Chromatographic Separation of Brain Lipids

2. ETHANOLAMINE-CONTAINING PHOSPHOLIPIDS*

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There have been several previous reports on the chromatographic behaviour of the ethanolamine-containing phospholipids on columns of alumina (Lea, Rhodes & Stoll, 1955; Rhodes & Lea, 1957) and silicic acid (Hanahan, Dittmer & Warashina, 1957; Rhodes & Lea, 1957; Macfarlane, Gray & Wheeldon, 1960). However, the lipid compositions of the two sources of ethanolamine-based phospholipid which have been most extensively studied, namely liver and egg yolk, present a relatively less complicated problem than brain tissue does.

It has been our experience with brain-lipid extracts that ethanolamine-containing phospholipids are not readily separated from phosphatidylserine, cerebroside and sulphatide on silicic acid columns; on alumina columns the elution patterns of ethanolamine-based phospholipids and sulphatide overlap. Egg yolk appears to be free from cerebroside and nearly free from phosphatidylserine; there have been no reports on its sulphatide content, if any. Liver seems to be the only animal tissue that is virtually free from cerebroside (Williams *et al.* 1945), although it does contain phosphatidylserine and there is some evidence for the presence of sulphatide (Green & Robinson, 1960).

In an earlier paper (Long & Staples, 1961) in which a brain-lipid extract was used, we reported the separation of cerebroside from aminophospholipid by gradient-elution chromatography on an alumina column. Continued gradient elution has now been found to produce a peak containing the whole of the ethanolamine-based phospholipid, completely free from phosphatidylserine, which latter remained firmly adsorbed on the alumina under these conditions. The eluted ethanolamine-containing lipid was, however, contaminated with sulphatide. Rechromatography of the ethanolamine-containing fractions on silicic acid gave a peak containing only a mixture of phosphatidylethanolamine and ethanolamine plasmalogen. A further fraction containing lysophosphatidylethanolamine and sulphatide could later be eluted.

A preliminary report of some of this work has already been published (Long & Staples, 1960).

* Part 1: Long & Staples (1961).

EXPERIMENTAL

Purification of solvents, specifications of silicic acid and of alumina (type *B*), preparation of rat-brain-lipid extracts, general chromatographic procedures and analytical methods for galactose, sulphate, amino nitrogen, total nitrogen and total phosphorus were as described by Long & Staples (1959, 1961). Acyl ester was determined by the hydroxamate procedure of Shapiro (1953), by using purified ovolecithin (Rhodes & Lea, 1957) as a standard, with the assumption of an acyl ester:phosphorus ratio of 2·0:1; the fatty acid:phosphorus ratio of the ovolecithin had the theoretical value of 2·0:1.

Additional analytical methods

Long-chain aldehydes. A sample of lipid, containing not more than the equivalent of 0·08 μmole of aldehyde, was decomposed with 90% (v/v) acetic acid and treated with Schiff's reagent. The procedure used was a modification of the method of Leupold & Büttner (1953), devised by Dr G. H. Sloane-Stanley of this Department (personal communication).

Potassium. The lipid sample containing not more than 0·15 μg.atom of potassium, was evaporated to dryness in a Pyrex-glass test tube and then ashed at 170° for 2 hr. with 0·1 ml. of 72% $HClO_4$. After dilution with water to 3 ml., the potassium content was determined with a flame photometer (Evans Electroselenium Ltd.) with a standard (0·15 μg.atom of K^+) and a blank subjected to the same procedure.

Ethanolamine and serine. The principle of Axelrod, Reichenthal & Brodie (1953) was used with several modifications.

The lipid sample, containing not more than 0·4 μmole of ethanolamine or 0·5 μmole of serine, was evaporated to dryness in a glass-stoppered 10 ml. centrifuge tube, and 0·5 ml. of 6N-HCl was added. The stoppered tube was heated on a boiling-water bath for 2 hr., cooled and treated with 0·8 ml. of methanol and 1·6 ml. of $CHCl_3$. The contents were mixed thoroughly with a Pasteur pipette and the lower $CHCl_3$-rich phase, containing fatty acids, fatty aldehydes, sphingosine and cholesterol, was withdrawn and discarded. The aqueous methanolic phase was washed twice in the same way with 1 ml. portions of $CHCl_3$–methanol (17:3, v/v). The final aqueous methanolic phase was then evaporated to dryness in a current of air or N_2 on a warm-water bath.

The dry residue was dissolved in water (1·2 ml.) and treated with 0·3 ml. of aqueous 5% (w/v) $NaHCO_3$ and 0·1 ml. of a 5% (v/v) solution of 1-fluoro-2:4-dinitrobenzene in ethanol. The tube was heated on a water bath

at 75–80° for 75 min. After cooling, 4 ml. of $CHCl_3$ was added to the mixture. The tube was then vigorously shaken and centrifuged. A 1 ml. sample of the upper aqueous phase was taken for the determination of serine, and a 3 ml. sample of lower phase for ethanolamine.

The $CHCl_3$ phase (3 ml.), containing dinitrophenylethanolamine, was evaporated to dryness in a current of air. The residue was dissolved in 4 ml. of 6N-HCl and extracted once with 5 ml. of light petroleum (b.p. 40–60°), the upper phase being discarded. The extinction of the strongly acidic lower phase was measured at 420 mμ in a Unicam SP. 1400 prism absorptiometer, and compared with a standard (0·4 μmole of ethanolamine) and a blank subjected to the same procedure.

The aqueous phase (1 ml.), containing dinitrophenylserine, was acidified with 0·5 ml. of N-HCl, and shaken with 5 ml. of *iso*butyl methyl ketone. After centrifuging, 4 ml. of the upper phase was transferred to another 10 ml. glass-stoppered centrifuge tube, to which was then added 4 ml. of 0·1N-NaOH. The tube was shaken vigorously and centrifuged; 3 ml. of the lower alkaline phase was removed and treated with 0·5 ml. of 2N-HCl. The extinction of this solution was also read at 420 mμ, and compared with a standard (0·5 μmole of serine) and a blank subjected to the same procedure.

RESULTS

Chromatography of rat-brain lipids on alumina

The washed protein-free lipid from three rat brains (about 5 g. fresh wt.), dissolved in 25 ml. of chloroform–methanol (50:50, v/v), was applied to a column of alumina (13 g.; 17 cm. × 1 cm. diam.; type *B*, Long & Staples, 1961), equilibrated with the same solvent mixture. The column was run with chloroform–methanol (50:50, v/v) and three 25 ml. fractions were collected, in which were present the cholesterol and choline-containing phospholipids. Gradient elution was then begun. The mixing vessel contained 125 ml. of chloroform–methanol (50:50, v/v), and 150 ml. of chloroform–methanol–water (45:45:10, by vol.) was placed in the reservoir. Eleven 25 ml. fractions were collected. The water content of the eluting solvent rose from 0 to 7 % (by vol.), and the fractions contained all the cerebroside and most of the sulphatide present in the lipid sample. The first traces of amino nitrogen appeared in fraction 12 (Long & Staples, 1961). A second gradient was then applied. The mixing vessel contained 125 ml. of chloroform–methanol–water (46·5:46·5:7, by vol.), and 200 ml. of chloroform–methanol–water (43:43:14, by vol.) was placed in the reservoir. Nine more 25 ml. fractions were collected, the water content of the eluting solvent increasing from 7 to 12·5 % (by vol.).

Each fraction was analysed for amino nitrogen, total nitrogen, ethanolamine, serine, total phosphorus, acyl ester, aldehyde, galactose and sulphate. Some of the analytical values for fractions 9–23 in a typical experiment are shown in Fig. 1. Amino-nitrogen analyses (not shown), by the ninhydrin method (Moore & Stein, 1948), were always made first, and gave an indication of the amounts of aminophospholipid present in the fractions. These values served as a guide for calculating the volume of each fraction to be taken for the exact determination of the other constituents. In Fig. 1 the discontinuous curve on the left shows the trailing edge of the elution of cerebroside, details of which are to be found in Fig. 4 of Long & Staples (1961).

In the individual fractions comprising the aminophospholipid elution curve (fractions 12–23), close agreement was found for the contents of ethanolamine, total phosphorus and total nitrogen, except in the very early fractions where the total-nitrogen values were high because of the simultaneous elution of sulphatide. When allowance was made for the nitrogen content of the sulphatide (calculated from the sulphate determinations), summation of the individual analytical values for fractions 12–23 gave the following data: total phosphorus, 106·1 μg.atoms; total nitrogen, 103·9 μg.-atoms; ethanolamine, 101·3 μmoles. The crude lipid applied to the column contained 116 μmoles of ethanolamine (i.e. 26·3 μmoles/g. of fresh brain), indicating a recovery of 88 %. In three similar experiments, the ethanolamine recoveries were 89, 99 and 100 %. In no case has any serine been found

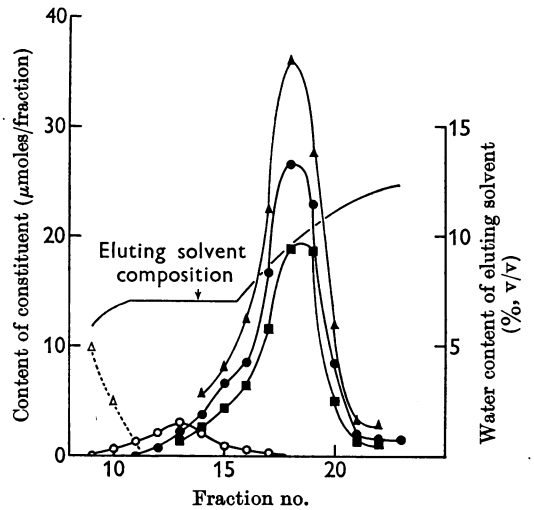

Fig. 1. Part of the elution curve of rat-brain lipid applied to an alumina column (13 g.; 17 cm. × 1 cm. diam.), with gradient elution whereby the water content of the chloroform–methanol (50:50, v/v) solvent was increased from 0 to 12·5 % (by vol.). The lipid was derived from 4·46 g. of rat brain (moist wt.). Volume of each fraction, 25 ml. △, Galactose; ○, sulphate; ●, ethanolamine; ■, aldehyde; ▲, acyl ester.

in these fractions, although in the experiment recorded in Fig. 1 the serine content of the lipid applied to the column was 35·2 μmoles (i.e. 7·9 μmoles/g. of fresh brain). This observation accords with the equivalence of total nitrogen and ethanolamine contents.

The acyl ester:phosphorus ratio for fractions 12–23 averaged 1·22:1, and the mean aldehyde:phosphorus ratio was 0·68:1. However, there were variations between experiments, aldehyde:phosphorus ratios from 0·48:1 to 0·70:1 having been obtained. For a mixture of phosphatidylethanolamine and ethanolamine plasmalogen, the theoretical value for the ratio (aldehyde+acyl ester):phosphorus is 2·0:1. In the experiment shown in Fig. 1, the observed average value for this ratio was 1·90:1, indicating the presence of lyso compounds. In the early fractions, however, it sometimes exceeded 2·0:1, suggesting the presence of some additional acyl ester. It may therefore be concluded that, although the bulk of the material eluted in the aminophospholipid peak consists of a mixture of phosphatidylethanolamine and ethanolamine plasmalogen, the fractions also contain some sulphatide and ethanolamine lysophospholipid as well as some 'excess ester'.

Chromatography on silicic acid of the rat-brain ethanolamine-containing lipids eluted from an alumina column

Silicic acid chromatography of the ethanolamine-containing fractions from the alumina column, by means of gradient elution with increasing content of methanol in chloroform, seemed likely to separate the 'excess ester' and lysophosphatidylethanolamine as fast- and slow-running fractions respectively from the mixture of phosphatidylethanolamine and ethanolamine plasmalogen, but it appeared probable that the separation of sulphatide from the latter two substances might present a more difficult problem. We have already reported (Long & Staples, 1961) that, on silicic acid, sulphatide is somewhat less readily eluted than are the ethanolamine-based phospholipids. However, it was first necessary to determine whether the sulphatide should be applied to the silicic acid column in the form of the free acid or as the potassium salt in order to effect the better separation. To test this point, those fractions eluted from an alumina column that contained ethanolamine-based lipid together with the bulk of the sulphatide, the latter in the free acid form (Long & Staples, 1961), were combined and evaporated to dryness. The residue was dissolved in chloroform–methanol (98:2, v/v) and divided into two equal portions.

One sample, which was untreated and in which the sulphatide was present as the free acid, was chromatographed directly on silicic acid (8 g.; 17 cm. × 1 cm. diam.) by gradient elution, whereby the content of methanol in the chloroform was raised from 2 to 14% (by vol.). The result of this experiment is shown in Fig. 2 (*a*).

The other portion was treated with methanol (0·5 vol.) and 0·1 M-potassium chloride (0·3 vol.). The mixture was shaken and centrifuged, and the upper aqueous methanolic phase was discarded. The lower phase was washed twice with an equal volume of methanol–0·1 M-potassium chloride–chloroform (48:47:3, by vol.). In this way the sulphatide was converted into its potassium salt. Finally the chloroform-rich lower phase was evaporated to dryness, and the residue was dissolved in chloroform–methanol (98:2, v/v) and chromatographed on silicic acid, with the same conditions as described for the untreated portion. The result of this experiment is shown in Fig. 2 (*b*).

Figs. 2 (*a*) and 2 (*b*) show that the elution pattern of the ethanolamine-based lipids is practically the same in both cases, as would be expected. However, the elution of sulphatide was more difficult to

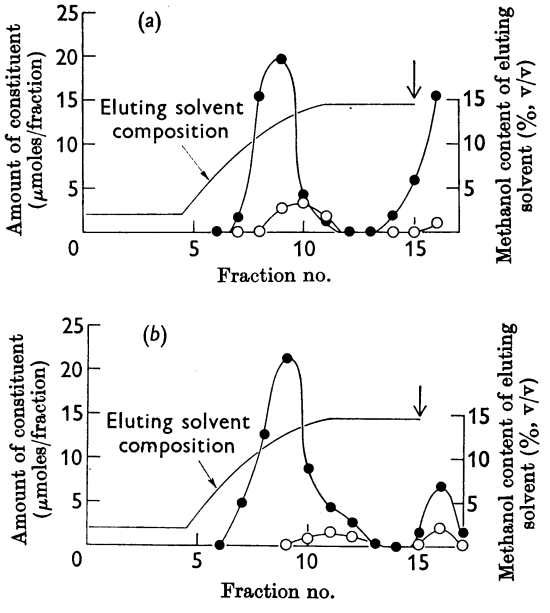

Fig. 2. Silicic acid chromatography of lipid material obtained from fractions 12–23 eluted from an alumina column of the type shown in Fig. 1. Gradient elution was used whereby the methanol content of the chloroform–methanol solvent was increased from 2 to 14% (by vol.). At arrow (fraction 15), the solvent was changed to methanol–chloroform–water (70:25:5, by vol.). Dimensions of silicic acid column: 8 g.; 17 cm. × 1 cm. diam. Volume of each fraction, 25 ml. ●, Ethanolamine; ○, sulphate. The sulphatide component was applied as (*a*) free acid, (*b*) potassium salt.

achieve when the latter was applied to the column as the potassium salt (Fig. 2*b*). In both cases the main sulphatide peak eluted during the application of the gradient was free from potassium. When the solvent was changed after fraction 15 to methanol–chloroform–water (70:25:5, by vol.), a small additional amount of sulphatide was eluted in each case, together with the ethanolamine-containing lysophospholipid. In the experiment of Fig. 2 (*b*), potassium was also eluted in this fraction, and was equivalent in amount to the total quantity of sulphatide applied to the column.

In view of these results, it appeared probable that sulphatide, applied as the potassium salt, would not be eluted from a silicic acid column by a solvent containing up to 9% (v/v) of methanol in chloroform, whereas the phosphatidylethanolamine and ethanolamine plasmalogen would be eluted completely under these conditions. Accordingly, a mixture of ethanolamine-containing lipid and sulphatide, obtained from an alumina column, was washed with aqueous potassium chloride as described for the experiment of Fig. 2 (*b*). The lipid was dissolved in chloroform–methanol (98:2, v/v) and applied to a silicic acid column. After elution of the 'excess ester' in two 25 ml. fractions with chloroform–methanol (98:2, v/v), gradient elution was applied in which the mixing vessel contained 125 ml. of chloroform–methanol (98:2, v/v), and 150 ml. of chloroform–methanol (88:12, v/v) were placed in the reservoir. In this way the methanol content of the eluting solvent was raised from 2 to 9% (by vol.), and after this solvent had passed through the column a further quantity of chloroform–methanol (91:9, v/v) was run in, until 14 more 25 ml. fractions had been collected. An ethanolamine-containing peak was eluted (Fig. 3). This peak was free from sulphatide, and contained in fractions 9–14 the following amounts of constituents: ethanolamine, 37·9 μmoles; total phosphorus, 40·4 μg.atoms; total nitrogen, 36·4 μg.-atoms; ester, 53·1 μmoles; aldehyde, 28·5 μmoles; indicating a mixture of phosphatidylethanolamine and ethanolamine plasmalogen in the ratio of about 30:70. Further, analyses of the individual fractions showed that the composition of the eluted material was constant. In fractions 15 and 16, some ethanolamine-containing lysophospholipid was eluted, as judged from the (acyl ester + aldehyde): phosphorus ratio of 1·02:1.

When the solvent composition was changed after fraction 16 to chloroform–methanol (80:20, v/v), 7·06 μmoles of sulphate were eluted in the next seven fractions, out of 7·87 μmoles applied to the column. This material was accompanied by the remainder of the ethanolamine-containing lysophospholipid (ethanolamine, 6·64 μmoles; total phosphorus, 6·83 μg.atoms; ester, 6·00 μmoles; aldehyde, 1·51 μmoles). Out of 48·4 μmoles of ethanolamine applied to the silicic acid column, 46·4 μmoles were recovered, i.e. 96%.

Stability of ethanolamine-containing phospholipids on an alumina column

In early experiments with alumina columns, the usual practice was to elute the cerebroside on the first day and the ethanolamine-containing lipids on the following day. Under these conditions, the first fraction collected on the second day consistently showed an abnormally high content of acyl ester (acyl ester:phosphorus ratio, approx. 6:1), although the contents of total phosphorus, total nitrogen, ethanolamine and aldehyde were as expected. This suggested that some type of lipid decomposition had occurred during storage for 17 hr. on the alumina column. In order to test the stability of the ethanolamine-containing lipids under these conditions, a sample of the mixture of phosphatidylethanolamine and ethanolamine plasmalogen, prepared as described in the last section, and containing 65 μmoles of ester and 31·8 μmoles of aldehyde, was dissolved in chloroform–methanol (50:50, v/v) and applied to an alumina column (13 g.). At intervals during 6 weeks, the column was eluted with chloroform–methanol (50:50, v/v)

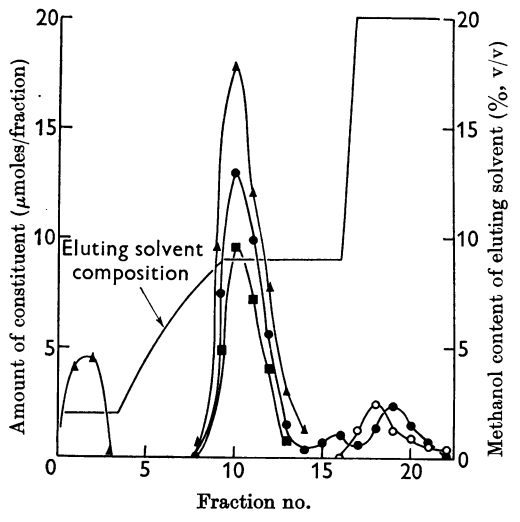

Fig. 3. Silicic acid chromatography of lipid material obtained from fractions 12–23 eluted from an alumina column of the type shown in Fig. 1. The sulphatide component was applied as the potassium salt. Gradient elution was used whereby the methanol content of the chloroform–methanol solvent was increased from 2 to 9% (by vol.). After fraction 16, the solvent was changed to chloroform–methanol (80:20, v/v). Dimensions of silicic acid column: 8 g.; 17 cm. × 1 cm. diam. Volume of each fraction, 25 ml. ▲, Acyl ester; ●, ethanolamine; ■, aldehyde; ○, sulphate.

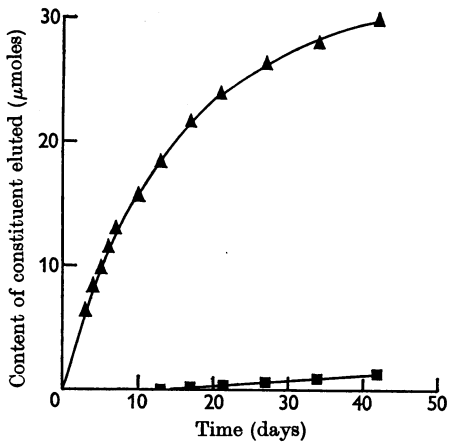

Fig. 4. Decomposition of a mixture of purified phosphatidylethanolamine and ethanolamine plasmalogen on a column of alumina (13 g.; 17 cm. × 1 cm. diam.). The total content of constituent (ordinate) is calculated by summation of the individual quantities eluted on the days of analysis (abscissa). Lipid applied to column contained 65 μmoles of acyl ester and 31·8 μmoles of aldehyde. ▲, Acyl ester; ■, aldehyde.

and a 25 ml. fraction was collected; the 'hold-up volume' of the column was 8 ml. When these fractions were analysed, no phosphorus was found, but acyl ester was present in measurable quantity in all cases. After about 2 weeks a trace of aldehyde was also eluted. The results are shown in Fig. 4.

Subsequent elution of the column with increasing content of water from zero up to 14% (by vol.) in the chloroform–methanol (50:50, v/v) solvent led to the very slow elution of ethanolamine-containing lipid which was not further characterized.

It will be seen that, although the ethanolamine-containing lipids do decompose on an alumina column, the rate is quite low.

DISCUSSION

In the work described, no attempt has been made to separate phosphatidylethanolamine from ethanolamine plasmalogen. The possibility of effecting this separation chromatographically seems rather remote, since the difference in properties due to the replacement of one acyl group by an αβ-unsaturated ether group would probably be no greater than that due to differences in chain length or degree of unsaturation of those component fatty acids which are present in the phosphatidylethanolamine. The ethanolamine-containing lipids isolated from rat brain in several individual experiments have shown aldehyde:phosphorus ratios varying from 0·48:1 to 0·70:1; the ester:phosphorus ratios varied in an inverse manner.

The isolation of the ethanolamine-containing phospholipids from the lipid mixture present in brain falls into two parts, namely separation from (*a*) cerebroside and phosphatidylserine, and (*b*) sulphatide. On an alumina column, gradient elution with increasing content of water in chloroform–methanol (50:50, v/v) has been found effective in achieving the separation from cerebroside (see also Long & Staples, 1961). The presence of phosphatidylserine unexpectedly presented no difficulty, since this substance is very firmly bound to alumina (Long, Shapiro & Staples, 1960) and is not easily extracted from it. The ethanolamine-containing phospholipid eluted from an alumina column is contaminated with sulphatide and also with small amounts of 'excess ester' produced by lipid decomposition (see below) as well as lyso compounds.

Separation of the mixture of phosphatidylethanolamine and ethanolamine plasmalogen from the aforementioned contaminants was achieved on a silicic acid column, since the 'excess ester' was readily eluted with chloroform–methanol (98:2, v/v), while the sulphatide and lysophosphatidylethanolamine were rather strongly retained.

The source of the 'excess ester' was at first attributed to the breakdown of some of the ethanolamine-containing lipid on the alumina column. However, a later experiment with purified material (Fig. 4) showed that the ethanolamine-containing lipids were fairly stable under these conditions unless there was very prolonged contact. We believe that the nature of the decomposition is most simply expressed by the following transesterification:

Phosphatidylethanolamine + methanol →
lysophosphatidylethanolamine + methyl ester.

This conclusion is largely based on the behaviour of phosphatidylserine (Long *et al.* 1960, and unpublished observations), which is extremely labile in contact with alumina.

It may be calculated from Fig. 4 that in 2-day experiments the quantity of 'excess ester' produced by breakdown of the ethanolamine-based phospholipids would not exceed 3·3% of the amount present in these lipids, whereas in a 1-day experiment it would amount to about 1%. The average values observed for 'excess ester' were 38·8 and 16·7% respectively. These results are consistent with the likelihood that the 'excess ester' is in fact largely derived from the decomposition of phosphatidylserine.

Since the decomposition scheme for the ethanolamine-containing phospholipids referred to in the last paragraph probably takes place to a very limited extent only under the chromatographic conditions employed in 1-day experiments on an alumina column, this mechanism cannot be held

responsible for the appreciable quantity of lysophosphatidylethanolamine found to be present. Although it is possible that some breakdown of phosphatidylethanolamine occurs during the process of evaporating the fractions from the alumina column in preparation for silicic acid chromatography, this seems unlikely to be extensive. It is probable therefore that lysophosphatidylethanolamine is a normal constituent of rat brain, and is not an artifact produced by experimental manipulations.

SUMMARY

1. Chromatography of mixed rat-brain lipids on an alumina column by a gradient method, in which the water content of a chloroform–methanol solvent was raised from 7 to 12·5% (by vol.), resulted in the elution of a peak containing all the ethanolamine-based phospholipids.

2. The main components were phosphatidylethanolamine and ethanolamine plasmalogen, but smaller quantities of sulphatide, lysophosphatidylethanolamine and 'excess ester' were also present.

3. Chromatography of this partially purified material on silicic acid, whereby the methanol content of a chloroform–methanol solvent system was gradually raised from 2 to 9% (by vol.), produced a satisfactory separation into the following fractions: (*a*) 'excess ester'; (*b*) a mixture of phosphatidylethanolamine and ethanolamine plasmalogen; (*c*) a mixture of sulphatide and lysophosphatidylethanolamine.

4. Phosphatidylethanolamine and ethanolamine plasmalogen have been found to be moderately stable when in contact with alumina.

We wish to express our thanks to Dr G. H. Sloane-Stanley for making available his unpublished procedure for aldehyde determination, and to Dr G. C. R. Morris for help with the potassium determinations.

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The Effect of Staphylococcal Leucocidin on the Leucocyte

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Staphylococcal leucocidin consists of two proteins that act synergistically to kill the leucocytes of humans and rabbits (Woodin, 1960). Observations on the cytotoxicity of leucocidin have been restricted to the use of simple viability tests such as the ability of leucocidin to inhibit respiration or produce morphological changes in susceptible cells. The present paper describes some biochemical changes in the leucocyte treated with leucocidin.

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EXPERIMENTAL

Materials

Leucocytes. These were obtained from peritoneal exudates 5–10 hr. after injection of 0·85% NaCl. The exudate, to which heparin (10 mg./1000 ml.) was added, was filtered through surgical gauze and centrifuged at 200*g* for 5 min. The cells were then washed three times by centrifuging at 200*g* for 5 min. in Hanks medium or a Krebs–Ringer solution (see below) and suspended in one of these media, and a sample was taken for counting. The data in this paper, unless otherwise stated, refer to rabbit leucocytes.