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Determination of Volatile Fatty Acids in Plasma after Ethanolic Extraction

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1. A new rapid micro-method for measuring plasma volatile fatty acids is described. The volatile fatty acids are extracted from plasma with ethanol in the presence of a known quantity of internal standard (sodium isobutyrate). After evaporation of the ethanolic solution of the sodium salts, the residue is dissolved in a dilute solution of orthophosphoric acid to permit analysis by g.l.c. 2. A technique of g.l.c. analysis is described which permits the separation of all the volatile fatty acids from the other plasma constituents at temperatures below 100°C in 5min. 3. Steam-distillation techniques are unsatisfactory when the acetic acid concentrations in the plasma are below 0.2mM. Heating of a number of plasma constituents in acid conditions gives rise to acetic acid. 4. The binding of volatile fatty acids to plasma proteins was studied; this binding is negligible for acetic acid, but increases with the length of the fatty acid carbon chain. 5. The limits of use of the method and the physiological implications are discussed.

There is considerable interest in measuring the concentration of volatile fatty acids in blood, especially in ruminants, in which these acids provide the major energy substrates (Annison & Armstrong, 1970). However, this measurement is not widely practised, since the techniques that have been proposed are rather long. Most of the techniques used at present to measure the concentration of plasma volatile fatty acids (Annison, 1954; Mahadevan & Zieve, 1969; Perry *et al.*, 1970; Ross & Kitts, 1971) use steam distillation followed by g.l.c. analysis. The volumes used are in all cases greater than 5ml of plasma and this limits the methods to use with large animals.

In Annison's (1954) method the pretreatment of the samples is relatively time-consuming and involves extraction with metaphosphoric acid and concentration of the filtrate. Other authors (Perry *et al.*, 1970; Ross & Kitts, 1971) have found it simpler to use steam distillation of the complete plasma after addition of an anti-foaming agent. The major inconvenience of these methods is not so much the preparation of the plasma as the steam distillation itself. The relatively low pH values that are necessary could theoretically cause decomposition of the most fragile acetyl groups on boiling and give as a result excess of acetic acid (Tabone, 1941). This could explain why Mahadevan & Zieve (1969) found a higher concentration of acetic acid from distillation of a chloroform–methanol extract of plasma than from direct chromatographic analysis of the extract. Similarly, Kurtz *et al.* (1971), who used steam distillation in a concentrated H_2SO_4 medium to measure the concentration of plasma volatile fatty acids, obtained very high concentrations of acetic acid which were unrelated to the normal values obtained for human plasma.

Erwin *et al.* (1961) considerably simplified the method by extracting plasma without steam distillation. However, the deproteinization by a mixture of sodium tungstate and H_2SO_4 introduced a large quantity of mineral salts. This makes the redissolution of samples after concentration in alkaline conditions difficult, particularly when small volumes are involved. Certain organic solvents that are miscible with water have the property of denaturing and precipitating the plasma proteins and are therefore suitable extracting agents.

The present paper describes a new rapid method of measuring the concentration of plasma volatile fatty acids by using extraction with ethanol and a new technique for separating the volatile fatty acids by g.l.c. During this work, possible binding of volatile fatty acids to plasma proteins was investigated. In addition, the proposed method was compared with many different procedures involving steam distillation.

Experimental

Chemicals

Volatile fatty acids, ethanol, chloroform and 85% (w/v) orthophosphoric acid of high purity were obtained from Prolabo, Paris, France. Bovine albumin and γ-globulins were purchased from Sigma, St. Louis, Mo., U.S.A. and acid-washed Chromosorb P (60–80 mesh) was obtained from Johns–Manville, Manville, N.J., U.S.A. Carbowax 20M was obtained from Supelco, Bellefonte, Pa., U.S.A.

Apparatus

The chromatograph Girdel 75E1 (Suresnes, France) used was equipped with a flame ionization detector. For ultrafiltration work CF 50 cones (Centriflo) from Amicon (Meca Vigor, Paris, France) were used together with a Sorvall centrifuge.

Animals and sampling

Sherman rats weighing 200–300g were fed *ad libitum* with a balanced laboratory diet. Blood samples were obtained from either the aorta or the portal vein under diethyl ether anaesthesia. Ruminant samples were obtained from the jugular vein of sheep and cattle fed *ad libitum* with hay.

Results

Analysis of volatile fatty acids by g.l.c.

The g.l.c. analysis of volatile fatty acids after a simple ethanolic extraction requires stable highly sensitive columns which permit elution of volatile fatty acids at temperatures below 100°C.

Choice of the stationary phase. Ottenstein & Bartley (1971*a*) showed that the separation of isobutyric acid and propionic acid is improved by decreasing the polarity of the column. However, there must be sufficient polarity to adequately separate isobutyric acid from butyric acid. The maximum possible decrease in the polarity of the column is also advantageous, since shorter retention times are obtained. The porous polymers (poly-aromatic resins) have too low a polarity to permit separation of isobutyric acid from butyric acid, and the high elution temperature that is necessary limits their use to ruminal or caecal contents. A large number of polyesters and also Carbowax (polyethylene glycol) have been used but all have too great a polarity to separate isobutyric acid from propionic acid. To remedy this, Ottenstein & Bartley (1971*a*) synthesized a new product (SP-1200), which was of low polarity and very efficient at high temperatures. The most efficient support for any of the stationary phases are the diatomites (Chromosorb P and W).

Problems encountered. The principal difficulty is the interaction of the volatile fatty acids with the column, which is very strong for acetic acid. Two phenomena may be observed: tailing of the peaks, and adsorption with the appearance of ghost peaks on subsequent injection of samples. To avoid these problems, Metcalfe (1960) incorporated phosphoric acid into the stationary phase. Byars & Jordan (1964) have used terephthalic acid with Carbowax 20M and also a combination of Carbowax and 2-nitroterephthalic acid. These acids have the property of deactivation of the adsorption sites.

The support can certainly contribute to adsorption. The polar compounds have also a tendency to react with the stainless-steel walls of the column (Hrivnak, 1970). Ottenstein & Bartley (1971*b*) have shown that this adsorption was greatest at the injection port and at the beginning of the column. Adsorption can also occur on the glass wool at the entry to the column, but the addition of phosphoric acid to the glass wool suffices to deactivate it. Although orthophosphoric acid can remove adsorption at the head of the column the necessity of its incorporation into the stationary phase depends on the materials used. Nevertheless, all injections must use water acidified with orthophosphoric acid.

Description of the technique. Columns (2m × 2.2mm) internal diam. were used for the analysis. The rate of flow of the carrier gas (N_2) was 25ml/min, and the oven temperature was 90°C. The temperature of the detector–injector block must not rise above 130°C, otherwise excessive heating of the column end by the detector causes the formation of peaks owing to Carbowax decomposition. It was found that Chromosorb P, when coated with Carbowax, does not adsorb the volatile fatty acids strongly, making unnecessary an addition of acid to the stationary phase. The molecular weight of Carbowax (4M, 20M) has negligible influence. The percentage of the stationary phase that remains fixed to Chromosorb P after conditioning the mixture determines the quality of the separations.

In all experiments, Chromosorb P was coated with Carbowax in the following way: for 10g of Chromosorb P the Carbowax was dissolved in 17ml of chloroform and the chloroform–Carbowax solution was mixed with the support by using a glass agitator. Drying the mixture at 40°C gave a quantitative coating.

The maximum percentage of stationary phase necessary to obtain separation of isobutyric acid and propionic acid is 3% (w/w). Even with low percentages of the stationary phase (2–5%) a relatively long conditioning of the column is necessary before high sensitivity can be obtained and thus only very small quantities of Carbowax remain bound to the support. These results show that a low final degree of coating is necessary to obtain efficient separation. Heating the mixture of support plus stationary phase before the column is filled gives an adequate degree of coating and stability of the columns after conditioning for 24h. At the same temperature, the phase fixed on the Chromosorb P is much more stable under N_2 than in air.

The quality of the chromatograms was similar whether the initial coating was 5, 10 or 15% (w/w), so long as the length of the heat treatment is adjusted (between 80 and 100°C) to obtain a final degree of coating of about 1% (w/w). However with 5% (w/w) coating the reproducibility of the columns and the quality of separation appeared to be best when heating for 48h at 80°C was used.

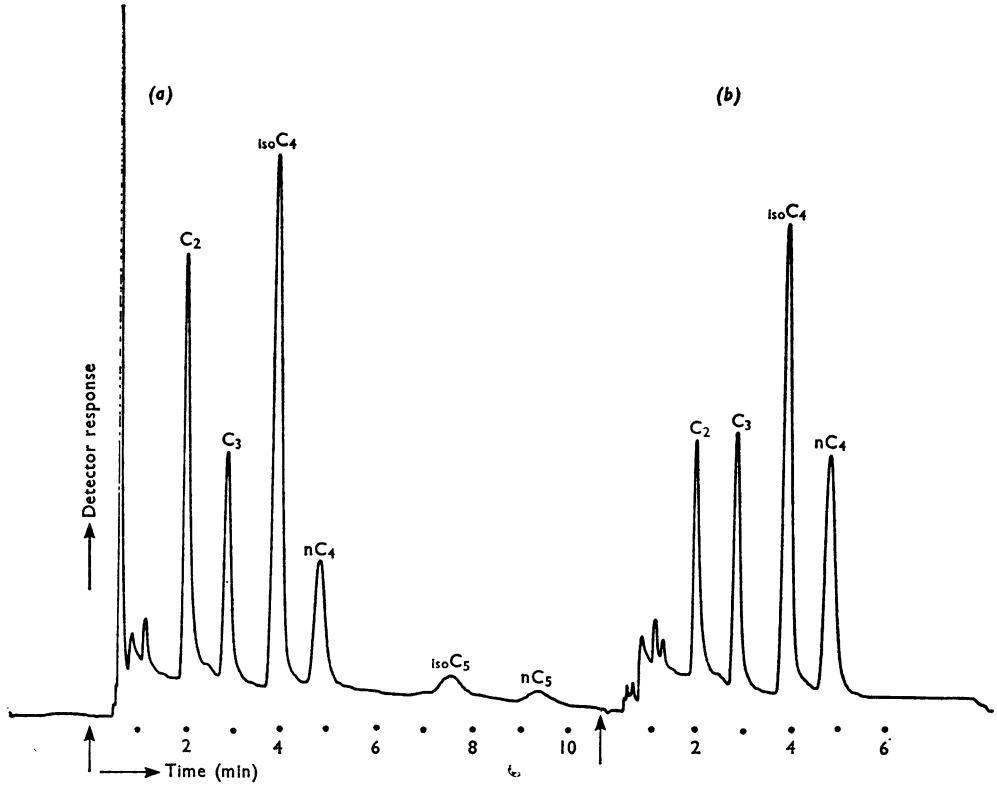

Fig. 1. *G.l.c. analysis of volatile fatty acids*

(*a*) is a g.l.c. analysis of a sample from 200μl of rat plasma (obtained from the portal vein) to which 20μl of sodium isobutyrate ($_{Iso}C_4$) (4mM) had been added. (*b*) is a g.l.c. analysis of a sample from 200μl of a standard mixture of volatile fatty acids: acetic acid (0.5mM), propionic acid (0.25mM) and butyric acid (0.25mM). The standard solution was subjected to the same treatment as the plasma. A stainless-steel column (2m×2.2mm internal diam.) containing 5% (w/w) Carbowax 20M on acid-washed Chromosorb P (60–80 mesh) was used. The 5% (w/w) Carbowax on Chromosorb P was treated for 48h at 80°C before the column was filled. The flow rate was 25ml/min, the oven temperature was 90°C and that of the detector–injector block was 125°C.

Before using the column, numerous injections (1μl) of 10% (v/v) H_3PO_4 must be made to suppress adsorption. Column aging may be detected by the appearance of tailing of the acetic acid peak as well as poor separation between isobutyric acid and butyric acid. The main inconvenience of the columns is thus their relatively short life (500 analyses). Their use is justified by the apparent previous lack of any mixture permitting operation at high sensitivities and at sufficiently low temperatures to avoid elution of other plasma constituents. This is why SP-1200 could not be used, although it is efficient for the separation of volatile fatty acids in a very short time period. Chromosorb P (AW) appears to give the best separation of volatile fatty acids consistent with the lowest temperatures. Chromosorb W with 5% (w/w) Carbowax does not permit separation of isobutyric acid from propionic acid.

Calculations. Measurement of peak height gives a result as satisfactory as a disc integrator when, as in the present case, chromatography gives very sharp peaks (see Fig. 1). However, the use of standard solutions at concentrations similar to those of the samples, undergoing every stage of the treatment in the presence of an internal standard, is necessary to obtain quantitative results. With repeated injection, the width of the acetic acid peaks may be irregular. Frequent passages of the standards after about 10 samples have been analysed are therefore necessary.

Preparations of samples

Heparinized blood samples were centrifuged immediately after collection. Then 20μl of internal standard and 200μl of plasma were placed in an Eppendorf centrifuge microtube and 1ml of pure

ethanol was added. Sodium isobutyrate, at a concentration of 4mM, was used as internal standard since it is not normally present in the plasma. The internal standardization becomes increasingly exact when it is subjected to all the treatments of the samples; it becomes indispensible when there are special treatments such as evaporation and redissolution of the dry residue. After agitation and centrifugation, the supernatant is transferred to a 4ml flask and made alkaline with 20μl of 0.2M-NaOH. It is then evaporated in a very slight air current at 20°C. The dry residue is redissolved in 15μl of water a few minutes before analysis. Just before injection on the column, 5μl of 25% (v/v) orthophosphoric acid are added. Acidification at the last moment avoids the risk of volatilization of the volatile fatty acids and of micellar separation of isobutyric acid, which is poorly soluble in acid conditions. Thus a solution with a concentration 10 times greater than that of the original samples is obtained.

A volume of 20μl of sample is used to rinse the micro-syringe and inject 1μl into the apparatus. When the acetate plasma concentration is less than 0.2mM, the samples should be concentrated 20-fold by using 0.5 or 1ml of plasma.

The evaporated samples should be analysed within 24h: beyond this time unexplained disappearances of isobutryic acid may occur. Alternatively the plasma, or the ethanolic extract, may be kept at −20°C for several days. In the method described above, the samples must never be evaporated at temperatures above 25°C. Heating to 80°C in alkaline conditions causes the degradation of many organic substances to acetic acid, especially those containing acetyl groups and sugars. For example, a sample of rat plasma which normally contains 0.2mM-acetic acid appears to contain more than 1mM-acetic acid if alkaline hydrolysis is used, and several mM-acetic acid if the evaporated sample is kept in an incubator at 80°C for more than 12h. Increasing the concentration of NaOH above 0.5M results in a poor recovery of the internal standard by decreasing the solubility of isobutyric acid in water.

Study of the extraction procedure

Protein binding. Solvents immiscible with water were not considered for the extraction procedure so as to eliminate the risk of selective solubilization of the volatile fatty acids as well as to simplify the treatment of samples. Bull & Breese (1967) reported that ovalbumin binds volatile fatty acids. The binding increases with the length of the carbon chain and with the pH. It was therefore essential to determine any binding of volatile fatty acids to plasma proteins and to check their redissolution in ethanol, whether in the free or bound form.

For studies on the binding of volatile fatty acids to proteins the large and small protein molecules are separated without denaturation by using a conical ultrafilter which allows the passage of substances with molecular weights of less than 50000. The capacities of ruminant plasma proteins (jugular blood of sheep) to bind volatile fatty acids were examined. Exact quantities of volatile fatty acids were added to the plasma at pH7.35. The recovery was compared after either ultrafiltration or ethanolic extraction. The binding of acetic acid and propionic acid to proteins is almost negligible at physiological pH values. In sheep the binding is about 30% for butyric acid and 60% for valeric acid (Table 1). A similar study with rat plasma showed that the binding of butyric acid is low (about 10%) but is greater for valeric acid

Table 1. *Percentage recovery of volatile fatty acids after ethanolic extraction or ultrafiltration of plasma*

Ultrafiltration through conical ultrafilters was done by centrifugation at 1000*g*. Filtration was terminated when 30–40% of the sample had passed through the filter. Then the ultrafiltrate was treated in the same way as the plasma. The results of two successive experiments are given separately; the second gives the results of ultrafiltration only, with plasma obtained from another sheep. Each value is the mean±S.E.M. with the number of analyses in parentheses.

		Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)	Valeric acid (mM)
	Added standard (mM) ...	0.500	0.250	0.250	0.250
Ethanolic extraction	Plasma	0.696±0.018 (6)	0.013±0.001 (5)	0.013±0.002 (6)	0
	Plasma+added standard	1.202±0.007 (6)	0.267±0.003 (6)	0.270±0.003 (6)	0.246±0.003 (6)
	% recovery	101.2	101.6	102.8	98.4
Ultrafiltration	Plasma	0.688±0.008 (6)	0.009±0.002 (6)	0	0
		1.610±0.059 (6)	0.037±0.002 (6)	0.021±0.002 (6)	0
	Plasma+added standard	1.200±0.016 (8)	0.259±0.004 (8)	0.167±0.004 (8)	0.094±0.003 (8)
		2.144±0.037 (6)	0.277±0.006 (6)	0.196±0.005 (6)	0.092±0.007 (6)
	% recovery	102.4	100.0	66.8	37.6
		106.8	96.0	70.0	36.8
	Mean percentage of binding	0	2	31.6	62.8

Table 2. *Concentration and percentage of volatile fatty acids by various techniques of treatment of rat plasma (from arterial and portal blood)*

Sodium isobutyrate was used in all cases as internal standard. The results are the mean values±S.E.M. with the number of analyses in parentheses. The percentages of each volatile fatty acid are given for portal plasma only.

	Arterial acetic acid	Portal acetic acid		Portal propionic acid		Portal butyric acid	
	Concentration (mM)	Concentration (mM)	Percentage	Concentration (mM)	Percentage	Concentration (mM)	Percentage
Ethanolic extraction (10)	0.212±0.005	0.589±0.010	62.9	0.237±0.002	25.3	0.111±0.001	11.8
Steam distillation							
Acidification by orthophosphoric acid to pH1.4 (6)	0.404±0.007	0.689±0.015	66.8	0.237±0.003	22.9	0.106±0.001	10.3
Acidification by orthophosphoric acid to pH3.5 (6)	0.243±0.004	0.543±0.009	61.1	0.239±0.003	26.9	0.107±0.001	12.0
Deproteinization by metaphosphoric acid filtrate pH1.4 (6)	0.391±0.025	0.675±0.015	65.0	0.249±0.006	23.9	0.115±0.002	11.1
Deproteinization by metaphosphoric acid filtrate pH3.5 (4)	0.267±0.005	0.586±0.016	62.0	0.250±0.004	26.4	0.110±0.002	11.6

(40%). This variation must depend on the binding on protein sites since ethanol, which denatures proteins, extracts the bound butyric acid and valeric acid.

To identify the protein fraction responsible for volatile fatty acids binding, these experiments were repeated with two samples of bovine plasma protein, namely albumin (Cohn fraction 5) and γ-globulins (Cohn fraction 2). The binding of volatile fatty acids by albumin is greater at the most acid pH, and the binding increases with the length of the fatty acid carbon chain.

With unesterified fatty acids binding sites for volatile fatty acids do not exist on γ-globulins. The denaturation of proteins by ethanol is sufficient to destroy the protein sites and extract the volatile fatty acids.

Comparative study of different methods of measurement of volatile fatty acids. Volatile fatty acids are not the sole substances extracted by ethanol. The product injected into the chromatograph is therefore a complex organic mixture which may undergo pyrolysis leading to the production of acetate and interfering substances. For this reason, a comparison was made between ethanolic extraction and steam distillation with a Markham still, after different treatments of the samples by using plasma taken from the aorta and the portal vein of rats (Table 2). For propionic acid and butyric acid all the techniques give similar results. For acetic acid, the pH has a very strong influence when methods of extraction by steam distillation were used. It appears that a very low pH causes hydrolysis of the acetyl groups, which is important in rat. This effect seems to be less predominant in the ruminant, as verified with cow plasma.

The coefficient of variation under the most exacting conditions (measurement with 200 µl of plasma) was usually between 1 and 3%. The precision could be improved, since the limiting factors are the measurement of peak heights (or the integral) and of the 20 µl of the internal standard. As isobutyric acid is completely soluble only immediately after acidification, any delay in injection into the chromatograph will result in a risk of considerable error.

Discussion

Gas–liquid chromatography analysis

Ethanolic extraction of blood volatile fatty acids also removes most of the other compounds with low molecular weight and will give satisfactory results only if the chromatographic analysis of the extract is sufficiently selective for volatile fatty acids. Analytical conditions permitting elution of compounds with higher boiling points than those of volatile fatty acids (i.e. excessive temperatures and a stationary phase of insufficient polarity) must therefore be avoided. In addition, at too high a temperature, pyrolysis may cause the production of acetic acid. The fact that the concentration of volatile fatty acids in the injected samples is below 1 g/litre and the low response coefficient of the flame ionization detector for these acids (for acetic acid in particular) make high sensitivity important.

The choice of support is very important in order to decrease the elution temperature. With an equivalent elution time, the use of Chromosorb P permits analysis at a temperature about 30°C lower than is necessary with Chromosorb W, when they are coated with Carbowax. The percentage of Carbowax determines both the degree of separation of the isobutyric acid (internal standard) from the propionic acid and from the butyric acid as well as the retention time and elution temperature. The heat treatment of the stationary phase before and after filling the column leads to a final coating of about 1% (w/w) 20M Carbowax. Although repeated injections of acidified water lead to a progressive deterioration of the column (which would no doubt be slower with higher degrees of coating) the heat treatment of Carbowax-coated Chromosorb P gives very reproducible results and rapidly conditioned columns.

When adsorption occurs only at the inlet of the column, injections of water acidified with H_3PO_4 may suppress this phenomenon. However, on Chromosorb P coated with Carbowax, some adsorption on to the interior of the column remains since low tailing is observed for acetic acid. In this case, incorporation of acid (orthophosphoric or terephthalic) may be considered, although this treatment must not permit elution of other organic acids such as lactic acid and 3-hydroxybutyric acid.

The use of other stationary phases and supports under the same conditions as Carbowax and Chromosorb P may be envisaged. A comparison of the final degrees of coating obtained would permit the choice of the most stable and efficient stationary phase and support, in particular for low concentrations of volatile fatty acids. Polyesters fixed on Chromosorb W are often used for the separation of volatile fatty acids, even at dilute concentrations, when highly purified extracts are available (Perry *et al.*, 1970; Ross & Kitts, 1971; Gibbs *et al.*, 1973). It has been noted that several organic acids (medium-chain fatty acids and lactic acid) are eluted on Chromosorb W (AW) coated with 5% (w/w) neopentylglycol succinate plus 1% (w/w) orthophosphoric acid, after ethanolic extraction of plasma. On SP-1200 (Ottenstein & Bartley, 1971*a*) the separation of volatile fatty acids is quite satisfactory at the lowest temperature used (120°C), but elution of lactic acid gives a broad peak with a high yield.

With Carbowax on Chromosorb P, organic acids such as lactic acid are not eluted. However, the number of analyses which can be carried out per day (50 per column) may be limited by the elution of medium-chain fatty acids if these are present in large quantities.

The choice of internal standard is particularly important. Although isobutyric acid is certainly the ideal internal standard for this type of g.l.c., its use poses a number of problems. Its solubility in acid conditions is definitely lower than that of propionic acid and butyric acid, which makes its redissolution difficult at the moment of injection. The only satisfactory technique is too acidify and then inject immediately before micellar separation occurs. In addition, certain samples of blood from the alimentary canal contain isobutyric acid which makes necessary a different internal standard such as isovaleric acid or 2-methylbutyric acid. The use of these acids makes the solubilization before injection even more difficult. For a micro-method of measurement, the choice of an internal standard other than a fatty acid would be difficult since there must be an adequate check of the success of redissolution.

The precision of measurement appears to depend entirely on a good recovery of the internal standard. The determination of peak height and peak area appears quite satisfactory even when the base line is not stable, so long as its drift is taken into account or by use of an electronic integrator. The recovery of isobutyric acid is easier when the sample volume is larger, although this is the most exacting stage when only 200 μl of plasma is used. With a larger volume of liquid in the sample-redissolution flask, isobutyric acid shows complete solubility.

Extraction procedures

Most extraction procedures cannot be used to measure plasma volatile fatty acids. Mahadevan & Zieve (1969) used a treatment which would be justified if the acetic acid was strongly bound to proteins, which is not the case. Similarly deproteinization of samples by a sodium tungstate–H_2SO_4 mixture (Erwin *et al.*, 1961) has no advantage over ethanolic extraction. On the contrary, this makes redissolution of the volatile fatty acids after concentration difficult, owing to the considerable mineral residue. The extraction sequence proposed by Gibbs *et al.* (1973) for the measurement of volatile fatty acids in urine appears hazardous, since it involves a complete evaporation of undissociated volatile fatty acids in organic solution. Steam distillation cannot be used for the extraction of volatile fatty acids at low concentrations owing to the risk of hydrolysis of various acetyl groups. It is a laborious technique which only gives satisfactory results with samples from ruminants.

The ethanolic-extraction procedure does not give pure solutions of volatile fatty acids but it is possible to recover them after g.l.c. to calculate their specific radioactivities. However, there must be increased coating of the columns with Carbowax to obtain a longer retention time.

Binding of volatile fatty acids by proteins

Acetic acid is one of the most diffusible metabolites, so that it is not surprising that binding to plasma proteins, linked to transport phenomena, is negligible

for this acid. In contrast, the binding of butyric acid suggests a possible mechanism regulating its transport, particularly since most of the butyric acid is removed by the liver and therefore the low percentage remaining in the peripheral blood may be entirely in the bound form. As for the long-chain fatty acids, binding of volatile fatty acids is limited to albumin.

It is clear that the very incomplete nature of earlier work in the field of volatile fatty acids resulted essentially from the lack of a simple and precise micro-method of measurement. Considering the nutritional aspects, in omnivorous monogastrics the volatile fatty acids are not thought to be important, although the caecal fermentations vary considerably according to the physicochemical composition of the diet. In monogastrics the role of the volatile fatty acids in hepatic metabolism has remained largely unknown. For acetate, comparable concentrations were found in the blood of germ-free and conventional animals (Annison *et al.*, 1968) and the role of endogenous acetate was discussed but has not been demonstrated conclusively (Palmquist, 1972). The physiological significance of the production of acetate and ketone bodies is of considerable interest.

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