were fed once daily on rations of horse flesh (1 part) and flaked maize (3 parts) with small supplements of cod-liver oil and minerals. The total VFA concentration in dog urine appeared to be of the same order as that found in sheep urine (Table 8), but the proportion of formate was higher than that normally found in sheep urine.

Examination of alimentary tract contents for VFA's. Samples of rumen and abomasal contents were taken from two sheep fitted with permanent fistulae in the rumen and in the anterior part of the duodenum just behind the pyloric sphincter. A further sheep fitted with fistulae in the caecum and ileum was available, and samples were obtained from both organs. In another experiment, the gut from a freshly slaughtered sheep was immediately tied off into the following sections: (a) duodenum

and jejunum, (b) ileum (c) caecum, and (d) the upper third of the colon. This experiment was repeated, but attention was confined to the small intestine, which was divided roughly into duodenum and jejunum, and ileum. Samples were taken from each section, and these and the other alimentary tract contents were analysed for VFA's. Liquid samples were mixed with an equal volume of $N-H_2SO_4$ saturated with $MgSO_4$, $7H_2O$ and semi-solid material was treated with an equal weight of the same reagent. The suspensions were filtered and 2–5 ml. of filtrate were distilled in the Markham still. The distillates were titrated with 0.01 N-NaOH under CO_2 -free conditions and retained for chromatographic analysis.

Appreciable quantities of formate were present only in the small intestine (Table 9), where the total amounts of formate found in these experiments were in the range 0.8–1.0 m-mole.

VFA's of gastric juice. Four samples were collected over 30 min. periods for 2 hr. after the animal had been fed 500 g. of fresh lucerne. Since the concentrations of VFA were low, the distillates

Table 6. *VFA content of cerebrospinal fluid (c.s.f.) of sheep*

Expt.	Sheep	Sample	Total VFA (m-moles/l.)	Molecular proportions of VFA's		
				Formate	Acetate	Propionate
1	8	C.s.f.	0.28	18	82	0
	23	C.s.f.	0.16	10	90	0
2	8	C.s.f.	0.55	19	81	0
	8	Blood	1.90	—	—	—
	23	C.s.f.	0.46	25	75	0
	23	Blood	1.05	16	83	1

Table 7. *VFA content of sheep urine collected during a 24 hr. period*

Animal fed once daily (600 g. hay/day). Sampling commenced after 24 hr. fasting, and animal fed 6 hr. after experiment started.

Sampling time (hr.)	Vol. of urine (ml.)	Total VFA (m-moles/l.)	Molecular proportions of VFA's	
			Formic	Acetic
1	25	0.18	9	91
2	15	0.17	12	88
3	40	0.22	17	83
4.5	53	0.22	—	—
5.5	42	0.28	21	79
6.5	24	0.28	15	85
7.5	33	0.28	—	—
9.0	13	0.28	0	100
11.5	60	0.19	—	—
14.5	115	0.21	8	92
24.0	120	0.24	11	89

Table 8. *VFA content of dog urine*

Dog	Sex	Sampling time (hr. after feeding)	Total VFA (m-moles/l.)	Molecular percentages of VFA's		
				Formic	Acetic	Propionic
1	m	1	0.40	53	47	0
2	m	1	0.60	57	43	0
3	f	2	1.09	62	38	0
4	m	4.5	0.64	48	49	3
5	m	23	0.53	51	49	0
6	m	1	0.68	53	47	0

from the four samples were pooled for chromatographic analysis. Formate and acetate were present in similar proportions to those usually found in blood, although the total VFA levels were much lower than those of blood (Table 10).

VFA utilization by the tissues

The mammary gland of the goat. A lactating goat prepared with both an exteriorized carotid artery and an exteriorized mammary vein by Dr J. L. Linzell for another purpose was used in these experiments. Blood samples from the carotid artery and mammary vein were withdrawn simultaneously during the recording of arterial blood pressure while the animal was standing quietly. The

results of VFA analyses on these samples, together with analyses of blood gases, sugar and haemoglobin supplied by Dr Linzell, are shown in Table 11. These figures corroborate that the animal was undisturbed and show that milk secretion was taking place. In the excited animal the uptake of milk precursors may cease (Graham, Kay & McIntosh, 1936) or the blood may become concentrated or diluted during its passage through the gland (Powell & Shaw, 1942). The results of the blood VFA analyses indicate that only a small amount of formate was taken up by the udder, although the acetate level fell 0.7–1.2 m-moles/l. McClymont (1951*b*) reported the uptake of acetate (0.2–1.0 m-mole/l.) from blood by the lactating bovine udder.

Table 9. *VFA content of various sections of the alimentary tract of the sheep*

Expt.	Sample	Total VFA (m-moles/l.)	Molecular proportions of VFA's						
			Formic	Acetic	Propionic	iso- Butyric	Butyric	isoValeric and 2-methyl- butyric	n-Valeric
1	Rumen	83	0	57	21	2	14	3	3
	Abomasum	5	2	72	16	2	6	1	1
2	Rumen	97	1	66	20	2	8	2	1
	Abomasum	8	1	80	14	1	4	0	0
3	Duodenum and Jejunum	0.8	14	76	7	0	3	0	0
	Ileum	3.8	22	77	1	0	0	0	0
	Caecum	19.5	1	72	16	3	5	2	1
	Colon	6.5*	0	72	16	2	6	2	2
4	Duodenum and Jejunum	7.0	22	73	3	0	1	0	1
	Ileum	12.0	21	73	3	0	2	0	1
5	Ileum	3.3	19	70	8	1	2	0	0
	Caecum	100	2	72	15	2	6	1	2
6	Ileum	4.0	7	84	9	0	0	0	0
	Caecum	20	1	78	15	1	3	1	1

* In m-moles/kg.

Table 10. *VFA content of sheep gastric juice*

Sampling period (min. after feeding)	Volume collected (ml.)	Total VFA (m-moles/l.)	VFA content of pooled samples	
			Formate	Acetate
5–35	16	0.30	} 18	} 82
35–65	15	0.22		
65–95	14	0.22		
95–125	14	0.21		

Table 11. *The VFA's haemoglobin and sugar content of blood withdrawn simultaneously from the carotid artery and mammary vein of a lactating goat, and respiratory quotients calculated from the blood gases of the samples*

Expt.	Sample	Total VFA (m-moles/l.)	Formate (m-moles/l.)	Acetate (m-moles/l.)	Haemoglobin (g./100 ml.)	Sugar* (mg./100 ml.)	Respiratory quotient
1	Carotid	1.3	0.12	1.18	9.62	53	1.50
	Mammary vein	0.5	0.12	0.37	9.55	38	
2	Carotid	1.3	0.16	1.14	9.50	52	—
	Mammary vein	0.6	0.12	0.41	9.55	36	
3	Carotid	1.7	0.20	1.47	9.05	53	1.97
	Mammary vein	0.5	0.13	0.36	9.25	31	

* Determined by method of Shaffer & Hartmann (1920).

Table 12. *VFA content of samples of venous (jugular) and arterial (carotid) sheep blood withdrawn simultaneously*

Time after feeding (hr.)	Total VFA (m-moles/l.)		Molecular proportions of VFA's					
	Art.	Ven.	Formic		Acetic		Propionic	
			Art.	Ven.	Art.	Ven.	Art.	Ven.
Pre-feed	0.68	0.56	16	21	82	77	2	2
3	1.87	1.43	10	6	88	93	2	1
5	1.97	1.22	7	13	93	86	0	1
17	1.45	1.17	6	7	92	91	2	2

Table 13. *Analysis of blood VFA's of several animal species*

Animal	No. of samples examined	Source of blood	Range of total VFA's (m-moles/l.)	Individual VFA levels (molecular proportions)		
				Formic	Acetic	Propionic
Horse	3	Venous	0.27-0.96	4-45	54-73	1-4
Dog	6	Venous	0.18-0.48	31-53	45-66	1-4
Rabbit	3	Arterial	0.89-1.55	6-30	70-90	0-4
Cat	3	Arterial	0.26-0.46	36-54	41-60	3-5
Goat	6	Venous	0.51-1.61	10-30	69-88	0-2
Cattle	3	Venous	0.55-1.32	12-28	71-87	0-3
Human	5	Venous	0.28-0.32	23-34	64-77	0-4

The tissues of the head of the sheep. Carotid artery and jugular vein samples were taken simultaneously from a sheep with an exteriorized carotid artery at intervals after feeding and analysed for VFA's. The results of a typical experiment are shown in Table 12.

The uptake of appreciable quantities of acetate in some cases was indicated, confirming the results obtained by Reid (1950) in similar experiments, but it would appear that little, if any formate is utilized or produced by the tissues of the head.

Blood VFA's of other species

The blood of a number of other species was examined in order to extend the findings made with sheep blood. Complete VFA analyses were made on human blood (5 samples) and on the blood of dogs (6), rabbits (3), cats (3), horses (3), goats (6) and cattle (3). Formate, in addition to acetate, was present in every sample examined. Small amounts of propionate were also observed (Table 13). The occurrence of formate in blood is obviously not confined to ruminants.

DISCUSSION

The occurrence of appreciable amounts of formate in every blood sample examined, irrespective of source, was an unexpected finding since many recent investigations of the nature of blood VFA's have made no mention of this acid as a blood constituent (cf. McClymont, 1951*a*; Craine & Hansen, 1952). Although the chromatographic findings were confirmed by the isolation of formic acid from sheep blood, the possibility still exists that a precursor in blood gives rise to formic acid under the acid condi-

tions employed either to steam distil the deproteinized blood filtrates or to extract the blood VFA's with ether before chromatographic analysis.

The concentration of formate in sheep blood appeared to be fairly constant, since although the blood acetate rises sharply after feeding, due to increased acetate production in the rumen, there is little change in the level of formate. Examination of the blood VFA's of fasted sheep indicated that the formate concentration in blood fell only slightly during a 48 hr. fasting period, although the acetate levels were greatly reduced.

Analysis of the contents of each part of the alimentary tract of the sheep indicated that significant amounts of formate were present only in the small intestine. Since formate is virtually absent from abomasal contents, the acid must either (a) be present in one or more of the secretions into the small intestine, or (b) be liberated as a result of the chemical or enzymic processes which occur in this organ, or (c) arise by bacterial action. The latter possibility is unlikely, since the environment is not favourable for bacterial activity. The total amount of formate present in the small intestine is probably not sufficient to account for the major part of the blood formate unless the acid is rapidly absorbed into the circulation.

The absence of formate from sheep saliva was an unexpected finding in view of the presence of small amounts of acetate (0.3-2.1 m-moles/l.). It would appear that either formate is utilized by the secreting salivary glands, or that there is a threshold value for formate under normal conditions since, when formate is administered intravenously to sheep, the acid can be detected in the saliva (unpublished results). Salivary secretion in the

ruminant is of importance in the maintenance of fluid and mineral levels in the rumen, and the daily output of saliva is probably of the order of 4 l. (Scheunert & Trautmann, 1921). The results obtained for the acetate concentration in sheep saliva in the present studies suggests that 2-8 m-moles of acetate are returned each day to the rumen in this manner: this amount of acetate is not significant when compared with the acetate concentration in the rumen (50-100 m-moles/l.).

An examination of sheep urine showed that small amounts of formate and acetate were normally present. The daily output of VFA (0.15-1.5 m-moles) represents a negligible energy loss to the animal. The total VFA content of dog urine (0.4-1.0 m-moles/l.) was similar to that of sheep urine, but the proportions of formate and acetate were almost equal and similar to the relative concentrations in dog blood. The formate levels in the samples of dog urine examined (0.21-0.6 m-mole/l., i.e. 1.0-3.1 mg./100 ml.) were lower than those reported by Bastrup (2-22 mg./100 ml.) in 1947.

The toxic effects of methanol have been attributed to the formate produced by the oxidation of this substance in the tissues (cf. Wilson & Schild, 1952). The demonstration of formate as a normal blood constituent suggests that although this acid is undoubtedly produced by the *in vivo* oxidation of methanol (Kendall & Ramanathan, 1953) it is not responsible for the toxicity of this alcohol. In recent experiments (Annison & Hill, to be published) aqueous solutions (100-150 ml.) of sodium formate (5-8 m-moles/kg. body wt.) were administered to sheep by injection into the jugular vein over periods of 10-20 min. The animals showed no ill-effects and the blood formate level returned to normal in 3-4 hr. The collection and analysis of urine after the administration of formate indicate that about 20% of the acid was excreted in the urine during this period.

Formate metabolism in animal tissues has been the subject of many recent investigations. The utilization of formate in the synthesis of serine (Sievekitz & Greenberg, 1949; Kruhøffer, 1951), purines (Sonne, Buchanan & Delluva, 1948; Greenberg, 1951) and labile methyl groups (Sakami & Welch, 1950; du Vigneaud, Verly & Wilson, 1950; Berg, 1951) has been demonstrated. Berg (1953) has shown that pigeon-liver extracts incorporate [^{14}C]formate into the methyl groups of methionine, cystathionine, the β -carbon of serine and the 2- and 8-carbons of purines. Sperling, Maxwell & Oettingen (1953) injected [^{14}C]formate into rats and measured the excretion and distribution of radioactive carbon. A considerable portion of the radioactivity (80%) was excreted within 24 hr., largely as $^{14}\text{CO}_2$. Examination of the tissues a week later indicated that the depot fats and proteins possessed measur-

able activity. Formate is obviously readily utilized by animal tissues, and the fairly constant levels observed in sheep blood suggest that the acid is continuously produced by other metabolic processes. The present studies have been largely confined to ruminants, but the results shown in Table 12 indicate that the concentration of formate in the blood of each animal species investigated is of the same order.

SUMMARY

1. A method has been developed for the complete analysis of the volatile fatty acids (VFA's) present in 10-20 ml. of blood.

2. Formic acid has been identified as a constituent of sheep blood and normally accounts for 10-30% of the molecules of VFA present.

3. Chromatographic evidence was obtained indicating that formic acid was present in human blood and in the blood of goats, cattle, horses, rabbits, cats and dogs at concentrations similar to those observed in sheep blood.

4. No evidence was obtained for the uptake or production of appreciable amounts of formate by the tissues of the actively secreting mammary gland of the goat, or of the head of the sheep.

5. The examination of the VFA's found in the alimentary tract of the sheep suggested that the formate in the peripheral circulation was probably not derived from this source.

6. Small amounts of VFA were demonstrated in sheep urine (0.17-0.28 m-moles/l.). The relative proportions of formate and acetate were similar to those observed in blood. The daily output of VFA in the urine was in the range 0.15-1.5 m-moles, which represents a negligible energy loss to the animal. Larger amounts of VFA were found in dog urine (0.40-1.09 m-moles/l.), and the proportions of formate and acetate were similar to those observed in dog blood, i.e. approximately 1:1.

7. Investigation of the distribution of blood VFA's between cells and plasma in sheep and cattle indicated that formate is considerably concentrated in the cells.

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REFERENCES

- Annison, E. F. (1954). *Biochem. J.* **57**, 400.
- Annison, E. F. & Pennington, R. J. (1954). *Biochem. J.* **57**, 685.
- Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944). *J. exp. Biol.* **20**, 120.
- Bastrup, J. Th. (1947). *Acta pharm. tox., Kbh.*, **3**, 303.

- Berg, P. (1951). *J. biol. Chem.* **190**, 31.
 Berg, P. (1953). *J. biol. Chem.* **205**, 145.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.
 Craine, E. M. & Hansen, R. G. (1952). *J. Dairy Sci.* **35**, 631.
 Du Vigneaud, V., Verly, W. G. & Wilson, J. E. (1950). *J. Amer. chem. Soc.* **72**, 2819.
 Elsdon, S. R. (1946). *Biochem. J.* **40**, 252.
 Friedemann, T. E. (1938). *J. biol. Chem.* **123**, 161.
 Graham, W. R., Kay, H. D. & McIntosh, R. A. (1936). *Proc. Roy. Soc. B*, **120**, 319.
 Grant, W. M. (1948). *Analyt. Chem.* **20**, 267.
 Grauer, A., Mendl, I., Strauss, E. & Neuberg, C. (1950). *Exp. Med. Surg.* **8**, 301.
 Greenberg, G. R. (1951). *J. biol. Chem.* **190**, 611.
 James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
 Kendall, L. P. & Ramanathan, A. N. (1953). *Biochem. J.* **54**, 424.
 Kruhoffer, P. (1951). *Biochem. J.* **48**, 604.
 McClendon, J. F. (1944). *J. biol. Chem.* **154**, 357.
 McClymont, G. L. (1951a). *Aust. J. agric. Res.* **2**, 92.
 McClymont, G. L. (1951b). *Aust. J. agric. Res.* **2**, 158.
 Meyerstein, W. (1942). *J. Physiol.* **101**, 5P.
 Moyle, V., Baldwin, E. & Scarisbrick, R. (1948). *Biochem. J.* **43**, 308.
 Phillipson, A. T. (1947). *J. exp. Biol.* **23**, 346.
 Phillipson, A. T. (1948). *Nutr. Abstr. Rev.* **17**, 12.
 Powell, R. C. & Shaw, J. C. (1942). *J. biol. Chem.* **146**, 207.
 Reid, R. L. (1950). *Aust. J. agric. Res.* **1**, 338.
 Sakami, W. & Welch, A. D. (1950). *J. biol. Chem.* **187**, 379.
 Scarisbrick, R. (1952). *Biochem. J.* **50**, xxiv.
 Scheunert, A. & Trautmann, A. (1921). *Pflüg. Arch. ges. Physiol.* **193**, 33.
 Shaffer, P. A. & Hartmann, A. F. (1920). *J. biol. Chem.* **45**, 365.
 Sievekitz, P. & Greenberg, D. M. (1949). *J. biol. Chem.* **180**, 845.
 Somogyi, M. (1930). *J. biol. Chem.* **86**, 655.
 Sonne, J. C., Buchanan, J. M. & Delluva, A. M. (1948). *J. biol. Chem.* **173**, 69.
 Sperling, F., Maxwell, E. S. & Oettingen, W. F. (1953). *Amer. J. Physiol.* **174**, 33.
 Weichselbaum, T. E. & Somogyi, M. (1941). *J. biol. Chem.* **140**, 5.
 Wilson, A. & Schild, H. O. (1952). *Clark's Applied Pharmacology*, 8th ed. London: J. and A. Churchill, Ltd.
 Woods, D. D. (1936). *Biochem. J.* **30**, 515.

The Estimation of Serum Lipoproteins. A Micromethod Based on Zone Electrophoresis and Cholesterol Estimations

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Investigations in recent years have indicated that the lipids do not exist 'free' in serum, but are present in combination with protein; the position has been adequately summarized by Russ, Eder & Barr (1951). The development of physical methods in recent years allowed many different approaches towards elucidation of the nature of these complex serum lipoproteins.

The elegant low-temperature ethanol fractionation procedure applied to plasma by Cohn and co-workers demonstrated that almost all the plasma lipids could be accounted for in two separate protein fractions, termed IV.1 and III.0 (Gurd, Oncley, Edsall & Cohn, 1949; Oncley, Gurd & Melin, 1950). These fractions contained largely the α - and β -globulins respectively and hence the lipid fractions were termed the α - and β -lipoproteins.

A second approach to the problem was produced by the ultracentrifuge studies of Gofman, Lindgren & Elliott (1949). Subsequent comprehensive studies from this school (Lindgren, Elliott & Gofman, 1951) suggest that by this procedure the β -lipoprotein component can be shown to be a mixture of molecular species of varying densities.

A third method for the study of serum lipoproteins is zone electrophoresis as developed by Wieland & Fischer (1948), Durrum (1950), Turba & Enenkel (1950), Cremer & Tiselius (1950), Kunkel & Slater (1952). This recent, but already well-known technique, permits isolation of each electrophoretic component in a fairly pure state. Thus zone electrophoresis should be an ideal method for the study of protein-lipid relationships in serum.

Fasoli (1952) separated the plasma proteins by paper electrophoresis using the method of Flynn & de Mayo (1951) in which the paper was stained with sudan III, and showed the presence of two lipid bands corresponding with the α - and β -globulins. Roughly the same results were obtained by Rosenberg (1952) employing sudan IV as the staining reagent, while Durrum, Paul & Smith (1952) used oil red O, and Swahn (1952, 1953) used sudan black.

Kunkel & Slater (1952) separated the lipoproteins from 2 to 5 ml. sera by zone electrophoresis in a starch block and analysed the various fractions for cholesterol and phospholipids. This method confirmed the presence of the two major lipoprotein constituents, and the results compared favourably with the lipid-staining techniques.