Separation of the Lower Fatty Acids as Anions by Paper Chromatography

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The work of Martin & Synge (1941) on the separation of mixtures of amino-acids by partition chromatography on silica gel led to the application of this method to the analysis of fatty acid mixtures by several workers. Lester Smith (1942) separated a mixture of formic, acetic, propionic, *n*-butyric and *n*-valeric acids into its separate components, and developments of this work by Elsden (1946), Ramsey (1948), Ramsey & Patterson (1945, 1948), Peterson & Johnson (1948) and Moyle, Baldwin & Scarisbrick (1948) have furnished methods for the quantitative analysis of fatty acids containing 1–10 carbon atoms. This work has been reviewed recently by Elsden (1950).

Substitution of the silica support by filter paper (Consden, Gordon & Martin, 1944) has found widespread application in the qualitative and approximate quantitative analysis of mixtures of closely related compounds. Paper chromatography possesses several advantages over partition chromatography on silica, not least of which are the small amounts of material required for an analysis and the ease of operation of the chromatogram. In attempting to apply paper chromatography to the separation of the lower fatty acids, the volatility of some members and the low solubility in water of others are limiting factors. Fink & Fink (1949) have overcome these unsuitable physical properties by the use of the corresponding potassium hydroxamate derivatives, but the difficulties involved in their preparation is a great disadvantage.

Since various workers (e.g. Goodall & Levi, 1946; Lederer, 1948; Linstead, 1949) have described the separation of various groups of anions and cations by paper chromatography, the present author considered that the fatty acid anions might be similarly separated. Using *n*-butanol or *n*-butanol containing ethanol as the mobile phase in the presence of ammonia, it has been possible to separate the formate, acetate, propionate, *n*-butyrate, *n*-valerate, *n*-caproate, and *n*-octanoate ions. Partial resolution of the *n*- and *iso*-C_4, C_5 and C_6 ions was also achieved.

METHODS

Solvents. All solvents were distilled before use.

Paper support. Whatman no. 1 filter paper sheets were used throughout.

Apparatus. The apparatus was similar to that described by Flood, Hirst & Jones (1948) except that a battery jar was substituted for the bell jar and the ground-glass flange formed the top instead of the base of the apparatus. By this means the atmosphere inside the chamber was less seriously disturbed when the papers were introduced and removed.

Procedure. A small circular spot of solution (2 μl.) containing the Na salts of the acids is placed on the starting line of the paper with the aid of a fine capillary tube. The chromatogram is allowed to run for a convenient time (approx. 20 hr. for most mixtures; the solvent boundary is then approx. 20 cm. below the starting line). The paper is then removed and dried, either in a steam oven for 5 min. or at room temperature for 1 hr. The positions of the ions are then revealed by spraying with a 0·04% (w/v) aqueous solution of bromothymol blue adjusted to pH 7·5 with NaOH. The anions give yellow spots and the cations deep-blue spots on a green ground. Alternative sprays such as bromocresol green, chlorophenol red and bromocresol purple are also useful. A disadvantage common to all the indicators used in the present work is the rapid fading of the spots due to the whole paper assuming the acid colour of the indicator, presumably due to the action of the CO_2 in the air.

RESULTS

Separation of the formate, acetate, propionate, *n*-butyrate, *n*-valerate, *n*-caproate and *n*-octanoate ions was readily achieved using as the solvent system *n*-butanol-aqueous ammonia or *n*-butanol-ethanol-aqueous ammonia and developing with the alcoholic phase. The R_F values of these ions and of the *iso*butyrate, *iso*valerate and *iso*caproate ions in these solvents are shown in Table 1. It was noticed that the R_F values of all the ions diminished gradually with the increasing age of the solvents and consequently a different standard of reference has been used. The ratio between the distance travelled by an anion and the distance travelled by the *n*-octanoate ion gives a fairly constant value (R_C), characteristic of the anion and independent of the age of the solvent. The advantages of using this kind of standard rather than R_F values have been discussed previously in the case of sugars and methylated sugar derivatives (Brown, Hirst, Hough, Jones & Wadman, 1948; Hirst, Hough & Jones, 1949). Table 2 shows the extreme R_C values obtained corresponding to the R_F values in the adjacent columns. The R_C values did not fall with the increasing age of the solvent, but varied haphazardly between the limiting values

Table 1. *R_F values of fatty acid ions on Whatman no. 1 paper at 15°*

(The values recorded are the averages of the initial values obtained for different preparations of the same solvent mixtures.)

	Solvent systems (by vol.)		
	n-Butanol 50 %, 1·5 N-aqueous ammonia 50 %	*n*-Butanol 50 %, 3·0 N-aqueous ammonia 50 %	*n*-Butanol 40 %, ethanol 10 %, 3·0 N-aqueous ammonia 50 %
Formate	0·09	0·08	0·14
Acetate	0·10	0·09	0·16
Propionate	0·19	0·18	0·26
n-Butyrate	0·33	0·30	0·39
*iso*Butyrate	0·31	0·28	0·37
n-Valerate	0·45	0·41	0·58
*iso*Valerate	0·39	0·36	0·55
n-Caproate	0·61	0·55	0·71
*iso*Caproate	0·60	0·54	0·69
n-Octanoate	0·74	0·69	0·80

Table 2. *Comparison of R_F and R_C values of fatty acid ions on Whatman no. 1 paper at 15°*

(Initial R_F values were obtained from the first 'run' of the chromatogram using a freshly prepared solvent mixture; final values obtained after about 14 days. The values are the averages of at least five separate determinations.)

	Solvent systems (by vol.)					
	n-Butanol 50 %, 1·5 N-aqueous ammonia 50 %		*n*-Butanol 50 %, 3·0 N-aqueous ammonia 50 %		*n*-Butanol 40 %, ethanol 10 %, 3·0 N-aqueous ammonia 50 %	
	R_F	R_C	R_F	R_C	R_F	R_C
Formate	0·09→0·06	0·10–0·12	0·08→0·06	0·10–0·11	0·14→0·10	0·15–0·17
Acetate	0·10→0·07	0·11–0·13	0·09→0·07	0·12–0·13	0·16→0·13	0·17 0·20
Propionate	0·19→0·14	0·22–0·25	0·18→0·13	0·23–0·26	0·26→0·19	0·29–0·32
n-Butryate	0·33→0·22	0·37–0·44	0·30→0·21	0·37–0·43	0·39→0·29	0·45–0·49
*iso*Butyrate	0·31→0·21	0·36–0·42	0·28→0·20	0·35–0·41	0·37→0·27	0·41–0·46
n-Valerate	0·45→0·33	0·56–0·61	0·41→0·32	0·56–0·60	0·58→0·44	0·67–0·72
*iso*Valerate	0·39→0·28	0·48–0·53	0·36→0·26	0·46–0·52	0·55→0·41	0·63–0·69
n-Caproate	0·61→0·50	0·82–0·85	0·55→0·46	0·79–0·81	0·71→0·56	0·86–0·89
*iso*Caproate	0·60→0·50	0·81–0·85	0·54→0·46	0·78–0·81	0·69→0·56	0·85–0·86
n-Octanoate	0·74→0·59	1·0	0·69→0·57	1·0	0·80→0·65	1·0

shown. The decreases in the R_F values correspond to a period of approximately 14 days from the initial mixing of the alcohol and ammonia. After this period the positions of the anions were revealed only with difficulty on spraying with the indicator solution.

Effect of cations on the R_F and R_C values of the anions

The R_F and R_C values of any given anion appeared to be independent of the salt initially used. Thus the movements of the anions down the paper were substantially the same whether the ammonium, potassium or sodium salts were used. On the other hand, the movements of the cations themselves appeared to be slightly greater as the chain length of the anion increased. Since the R_F values of the cations were of the order of 0·02, however, this difference is negligible. In general the sodium ion moved faster than the potassium ion.

Effect of concentration on the movements of the anions

The variation of the concentrations of the salt solutions from 0·25 to 1·0 % (w/v) did not affect the distances travelled by the ions. This is important in view of the observation (Smith & White, 1929) that the partition coefficients of the volatile fatty acids between water and organic solvents vary with concentration. This variation was shown by Elsden (1946) to affect the R values on the silica partition chromatogram. Concentrations greater than about 1·5 % gave rise to elongated spots on spraying.

Quantities of ions equivalent to 5 μg. of the corresponding salts can be detected on the developed chromatograms. The usefulness of this method for the detection of minute quantities of the acids is thus apparent.

Separation of artificial mixtures of anions

The distances moved by the anions were independent of the presence of other anions. Mixtures of the sodium salts of as many as six acids gave separate spots with R_F values equal to those of the individual sodium salts run independently on the same paper. Only the formate and acetate ions proved difficult to separate completely into two distinct spots. This can be achieved, however, by running the chromatogram for approx. 60 hr. instead of the usual 20 hr.

Separation of isomers

The separation of the *n*- and *iso*-C_4, C_5 and C_6 ions was incomplete in 20 hr. By running the chromatogram for approx. 60 hr., complete separation of the C_4 and C_5 isomers could be achieved. The high R_F values of the *n*- and *iso*-C_6 ions would necessitate the use of excessive lengths of paper to separate these isomers completely on a one-dimensional chromatogram.

SUMMARY

1. The separation of the lower fatty acids as anions by paper chromatography is described using for development mixtures of alcohols with aqueous ammonia. Separation of the *n*- and *iso*-C_4 and C_5 anions has also been achieved.

2. The anions and cations from quantities of salts as low as 5 μg. can be detected on the developed chromatograms.

REFERENCES

Brown, F., Hirst, E. L., Hough, L., Jones, J. K. N. & Wadman, W. H. (1948). *Nature, Lond.*, **161**, 720.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Elsden, S. R. (1946). *Biochem. J.* **40**, 252.
Elsden, S. R. (1950). *Biochem. Soc. Symp.* **3**, 74.
Fink, K. & Fink, R. M. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 654.
Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1948). *J. chem. Soc.* p. 1679.
Goodall, R. R. & Levi, A. A. (1946). *Nature, Lond.*, **158**, 675.
Hirst, E. L., Hough, L. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 928.
Lederer, M. (1948). *Nature, Lond.*, **162**, 776.
Lester Smith, E. (1942). *Biochem. J.* **36**, xxii.
Linstead, R. P. (1949). *Nature, Lond.*, **163**, 598.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
Moyle, V., Baldwin, E. & Scarisbrick, R. (1948). *Biochem. J.* **43**, 308.
Peterson, M. H. & Johnson, M. J. (1948). *J. biol. Chem.* **174**, 775.
Ramsey, L. L. (1948). *J. Ass. off. agric. Chem., Wash.*, **31**, 164.
Ramsey, L. L. & Patterson, W. I. (1945). *J. Ass. off. agric. Chem., Wash.*, **28**, 644.
Ramsey, L. L. & Patterson, W. I. (1948). *J. Ass. off. agric. Chem., Wash.*, **31**, 139.
Smith, H. W. & White, T. A. (1929). *J. phys. Chem.* **33**, 1953.

Cholesterol Metabolism

2. CHOLESTEROL METABOLISM IN THE RAT

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The experiments to be described arose from an observation by Cook (1937) that when cholesterol in a fat-containing diet was fed to rats there was a loss of the sterol. Cook (1938) constructed a lipid and sterol balance sheet and showed that there was an apparent correlation between the amount of cholesterol metabolized and the increase of fatty acids excreted in the faeces. An acid was isolated from the faecal lipids, but it could not be stated definitely that it was formed from the breakdown of cholesterol. Polgar (1948) investigated the fatty acids present in the tubercle bacillus separating them by the fractional crystallization of their acetol esters ($R.CO_2.CH_2.CO.CH_3$) and treating the latter with reagents for ketones such as semicarbazide or 2:4-dinitrophenylsemicarbazide. It was decided to apply these methods to the study of the acids produced in the metabolism of cholesterol. Different types of fat were administered with the cholesterol to ascertain if these influenced its metabolism.

There is little exact information on the changes which cholesterol undergoes in the animal body and this work is part of a larger study of the metabolism of this compound in various animal species.

METHODS

Animals. Piebald male rats about 6 months old and of average weight 300 g. obtained from the Rowett Institute, Aberdeen, were used in all experiments. The animals were housed in a room maintained at a temperature of 22° ± 1° in metabolism cages fitted with Hopkins's separators, the urine and faeces being collected separately. The food was weighed daily, moistened and placed in containers, any food spilled being returned to the dish. The animals were allowed free access to water.