The Application of Counter-Current Distribution to the Separation of Phospholipins

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Counter-current distribution between two immiscible solvent phases, as first developed by Craig (1944), offers advantages over both direct and partition chromatography for analytical and preparative work in the field of phospholipins (Lovern, 1949). The only recorded use of the method for such purposes appears to be in a series of papers from one laboratory and deals with vegetable phosphatides (Scholfield, Dutton, Tanner & Cowan, 1948; Scholfield, McGuire & Dutton, 1950; McGuire & Earle, 1951). These workers obtained varying degrees of separation of constituents by distribution between hexane and aqueous methanol or ethanol in a metal apparatus of essentially the original Craig pattern. Various developments in design of apparatus (Lochte & Meyer, 1950; Craig, Hausmann, Ahrens & Harfenist, 1951; Lathe & Ruthven, 1951) have considerably extended the scope of the method, and recently the similar 'cascade distribution' (Kies & Davis, 1951) offers an alternative technique.

The author has studied the applicability of the counter-current technique to the separation of the constituents of the crude glycerophosphatide fraction of the lipids of ox brain, using light petroleum (b.p. 40–60°) and aqueous ethanol as solvents. Preliminary tests, both in a metal apparatus of the original Craig pattern and in a series of separating funnels, suggested that phospholipins are likely to give trouble with apparatus in which all equilibrations and transfers are performed by one operation. They appear to have a considerable effect on the solubility of one solvent in the other. Thus, although the two phases are saturated with each other at the start of the experiment, the relative volumes of the two phases alter appreciably as fractionation proceeds along a series of vessels. This could lead to trouble with mixed phase transfers in an automatic apparatus. Moreover, at certain stages of the separation—usually rather late stages—it occasionally happens that the contents of one vessel will form a very stable emulsion whereas all the rest separate rapidly. This may occur only once or twice in the course of hundreds of transfers, and it can easily be dealt with in a procedure based on individual transfers, but would pass unnoticed in an automatic apparatus. For these reasons, in spite of the great expenditure of time required, the use of a series of

conical flasks has been adopted. They permit the use of any desired volumes and are thus ideal for preparative work. Separating funnels are more easily manipulated, but involve the danger of contamination with stopcock grease. Beroza (1951) prefers bottles to funnels for the same reason.

EXPERIMENTAL

The main experiment was carried out with the total ether-soluble fraction of the crude phospholipins from ox brain. It would, therefore, contain all those lipids based on glycerophosphoric acid (except part of the fully saturated ones, e.g. dipalmityl lecithin), but should be almost free from sphingolipins. Subsidiary trials, for comparative purposes, were made with smaller amounts (about 7 g. each) of crude preparations of (a) phosphatidyl choline, (b) phosphatidyl ethanolamine and (c) phosphatidyl serine. Preparation (a) contained much (b), (b) contained minor amounts of (a), and (c) contained a little (b). Preparations (b) and (c) were made by the method of Folch (1949) from the 'kephalin' of mixed ox brain and spinal cord lipids. All products contained plasmalogen, a constituent which seems to have been ignored by Folch (cf. also Klenk & Böhm, 1951). They were free from acetone-soluble lipids but had not been purified by dialysis.

Ox-brain glycerophosphatide (120 g.) was dissolved in 300 ml. of light petroleum saturated with 85% (v/v) ethanol in the first of a series of twenty conical flasks, the other flasks likewise containing 300 ml. of light petroleum saturated with 85% ethanol. Batches of 300 ml. of 85% ethanol saturated with light petroleum were passed in succession through the train of flasks, with thorough shaking at each stage. Transfer from flask to flask was effected by suction. The successive ethanol extracts were removed as a series of fractions from the end of the series, the process corresponding roughly to elution chromatography. The operation was continued until the weight of lipid in each fraction was very small and only a small proportion of the total lipid remained in the petroleum phase. This gave 62 eluted fractions accounting in all for 93.3% of the total material. The contents of each petroleum fraction were separately recovered, giving a further 20 fractions containing the remaining 6.7% of the original lipid.

The supplementary trials with crude phosphatidyl serine and crude phosphatidyl ethanolamine were run in the same manner, using 100 ml. batches of each solvent phase, in a series of twenty conical flasks, except that only 20 ethanol fractions were run through the train in the former case and, inadvertently, 19 instead of 20 fractions in the latter. The eluted material amounted to 83.3% of the total for phosphatidyl serine and 69% for phosphatidyl ethanol-

amine. The crude phosphatidyl choline was treated differently. It was partitioned between batches of 100 ml. each of light petroleum and 90% (v/v) ethanol in a series of sixteen flasks, but no fractions were run off at the end of the series. Instead, the ethanol phases were allowed to remain successively one flask nearer the first in the series, until all flasks contained both phases. Then the entire contents of each flask were recovered as a single fraction. This corresponds to development of a chromatogram until the solute just reaches the bottom of the column, followed by cutting of the column into sections for extraction.

Grünberg (1938) which, although less reliable than that of Ehrlich, Taylor & Waelsch (1948), involves a simpler technique adequate for observing the progress of separation among a large number of fractions. CHCl_3 was used instead of amyl alcohol as the extractant, since it gives much lower blank values. Comparison was against a standard of pure palmitaldehyde, used as such and not as its glyceryl acetal. Palmitaldehyde was kindly supplied by Mr G. I. Gregory, of the Department of Organic Chemistry, University of Bristol. Phosphorus was determined on certain fractions, using Allen's (1940) method.

Fig. 1. Ethanol phase in the distribution of crude ox-brain glycerophosphatides between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

All fractions were examined for choline, ethanolamine, serine and plasmals (higher fatty aldehydes). Inositol was not determined. Paper chromatography (Chargaff, Levine & Green, 1948; Brante, 1948) was used for qualitative examination of the bases present. Choline was determined after refluxing for 2 hr. with ethanolic 0.5 N-KOH, followed by addition of water, acidification with HCl, removal of fatty acids with ether and evaporation of the aqueous solution to dryness on the steam can under vacuum, to remove excess HCl. The residue was made up to known volume with water (saturated with CHCl_3 to prevent microbial destruction if any appreciable delay should occur) and the choline estimated by Glick's (1944) method, combined with the use of Beattie's (1936) aqueous reineckate solution and Winzler & Meserve's (1945) ultraviolet absorption technique. Ethanolamine and serine were determined after refluxing for 6 hr. with 6 N-HCl, removal of fatty acids with ether, evaporation to dryness and solution in water to known volume as for choline. The ethanolamine and serine content was then determined by a combination of the methods of Artom (1945) and Burmaster (1946), permittit being replaced by the synthetic resin Zeo-Karb 215. Plasmals were determined by the method of Feulgen &

RESULTS AND DISCUSSION

Fig. 1 shows the results obtained for the 62 fractions of the ethanol phase of the ox-brain glycerophosphatides, for yield (percentage of total ethanol-phase lipid) and content of the three nitrogenous components and of plasmals, all expressed as percentages. Fig. 2 shows similar results for the 20 fractions of the petroleum phase, except that serine and ethanolamine have been given as total amino base. This was predominantly ethanolamine. The results obtained on the crude phosphatidyl choline are shown in Fig. 3, those for the ethanol phase of crude phosphatidyl ethanolamine in Fig. 4 and for the petroleum phase of the same fractionation in Fig. 5. Fig. 6 shows the data obtained on the ethanol phase of the crude phosphatidyl serine, most of the petroleum phase fractions being too small for analysis. In Figs. 1 and 4 the plasmal scale is half that for the other components, but in the other diagrams it has proved convenient to use the

same scale for all components. To facilitate comparison of the various Figures, fraction numbering in all cases has been arranged so that number 1 represents the fraction most preferentially soluble in the ethanol phase.

Fig. 2. Petroleum phase in the distribution of crude ox-brain glycerophosphatides between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

Fig. 3. Combined phases in the distribution of crude phosphatidyl choline between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

Considering first the main experiment, the fraction-yield curve in Fig. 1 indicates a whole series of overlapping fractions and various changes in appearance of the fractions also suggest a complex distribution pattern. Fractions 1 and 2 were

deep orange in colour and only semi-solid at room temperature. Fraction 3 was a little paler and more solid. Fractions 4-7 were yellow and quite firm solids, fractions 8-12 were cream-coloured firm solids, fractions 13 and 14 were yellow soft semi-

Fig. 4. Ethanol phase in the distribution of crude phosphatidyl ethanolamine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

Fig. 5. Petroleum phase in the distribution of crude phosphatidyl ethanolamine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

solids, fraction 15 was again cream coloured and somewhat firmer, fractions 16-57 were cream-coloured firm solids and fractions 58-62 were yellowish brown firm solids.

As might be expected from the known solubility properties of the phospholipins, phosphatidyl choline is removed by the ethanol preferentially to most of the other constituents, but the curve indicates the presence of derivatives of considerably different partition coefficients, varying presumably in fatty acid components. Some of these, e.g. the small proportion of the total phosphatidyl choline represented by fractions 30–37, are markedly different from the main fraction represented by fractions 1–7. Phosphatidyl choline is not completely extracted until fraction 49.

Fig. 6. Ethanol phase in the distribution of crude phosphatidyl serine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

Figs. 3 and 4 likewise show the early extraction of phosphatidyl choline by the ethanol. The lipid preparations in question had, of course, already been separated on the basis of relative solubility in ethanol, and as the starting material (mixed brain and spinal cord as against brain only) was also different, the curves would not be expected to duplicate very closely those in Fig. 1. It may be significant that the long tail-off in the extraction of phosphatidyl choline appears in Fig. 4 for the less ethanol-soluble preparation, but is not seen in Fig. 3. On the other hand, Fig. 3 shows evidence of at least two phosphatidyl cholines, one represented by fractions 1–4, and one by fractions 5–7. These may well correspond to fractions 1–12 and 13–18 respectively in Fig. 1.

It might be expected that the curve for ethanolamine would be particularly complex, since this base is present in two lipid classes, phosphatidyl ethanolamine and plasmalogen. In Fig. 1, from fraction 1 to about fraction 22, the ethanolamine curve shows violent fluctuations, with indications of some large main fractions, e.g. fractions 13–22 inclusive. In these first 22 fractions, apart from the earliest ones and, apparently, fraction 17, the ethanolamine is derived from both the above types of phospholipin. A plasmal content of 7.5%, about the mean level of fractions 8–22, would account for an ethanolamine content of about 1.8%, whereas the total ethanolamine content of these fractions is

of the order of 2.5%. The plasmal curve covering these same 22 fractions shows evidence of more than one plasmalogen, presumably involving a different aldehyde. There is considerable discrepancy in the literature as to the range of aldehydes present in brain plasmalogen (Thannhauser, Boncoddio & Schmidt, 1951; Leupold, 1950). At the time the present work was completed it had not been reported that plasmalogens may have a base other than ethanolamine, but some of the results, discussed later, suggest that this must be the case. A very recent paper by Klenk & Böhm (1951) provides evidence for a plasmalogen containing serine instead of ethanolamine. In spite of the relatively inaccurate technique, there are clearly three distinct plasmalogen fractions represented roughly by fractions 1–11, 12–17 and 18–22 respectively.

From fractions 23 to 44 inclusive, the ethanolamine curve shows only small fluctuations, some within the limits of experimental error. Over this same range of fractions, the plasmal curve climbs steadily to a roughly constant level of 17%, which is then maintained from about fractions 42 to 51. Some of the peaks and depressions in the plasmal curve over the fraction range 23–44 can be correlated with similar features in the ethanolamine curve, e.g. the drop in both curves for fractions 35 and 36, but many of the smaller irregularities in the plasmal curve may be due to experimental error. A plasmal level of 17% requires about 4% of ethanolamine, fully as much as the total present. Although the estimation of natural plasmalogen against free palmitaldehyde is not likely to give an accurate figure for the plasmal content of the fractions, its approximate correctness was confirmed by estimation of the acetone-soluble lipid set free when an ether solution of the fraction was emulsified with a solution of mercuric chloride, which rapidly breaks the acetal linkage in plasmalogen (Feulgen, Imhäuser & Behrens, 1929).

From fraction 45 onwards the ethanolamine curve again shows considerable fluctuations with evidence of at least three main components: fractions 45–48, 49–57 and 58–62 respectively. Some fractions show trends in the ethanolamine curve in this range corresponding to similar trends in the plasmal curve, e.g. the very low value for fraction 57, but most of them are entirely unconnected, e.g. the ethanolamine peak at fraction 54 is associated with a pronounced minimum on the plasmal curve. There is, rather, evidence in fractions 45 to at least 57 of the occurrence of phosphatidyl ethanolamines different from those of fractions 1–22, a point referred to again in considering the serine curve. The plasmal curve shows clear evidence of more than one fraction over this later range, the minimum between fractions 51 and 58 being outstanding.

The results on crude phosphatidyl choline (Fig. 3) show a much closer parallelism between the distribution of plasmalogen and ethanolamine. They agree with Fig. 1 in suggesting more than one plasmalogen (fractions 1-4, 5-14, 15 and 16). Again it is in the earlier fractions that the highest ratios of phosphatidyl ethanolamine to plasmalogen are found. At the peak of the plasmal curve there is still a considerable excess of ethanolamine (4%) over that required by the plasmal (about 2.4%), but at fraction 16 the ratio of plasmal to ethanolamine is just right for plasmalogen.

In Fig. 4, representing a relatively less ethanol-soluble lipid than Fig. 3, there is evidence of two plasmalogens, the plasmal curve corresponding fairly well to the later part of that in Fig. 1 (from fraction 17 onwards). The ethanolamine curve does not run parallel with it and, as in the other cases, ethanolamine differs from choline in not showing any tendency to elimination as fractionation proceeds. Fig. 4 emphasizes that lipids rich in ethanolamine tend to be rich in plasmalogen, but it also provides direct evidence that not all this plasmal can be present in ethanolamine-containing plasmalogen. Some of the fractions contain about 36% of plasmal, requiring about 8.6% of ethanolamine, whereas only 5% is present. At fraction 16 the ratio of the two substances is about right for the accepted plasmalogen structure. In the earlier fractions there is a great excess of ethanolamine, obviously present as phosphatidyl ethanolamine.

In the partition of the crude phosphatidyl ethanolamine the process was not carried near to completion, and Fig. 4 should be compared with the early part of Fig. 5. For fractions 1-7 (Fig. 5) the plasmal content of about 20% requires about 4.8% of ethanolamine, near to the 4.5% which is present. The later fractions in Fig. 5, however, tell a different story. They contain 10-15% of plasmal, requiring about 3% of ethanolamine, whereas only about 1% is found.

The crude phosphatidyl serine, Fig. 6, contains very little plasmalogen, and where ethanolamine is present it is more than adequate to account for the plasmal. But in one or two fractions no ethanolamine at all could be detected, yet plasmal in about the same amount as in the neighbouring fractions was still present.

The serine curve in Figs. 1 and 6 resembles the ethanolamine curve in being extended over the full range of fractions. In Fig. 1 it shows only minor fluctuations from fractions 1 to 17, though with a definite minimum in fractions 11 and 12, after which there is a sudden increase followed by a fairly general rise with minor fluctuations to fraction 44. From here until fraction 57 the serine curve exhibits a series of abrupt rises and falls rivalling those of ethanolamine over the same fraction range, the

two curves being sometimes in phase and sometimes out of phase. In general, the similar behaviour of phosphatidyl ethanolamine and phosphatidyl serine is not surprising, in view of the extreme difficulty in separating these lipids by the classical methods. The curves in Fig. 1 suggest that they have a similar assortment of fatty acids and that both contain representatives, appearing in fractions 45 onwards, considerably different in fatty acid composition from those appearing in the earlier fractions. Klenk & Böhm (1951) emphasize the complex range of fatty acids present in both phosphatidyl ethanolamine and phosphatidyl serine, but report great differences in this respect between the two lipids. However, they mention that intermediate fractions, discarded in the preparation of purified fractions, may have influenced this. It was noted that fractions 45-62 (especially fractions 60-62) were much more slowly broken down by acid hydrolysis than were all the earlier fractions. The generally slower rise of the serine curve until it meets the ethanolamine curve at fraction 45 is in line with the relative solubilities of the two lipids in ethanol (Folch, 1948).

The serine curve in Fig. 6 exhibits a steep initial rise instead of the long slow rise in Fig. 1. There are several peaks and depressions, the agreement between Figs. 1 and 6 showing that there must be a number of phosphatidyl serines, differing in their fatty acid components.

In all cases the combined derivatives of choline, ethanolamine and serine can account for only a portion of the total fraction, e.g. ranging for Fig. 1 from 48 to 89%, with a value of about 60% for the majority of the fractions. Phosphorus determinations, made on every fifth fraction in Fig. 1, ranged from 2.0 to 3.7%, with most in the range 2.5-3.5%, thus corresponding quite well with the values obtained from the nitrogenous derivatives. Part of the balance in some fractions may consist of inositol lipids, but a much greater factor seems to be the presence of lipids of the wax ester type. All the fractions in Fig. 1 yield appreciable amounts (ranging from 3 to 15%, with the lowest values in the early fractions) of unsaponifiable matter, by ether extraction of alkaline hydrolysates. According to Feulgen *et al.* (1929) the acetal linkage of plasmalogen is completely resistant to 5 hr. boiling with 5% aqueous sodium hydroxide, and ether extraction of the solution should leave the aldehyde in the aqueous layer as the sodium salt of plasmalogenic acid. In the author's laboratory, where ethanolic 0.5N-potassium hydroxide is routinely used for alkaline hydrolysis, it has been found that small, variable amounts of plasmal are set free in 2 hr. refluxing. Accordingly, unsaponifiable matter has been determined after removal of plasmal with mercuric chloride. The unsaponifiable matter from

all fractions had a similar appearance, being a soft paste at room temperature and a mobile liquid when warm. It had an iodine value ranging from 70 to over 100 in the various fractions. The total product was submitted to fractional distillation at 0.1 mm. pressure, but only a few drops distilled over the range 120–180°, when distillation was stopped. Distillate and residue were recombined and acetylated. The product had a saponification equivalent of 411.3, corresponding approximately to the acetate of a C_{25} monohydric alcohol. There would almost certainly be a mixture of homologues present, probably those of an even number of carbon atoms. Such alcohols, presumably esterified with fatty acids, do not seem to have been reported previously in the crude phospholipin fraction of ox brain.

Turning to Fig. 2, and comparing it with Fig. 5, it can be seen that the greater part of the material recovered from the petroleum phase has remained virtually unmoved by the ethanol. Following the initial fractions containing material closely resembling the final fractions of the ethanol phase, especially marked in the incomplete procedure represented in Fig. 5, there is a long run of very small fractions and then an abrupt rise.

The nature of the last few fractions is obscure. In Fig. 2 appreciable amounts of choline are shown, which cannot be present as phosphatidyl choline. The obvious suggestion would be small amounts of sphingomyelin but these fractions do not have the solubility properties of sphingomyelin. They are firm solids readily soluble in cold ether or light petroleum. They are quite insoluble in ethanol. They also contain appreciable amounts of plasmalogen and ethanolamine. It should be noted that they account for only a very small proportion (less than 0.5 %) of the total for the crude glycerophosphatides of ox brain, but considerably more—about 8 %—of the crude phosphatidyl ethanolamine of the mixed brain and spinal cord. The very small fractions covering the long flat minimum of the petroleum phase—yield curve are different again, being soft gummy products which become liquid on warming.

It seems clear that such a complex mixture as the total glycerophosphatides of ox brain requires a far

longer fractionation chain than 20 units to effect sharp separation of major constituents, and that even under the best conditions there would be poor separation of some of them, e.g. of serine-containing from ethanolamine-containing lipids, unless other solvent pairs should give better results. However, in Fig. 1, fractions 1–20 contain most of the phosphatidyl choline with relatively small proportions of lipids containing serine and ethanolamine. Fractions 30–45 should give a fraction consisting mainly of plasmalogen and phosphatidyl serine, and fractions 58–62 should contain an even purer plasmalogen. When it is remembered how markedly phospholipins influence the solubility properties of other phospholipins it is not surprising that sharp separation is not achieved by the relatively simple fractionation procedure used in these experiments.

SUMMARY

1. The crude glycerophosphatides of ox brain, and crude preparations of phosphatidyl choline, phosphatidylethanolamine and phosphatidylserine, have been partitioned between aqueous ethanol and light petroleum, employing the technique of counter-current distribution.

2. The results suggest a useful degree of separation of phosphatidyl choline from similar lipids containing ethanolamine and serine and of plasmalogen from lipids other than phosphatidyl serine. Separation of phosphatidyl ethanolamine from phosphatidyl serine is poor.

3. There is evidence that the plasmalogens are a complex group which may include derivatives with a base other than ethanolamine, as well as a range of aldehydes. The glycerophosphatides containing choline, ethanolamine and serine all show evidence of multiplicity of fatty acid composition.

4. The crude phospholipins of ox brain contain substances not so far classifiable, including choline-containing lipids different from lecithin and sphingomyelin, appreciable amounts of what appear to be wax esters, and gummy lipids of unknown composition.

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Estimation of Nucleic Acids in Tissue from the Nervous System

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The starting point of this investigation was the report of Davidson & Waymouth (1944) that white matter of the brain of the sheep contained a higher concentration of protein-bound phosphorus, i.e. phosphorus remaining after removal of lipid phosphorus and acid-soluble phosphorus, than grey matter. If most of this protein-bound phosphorus were present as nucleic acid, there remained the possibility that nucleic acid might be an important constituent of the myelin sheath of a mammalian nerve fibre. Since no nucleic acid can be detected in the myelin sheath by the usual histochemical means, it seemed worth while to investigate the matter further. Attempts were made to estimate the concentration of deoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) in different parts of the nervous system by two methods at present widely used, that of Schmidt & Thannhauser (1945) and that of Schneider (1945). Although recovery experiments showed that added DNA, PNA, or mixtures of the two, could be recovered quantitatively from brain extracts by either method, it soon became apparent that the two methods gave widely diverging results when they were applied to white matter and grey matter of brain and to peripheral nerve.

It was decided, therefore to investigate the validity of these methods for tissue from the nervous system. The conflicting results are due, in part, to the presence of considerable amounts of a phosphorus-containing compound in brain and spinal cord, particularly in the myelin-rich white matter. The phosphorus of this compound is not removed by ice-cold 10% (w/v) trichloroacetic acid (TCA) nor is it removed by the usual ethanol-ether treatment for the extraction of lipids. This substance is probably the same as the inositol-containing

trypsin-resistant lipid-protein complex isolated from white matter by Folch & Le Baron (1951). Dr J. Folch, McLean Hospital, Waverley, Mass., in experiments as yet unpublished, has obtained results similar to those reported here.

In addition, it is shown that TCA extracts of brain and nerve tissue, prepared as described by Schneider (1945), contain chromogenic material that interferes with the colour reactions of Dische (1930) for DNA and Meibbaum (1939) for PNA. Folch (1951) suggests that this interference may be caused by traces of the complex lipid substance, strandin, isolated from brain by Folch, Arsove & Meath (1951). A preliminary account of these experiments has already appeared (Rossiter, Logan & Mannell, 1951).

METHODS

Standards

The same DNA and PNA standards were employed throughout this work. Results obtained for each of the methods are comparable inasmuch as they are all given in terms of the P content of these standards. The DNA standard was a preparation of calf sodium thymonucleate prepared by the

Table 1. *Absorption characteristics and phosphorus content of standard preparations of DNA and PNA*

	$\lambda_{\text{max.}}$ (m μ .)	ϵ (P) at $\lambda_{\text{max.}}$	$\lambda_{\text{max.}}$ (m μ .)	ϵ (P) at $\lambda_{\text{min.}}$	P (%)
DNA	259	7300	230	2900	8.3
PNA	258	9300	228	3500	8.5

method of Mirsky & Pollister (1942), and kindly provided by Dr G. C. Butler, Department of Biochemistry, University of Toronto. The PNA standard was a preparation of yeast sodium ribonucleate selected from a number of commercial preparations examined. Table 1 shows that the absorption