APPENDIX

Correction Procedure

By H. R. CAMA, F. D. COLLINS and R. A. MORTON
Biochemistry Department, University of Liverpool

(*Received* 7 *February* 1951)

A correction procedure can be developed which makes use of all the wavelengths at 2 mμ. intervals from 310 to 340 mμ. As only three wavelengths are necessary in order to correct for linear irrelevant absorption, thirteen degrees of freedom are available for the determination of the error. The following formulae are derived by the method of least squares (see Whittaker & Robinson, 1924).

Let E be the measured extinction at wavelength λ and let α be the ratio of the absorption of pure all-*trans*-vitamin A λ to the absorption at wavelength $\lambda_{max.}$ Then at wave-at length λ

$$E=\alpha\bar{E}+a\lambda+b,$$

where $\bar{E}$ = extinction actually due to vitamin A at $\lambda_{max.}$ and a and b are constants.

By the method of least squares

$$\bar{E}=\frac{\begin{vmatrix}\Sigma\alpha E & \Sigma\alpha\lambda & \Sigma\alpha\\ \Sigma\lambda E & \Sigma\lambda^2 & \Sigma\lambda\\ \Sigma E & \Sigma\lambda & 16\end{vmatrix}}{\begin{vmatrix}\Sigma\alpha^2 & \Sigma\alpha\lambda & \Sigma\alpha\\ \Sigma\alpha\lambda & \Sigma\lambda^2 & \Sigma\lambda\\ \Sigma\alpha & \Sigma\lambda & 16\end{vmatrix}}.$$

The variance of E will be

$$\sigma^2_{\bar{E}}=\frac{\begin{vmatrix}\Sigma\lambda^2 & \Sigma\lambda\\ \Sigma\lambda & 16\end{vmatrix}\begin{vmatrix}\Sigma\alpha^2 & \Sigma\alpha\lambda & \Sigma\alpha & \Sigma\alpha E\\ \Sigma\alpha\lambda & \Sigma\lambda^2 & \Sigma\lambda & \Sigma\lambda E\\ \Sigma\alpha & \Sigma\lambda & 16 & \Sigma E\\ \Sigma\alpha E & \Sigma\lambda E & \Sigma E & \Sigma E^2\end{vmatrix}}{13\times\begin{vmatrix}\Sigma\alpha^2 & \Sigma\alpha\lambda & \Sigma\alpha\\ \Sigma\alpha\lambda & \Sigma\lambda^2 & \Sigma\lambda\\ \Sigma\alpha & \Sigma\lambda & 16\end{vmatrix}^2}.$$

In the case of vitamin A acetate in *cyclo*hexane,

$$\bar{E}=\frac{21760\Sigma\alpha E-19{\cdot}824\Sigma\lambda E-13488\Sigma E}{1297{\cdot}422}.$$

In the case of vitamin A alcohol in *cyclo*hexane,

$$\bar{E}=\frac{21760\Sigma\alpha E+2{\cdot}848\Sigma\lambda E-20735{\cdot}36\Sigma E}{1461{\cdot}4985}.$$

The above derivation can be generalized and would include the Morton-Stubbs equations.

REFERENCE

Whittaker, E. T. & Robinson, G. (1924). *The Calculus of Observations.* London: Blackie and Son Ltd.

Separation and Estimation of Saturated C_2–C_7 Fatty Acids by Paper Partition Chromatography

By R. L. REID and M. LEDERER
Division of Animal Health and Production, C.S.I.R.O., McMaster Animal Health Laboratory, Sydney, Australia

(*Received* 14 *February* 1951)

Investigations at this laboratory, involving many analyses of mixtures of lower fatty acids in rumen contents and in blood of sheep, indicated the need for more convenient methods than those at present available. Preliminary investigations have indicated that paper chromatography offers a solution to this problem.

One of us (R.L.R.) had previously used the silica-gel partition-chromatographic methods of Elsden (1946) and of Moyle, Baldwin & Scarisbrick (1948). Elsden's method satisfied a long-felt need, but it has certain disadvantages. Moyle *et al.* (1948) found that the use of buffered columns obviated most of the disadvantages of Elsden's method, and gave extremely accurate results. However, it is tedious, and this is an important limiting factor in routine analyses. Particularly is this so when more than three acids are to be determined, and hence two or more columns must be used.

The method of Peterson & Johnson (1948) also employs silica-gel columns, but is likewise tedious because more than one column must be used to effect separation of the acids and many titrations are necessary.

Paper chromatography has recently been applied to the separation and quantitative determination of relatively non-volatile organic acids (Lugg & Overell, 1947, 1948) and to the separation of aromatic monocarboxylic acids (Lederer, 1949*b*). The volatility of the lower fatty acids, however, limits the direct application of paper-chromatographic techniques for their separation and identification. Fink & Fink (1949) overcame this difficulty by chromatographing the acids as their hydroxamate derivatives and spraying the developed chromatogram with ferric chloride. The derivatives were then visible as purple spots on a yellow background, but accurate quantitative determination was not possible.

We have now devised a paper partition-chromatographic procedure by which we can separate, identify and estimate lower fatty acids from C_2 to C_7 in the one procedure and with sufficient accuracy for most biological purposes. Since our work was completed, Brown & Hall (1950; cf. Brown, 1950) have published a very similar method for the separation and identification of lower fatty acids, but their findings differ from ours in certain aspects.

Lactic and formic acids cannot be separated from acetic by our technique, or by that of Brown & Hall (1950). These two acids, when present in significant amounts, must be removed by preliminary distillation by a method such as that of Friedemann (1938). When determining volatile fatty acids in rumen liquor, in which lactic and formic acids are not usually present in significant quantities, a single Markham steam distillation (McAnally, 1944) is adequate.

The chromatographic technique which we employ is the ascending development for 20–24 hr. as described by Williams & Kirby (1948). The solvent is butanol saturated with aqueous 1·5 N-ammonia, which was used previously by one of us in the chromatography of inorganic anions (Lederer, 1949*a*) and also by Brown & Hall (1950). The acids are run on the chromatogram in the form of their ammonium salts. Unlike Brown & Hall (1950), we have found that the use of sodium salts is unsatisfactory because, under most conditions, the spot given by the sodium ion partly or completely obliterates that given by acetate.

After development and air-drying, the paper is sprayed with bromocresol purple in ethanol containing formaldehyde. The inclusion of formaldehyde in this reagent increases the subsequent spot definition. Formaldehyde reacts with the ammonium ion to form the weak base hexamethylenetetramine. The free acids, after exposure of the chromatogram to ammonia, appear as regular yellow spots on a purple background. In our hands, bromocresol purple has proved superior to several other indicators, including bromocresol green and bromothymol blue, which were used by Brown & Hall (1950). It is more sensitive to low concentrations of the acids and gives spots which are more sharply defined and usually more stable; these considerations, as will be seen, are important in quantitative analysis.

Of the methods of quantitative analysis investigated, the only one found suitable was the calculation of acid content from spot area, the latter being related linearly to the logarithm of the former. The use of this technique was suggested by Fisher, Parsons & Morrison (1948) for the quantitative determination of amino-acids and sugars. Bryant & Overell (1951) have successfully used the relationship in the estimation of relatively non-volatile organic acids, and their observations are confirmed in our work with lower fatty acids.

EXPERIMENTAL

Reagents

Solvent system. Redistilled *n*-butanol is saturated with an equal volume of aqueous 1·5 N-NH_3.

Indicator solution. This is a solution of 0·04% (w/v) bromocresol purple in a 1:5 (v/v) dilution of B.P. formalin in ethanol. The pH of this solution is adjusted to approximately 5·0 by the addition of 0·1 N-NaOH.

General procedure

Development of chromatogram. Sheets of Whatman no. 1 (special chromatographic) paper (12 in. (30 cm.) square) are exposed to an atmosphere of concentrated aqueous NH_3 in a closed vessel for 3–4 hr. immediately before use. Samples of approximately 5 μl., containing the fatty acids in the form of their ammonium salts, are placed 3–4 cm. apart along a 'starting line' 3 cm. from the bottom of the paper. Each sheet is stapled in the form of a cylinder which is then stood in a 0·5–0·7 cm. layer of butanol phase of the solvent in a convenient jar or tank with close-fitting lid. The aqueous solvent phase is transferred to a beaker or beakers which are stood in the butanol phase. The same solvent mixture can be used for at least four chromatograms. A development time of 18 hr. is sufficient for complete separation of the acids, the solvent reaching the top of the paper in 18–20 hr. The time of development is not critical and can be extended to at least 24 hr. if necessary. When development is complete, the paper is removed and allowed to dry at room temperature.

Spraying technique. Several attempts were made to control the spraying intensity in an effort to obtain even spraying of the paper. With light spraying, any unevenness causes erratic quantitative results. We found, however, that the best spot definition was obtained with an intensity of spraying sufficient to allow complete and even penetration of indicator solution to the reverse side of the paper. For this purpose an ordinary atomizer proved as efficient as any other technique tested.

Development of acid spots. After holding the sprayed chromatogram for 2–3 min. in an atmosphere saturated with a 3% (v/v) dilution of aqueous NH_3 (sp.gr. 0·880) (removing it for a few seconds at brief intervals), the acids appear as bright-yellow spots on a stable purple background (Fig. 1).

Quantitative analysis. The acid spots are traced on to graph paper, ruled in 0·125 in. (approx. 3 mm.) squares, and an estimate of their area is thus obtained. Two standard solutions, containing known concentrations of the ammonium salts of the fatty acids under investigation, must be chromatographed on the same sheet with each test solution.

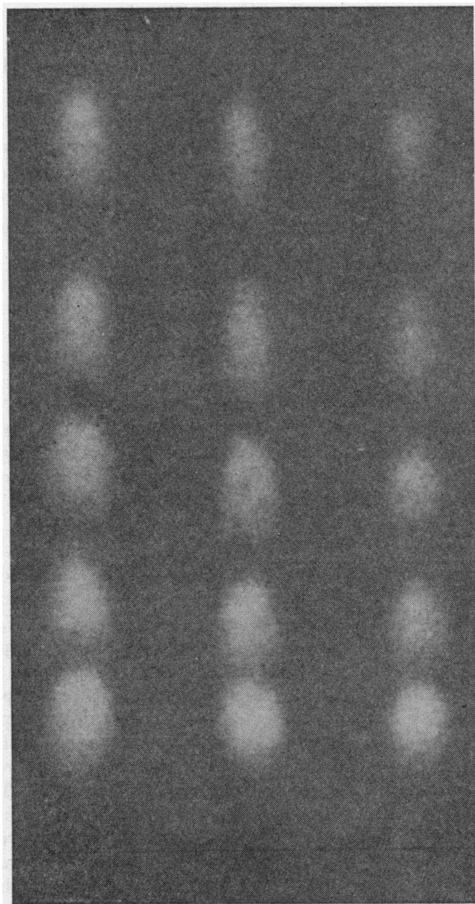

Fig. 1. Separation of C_2–C_6 fatty acids from 5 μl. samples of mixed solutions of their ammonium salts. Concentration of acids (μg./5 μl.) from left to right: acetic: 93, 78, 62; propionic: 77, 64, 51; *n*-butyric: 76, 63, 50; *iso*valeric: 68, 57, 45; *n*-hexanoic: 64, 54, 43 respectively. The chromatogram was run for 18 hr. on Whatman no. 1 (special chromatographic) paper, with *n*-butanol-aqueous ammonia as the developing solvent (see Text).

Due to the direct relationship between spot area and logarithm of acid content, the spot areas given by these standards can be used to plot regression lines for each acid, from which the acid content of unknown spots can be read directly.

Normally we run two standards with six or seven different unknowns on each of four sheets. Calculations are thus based on the mean of quadruplicate estimates and variations from sheet to sheet are automatically accounted for. The concentrations of each acid in the standard solutions are so adjusted that test solutions fall within their limits.

RESULTS

R_F *values*

The R_F values of the ammonium salts of lower fatty acids, from formic to nonanoic (pelargonic), are shown in Table 1. Acids from acetic to heptanoic (oenanthic) (C_2–C_7) separate completely, but heptanoic acid (C_7) is inseparable from octanoic (caprylic; C_8) and from nonanoic acid (C_9). Octanoic acid can be estimated in the presence of hexanoic (caproic) acid (C_6) but not when heptanoic or nonanoic acids are also present. Formic and lactic acids cannot be separated from acetic acid, but preliminary steam distillation (Friedemann, 1938) prevents their interference in acetic acid determinations. *n*-Valeric and *iso*valeric acids are inseparable. The ammonium salts of all dicarboxylic acids investigated did not move in this solvent.

Table 1. R_F *values of ammonium salts of fatty acids and of other organic acids*

(Development with *n*-butanol-aqueous ammonia by upward running technique with Whatman no. 1 filter paper, see Text.)

	R_F values	
Acid	Single acids in solution	Mixture of acids in solution
Formic	0·10	—
Acetic	0·11	0·10–0·11
Propionic	0·19	0·18–0·20
n-Butyric	0·29	0·29–0·32
n-Valeric	0·41	—
*iso*Valeric	0·40	0·39–0·44
n-Hexanoic (caproic)	0·53	0·53–0·59
n-Heptanoic (oenanthic)	0·62	0·62–0·64
n-Octanoic (caprylic)	0·65	0·64–0·66
n-Nonanoic (pelargonic)	0·67	—
Lactic	0·10	—
Pyruvic	0·01	—
Malic	0·0	—
Fumaric	0·0	—
Maleic	0·0	—
Oxalic	0·0	—
Malonic	0·0	—
Succinic	0·0	—
α-Ketoglutaric	0·0	—

The R_F values reported by Brown & Hall (1950) for fatty-acid anions, put on the chromatogram in the form of their sodium salts, are similar to those observed by us for ammonium salts. On the contrary, we have observed consistently lower R_F values when mixtures of sodium salts are used, e.g. acetic acid 0·08, propionic acid 0·12–0·14, butyric acid 0·21–0·26, *n*-valeric acid 0·37 and hexanoic acid 0·53. The reasons for this discrepancy are not clear.

Quantitative analysis

Before adopting a method involving calculation of acid content from spot area, we investigated a number of other possible techniques:

(*a*) *Titration of eluted acids.* The efficiency of a titration technique depends upon the neutralization of ammonia adsorbed on the filter paper during the development of the chromatogram, on quantitative elution of the ammonium salts from the paper and on their conversion into free acids. We found that the salts could, apparently, be eluted with distilled water and that the removal of ammonia was accomplished through the formation of hexamethylenetetramine in the presence of formaldehyde. The position of the ammonium salts at any time during development of the chromatogram could be accurately predicted from known R_F values. After 18 hr. development, the chromatogram was removed from the solvent and 1 in. (2·5 cm.) squares of paper, in the predicted position of each acid, were cut out immediately and placed in 5 ml. distilled water. After 5 min., 5 ml. of a 1:5 (v/v) aqueous dilution of B.P. formalin (exactly neutralized) were added and the free fatty acids were then titrated.

Recoveries, although not complete, showed a regular trend which was dependent on the amount of fatty acid present. It was apparent that analyses would have to be based on a standard recovery curve obtained with standard solutions run on the same chromatogram, and that this curve would vary from sheet to sheet. The technique was finally abandoned.

(*b*) *Determination of ammonia.* An indirect method based on the determination of the ammonia-nitrogen content of the ammonium salts, by reaction with Nessler's reagent after elution from paper, was also rejected. Ammonia adsorbed on the paper during development of the chromatogram caused high blanks, even after drying the chromatogram in a hot-air oven. These blanks varied from point to point on the paper, being highest near the bottom of the chromatogram.

(*c*) *Measurements of electrical conductivity.* These were made on aqueous solutions of the eluted salts, but also proved unsatisfactory.

(*d*) *Spot-area measurements—incorporation of indicator in mobile phase.* In our earlier experiments, when it appeared that variations in spraying intensity over the chromatogram affected the validity of spot-area measurements, we tried incorporating indicators in the mobile phase. With most indicators tested, exposure of the chromatogram to air after development was accompanied by the spontaneous appearance of acid spots, thus obviating the necessity for spraying with formaldehyde. The rate of appearance and stability of the spots depended largely on the pH range of the indicator, i.e. on the rate of colour change as ammonia evaporated from the paper. Indicators with a pH range within the limits 4·4–8·0 gave well defined spots which did not fade rapidly, e.g. bromocresol purple, phenol red, chlorophenol red, rosolic acid, neutral red and methyl red.

The principal objection to this technique is the fact that the R_F values of most indicators in this solvent are too low (Lederer, 1950). For instance, in phenol red ($R_F = 0·20$) ammonium acetate appeared as a bright-yellow spot on a red background, but ammonium propionate and salts of higher acids were not covered. In rosolic acid ($R_F = 0·40$) only ammonium acetate, propionate and butyrate could be detected as yellow spots on an orange background.

Of the indicators tested, neutral red ($R_F = 0·66$) and methyl red ($R_F = 0·59$) alone were satisfactory. In neutral red (0·05% (w/v) in mobile phase), magenta spots were visible on an orange background after 5–8 min. exposure to air. In methyl red (0·05% (w/v) in mobile phase), red spots appeared on a yellow background after 2–3 min. Acids from C_2 to C_6 were thus detectable, but the spots were not sufficiently well defined to allow accurate calculation of their area.

(*e*) *Spot-area measurements after spraying with bromocresol purple.* This technique was adopted only after exhaustive studies of those already outlined. Spot-area measurements may give highly inaccurate results if subsequent calculations of acid content are made from a single standard regression line obtained independently. The extent of inaccuracy depends primarily upon variations in intensity of spraying from chromatogram to chromatogram. The order of accuracy is increased enormously by chromatographing two standard solutions simultaneously with each unknown fatty-acid solution, and by calculation from the means of quadruplicate spot-area measurements.

The validity of this technique has been tested by two independent observers—one of us (R.L.R.) and another worker in this laboratory, who had previously had only a few days' experience with the technique. In each instance 5 μl. of each of six dilutions of a standard solution were run on each of four sheets, being placed on each sheet in random order to minimize bias in tracing. After development, the chromatograms were sprayed, spots traced and areas measured in the usual way. Differences between sheets were statistically significant for all acids of both observers. Calculation of the regression of spot area on logarithm of acid content from the data of the last experiment carried out by each observer gave regression coefficients which are included, together with their standard errors, in Table 2.

Table 2. *Regression of spot area on logarithm to base* 10 *of acid content* (*μg./5 μl.*)

(S_b is standard error of regression coefficient b.)

	Observer *A*		Observer *B*	
Acid	Regression coefficient (b) (sq.in.)	S_b	Regression coefficient (b) (sq.in.)	S_b
Acetic	+0·576	0·056	+0·458	0·064
Propionic	+0·631	0·046	+0·465	0·099
n-Butyric	+0·590	0·069	+0·599	0·114
*iso*Valeric	+0·562	0·068	+0·810	0·197
n-Hexanoic	+0·565	0·075	—	—
n-Heptanoic	+0·693	0·053	—	—

Table 3. *Deviations of predicted from actual acid concentrations*

		Two standards on each sheet				Three standards on each sheet			
Acid	Range of concn. (μg./5μl.)	No. of concn.	Mean deviation (μg./5μl.)	Standard deviation (μg./5 μl.)	Error at mid-point of range (%)	No. of concn.	Mean deviation (μg./5μl.)	Standard deviation (μg./5 μl.)	Error at mid-point of range (%)
Acetic	34–93	8	−1·56	2·74	4·3	6	−2·77	2·88	4·5
Propionic	16–77	8	−1·46	1·71	3·7	6	−0·77	1·71	3·7
n-Butyric	16–63	7	+0·10	0·88	2·2	5	+0·04	0·82	2·1
n-Valeric	17–57	6	−1·18	1·16	3·2	4	−0·90	1·23	3·3
n-Hexanoic	31–71	7	−2·00	2·09	4·1	5	−0·66	1·58	3·1
n-Heptanoic	34–69	4	−1·85	1·55	3·0	3	−1·00	1·03	2·0

In each experiment the greatest and least dilutions were then treated as standards to fit a regression line from which the acid concentrations in the intermediate dilutions were predicted. In all experiments, the deviations of predicted from actual acid concentrations were independent of concentration over the range studied. Mean deviations and standard deviations calculated from the data of the first observer are given in Table 3. At the midpoints of the ranges of concentration studied, the standard deviations represent an error of 2–5 % for each acid. The errors calculated from the data obtained by the second observer were similar.

A partial explanation of the persistently negative mean differences appears to be that tracings of the small spots given by the lower standards (in which the acid concentrations were below the ranges given in Table 3) tended to give areas slightly larger than the theoretical. This personal error was most pronounced for higher acids, where the higher R_F values resulted in a more diffuse margin around the spots given by low concentrations of these acids.

Because of this, the effect on prediction errors of using three standards on each sheet was calculated from the same data, with an intermediate dilution in each series as the third standard. Mean deviations of predicted from actual concentrations were lower for each acid except acetic, but the effect on percentage error was not such as to warrant the use of three standards on each sheet as a routine procedure (Table 3).

Errors calculated similarly from the means of triplicate estimates were no higher for the first observer, but were higher for the second observer (see Table 2).

It should be noted that the ranges of concentration given in Table 3 do not define the limits between which the relationship between spot area and logarithm of acid content holds, as the standard solutions are not included. Table 4 shows the minimum amounts of each acid which we were able to detect qualitatively and estimate quantitatively by this method.

Table 4. *Minimum concentrations of acids required for detection and for quantitative estimation*

Acid	For detection (μg./5 μl.)	For quantitative estimation (μg./5 μl.)
Acetic	3	10
Propionic	3	11
n-Butyric	4	11
n-Valeric	6	13
n-Hexanoic	6	13
n-Heptanoic	7	14

Influence of some experimental conditions

Filter paper. Whatman no. 2 paper (untreated) was satisfactory for qualitative analysis, but trailing of acids, which interfered with quantitative analysis, was often observed. This trailing was not observed on Whatman no. 1 (special chromatographic) paper.

Volume of sample. Delivery of 5 μl. portions from a microsyringe is unnecessarily tedious. Micropipettes, made from calibrated thermometer tubing, are accurate and convenient. Alternatively, a haemocytometer pipette can be used. The actual volume of solution taken for analysis is, within limits, immaterial provided that the same volume is used for both standard and unknown solutions. For this reason, an appropriate calibration mark on a micropipette can be chosen to deliver a volume of

approx. 5 μl. and this volume then adhered to throughout the analysis.

Unsuitability of sodium salts. Solutions of sodium salts of fatty acids were unsatisfactory, both for qualitative and for quantitative analysis. The concentration of salts in the solution put on the chromatogram should be moderately high, otherwise acids which constitute a small fraction of the total may not be detectable in a qualitative analysis or measurable in a quantitative analysis (e.g. valeric acid in rumen liquor). Under these conditions, when relatively large amounts of sodium are present, the sodium spot overlaps or obliterates the acetate spot and prevents its quantitative determination. On several occasions we were unable to detect acetate, even though present in appreciable amounts. Acetate could be detected only when the total fatty acid content, and hence the content of Na^+, was small. This is a serious limitation in both qualitative and quantitative analysis.

Loss of acids before development. Loss of acids through evaporation may occur during the time taken to put eight or nine 5 μl. samples on to the chromatogram, particularly at higher room temperatures (25°). This loss is presumably due to displacement of fatty-acid anions from their combination with NH_4^+ by stronger acid radicals in the filter paper. It can be completely prevented by exposing the dry paper to an atmosphere saturated by aqueous 15N-ammonia for several hours before use. Similar losses, of course, do not occur during exposure of the developed chromatogram.

Measurement of spot area. The use of a planimeter gave errors as high as 40%, an observation which appears to be contrary to the experience of Fisher *et al.* (1948) with amino-acids and sugars, but which has been confirmed by Bryant & Overell (1951). As stated elsewhere, graph-paper tracings proved entirely satisfactory and convenient. Alternative satisfactory methods include the expression of spot area in terms of the mass of the filter paper enclosed by the spot (Bryant & Overell, 1951).

Applications of the method

Lower fatty acids in rumen contents of sheep. In our experience, if lactic acid is not present, a single steam distillation in the presence of an equal volume of acidified magnesium sulphate solution (McAnally, 1944) is sufficient to effect preliminary separation of the acids. The volume of rumen liquor taken should be 2–5 ml., depending on fatty-acid concentration. Total fatty acids are determined by titration of the distillate with barium hydroxide solution of suitable concentration in the absence of carbon dioxide, phenol red being used as indicator. If lactic acid is present, double steam distillation can be carried out by the method of Friedemann (1938) although, in our experience with rumen liquor containing up to 5 mg.mol. lactic acid in the presence of 10–12 mg.mol. lower fatty acids per 100 ml., the amount of lactic acid which distils over in a single distillation in the Markham apparatus causes an error of only 3–5% in the determination of acetic acid. For many practical purposes this error can be neglected, thus avoiding the necessity for double distillation.

After addition of excess barium hydroxide, the first 30 ml. of distillate is evaporated to dryness, the later stages of evaporation being carried out after transfer of the solution to a 10 ml., or smaller, centrifuge tube. Conversion of the barium salts to ammonium salts is effected by addition of a suitable known volume of saturated ammonium oxalate solution (usually 0·5–1·0 ml.). The concentration of ammonium salts in the final solution obviously depends on the solubility of ammonium oxalate, and is of the order of 2·0–2·5 μg.mol./5 μl.

After centrifuging to remove barium oxalate, 5 μl. portions of the supernatant solution, containing the ammonium salts of the fatty acids, together with excess ammonium oxalate, are transferred to paper for the subsequent analysis. Ammonium oxalate remains on the 'starting line' (cf. Table 1) and the presence of an oxalic acid spot in this position after spraying provides a check as to whether sufficient ammonium oxalate was originally added to precipitate all barium. Phenol red does not interfere with the analysis and usually moves to a position between ammonium acetate and ammonium propionate.

The percentage composition of the mixture of lower fatty acids in rumen liquor is remarkably constant under a wide range of dietary conditions, which facilitates the choosing of suitable standard solutions. As C_5 acids constitute only 3–6% of the fatty acids present, the amounts run on the chromatogram (6–12 μg.) are outside the range of the method. It is possible, however, to make a reasonably accurate estimate by direct visual comparison with suitable standards (6 and 12 μg./5 μl.). The amounts of C_6 acid present are too small to be detected by this method.

Several analyses, both by this method and by that of Moyle *et al.* (1948), have shown that although the latter method is the more accurate, results by the two methods are, consistently, in reasonable agreement (Table 5).

To date we have made seventy-two analyses of rumen liquor. Recoveries in sixty-six of these, based on the total acid titration of the first distillate, ranged from 91 to 108%, with a mean of 97·4 ± 4·2%. Recoveries would not be expected to exceed this value, as small amounts of acids higher than C_5 are not detected.

Fatty-acid metabolism in nematode parasites. The method has been used successfully by another

Table 5. *Comparison of present method with that of Moyle, Baldwin & Scarisbrick* (1948)

(Acids as mmol./100 ml. rumen liquor.)

Rumen liquor sample no.	Present method C_2	C_3	C_4	C_5	Total recovery (%)	Method of Moyle *et al.* C_2	C_3	C_4^+	Total recovery (%)
1	7·38	2·28	1·56	0·90	94	7·29	2·38	2·48	94
2	7·80	2·40	1·68	0·90	93	8·19	2·41	2·82	97
3	7·92	1·80	1·74	0·90	94	7·89	2·16	2·67	97
4	4·63	0·90	0·86	0·48	95·6	4·73	0·89	1·43	99·5
5	6·60	1·80	1·39	0·51	97·5	6·69	1·75	1·91	98
6	6·95	1·95	1·46	0·54	98	7·12	1·99	2·12	101

worker in this laboratory as a convenient approach to metabolic studies, involving fatty acids as high as hexanoic, in nematodes. Preliminary separation of the acids from homogenized tissue extracts by the method of Friedemann (1938), or by distillation as described by McClendon (1944), is followed by the same general procedure outlined herein for analyses of rumen liquor, but suitably modified to cope with the much smaller quantities of material available. Recoveries have been of the order of 95–102 %.

Limitations of the method

The principal limitations of the method have already been discussed. It is less accurate than that of Moyle *et al.* (1948), and isomeric acids and acids higher than C_7 cannot be separated. Nevertheless, in our hands, the method has proved considerably more convenient than its predecessors and, as any inaccuracy involved has usually been within the limits of normal biological variation, it is of little consequence.

The failure to separate acids higher than C_7 is not an intrinsic limitation to paper partition-chromatographic separation of these acids. We have observed considerably lower R_F values in pentanol, which suggests that higher fatty acids may be separable in higher-alcohol solvents. Isomeric acids have not so far been separated.

SUMMARY

1. A method is described by which steam-volatile saturated fatty acids can be separated, identified and estimated by paper-partition chromatography.

2. The acids are run upward on paper as their ammonium salts using n-butanol-aqueous ammonia as solvent system. The free fatty acids appear as yellow spots on a purple background after the developed chromatogram is sprayed with a bromocresol purple solution containing formaldehyde. Fatty-acid content can be calculated from spot area because of the direct relationship between spot area and the logarithm of acid content.

3. Fatty acids of the normal series from acetic to heptanoic can be separated, identified and estimated with an accuracy of ± 2–5% for each acid. Heptanoic acid cannot be separated from octanoic and nonanoic acids; octanoic acid can only be estimated in the absence of heptanoic and nonanoic acids. Isomeric acids cannot be separated.

We with to express our sincere appreciation to Miss M. C. McKevett, who carried out the numerous statistical analyses essential to the development of the method.

Our thanks are also due to Dr W. P. Rogers, Miss H. N. Turner, Mr J. Hogan and Mr W. K. Warburton for their assistance at various stages of this work. We are also indebted to Mr G. K Hughes for gifts of fatty acids.

Note. Since this manuscript was completed, a recent paper by Hiscox & Berridge (1950) has come to hand. These authors have separated volatile fatty acids on paper in a mobile phase of butanol, with ethylamine in the vapour phase. After spraying with bromocresol green they observed that the 'area of the spot gives a rough indication of the total quantity of fatty acid present' and have used the method to obtain 'roughly quantitative' results.

REFERENCES

Brown, F. (1950). *Biochem. J.* **47**, 598.
Brown, F. & Hall, L. P. (1950). *Nature, Lond.*, **166**, 66.
Bryant, F. & Overell, B. T. (1951). *Nature, Lond.* (in the Press).
Elsden, S. R. (1946). *Biochem. J.* **40**, 252.
Fink, K. & Fink, R. M. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 654.
Fisher, R. B., Parsons, D. S. & Morrison, G. A. (1948). *Nature, Lond.*, **161**, 764.
Friedemann, T. E. (1938). *J. biol. Chem.* **123**, 161.
Hiscox, E. R. & Berridge, N. J. (1950). *Nature, Lond.*, **166**, 522.
Lederer, M. (1949 *a*). *Aust. J. Sci.* **11**, 174.
Lederer, M. (1949 *b*). *Aust. J. Sci.* **11**, 208.

Lederer, M. (1950). *Science* (in the Press).
Lugg, J. W. H. & Overell, B. T. (1947). *Nature, Lond.*, **160**, 87.
Lugg, J. W. H. & Overell, B. T. (1948). *Aust. J. Sci. Res.* A, **1**, 98.
McAnally, R. A. (1944). *J. exp. Biol.* **20**, 130.
McClendon, J. F. (1944). *J. biol. Chem.* **154**, 357.
Moyle, V., Baldwin, E. & Scarisbrick, R. (1948). *Biochem. J.* **43**, 308.
Peterson, M. H. & Johnson, M. J. (1948). *J. biol. Chem.* **174**, 775.
Williams, R. J. & Kirby, H. (1948). *Science*, **107**, 481.

The Relative Rates of Oxidation of Lipovitellin and of its Lipid Constituent

By C. H. LEA and J. C. HAWKE
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

(*Received* 1 *March* 1951)

Unsaturated lipids in general, and phospholipins in particular, are known to undergo oxidation when exposed to the atmosphere, and such changes are liable to occur whenever lipid-containing substances are handled. The recent observation by Dam and co-workers (Dam, 1944 *a,b*; Dam & Granados, 1945 *a,b*) that fat peroxides and a yellow-brown discoloration can develop in tissues *in vivo*, particularly when the diet is deficient in tocopherols (which act as anti-oxidants) and rich in highly unsaturated fatty acids, lends added biochemical interest to a process hitherto considered mainly of importance in extracted lipids and 'dead' tissues.

Lipids, which in the isolated state are very susceptible to attack by oxygen, often show in the tissue an astonishing resistance; linseed oil, for example, remains unoxidized for long periods in the harvested flax-seed. Although the difference is probably connected with the fact that inhibiting substances such as phenolic anti-oxidants are only in part extracted with the lipid, the further possibility exists that the more reactive lipids may be stabilized against oxidation by existing in combination with other constituents of the tissues. Triglyceride fat, and particularly that present in the large fat storage depots, is mainly free, whilst much of the phospholipin occurs in combination with protein or (in less degree) carbohydrate in the cells. It seemed of interest, therefore, to compare the rates of oxidation, both in the presence and absence of catalysts, of phospholipin (*a*) in combination with protein, and (*b*) in the free state, either alone or admixed with the protein.

Lipoproteins in general tend to be ill defined, noncrystalline, labile substances, difficult to isolate, characterize and handle. Lipovitellin from egg yolk was chosen for the present work because its comparatively high stability and low degree of unsaturation favoured separation in an undecomposed and unoxidized state. Since the autoxidation of lipids can be greatly accelerated by exposure to light or by the presence of trace metal and other contaminants, or retarded by substances which function as anti-oxidants (inhibitors), the technique employed included precautions against interference by such factors.

EXPERIMENTAL

Preparation of lipovitellin from egg yolk

The method used was essentially that of Chargaff (1942), with modification where necessary to overcome the intractable emulsions encountered in the original procedure. Batches of 48 eggs, not more than 24 hr. old, were washed in 2% lysol and in ethanol, which was burned off before breaking. The yolks, after removal of the membrane and chalazae, were mixed with an equal volume of water and extracted by shaking five or six times with 800 ml. peroxide-free ether until the extract was practically colourless. Saturated NaCl solution (520 ml.) was then added to dissolve the lipoprotein, and the solution was dialysed through cellulose, first into water (16 l.) for several hours and then overnight into water (80 l.). All operations were carried out at 0–3° with precooled solvents and equipment. The very large quantities of water required for dialysis were prepared by mixed bed de-ionization of the laboratory distilled water supply. Cellulose casing was found to contain considerable quantities of water-soluble material and was thoroughly extracted before use.

The crude lipoprotein which separated was centrifuged, washed twice with 800 ml. water, suspended in 560 ml. water and shaken with 800 ml. ether. No ether could be separated at this stage, and 240 ml. saturated NaCl solution were added and the whole dialysed. During dialysis an ether layer separated and was discarded before centrifuging the protein. The processes of solution by NaCl, reprecipitation by dialysis, washing and extraction with ether were repeated four times, with omission of the ether extraction on the last occasion. The lipoprotein suspension obtained was held at 0° in an atmosphere of N_2, and its solids content determined by drying to constant weight *in vacuo* at 70°.