Studies on Phospholipids

10. ANOMALOUS COUNTERCURRENT DISTRIBUTIONS*

By F. D. COLLINS

Department of Biochemistry, University of Melbourne, Parkville N. 2, Victoria, Australia

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Separation of lipids by countercurrent distribution is not easy for the following reasons. If the total concentration of solute is too high (above 0.01 M) the partition coefficients and phase volumes alter because, in effect, a new solvent system is formed. Even if the concentrations are kept below 0.01 M separations of the phospholipids are difficult since the partition coefficients lie within a comparatively narrow range of values and some fractions are liable to form emulsions. Further, the phospholipids contain a mixture of fatty acids; a pure lecithin, for example, does not exist in Nature but only a family of lecithins, causing the distribution curves to be somewhat broader than required by theory (Collins, 1960*a*, 1963).

The countercurrent distribution of phospholipid derivatives (Collins, 1960*b*) is more successful than that of the native compounds as a wider range of partition coefficients is obtained, giving good separations (Collins & Shotlander, 1961*a*), and there is no emulsification. However, the complex phospholipids (Collins, 1960*b*) give distribution curves that are skewed and differ considerably from those expected theoretically. The purpose of the present paper is to examine the general problem of association and to show that this applies in the complex phospholipids.

EXPERIMENTAL

Materials

The rat-liver lipids were prepared as described by Collins & Shotlander (1961*a*) and various fractions were obtained by countercurrent distribution.

Methyl ester of dinitrophenylphosphatidylethanolamine. This was isolated after distribution in heptane-methanol (Collins & Shotlander, 1961*b*) and had molar proportions phosphorus:DNP groups:fatty acids 1:1:1.96.

Triglycerides. Neutral fat from rat liver was distributed in heptane-methanol and the triglycerides were located by the determination of ester groups and by showing the absence of phosphorus and sterols. The distribution corresponded closely to the theoretical one although it was somewhat broader.

Cholesterol. Total sterols were determined on a distribution of rat-liver lipids in carbon tetrachloride-methanol-water: two peaks were obtained with partition coefficients

of 0.043 and 0.43, which were assumed to correspond to esterified and free cholesterol respectively. Both curves agreed closely with those expected for a normal distribution in spite of the fact that they overlapped with other components, notably the free cholesterol curve with that corresponding to the complex phospholipid fraction.

Phosphatidic acid-like materials. These corresponded to fractions X, Y and Z of Collins (1959*a*) and were further characterized by Collins & Shotlander (1961*b*). The curves were always symmetrical.

Lecithin. This was prepared from egg-yolk lipids (Hanan & Jayko, 1955); it contained 3.44% of phosphorus and had the molar proportions phosphorus:choline:fatty acids 1:0.98:1.98.

Kephalin. β -Dipalmitoyl-DL- α -kephalin (synthetic grade B) was supplied by the California Corp. for Biochemical Research. Analysis by conversion of a portion of the substance into the methylated and DNP derivative followed by countercurrent distribution indicated a purity of about 85%.

Lysolecithin. This was a gift from Miss H. Doery of the Commonwealth Serum Laboratory and gave a single spot on paper chromatography in the system described by Lea, Rhodes & Stoll (1955).

Trioctyl phosphate. This was supplied by Albright and Wilson (Aust.) Pty Ltd.

Complex phospholipids. The mixed fraction (Table 2) was obtained from the methylated and dinitrophenylated rat-liver lipids by cold-acetone chromatography (Collins & Shotlander, 1960) and the separation of the acetone-soluble material into a non-polar and polar fraction was carried out as described by Collins & Wheeldon (1957). The polar fraction was used as such although it was heterogeneous, as shown by countercurrent distribution.

Further fractions of the complex phospholipids were obtained by countercurrent distribution of the mixed fraction in carbon tetrachloride-methanol-water and isolation of small subfractions as shown in Fig. 3.

Methods

The methods for the determination of phosphorus, DNP groups, choline and ester groups were those used by Collins & Shotlander (1961*a*). Sterols were determined by the procedure described by Cook (1958).

Countercurrent distribution. An all-glass countercurrent distribution machine (Towers) with 20 ml. in each phase and capable of 120 transfers was used. The solvent system was carbon tetrachloride-methanol-water (62:35:3.15, by vol.).

Partition coefficients. The variation of partition coefficient with concentration was measured by equilibrating 10 ml. of

* Part 9: Collins (1963).

each phase of carbon tetrachloride-methanol-water with a known amount of material, separating the two phases and determining the concentration of phosphorus or DNP groups in each phase. The partition coefficient was the ratio of the concentration in the top phase to that in the bottom. With the complex phospholipids the determinations were all recorded in terms of the DNP groups. The ratio of DNP groups to phosphorus did not vary greatly, but remained in the range 0.4-0.5 and was constant for each preparation.

Calculation of a relationship between partition coefficient and concentration. Anomalous distributions will be obtained if the material either associates with itself or another substance, or if it dissociates, and this can be represented by $A + B \rightleftharpoons C$.

Let a_1, b_1, c_1 be the concentrations of each substance in the upper phase and a_2, b_2, c_2 be their concentrations in the lower phase at equilibrium. The total quantity of each material will be written A and B . Hence

$$a_1 + a_2 + c_1 + c_2 = A \quad (1)$$

$$\text{and} \quad b_1 + b_2 + c_1 + c_2 = B \quad (2)$$

Let the partition coefficient of each compound be designated p_a, p_b and p_c ; hence

$$\frac{a_1}{a_2} = p_a, \quad \frac{b_1}{b_2} = p_b \quad \text{and} \quad \frac{c_1}{c_2} = p_c \quad (3)$$

Also because of the equilibrium

$$\frac{a_1 b_1}{c_1} = K_1 \quad \text{and} \quad \frac{a_2 b_2}{c_2} = K_2 \quad (4)$$

From equations (3) and (4) it follows that

$$p_a p_b = \frac{K_1}{K_2} p_c \quad (5)$$

From equations (1), (2), (3) and (4) it follows that

$$a_2 \left[1 + p_a + \frac{b_2}{K_2} (1 + p_c) \right] = A \quad (6)$$

$$\text{and} \quad b_2 \left[1 + p_b + \frac{a_2}{K_2} (1 + p_c) \right] = B \quad (7)$$

Subtracting equation (7) from (6) gives

$$a_2(1 + p_a) - b_2(1 + p_b) = A - B \quad (8)$$

and thus a quadratic equation for a_2 can be obtained

$$\frac{1 + p_a}{1 + p_b} \frac{1 + p_c}{K_2} a_2^2 + \left[(1 + p_a) - \frac{(1 + p_c)(A - B)}{K_2(1 + p_b)} \right] a_2 - A = 0 \quad (9)$$

the solution of which is

$$a_2 = - \left[(1 + p_a) - \frac{(1 + p_c)(A - B)}{K_2(1 + p_b)} \right] + \left\{ \left[(1 + p_a) - \frac{(1 + p_c)(A - B)}{K_2(1 + p_b)} \right]^2 + 4 \times \frac{1 + p_a}{1 + p_b} \frac{1 + p_c}{K_2} A \right\}^{\frac{1}{2}} \div 2 \times \frac{1 + p_a}{1 + p_b} \frac{1 + p_c}{K_2} \quad (10)$$

By using the relation (8) the corresponding solution for b_2 can be obtained.

If an equilibrium mixture as defined above is distributed between two phases the measured partition coefficient (P) will be either

$$P = \frac{a_1 + b_1 + c_1}{a_2 + b_2 + c_2} \quad (11)$$

(if the total amount of material in each phase is measured) or

$$P' = \frac{a_1 + c_1}{a_2 + c_2} \quad (12)$$

if only one component is measured.

In terms of a_2 and b_2 these equations become

$$P = \frac{p_a + \frac{b_2}{a_2} p_b + \frac{b_2 p_c}{K_2}}{1 + \frac{b_2}{a_2} + \frac{b_2}{K_2}} \quad (11a)$$

$$\text{or} \quad P' = \frac{p_a + \frac{b_2 p_c}{K_2}}{1 + \frac{b_2}{K_2}} \quad (12a)$$

These expressions can be expressed in terms of A and B by using the solutions for a_2 and b_2 .

It can be shown that as the values of A and B increase the value of P or P' tends towards p_c , and as the values of A and B decrease the value of P tends towards

$$\frac{p_a(1 + p_b)A + p_b(1 + p_a)B}{(1 + p_b)A + (1 + p_a)B}$$

and P' tends towards p_a .

The complete solutions are complex, but under certain conditions simplified solutions are possible. If $A = B$ and if

$$\frac{4(1 + p_a)(1 + p_b)(1 + p_c)K_2 B}{[(1 + p_a)(1 + p_b)K_2 + (1 + p_c)(A - B)]^2} \text{ is either } \ll 1 \text{ or } \gg 1$$

$$\text{then} \quad P = \frac{p_a(1 + p_b) + p_b(1 + p_a) + p_c(B/K_2)}{1 + p_b + 1 + p_a + (B/K_2)} \quad (11b)$$

or

$$\frac{p_a(1 + p_b) + p_b(1 + p_a) + p_c \left[\frac{(1 + p_a)(1 + p_b)K_2 B}{1 + p_c} \right]^{\frac{1}{2}}}{1 + p_b + 1 + p_a + \left[\frac{(1 + p_a)(1 + p_b)K_2 B}{1 + p_c} \right]^{\frac{1}{2}}} \quad (11c)$$

$$\text{and also then} \quad P' = \frac{p_a + \frac{B}{1 + p_b} \frac{p_c}{K_2}}{1 + \frac{B}{1 + p_b} \frac{1}{K_2}} \quad (12b)$$

$$\text{or} \quad \frac{p_a + \left[\frac{(1 + p_a)K_2}{(1 + p_b)(1 + p_c)} B \right]^{\frac{1}{2}} p_c}{1 + \left[\frac{(1 + p_a)K_2}{(1 + p_b)(1 + p_c)} B \right]^{\frac{1}{2}}} \quad (12c)$$

RESULTS

Table 1 shows the partition coefficients of a number of compounds which did not vary with concentration. On countercurrent distribution these materials all gave symmetrical or nearly symmetrical curves, as also did trioctyl phosphate, mixed triglycerides, cholesterol and phosphatidic acid-like fractions.

The partition coefficients of samples of the complex phospholipids were dependent on concentration, as shown in Table 2 and Fig. 1. The partition

coefficient tends to a low value at high concentrations and a high value at low concentrations. The experimental results fitted the equation

$$P = \frac{\alpha + \beta c}{1 + \gamma c} \quad (13)$$

Table 1. *Partition coefficients of some lipids in carbon tetrachloride-methanol-water and their independence of concentration*

Figures under 'Concn.' represent ranges of values.

Material	Partition coeff.	Concn. (μM)
Lecithin	1.17 ± 0.010 (7)	24.4-5680
Lysolecithin	3.14 ± 0.35 (8)	43-942
Dipalmitoyl-DL- α -kephalin	1.085 ± 0.041 (14)	44-4630
Methyl ester of dinitrophenyl-phosphatidyl-ethanolamine	0.24 ± 0.005 (7)	28-3070

Table 2. *Partition coefficients of complex phospholipid fractions at various concentrations in carbon tetrachloride-methanol-water, showing dependence on concentration*

Results represent ranges of values.

Material	Partition coeff.	Concn. (μM)
Mixed complex fraction	3.55-1.04	12-1096
Tubes 41-54 (see Fig. 3), A	6.63-0.87	7.4-888
Tubes 62-69 (see Fig. 3), B	2.91-1.46	7.8-872
Tubes 99-114 (see Fig. 3), C	6.7-2.0	10-1120

Fig. 1. Variation of partition coefficients with concentration for a sample of complex phospholipids (Fig. 2A). The experimental points are marked $\times$. The continuous line represents the curve $P' = (5.89 + 1.66c)/(1 + 2.55c)$.

where α , β and γ are constants and c is the concentration of DNP groups. The expression is of the same form as (12b), which was deduced by introducing certain simplifications into the general equation. The data shown in Fig. 1, which were calculated by the method of least squares, imply a partition coefficient of 5.89 for the undissociated molecule and of 0.65 for the associated molecule. The empirical relation (13) was used to calculate, for different values of c , the countercurrent distribution. The calculation, performed by an electronic computer, was made with a programme that paralleled the successive stages of an actual distribution. The results of these calculations are shown in Fig. 2 and may be compared with the experimental curves shown in Fig. 3. The calculated and experimental curves are similar in shape, with one edge much steeper than normal and the other much less steep. Also, the position of the maximum varies with concentration as found in practice. The experimental

Fig. 2. Theoretical countercurrent distributions calculated for concentration-dependent partition coefficients $P' = (p_a + p_c ac)/(1 + ac)$ for 120 transfers. Series A (—) were calculated for p_a 5.89, p_c 0.65, a 2.55 for values of c of 100, 50, 10 and 1 μmoles . Series B (---) were calculated for p_a 2.97, p_c 1.12, a 0.698 for values of c of 100, 50, 10 and 1 μmoles .

Fig. 3. Experimentally determined countercurrent distributions of the total complex phospholipid fraction from rat-liver lipids. Samples (curves left to right) contained 156, 42, 22, 6.9 and 0.83 μ moles respectively. Tubes marked A, B and C in the sample containing 156 μ moles were combined as shown and used for the results shown in Table 2.

curves are somewhat broader than the theoretical distributions, indicating heterogeneity; this is discussed below.

DISCUSSION

Anomalous countercurrent distributions were described by Haussman & Craig (1952), Ahrens & Craig (1952) and Haussman & Craig (1958), who realized that this must be due either to the partition coefficient changing with concentration or to the presence of unresolved mixtures. Purified lecithin gives broader curves than are required theoretically owing to the presence of a number of compounds with different fatty acids (Collins, 1963), whereas the skewed distributions obtained with oleic acid by Ahrens & Craig (1952) were ascribed to mixtures of position isomers of oleic acid. Haussman & Craig (1958) obtained distribution curves that were narrower than those required theoretically; this was due to the interaction of high concentrations of the solute with a component of the solvent mixture. Ahrens & Craig (1952) distributed fatty acids and found skewed curves due to association, but on the addition of acetic acid to the solvent mixture the distribution became normal.

The partition coefficients of lecithin, lysolecithin and the methyl ester of dinitrophenylphosphatidylethanolamine were constant over a range of concentration, even though they were all mixtures, owing to the presence of a variety of fatty acids. All these compounds gave symmetrical distributions, although the curves were broader than the normal curve. A number of other phospholipids give

broad but symmetrical curves (Collins & Shotlander, 1961*a*; Collins, 1960*b*) and it is reasonable to assume that here also the partition coefficient is constant.

The complex phospholipid fraction showed a skewed distribution and the partition coefficient varied with concentration. When the empirical relationship (13) was used to calculate the distribution good agreement was obtained for the degree of skewness and dependence on concentration of the tube with maximum content. Thus it may be concluded that the skewed distribution is due to an association between two components. The complex phospholipids contain equal amounts of lecithin and dinitrophenylphosphatidylethanolamine (Collins, 1960*b*). The results given by Collins (1960*b*) showed that the dinitrophenylphosphatidylethanolamine in the complex fraction was different from the remainder of the dinitrophenylphosphatidylethanolamine; the complex phospholipids may therefore be an association between a phosphotriester of dinitrophenylphosphatidylethanolamine and lecithin. The complex phospholipids give skewed distributions representing an association and it follows that no further purification can be expected unless the association is prevented.

These conclusions about the nature of the complex phospholipids in solution have several consequences. Earlier work (Collins, 1959*a*) indicated that the partition coefficient of the rat-liver complex phospholipids was 2.5; this resulted by working with one rat liver in each experiment and thus always distributing approximately the same amount of material. Later work (Collins & Shotlander, 1961*a*) indicated that some preparations gave fractions whose partition coefficient was not 2.5, but only recently has the reason for these discrepancies become clear.

The complex phospholipid from sheep brain (Collins, 1959*b*) gave a nearly symmetrical curve on distribution in carbon tetrachloride-methanol-water; this indicates that no association or dissociation was taking place.

The comparison between the complex phospholipids of rat-liver mitochondria and microsomes by Collins & Shotlander (1961*b*), in which they conclude that 'the mitochondria lipids lack a component of the complex amino-phospholipids which is present in microsomes', may need modification. The two distributions shown in this paper were at different concentrations and part of the difference is due to the change in peak maximum with concentration. There may be significant differences but because of the dependence on concentration it is not possible to draw a clear conclusion at present.

In the particular case investigated above, eqn (13) held and this corresponds to the equation obtained by making certain simplifying assumptions. The

countercurrent distribution curve corresponding to this relation between the partition coefficient and concentration was skew with one side steeper than normal and the other less steep. In this example it was assumed that only one component of the equilibrium was measured and hence the term in p_b did not enter into this equation. This was because DNP groups and not phosphorus were measured but could also be the case if, for example, the second component was a proton or other small ion. Where both components were measured there are two possibilities. One is that both p_a and p_b are either greater or less than p_c and the other is that either $p_a < p_c < p_b$ or $p_a > p_c > p_b$. The first case will give the same type of skewed curve as in Fig. 2 and will be the sum of two distributions with their tails pointing in the same direction. The second case will correspond to the sum of two skew curves with their tails pointing in opposite direction. Where p_c is the mean of p_a and p_b the countercurrent distribution curve will be symmetrical but in the centre portion the slopes will be steeper than normal and the tails will be broader. This corresponds to the type of curve obtained by Haussman & Craig (1958).

No attempt has been made in this investigation to see if the general equations could give rise to any other types of curves; but it seems likely that all anomalous distributions may be explained in terms of a simple equilibrium $A + B \rightleftharpoons C$ and the corresponding partition coefficients p_a , p_b and p_c .

In view of the increasing range of materials such as protein and viruses (Bengtsson, Philipson & Albertsson, 1962) being examined by countercurrent distribution it may be important that the cause of anomalous distributions should be determined.

SUMMARY

1. The partition coefficients of several phospholipids, including lecithin, lysolecithin and phosphatidylethanolamine, were found to be independent of concentration.

2. The partition coefficients of the complex phospholipids were concentration-dependent and it

was concluded that this was due to an association of two components.

3. A mathematical analysis of the effect of an equilibrium on partition coefficients and on countercurrent distributions was made.

4. It was concluded that the two types of anomalous distributions were possible: (i) a skew distribution in which one side of the curve was steeper than normal and the other was less steep; (ii) a symmetrical curve in which the slopes in the centre portion were both steeper than normal.

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