[12] P. HELE, G. POPJÁK AND M. LAURYSSENS, *Biochem J.*, 65 (1957) 348.
[13] R. L. REID AND M. LEDERER, *Biochem. J.*, 50 (1951) 60.
[14] H. KAUFMANN AND W. H. NITSCH, *Fette u. Seifen*, 56 (1954) 154.
[15] H. J. DEUEL, JR., *Lipids*, Vol. I, Interscience Pub., New York, 1951, p. 195.
[16] D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. (London)*, *B*, 124 (1938) 397.
[17] F. X. HAUSBERGER, S. W. MILSTEIN AND R. J. RUTMAN, *J. Biol. Chem.*, 208 (1954) 431.
[18] F. X. HAUSBERGER AND S. W. MILSTEIN, *J. Biol. Chem.*, 214 (1955) 483.

Biochim. Biophys. Acta, 40 (1960) 110–118

THE IDENTIFICATION AND QUANTITATIVE ESTIMATION OF ETHANOLAMINE AND SERINE IN LIPID HYDROLYSATES

W. L. MAGEE*, R. W. R. BAKER AND R. H. S. THOMPSON
Department of Chemical Pathology, Guy's Hospital Medical School, London (Great Britain)

(Received August 12th, 1959)

SUMMARY

A paper chromatographic method for the separation and identification of minute amounts (0.01–0.10 μmole) of ethanolamine and serine in lipid hydrolysates is described. The chromatograms are stained directly with a ninhydrin reagent and the resultant coloured spots cut out and extracted for quantitative estimation. The method has been applied to the analysis of several phospholipid preparations. The results are compared with those obtained by a dinitrofluorobenzene method.

INTRODUCTION

The present study was undertaken in an attempt to find a suitable method for the quantitative analysis of phosphatidylethanolamine and phosphatidylserine in phospholipid fractions. It was felt that determination of ethanolamine and serine in hydrolysates of lipids represented the most practical approach to the solution of this problem. Several such assay procedures have been described, and the field was reviewed recently by DITTMER, FEMINELLA AND HANAHAN[1], who found that many of the available methods were inadequate.

A previous paper chromatographic procedure[2] for the micro estimation of nitrogenous phosphatide constituents has been found to give good results with standard ethanolamine and serine solutions, but DITTMER[1] *et al.*[1] and MUNIER[3] have proved that it is unsatisfactory when applied to lipid hydrolysates. The chromatographic method described in this paper is both rapid and convenient, and is suitable for the determination of from 0.01 to 0.10 μmole of each compound in lipid hydrolysates.

* Postdoctoral Fellow of the National Multiple Sclerosis Society.

MATERIALS AND METHODS

Preparation of cephalin fractions from human brain

Samples corresponding to fractions III and V of human brain cephalin were prepared by the method of FOLCH[4]. Fraction III contained a large proportion of the phosphatidylserine present in the original cephalin preparation. Fraction V was rich in phosphatidylethanolamine. Both fractions, however, were mixtures of the two phosphatides and other lipid materials. The individual fractions were freed from non-lipid contaminants either by dialysis or by the washing procedure described by FOLCH, LEES AND SLOANE-STANLEY[5], and were emulsified with water, freeze-dried and stored *in vacuo* over P_2O_5.

Fraction V of human brain cephalin was further purified by chromatography on a column of silicic acid. The sub-fraction rich in ethanolamine obtained in this way was stored at —10° in chloroform solution.

Preparation of phosphatidylethanolamine fraction from ox heart

This was prepared by Dr. G. R. WEBSTER in this laboratory by fractionation of a lipid extract obtained from ox heart on a column of silicic acid.

Ethanolamine and serine standard solutions

Ethanolamine and d,l-serine were obtained from L. Light & Co. Ltd. The ethanolamine was redistilled, and the fraction boiling at 170–171° (760 mm) was used in all experiments. Stock solutions, 0.01 *M*, were made up in water and stored at 0°. Both reagents gave single ninhydrin-reactive spots on paper chromatograms.

Hydrolysis procedure

A phospholipid sample weighing from 15 to 40 mg was placed in a Pyrex test tube (15 × 125 mm) and 4 ml of 2 *N* H_2SO_4 added. The tube was sealed in a flame and then immersed in a boiling water-bath for 12 h. After cooling, the tube was opened and its contents transferred to a 12 ml Pyrex centrifuge tube. The test tube was rinsed with 1 ml water in two portions and the washes added to the original hydrolysate. 3 drops of a 0.1 % aqueous solution of methyl red were added. With frequent stirring, sufficient solid $BaCO_3$ was next added to neutralise the hydrolysate, as indicated by the cessation of CO_2 evolution and the change in indicator colour. An excess of $BaCO_3$ could be added without harmful effect. In a few samples excessive frothing occurred, but this could be prevented by the addition of a few drops of ethanol. The sample was centrifuged and the supernatant transferred to a 10 ml volumetric flask. The precipitate was washed twice with 2.5 ml portions of water, and the washes were added to the original supernatant. The volume of the hydrolysate was adjusted to 10 ml, and the samples were filtered. The clear filtrate was stored in the cold (0–4°).

With each set of phospholipid samples hydrolysed, a blank (4 ml 2 *N* H_2SO_4) and serine and ethanolamine standard solutions (20 or 40 μmoles of each in 4 ml 2 *N* H_2SO_4) were carried through all the above operations.

Paper chromatography

A 1 foot square of Whatman 3MM paper was divided into eight lanes 1.25 in. wide. 1-in. margins were left on each side of the sheet. Because of the high sensitivity

of the ninhydrin reagent, it was essential that the chromatogram be handled as little as possible, and then only on the margins, particularly before colour development. The solutions to be chromatographed were applied to the paper in the centre of each lane along a base line 1 in from the bottom of the sheet. The volume applied was usually between 20 and 60 μl. The blank and serine standard solutions were applied first, as these could be dried in a blast of hot air. The volatility of ethanolamine at elevated temperatures made hot air drying impossible in the samples containing the amino alcohol.

The paper square was sewn into cylindrical form before being placed in a chromatographic chamber, and was run by the ascending technique. The solvent system employed was methyl ethyl ketone–methyl cellosolve–20% acetic acid, 40:15:20 (v/v/v). The methyl cellosolve used in this solvent was purified before use by the method of EASTOE[7] and was stored in the cold in dark bottles. The running time with this solvent was from 2 to 4 h. The papers were finally dried in air at room temperature.

Quantitative estimation of serine and ethanolamine

A ninhydrin–$SnCl_2$ reagent was made up immediately before use by a modification of the method of CLARKSON AND KENCH[8]. This was prepared by dissolving 4 mg of $SnCl_2 \cdot 2H_2O$ (Hopkin & Williams Ltd., A.R.) in 5 ml freshly-prepared 0.2 *M* citrate buffer, pH 5.0. To this was added 0.25 g ninhydrin (Hopkin & Williams Ltd.). The mixture was shaken for a few min, made up to 50 ml with isopropanol and shaken mechanically for a further 60 min.

The chromatogram was dipped in the reagent, drained free of excess liquid and placed in an oven at 50° for 90 min. After colour development the ninhydrin spots, together with appropriate areas from the blank lanes, were outlined with pencil using a template, and cut out. Thus an equal area of paper was taken for each sample. The pieces, handled with forceps, were shredded with scissors and placed in glass-stoppered test tubes. To each, 5 ml of 50% aqueous ethanol (made with redistilled ethanol) was added, and the tubes were shaken gently for 30 min. The contents of the tubes were filtered and the optical density of the filtrate was measured in 1 cm cells at 570 mμ in a spectrophotometer against 50% aqueous ethanol.

RESULTS AND DISCUSSION

Fig. 1 shows a representative chromatogram for which the running time was 3 h. The base line and solvent front are indicated by solid lines. Lanes A and B contained 0.06 μmole of ethanolamine and serine respectively. An aliquot of an hydrolysate of a sample of Fraction III from human brain cephalin was applied to the chromatogram in lane C. The serine spot is clearly visible, but a very faint ethanolamine spot seen on the original chromatogram is not apparent in the reproduction. The amount of ethanolamine present was less than that which could be determined accurately. In order to assay the ethanolamine content of the sample, it was necessary to apply a three-fold volume of the hydrolysate to the paper (lane D). The ethanolamine spot (circled) is now readily seen, and the quantity of ethanolamine present was sufficient for an accurate determination. Although the concentration of serine in lane D is much greater than that usually assayed, the separation of the two compounds is

still distinct. An aliquot of an hydrolysate of fraction V from human brain cephalin was applied to the chromatogram in lane E. The hydrolysate contained approximately 10 times as much ethanolamine as serine. Both compounds were present in the single aliquot at concentrations suitable for analysis.

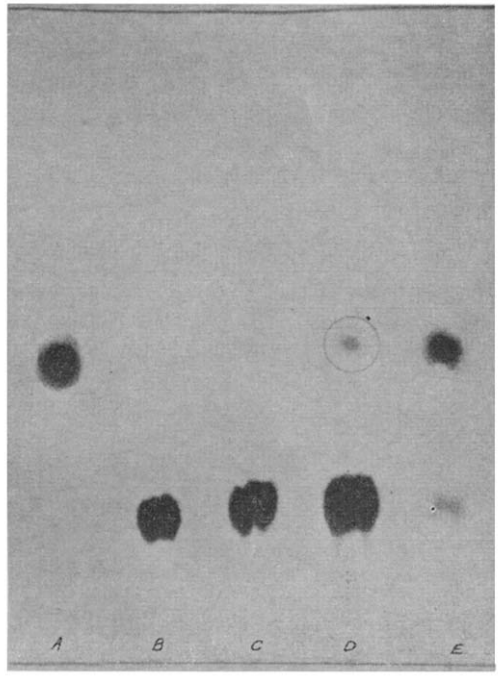

Fig. 1. A representative chromatogram. A, 0.06 μmole ethanolamine; B, 0.06 μmole serine; C and D, 20 and 60 μl respectively of an hydrolysate of fraction III from human brain cephalin; E, 20 μl of an hydrolysate of fraction V from human brain cephalin.

With the chromatographic system described the spots obtained are discrete, with little tailing. The short running time required for good separation is an advantage. The R_F values obtained for ethanolamine and serine were (mean $\pm$ S.E.M. for 26 consecutive chromatograms) 0.47 $\pm$ 0.006 and 0.22 $\pm$ 0.003 respectively.

As was reported by DITTMER *et al.*[1] for the solvent systems described by LEVINE AND CHARGAFF[2], it was found in this investigation that traces of salts or hydrochloric acid in the hydrolysates resulted in poor separation. As a consequence, the hydrolysis procedure outlined above using dilute sulphuric acid followed by neutralization with solid $BaCO_3$ was devised.

Although an appreciable loss of both ethanolamine and serine occurred during the hydrolysis procedure and the subsequent experimental manipulations, this loss was quite reproducible. It was compensated for by the fact that the standard solutions were carried through the same procedure.

In Table I the colour yields of ethanolamine and serine standards run on five separate chromatograms are recorded. The colour yield of ethanolamine on a molar basis is less than half that obtained with serine. The relationship between optical density and concentration is not perfectly linear, especially for ethanolamine. Thus

it is necessary to run an appropriate set of standards for each determination. This can be done conveniently on a single chromatogram.

As can be seen from Table I, the optical densities of the standards varied considerably from one chromatogram to another. However, the colour yield of the individual compounds in both the standard solutions and the lipid hydrolysates varied correspondingly on the same chromatogram. Thus the calculated concentration of each compound in the lipid hydrolysates was considerably more consistent from one determination to the next than the figures in Table I would suggest. It is, of course, essential to apply both standards and unknowns to the same chromatogram.

TABLE I

COLOUR YIELD OF ETHANOLAMINE AND SERINE STANDARDS

(Optical density/cm at 570 mμ)

Expt. No.	*Ethanolamine (μmole)*			*Serine (μmole)*		
	0.02	*0.06*	*0.10*	*0.02*	*0.06*	*0.10*
1	0.037	0.092	0.133	0.086	0.217	0.356
2	0.035	0.082	0.119	0.068	0.193	0.319
3	0.032	0.083	0.125	0.066	0.192	0.310
4	0.031	0.069	0.118	0.070	0.192	0.317
5	0.031	0.087	0.140	0.070	0.213	0.332
Mean	0.033	0.083	0.127	0.072	0.201	0.327

It is possible to increase considerably the intensity of the ninhydrin spots on the chromatograms by increasing the temperature during colour development. With higher temperatures, however, the results were less reproducible, particularly for ethanolamine, where a marked fall in colour yield at higher concentrations was observed. Higher temperatures during colour development also resulted in very high blank readings. Using the conditions described above, blank readings for both ethanolamine and serine were of the order of 0.010 optical density unit.

In Table II the ethanolamine and serine concentrations of several phospholipid fractions are shown. The values obtained with the ninhydrin procedure described above are compared with those obtained on the same hydrolysates by means of a dinitrofluorobenzene (DNFB) method[9]. The reproducibility of the chromatographic method is indicated by the triplicate analyses on purified fraction V from human brain.

The concentrations of serine as determined by the chromatographic method were consistently lower than those obtained by the DNFB method. This is particularly apparent in the hydrolysates containing very little serine and considerable ethanolamine. We suggest that this is due to the fact that any phosphoethanolamine formed during hydrolysis would be determined as serine by the DNFB method[1]. With the chromatographic conditions used here, phosphoethanolamine has an R_F of 0.14. The colour yield of phosphoethanolamine with ninhydrin is low[10], particularly on the chromatograms. Thus this compound was not detected in the lipid hydrolysates with the ninhydrin stain. It is probably present in the hydrolysates in low and variable concentrations and could perhaps be estimated on the chromatograms by phosphorus analysis, although this was not attempted.

Choline does not react with the ninhydrin reagent. The presence of choline in the hydrolysates did not interfere with the separation of ethanolamine and serine unless it was present on the chromatograms at very high concentration (0.5 μmole or greater); it then appeared to impede the movement of the ethanolamine spot.

It is felt that a chromatographic separation of ethanolamine and serine before analysis considerably reduces the possibility of interference in their estimation by other ninhydrin-reactive substances likely to be present in lipid hydrolysates.

TABLE II

ANALYSIS OF PHOSPHOLIPID FRACTIONS

Phospholipid	*Sample No.*	*Method of analysis*	*Composition*	
			Ethanolamine %	*Serine* %
Crude fraction III (human brain)	1	DNFB	0.30	7.42
		Ninhydrin	0.47	6.94
	2	DNFB	0.29	7.94
		Ninhydrin	0.35	7.48
Purified fraction V (human brain)	1	DNFB	7.90	0.80
		Ninhydrin a	7.80	0.65
		Ninhydrin b	7.85	0.50
		Ninhydrin c	7.70	0.51
"Phosphatidylethanolamine" (ox heart)	1	DNFB	6.30	0.69
		Ninhydrin	6.34	0.25

ACKNOWLEDGEMENTS

We wish to thank Dr. G. R. WEBSTER for his gifts of the ox heart phosphatidylethanolamine fraction and of a sample of phosphoethanolamine, and for his many helpful suggestions. We wish also to thank the Medical Research Council for a grant towards the expenses of this work.

REFERENCES

1 J. C. DITTMER, J. L. FEMINELLA AND D. J. HANAHAN, *J. Biol. Chem.*, 233 (1958) 862.
2 C. LEVINE AND E. CHARGAFF, *J. Biol. Chem.*, 192 (1951) 465.
3 R. MUNIER, *Bull. soc. chim. biol.*, 33 (1951) 862.
4 J. FOLCH, *J. Biol. Chem.*, 146 (1942) 35.
5 J. FOLCH, M. LEES AND D. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
6 D. J. HANAHAN, J. C. DITTMER AND E. WARASHINA, *J. Biol. Chem.*, 228 (1957) 685.
7 J. E. EASTOE, *Biochem. J.*, 61 (1955) 601.
8 T. W. CLARKSON AND J. E. KENCH, *Biochem. J.*, 62 (1956) 361.
9 J. AXELROD, J. REICHENTHAL AND B. B. BRODIE, *J. Biol. Chem.*, 204 (1953) 903.
10 C. E. DENT, *Biochem. J.*, 43 (1948) 169.