EFFECT OF SOLUTE CONCENTRATION ON COUNTERCURRENT DISTRIBUTION OF PHOSPHOLIPINS*

by

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It has been generally accepted on theoretical grounds that the best separation of a complex mixture by countercurrent distribution is obtained by working with dilute solutions. CRAIG[1] reckoned that 0.5 g of solute was the largest amount of material which could reasonably be introduced into his original all-metal twenty tube countercurrent apparatus, with a solvent capacity of 8 ml for each phase.

Countercurrent distribution has been applied in an attempt to separate the phospholipins of plants phosphatides by SCHOLFIELD, DUTTON, TANNER AND COWAN[7], SCHOLFIELD, MCGUIRE AND DUTTON[8] and MCGUIRE AND EARLE[6], and of ox brain by LOVERN[5]. The former group of workers quote figures of approximately 3 g of phospholipin dissolved in 70 ml of the lighter phase as the concentration used in their apparatus. LOVERN[5] dissolved 120 g of ox brain phosphatide in 300 ml of lighter phase and 7 g of purified phosphatides in 100 ml of lighter phase.

The present work was commenced to study the composition of crude phosphatide fractions from fish muscle. It was intended to use a replica of the original metal CRAIG apparatus[1] for pilot runs to choose solvent pairs. The distribution of the fraction between light petroleum: acetone-ethanol-water was found to vary extensively, depending on the concentrations used. Better separation of various constituents were obtained at very high concentrations.

METHODS

Preparation of phospholipin extract. The phospholipin used was one of the fractions available in a current series of studies on the lipids of fish muscle. It was derived from an acetone-extract of haddock muscle which, after purification with light petroleum, was taken up in ether and precipitated with a larger excess of acetone. The original crude acetone extract contained considerable phospholipin which could be separated by the subsequent precipitation. This crude phospholipin was purified by twice repeated reprecipitation from acetone, but in view of its origin would be expected still to contain some non-lipid impurity.

Countercurrent distribution. The countercurrent distribution was done in a 20 tube all-metal apparatus as described by CRAIG[1]. Each tube had a total capacity of approximately 16 ml allowing for 8 ml of each phase. The solvent pair was obtained by shaking together 2 vols light petroleum (B.P. 40–60° C), 2 vols 10:1 acetone-water, and 1 vol 10:1 ethanol-water. The two phases are referred to as light petroleum phase and acetone-ethanol phase respectively.

Analytical methods. The % of the total fraction in each tube was determined by direct weighing. Phosphorus was determined by the method of LEPAGE AND UMBREIT[4]: nitrogen by digestion with conc. H_2SO_4 with Hg and $K_2S_2O_8$ as catalysts: the samples were cleared with H_2O_2. Plasmals were

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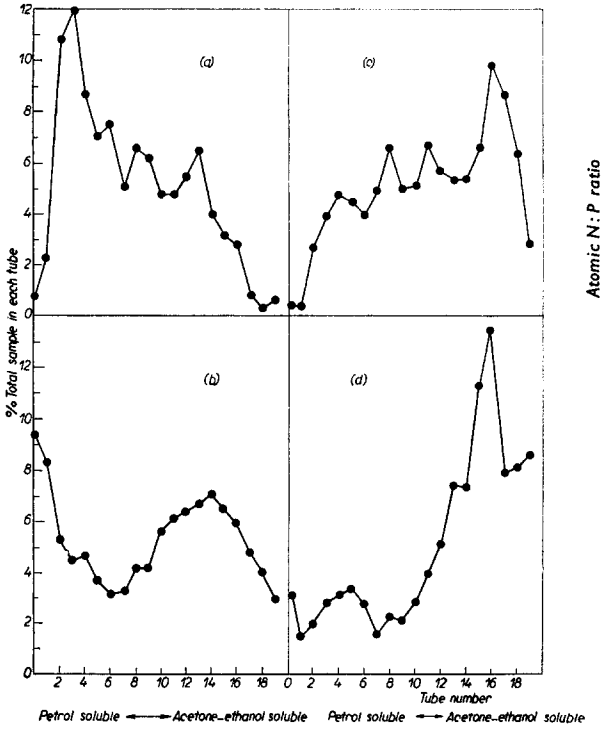

Fig. 1. Effect of solute concentration on countercurrent distribution of phospholipins between light petroleum and aqueous acetone-ethanol.
(a) = 3.74 g phospholipin in first two tubes ~ 75% in tube O.
(b) = 1.55 g phospholipin in tube O.
(c) = 0.54 g phospholipin in tube O.
(d) = 0.31 g phospholipin in tube O.

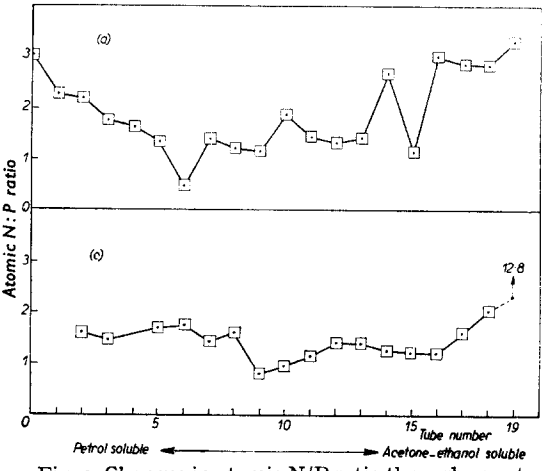

Fig. 2. Changes in atomic N/P ratio through counter current distribution of phospholipins between ligh petroleum and aqueous acetone-ethanol.
(a) = 3.74 g phospholipin in first two tubes ~ 75% in tube O.
(c) = 0.54 g phospholipin in tube o.

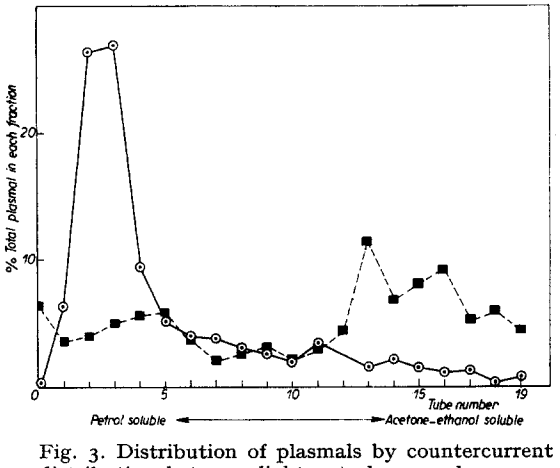

Fig. 3. Distribution of plasmals by countercurrent distribution between light petroleum and aqueous acetone-ethanol.
⊙——⊙ = 3.74 g phospholipin in first two tubes. (a) of Fig. 1.
■ --- ■ = 0.31 g phospholipin in first tube. (d) of Fig. 1.

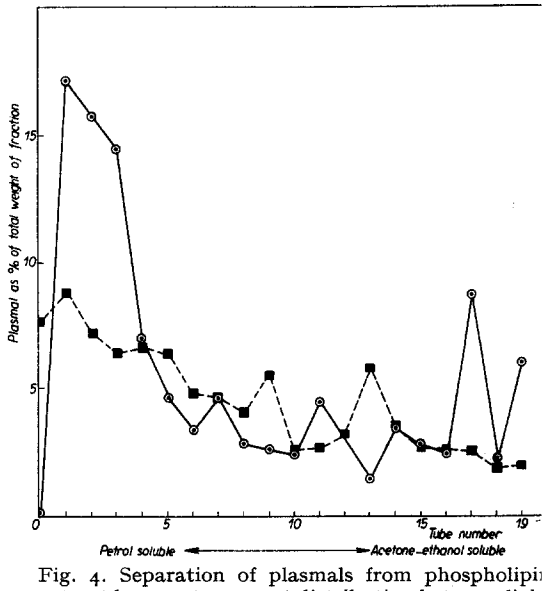

Fig. 4. Separation of plasmals from phospholipin extract by countercurrent distribution between ligh petroleum and aqueous acetone-ethanol.
⊙——⊙ = 3.74 g phospholipin in first two tubes (a) of Fig. 1.
■ --- ■ = 0.31 g phospholipin in first tube. (d) of Fig. 1.

determined in 1 ml of acetic acid with 10 ml of the fuchsin reagent of FEULGEN AND GRÜNBERG[3]. and 3 drops of 6% $HgCl_2$. The reaction was allowed to take place overnight and 4 ml of *n*-propyl alcohol then added to bring the dye into solution. The stoppered solution was allowed to stand for 10 minutes to clear and the colour then rapidly determined colorimetrically. The time of clearing needed to be carefully controlled as the propyl alcohol blank increases fairly rapidly. The *n*-propyl alcohol blank was found to be reasonable and the method more reproducible than extraction procedures where part of the colour tends in some samples to remain in the aqueous phase. Choline was estimated as described by LOVERN[5].

RESULTS

Fig. 1 shows the % of the total fraction in each tube after countercurrent distribution of four different concentrations of phospholipins. It can be seen that as the solutions become progressively more dilute, the bulk of the sample tends towards the acetone-ethanol phase. The peak in the light petroleum phase in the earlier tubes becomes successively smaller and flatter. The final peak in the acetone-ethanol phase becomes progressively larger and is shifted further in the direction of solubility in the lower phase. At all four concentrations the original sample has been separated into at least four different fractions.

Atomic N/P ratios showed marked fluctuations (Fig. 2). A fraction with an N/P ratio of less than 1 partitioned further into the acetone-ethanol in more dilute solution, as did a water soluble nitrogenous contaminant (N = 12 %).

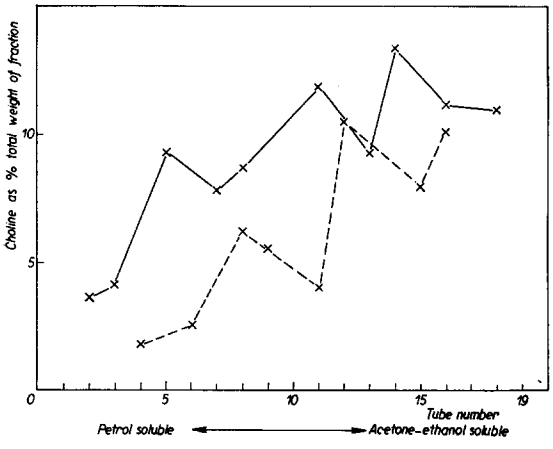

Fig. 5. Distribution of choline-containing phospholipins by countercurrent distribution between light petroleum and aqueous acetone-ethanol.
×——× = 3.74 g phospholipin in first two tubes. (a) of Fig. 1.
×---× = 0.54 g phospholipin in first tube. (c) of Fig. 1.

Fig. 3 shows the % plasmal in each tube expressed as a percentage of the total plasmal. Countercurrent distribution of the concentrated phospholipin extract has concentrated over 50 % of the total plasmal into two tubes at the light petroleum soluble end of the distribution. The more dilute extract shows the bulk of the plasmal distributed to the more acetone-ethanol soluble end. The underlying significance of these results is shown in Fig. 4. The fractions with the greatest plasmal content as a percentage of the total weight of the fraction were those highly soluble in light petroleum, *i.e.* fractions 1–3 at both concentrations; but in the concentrated extract the greatest weight of the fraction was also here and there has been a considerable separation of the plasmals from the other constituents.

Fig. 5 shows the effect produced by countercurrent distribution at higher concentrations for separation of the choline-containing phospholipins. At both high and low concentrations the phosphatidyl choline compounds tend towards the acetone-ethanol soluble phase. As at higher concentrations the greatest weight of the fraction tended towards the light petroleum phase (Fig. 1(a)), the phsophatidyl choline compounds obtained towards the acetone-ethanol soluble end were relatively purer.

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To obtain some idea of the nature of this effect 2.35 g and 0.5 g of phosphatide were each dissolved in 10 ml of light petroleum saturated with 85 % ethanol and shaken with 10 ml of 85 % ethanol in a measuring cylinder. In both cases the volume of light petroleum phase was reduced by 1 ml. The results are shown in Table I.

TABLE I

EFFECT OF CONCENTRATION ON THE DISTRIBUTION OF PHOSPHATIDE BETWEEN 1:1 LIGHT PETROLEUM AND 85% ETHANOL

Total Wt. Phosphatide g	*Phosphatide in light petroleum. g (1)*	*Phosphatide in 85% ethanol (2)*	*Ratio (1)/(2)*	*Plasmals as % phosphatide in light petroleum*	*Plasmals as % phosphatide in 85% ethanol*	*% Total plasmal in light petroleum phase*
2.35	1.44	0.91	1.58	4.4	2.9	71
0.5	0.19	0.35	0.54	6.3	2.8	55

The distribution ratio of the total phosphatide was increased three times at the higher concentration. The plasmal distribution was increased by 30 %. Choline-containing phosphatides were shown to be preferentially partitioned into the ethanol phase at all concentrations (Fig. 5). Serine and ethanolamine were present in this phosphatide mixture in negligible quantities and therefore the threefold increase in distribution ratio in the concentrated solution was to a considerable extent accounted for by the preferential retention in light petroleum of a phosphatide with an unidentified base. This explains the fact that although the % of the total plasmal retained in the light petroleum in the concentrated solution was greater, the plasmal content as a percentage of the phosphatide retained was reduced.

A countercurrent distribution through ten tubes only, between light petroleum and 85 % ethanol was done on the acetone-insoluble matter from unbleached groundnut phosphatides and unbleached soyabean lecithin*. It can be seen by comparison of graphs (e) and (g), Fig. 6 and graph (b) Fig. 1 that weight curves

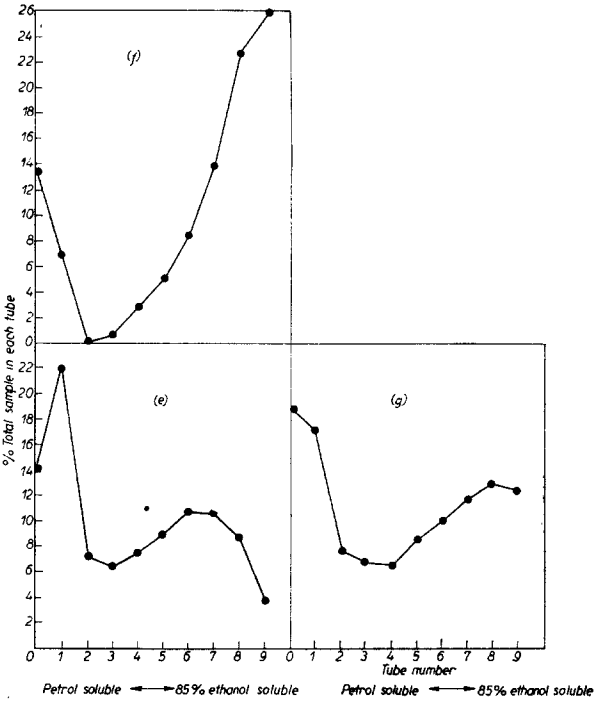

Fig. 6. Countercurrent distribution of groundnut phospholipins and soyabean lecithin between light petroleum and 85% ethanol.
(e) = 1.0 g groundnut phospholipin.
(f) = 0.14g groundnut phospholipin.
(g) = 0.8 g soyabean lecithin.

* The groundnut and soyabean phosphatides were kindly supplied by J. Bibby and Sons, Ltd., Liverpool.

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from different phosphatides resemble each other more closely at the same concentrations than the weight curves for the same phosphatide at different concentrations. In all cases increased concentration favoured the partition to the light petroleum soluble end of the distribution.

DISCUSSION

Countercurrent distribution between light petroleum and acetone-ethanol of phospholipin extracts from haddock muscle have been found to give widely varying distribution patterns depending on the concentration of the phosphatide. Preferential separation into the light petroleum fraction occurs at high concentrations, and with increasing dilution, more extract tends into the acetone-ethanol-water phase. These observations have also been found to apply to the total phospholipins of groundnut and soyabean when distributed between light petroleum and 85 % ethanol. This effect of concentration would therefore appear to be fairly general for widely differing phospholipin compositions.

Theoretical explanation for these observations is difficult owing to the complexity of the mixtures and to the fact that solutes do not obey partition laws at high concentrations (CRAIG[2]). The changes in distribution at varying concentrations of this particular sample of acetone-insoluble phosphatides from haddock muscle, would not appear to be caused by changes in intersolubility of the solvent pair. The volume changes of the solvent pair were small and the same for both concentrated and dilute phosphatide solutions. It should be noted however that 20 g of acetone-soluble 'lecithins' obtained from haddock muscle rendered completely miscible 80 ml of light petroleum and 80 ml of 85 % ethanol which had been previously saturated with each other. Certain classes of phosphatide can therefore have extremely serious effects on the intersolubility of the solvent pair.

It would appear from Table I that the partition ratios of some phosphatide-like substances have a greater rate of change with concentration than others. It might be expected that after one transfer the concentration would be so reduced that any efficient separations by the use of the effect would not be obtained. There are, however, so many examples of one phosphatide influencing the solubility of another in various solvents that if a large percentage of one or more substances is removed in the first transfer the partition of others may remain permanently altered. It is also possible that at high concentrations the effect of solubilities of the various phospholipin fractions in each other are becoming of relatively more importance than the partition between the solvent pair. Difficulties with emulsions were not encountered with concentrated solutions.

The significance of the present results lies in the fact that quite striking separations of some constituents have been obtained at high concentrations, notably the separation of the plasmal-containing from the choline-containing phospholipins. This introduces another possible means of separation without the search for a different solvent pair. It is also important that results from different laboratories, or on different samples of phosphatide, should not be compared unless the concentrations of the original solute are of the same order.

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SUMMARY

Countercurrent distribution of the crude phosphatides obtained from an acetone extract of haddock muscle, between light petroleum and acetone-ethanol, has been shown to vary greatly with the concentration of the extract. Increasing the concentration of the phospholipin extract has been found to favour distribution into the light petroleum phase. This effect would appear to be of a more general application, as it has been found to apply to the countercurrent distribution of phospholipins from groundnut and soyabean between light petroleum and 85% ethanol.

Countercurrent distribution of the phospholipins of haddock muscle at high concentrations was found to be advantageous for the separation of some constituents of the mixture. for example separation of the plasmal-containing from the choline-containing phosphatides.

RÉSUMÉ

Nous avons montré que la distribution des phosphatides bruts d'un extrait acétonique de muscle d'aiglefin entre l'éther de pétrole et un mélange acétone-éthanol (dans un appareil à contre-courant) varie beaucoup avec la concentration de l'extrait. Lorsque la concentration de phospholipine dans l'extrait augmente. le passage dans la phase éther de pétrole est favorisé. Il semble que cet effet soit d'une application plus générale; nous avons trouvé, en effet, qu'il s'applique à la distribution (dans l'appareil à contre-courant) de phospholipines de pistache et de fève de soya entre l'éther de pétrole et l'éthanol à 85%.

La distribution (dans l'appareil à contre-courant) des phospholipines de muscle d'aiglefin en concentration élevée peut servir à séparer certains constituants du mélange; par example, l'on sépare ainsi les phosphatides contenant du plasmal d'avec ceux qui contiennent de la choline.

ZUSAMMENFASSUNG

Es wurde gezeigt, dass die Gegenstromverteilung roher, aus dem Acetonextrakt von Schellfischmuskeln erhaltener Phosphatide zwischen Leichtpetroleum und Aceton-Alkohol sehr stark mit der Konzentration des Extraktes variert. Es wurde gefunden, dass ein Ansteigen der Konzentration des Phospholipinextraktes den Übergang in die Leichtpetroleumphase begünstigt. Da sich dieser Effekt — wie gefunden wurde — auf die Gegenstromverteilung der Phospholipide der Erdnuss und der Sojabohne zwischen Leicht-petroleum und 85% Alkohol anwenden lässt, würde es scheinen, dass er allgemeiner anwendbar wäre.

Es wurde gefunden, dass die Gegenstromverteilung der Phospholipine von Schellfischmuskeln bei hohen Konzentrationen vorteilhaft ist zur Abtrennung einiger Bestandteile aus der Mischung, z.B. zur Trennung der plasmal- und cholinenthaltenden Phosphatide.

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