The Application of Counter-Current Methods to the Fractionation of Lipid Material from Brain

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(Received 11 September 1952)

The fractionation of lipid mixtures by classical methods based on their differential solubilities in organic solvents is an elaborate and lengthy procedure, frequently yielding products of doubtful purity. For this reason alternative methods of purification were sought. Attempts to apply adsorption chromatography were not encouraging in our hands, as the separation was poor, and invariably about 25% of the material remained firmly adsorbed to the column, resisting elution by all the usual solvents. Partition chromatography on silica gel was unsatisfactory, as the phospholipins altered the equilibrium between stationary and mobile phases, and completely upset the columns.

Separation of lipids by the methods of counter-current distribution (Craig, 1944, 1950) seemed to avoid these difficulties. Partition between solvents is not complicated by a supporting medium. Recently, Lovern (1952) has applied the counter-current method to the fractionation of brain phospholipins.

The investigations reported here cover the development of a basic solvent system for the distribution of brain lecithin, kephalin, and mixed sphingomyelin and cerebrosides. This solvent system was then applied to the distribution of crude brain lipid, and modified for the study of certain fractions. The identity of the fractions obtained was then sought by various analytical methods, and from these results the positions of some of the more clearly defined lipids in the distributions have been indicated.

Part of this work has been presented at a meeting of the Biochemical Society on 24 October 1952 (Cole, Lathe & Ruthven, 1953a).

METHODS

Calculations. The methods of Williamson & Craig (1947), and of Nichols (1950), have been used for calculating theoretical curves, determining the relationship between distribution constants and the position of peaks, and for judging the effect of changing the proportion of phases. Distributions are uniformly charted in this paper with the polar plates to the left.

Solvents. With the exception of methanol, which has been of a grade which contained less than 0.05% acetone

(Burroughs Ltd.), methylene chloride which was 'laboratory grade', and carbon tetrachloride, which was 'pure' or 'redistilled', all solvents have been of A.R. standard, or of comparable purity. Ethers have been found to be peroxide free.

Preparation of crude brain lipid. 'Homogenized' adult human brain was dehydrated at room temperature by three consecutive 24 hr. extractions with acetone, using 1 l. of solvent/kg. of wet tissue at each extraction. The dehydrated residue, from which most of the neutral fat and cholesterol had been removed, was extracted three times with a mixture of 2 vol. of methanol and 1 vol. of CHCl_3 (2.5 l. of mixture/kg. of wet tissue). The CHCl_3 -methanol and acetone extracts were taken down separately under vacuum at less than 50°, and combined.

Preparation of brain lipid fractions

Ethanol-soluble, ethanol-insoluble, and ether-insoluble fractions of brain lipid, customarily called lecithin, kephalin, and mixed sphingomyelin and cerebrosides, respectively, were prepared. The method was adapted from that of Folch (1942) for the isolation of kephalins.

Lecithin. Minced brain tissue was dehydrated by homogenizing twice with acetone (3.8 ml./g. wet tissue). The extracts were discarded. After extracting the residue with ethanol (4 ml./g. tissue), the solvent was separated, and taken to dryness. The solvent residue was dissolved in a little light petroleum (b.p. 40–60°) and poured into a large excess of acetone. By suspending the precipitate in ether, filtering, and pouring the filtrate into a large volume of ethanol, an acetone-insoluble, ether- and ethanol-soluble lipid fraction (lecithin) was obtained.

Kephalin. The ethanol-insoluble residue of dehydrated brain was extracted twice with light petroleum (about 4 ml./g. tissue). Following concentration of the extracts under vacuum, the oily residue was suspended in wet ether (containing 1%, v/v, water), and centrifuged after standing 8 weeks at -12°. The ether-soluble portion was reduced in volume, and 5 vol. of ethanol were added. The acetone-insoluble, ether-soluble, ethanol-insoluble residue was regarded as kephalin.

Mixed sphingomyelin and cerebroside. Two preparations were used. Preparation 'a' was the white, ether-insoluble, material separated off in the isolation of kephalin.

Preparation 'b' came from crude methanol- CHCl_3 extract of brain. After concentration, a little ether was added, and the resulting milky mixture was poured into a large volume of wet ether (about 2 l./100 g. of original tissue) with mechanical stirring. The solution was kept at 0° overnight, the supernatant decanted, and the residue centrifuged. A layer of white, non-adhesive, precipitate formed above a thick

yellow oil in the bottom of the centrifuge tube. The precipitate was removed from the oil with a spatula, washed with ether, and used as preparation 'b'.

Distribution of lipid and isolation of fractions

All the counter-current distributions were made in the glass and polythene apparatus described by Lathe & Ruthven (1951). In this apparatus the lower phases move, and in the work reported here (except the system in Fig. 9) lower layer is always the non-polar phase.

The lipid to be distributed (usually 0.5–2 g.) was dissolved in the appropriate solvent system, using 20 ml. of each phase when the volume ratio was 1:1, and volumes of 10 ml. of one phase and 40 ml. of the other when the ratio was 1:4. At each plate of the distribution the two phases were equilibrated by 50–60 inversions in 1 min. The contents of those few tubes which emulsified were drawn off, centrifuged, and the phases placed in the appropriate tubes.

Distributions were expanded to 49 plates (tubes), unless otherwise stated, after which both phases of each tube were removed together by suction, via a narrow polythene tube passed through a side arm. The solvent from each tube was then reduced in volume at low pressure and at a temperature below 50°, and the residue was dissolved in a minimum amount of methanol-ether or methanol-CHCl₃, as appropriate, and transferred to a tared 25 ml. wide-mouthed, screw-capped bottle. The contents were taken almost to dryness by warming under a stream of N₂, and then dried overnight in a vacuum desiccator over H₂SO₄. The bottles were capped after reweighing.

Treatment of individual fractions

Hydrolysis. The individual lipid fractions, each representing a single plate of a distribution, were hydrolysed separately. To the lipid (usually 10–100 mg.) in each wide-mouthed bottle were added 3 ml. of methanolic HCl (2 ml. of 11 N aqueous HCl added to 8 ml. methanol). The fifty bottles from one distribution were placed in a large Pyrex desiccator, equipped with a reflux condenser, and containing another 200 ml. of methanolic HCl. The whole apparatus was mounted on a steam bath, and its contents refluxed at 70–80°. After 4 hr. the methanolic HCl was removed with gentle suction, and the drying completed *in vacuo* over KOH.

Desalting. The apparatus was essentially the same as that described by Consden, Gordon & Martin (1947), except that 0.1 N-H₂SO₄ flowed through the anode compartment continually.

About 100 mg. of lipid hydrolysate, dissolved or suspended in 2 ml. of a mixture of equal parts of methanol and water, were desalted with rectified mains current. The current was measured and a resistance in series was adjusted to maintain the current at approximately 250 ma. The desalting was continued for about 10 min. after the current had reached 50–70 ma., with no resistance in series. If the current did not fall desalting was continued for about 1 hr. at 700–800 ma.

The desalted solutions were taken to dryness under vacuum and dissolved in the minimum of 50% (v/v) aqueous methanol ready for application to paper for chromatography.

Paper chromatography. Ascending and descending chromatograms were carried out using no. 4 Whatman paper, specially prepared for chromatography. The liquid

front in the ascending chromatograms was allowed to run for about 30 cm., and in the descending about 40 cm. The amount of hydrolysate put on the paper was usually equivalent to 1 mg. of original lipid; 20–40 µg. of standards were applied.

Paper chromatograms were run in three different solvent systems as follows (all percentages are v/v unless stated otherwise): (1) The top phase of a system containing 40% *n*-butanol, 10% glacial acetic acid, and 50% water (Hanes & Isherwood, 1949). (2) A homogeneous system of 60% *n*-butanol, 20% morpholine and 24% water, a system modified from that of Levine & Chargaff (1951). (3) A single-phase system containing 80% *tert*.-butanol, 20% water and 4% (w/v) picric acid (Hanes & Isherwood, 1949).

The first two solvent systems were used for bases and amino-acids. Spots were stained by dipping the air-dried papers in acetone containing 0.25% ninhydrin, and allowing them to dry at room temperature. In the butanol-morpholine system choline showed up as a white unstained spot against a faint pink background.

All three solvent systems were used to identify inorganic phosphate and phosphate esters. Phosphate spots were stained by spraying with a molybdate reagent, heating to 85° for about 5 min., exposing to steam for a few minutes, and leaving for several hours. The reagent, adapted from that described by Hanes & Isherwood (1949), was prepared by mixing 2.1 ml. of 72% (w/w) HClO₄, 1.0 ml. of 5 N-HCl, and 10 ml. of 5% (w/v) ammonium molybdate, and making up to 100 ml. with ethanol.

Quantitative estimation of constituents

Nitrogen, cholesterol, phosphorus. Lipid samples in methanol-CHCl₃ (2:1) were taken for estimation. After Se-H₂SO₄ digestion, N was estimated by the use of Nessler's reagent (King, 1946). Cholesterol was determined by the Liebermann-Burchard reaction, as described by King (1946). P was determined after HClO₄ digestion, as the reduced phosphomolybdate complex (King, 1946).

Galactose. The method employed was that described by Brand & Sperry (1941), with the exception that a sulphonated aliphatic wetting agent (Teepol) was used to emulsify the lipid, bromothymol blue served as the acid-base indicator, and ferroin-*o*-phenanthroline as the oxidation-reduction indicator. The method was checked with the stipulated controls and the calculations were based on recoveries of known galactose solutions, which averaged 87%. In view of the instability of galactose to alkali, parallel hydrolyses were done in 6 N-NaOH, instead of 6 N-HCl, to increase the specificity of the method.

Iodine numbers. A sample containing 3–5 mg. of lipid dissolved in 5 ml. CHCl₃ was estimated by the micromethod of Yasuda (1931), based on the method of Rosenmund & Kuhnnehn (1923), using pyridine sulphate dibromide reagent.

EXPERIMENTAL

Solvent systems

Emulsion formation, which is frequently troublesome in lipid distributions, was largely overcome by using a system in which the water content was low, and the difference between the specific gravities of the two phases was large. By mixing CCl₄ (62 vol.), methanol (35 vol.) and water (3.15 vol.) such a system was obtained. It divided into

Table 1. *Composition of solvent systems for counter-current distributions*

(Figures in the body of the table are volumes of individual components required to prepare system; LP = light petroleum, b.p. 40–60°.)

System	A	B	C	D
Volume ratio of upper to lower phase	1/1	1/4	1/1	1/1
Components	CCl ₄ 62 MeOH 35 H ₂ O 3.15	CCl ₄ 62 MeOH 35 H ₂ O 3.15	LP 31 CCl ₄ 31 MeOH 35 H ₂ O 3	CCl ₄ 25 CHCl ₃ 15 CH ₂ Cl ₂ 10 MeOH 40 H ₂ O 10

approximately 60 vol. of an upper phase and 40 vol. of a lower phase. From the specific gravities, and by an empirical determination of the amount of water necessary to divide the phases as above, the following percentage composition (v/v) was calculated:

	CCl ₄	MeOH	H ₂ O
Upper phase	46	54	4.75
Lower phase	88.5	11.5	0.75

This system is referred to as system *A* (Table 1) when the phases were of equal volume, and as system *B* when the volume of the lower phase was 4 times that of the upper. Other systems were devised from system *A* by raising the polarity through the addition of CHCl₃ and methylene chloride (system *D*, Table 1), or by lowering it through the addition of light petroleum (system *C*).

Distribution of brain-lipid fractions

As an aid in the interpretation of weight curves obtained from the distribution of crude brain lipid, preliminary distributions of crude brain fractions of lecithin (0.8 g.), kephalin (0.8 g.) and preparation 'b' of sphingomyelin and cerebroside (0.9 g.) were made in system *A*.

These distributions (Fig. 1a) showed that the kephalin fraction was largely polar (tubes 0–15), sphingomyelin and cerebroside were mainly in the tubes of intermediate polarity (16–33), while lecithin had peaks in both regions. The only very non-polar material occurred in the kephalin fraction, and was small in quantity. Preparation 'a' of sphingomyelin and cerebroside showed a polar fraction (Fig. 1b) in addition to the one of intermediate polarity found in preparation 'b' (Fig. 1a). This polar material may be due to the heavy yellow oil which was excluded from 'b'.

Since many overlapping fractions were present in the polar tubes, the material was redistributed in a system in which the lipid would be placed more centrally, where the degree of separation of solutes is maximal (Williamson & Craig, 1947). This could be done either by using a more polar solvent (system *D*) or by changing the ratio of phases (system *B*).

A distribution of tubes 0–18 (Fig. 1a) of kephalin in system *D* is shown in Fig. 2a. A relatively small shift of the fractions towards the higher-numbered tubes resulted. A similar result (Fig. 2b) was obtained when the polar fractions of preparation 'a' of sphingomyelin and cerebroside (tubes 0–11, Fig. 1b) were distributed in this system. Both distributions have peaks at tubes 3 and 9, but while the peaks are approximately equal in kephalin (Fig. 2a), the less polar was much more prominent in the distribution of

sphingomyelin and cerebroside. The polar fractions of lecithin could not be run in this solvent system because of emulsions.

Fig. 1. (a) Distributions (49 transfers) of lecithin (—), kephalin (.....), and preparation 'b' of sphingomyelin and cerebroside (---) in system *A*. (b) Distributions (39 transfers) of preparation 'a' of sphingomyelin and cerebroside in system *A*.

In order to avoid emulsification of these polar fractions the alternative procedure of varying the phase ratio (system *B*) was tried (Fig. 3). Although many fractions are common to the three curves some characteristic differences are found. In general, lecithin is most polar, kephalin less so

Fig. 2. Distributions in system D. (a) The polar part of cephalin (tubes 0-18 of Fig. 1a). (b) The polar part of preparation 'a' of sphingomyelin and cerebroside (tubes 0-11 of Fig. 1b).

Fig. 3. Distributions of the polar parts of lecithin (tubes 0-13 of Fig. 1a) (—), cephalin (tubes 0-18 of Fig. 1a) (....), and preparation 'a' of sphingomyelin and cerebroside (tubes 0-18 of Fig. 2b) (---) in system B.

and sphingomyelin and cerebroside least of the three. The non-polar material (tubes 45-49), present in all the distributions, may be due to decomposition products.

To increase the separation of the fractions of intermediate polarity (tubes 16-33) in system A, either more transfers or a new solvent system were needed. System A was therefore made less polar, by adding light petroleum. In a distribution, which is not reported in detail, this resulted in the splitting of the central fraction of lecithin, and led us to develop system C (Table 1), which was used later for the distribution of brain fractions of intermediate polarity.

Distribution of crude brain lipid

The scheme of fractionation, developed for the brain fractions, was applied to 2.9 g. of crude brain lipid. Some cholesterol, which crystallized out of the acetone solution in the cold (roughly one-third of the total) was inadvertently omitted from this distribution. The preliminary distribution of the lipid in solvent A (Fig. 4a) showed division into three main groups as follows:

Character	Tubes	% of whole
Very polar	0-4	15
Polar	5-17	15
Intermediate polarity	18-40	70

To secure better resolution of the polar fractions (tubes 0-17) these were redistributed in solvent B (Fig. 4c). The very polar fractions (tubes 0-4) were then found in tubes 0-8, and those that were previously in tubes 5-17, in one group, were distributed in two groups of fractions, 8-25, and 26-44.

Brain lipids of intermediate polarity in system A (Fig. 4a, tubes 18-40) were combined in two groups (tubes 18-27, and 28-40) and redistributed separately in the same system for 24 transfers (Fig. 4b₁ and 4b₂). The expected non-homogeneity of this material was confirmed by the difference in the two curves obtained, the more polar materials (curve b₁) having a skewed peak at tube 12 while the less polar (curve b₂) had a peak at 15.

At this point an unsuccessful attempt was made to separate the components of part of curve 4b₁ (tubes 0-15) by means of a new solvent system containing ethyl acetate. No distribution curve is shown since no increased separation was achieved, although this solvent displaced the lipid toward the non-polar end of the graph. The material was combined again, as also were tubes 8-24 of curves 4b₂, and the two distributed separately in system C (Fig. 4d).

System C provided better resolution. Four main groups of fractions were distinguishable, namely tubes 0-5, 6-26, 27-42, and 43-49. It was in keeping with the original source of the materials in these two distributions (i.e. tubes 18-27 and 28-40 of Fig. 4a) that the two more polar fractions should predominate in one distribution (d₁), and the less polar fractions in the other distribution (d₂). As will be seen later the less polar peaks occur in the positions of cholesterol and neutral fat.

Characterization of counter-current fractions from brain lipid

Chemical analyses, as well as comparison with the previously described counter-current distributions of brain lecithin, cephalin, and sphingomyelin and cerebroside, were used to identify the various fractions. The brain lecithin, cephalin, and sphingomyelin and cerebroside were

prepared after acetone precipitation, and therefore would not be expected to contain cholesterol, cholesterol esters, or neutral fat.

Neutral fat. The distribution constants of neutral fats in system *A* are approximately 0.1 (Cole *et al.* 1953*b*), from which it was calculated that they would be found with a peak in tubes 45–47, after 49 transfers. In Fig. 4*a* only a small amount of material is present in this position. This is in agreement with the low neutral fat content of brain.

present in tubes 7–49) a similar distribution was found for cholesterol in system *C* (Fig. 6*b*), the main bulk being in tubes 26–42. The small amount in this region in Fig. 6*a* is consistent with the manner in which this material was derived from the original distribution in system *A* (Fig. 4*a*).

Galactose and carbohydrate. Semi-quantitative Molisch tests showed that carbohydrate was present in tubes 24–30, in the distribution in system *A* (Fig. 4*a*), while galactose, or other alkali-labile reducing substances, were found in tubes

Fig. 4. (*a*) Distribution of crude brain lipid in system *A*. (*b*₁) Distribution (24 transfers) of central fractions of crude brain lipid (tubes 18–27 of Fig. 4*a*) in system *A* (—). (*b*₂) Distribution (24 transfers) of central fractions of crude brain lipid (tubes 28–40 of Fig. 4*a*) in system *A* (.....). (*c*) Distribution of the polar fractions of crude brain lipid (tubes 0–17 of Fig. 4*a*) in system *B*. (*d*₁) Distribution of central fractions of crude brain lipid (tubes 0–15 of Fig. 4*b*₁) in system *C* (—). (*d*₂) Distribution of central fractions of crude brain lipid (tubes 8–24 of Fig. 4*b*₂) in system *C* (.....).

Cholesterol. The partition coefficient of cholesterol in system *A* was found to be 0.40. This was obtained by equilibrating 40 mg. between 10 ml. of each phase, separating the phases, evaporating them, and weighing the residue. Cholesterol was calculated to give a peak at tube 35, in a distribution of 49 transfers.

Cholesterol was not determined in tubes 20–40 of the distribution of crude brain lipid in system *A* (Fig. 4*a*) owing to the omission, as mentioned previously, of some of the cholesterol. Instead, estimations were made on a parallel distribution which included all the cholesterol. The values found (Fig. 5) indicated that most of the cholesterol was in tubes 27–39 with a peak (70–80% cholesterol) at tube 35. This agrees with the position calculated from the partition coefficient. Although rather more spread (a trace was

25–33 with a peak at tube 29 (Fig. 5). This is considered to be the peak of cerebrosides, and this position is in good agreement with that of the central peak of the distribution of the ether-insoluble brain fraction (Fig. 1*a*). Another peak of alkali-labile reducing substances was found in the very polar tubes (Fig. 5).

Nitrogen and phosphorus contents. As most of the phospholipins have similar P contents, the usefulness of P analyses in distinguishing one phospholipin from another is limited, and the curve of P content serves merely to indicate the occurrence of non-phospholipin material. The N content of phospholipins is more variable, ranging, among mono-aminophospholipins, from 1.58% (phosphatidylserine) to 2.03% (phosphatidylethanolamine). Diaminophosphatides contain about 3.5% N, cerebrosides, 1.75% N.

The amount of N and P was determined in each tube of the distribution of crude brain lipid in system *A* (Fig. 4*a*). For comparison with the weight curve the P figures have been recalculated as phosphatides, assuming an average content of 3.85% P, and then expressed in the usual way, in curve *B* of Fig. 7, as a percentage of the whole weight of lipid. Curve *A* of Fig. 7 is the weight curve of non-cholesterol lipid, obtained by subtracting the cholesterol of each tube from the lipid in that tube. In the same way, the non-cholesterol, non-phospholipin material was found by subtracting curve *B* from *A*, and is shown as curve *C*. Curve *D* is the cerebroside fraction calculated from galactose.

Fig. 5. Distribution of crude brain lipid by weight (—) in system *A*, showing the position of cholesterol (---), alkali-labile reducing substances (----) and the iodine number of the non-cholesterol material (.....). (Bases are shown as follows: amino-acids (AA), ethanolamine (E), choline (C), serine (S).)

In the crude brain lipid distribution in system *A* (Fig. 7) the most polar tubes (0–5) were low in P. They were also very high in N content (12.8% in tube 3). In tubes 6–16 the phospholipin and non-phospholipin curves showed that there were at least two groups of material. In the more polar region (tubes 5–9) the P content was lower than that of phosphatides, while the N was substantially higher (in tubes 5, 7 and 9; 4.3, 3.5 and 2.0%, respectively). In the less polar part (tubes 10–14) the P content is consistent with the presence of phosphatidylserine. The N content was approximately 1.9%.

In the centrally placed fractions (tubes 17–35 of Fig. 7) it was necessary to account for the positions of lecithin, and sphingomyelin and cerebroside. The non-cholesterol, non-phospholipin curve (*C*) had two peaks in this region, at tubes 25 and 29. That at tube 29 coincides with the position of cerebroside (Fig. 7, curve *D*) but the peak at tube 25 has not been explained. The phospholipin curve (*B*) included both lecithin and sphingomyelin and it had two peaks at tubes 22 and 27. Sphingomyelin, a diaminophospholipin, might be distinguished from lecithin by the fact that, compared with lecithin, there is an extra atom of N for every atom of P. However, cerebroside also leads to excess N, since it is not included in the calculation of the phospholipin curve. This difficulty is overcome by expressing the N that is excess to P, as a percentage of the non-phospholipin, non-cholesterol material. Between tubes 26 and 32 the N

content of this material varied from 2.05 to 1.5%, with an average close to the N content of cerebroside. However, in tube 21 the same calculation leads to an N content of 4.9%. This is, of course, only an apparent N content since it is due to the excess N of a phospholipin the weight of which has already been taken into account in curve *B*. The high apparent N content of the material under curve *D* in the region of tube 21 suggests that this phospholipin peak is due to sphingomyelin.

Fig. 6. (a) Distribution of central fractions of crude brain lipid (tubes 0–15 of Fig. 4*b*₁) in system *C* by weight (—), showing the position of phospholipin (---) and non-phospholipin (.....). (b) Distribution of central fractions of crude brain lipid (tubes 8–24 of Fig. 4*b*₂) in system *C* by weight (—), showing the position of phospholipin (---), cholesterol (---) and non-phospholipin, non-cholesterol material (.....).

In order to confirm the relative position of these lipids a similar calculation was made for the curve of distribution in system *A* of preparation 'b' of mixed sphingomyelin and cerebroside (Fig. 8). As in the distribution in Fig. 7 the presence of sphingomyelin is indicated by a rise in the apparent N content (curve *D*) of the non-phospholipin material, in the region of tube 20. The two peaks of the non-phospholipin curve (*C*) are also in comparable positions to those of Fig. 7.

The effect of redistributing the more centrally placed materials of crude brain lipid (Fig. 4*b*) in solvent system *C* is

shown in Fig. 6a and b. Curves have been drawn for phospholipin ($P \times 100/3.85$), cholesterol, and non-cholesterol, non-phospholipin material. Apart from the cholesterol, most of the material (previously tubes 17–40) was displaced to the more polar end (tubes 8–25). The non-phospholipin material was fairly widely spread. The identification of individual fractions was not attempted.

Fig. 7. Recalculation of the distribution of crude brain lipid in system A (Fig. 4a) showing (A) the non-cholesterol lipid (—), (B) phospholipin (---), (C) non-cholesterol, non-phospholipin (.....) and (D) cerebroside (-·-·-·).

Fig. 8. Distribution of the central fraction of preparation 'b' of sphingomyelin and cerebroside in system A by weight (—), showing (B) phospholipin (---), (C) non-phospholipin (.....) and (D) the apparent N_2 content of the non-phospholipin material (-·-·-·).

Degree of unsaturation. It appeared that some indication of the relative distribution of unsaturation could be obtained from iodine numbers, even though all of these would be depressed to some extent by oxidation. Alternate tubes of the distribution of crude lipid in system A (Fig. 4a) were examined and after subtracting the iodine uptake due to cholesterol, the iodine number of the non-cholesterol material was obtained, and is shown in Fig. 5. The most polar tubes (0–4) were the most saturated, tubes 6–15 were the most unsaturated, while those of the central peak (tubes 20–30) were of intermediate character.

Identification of bases. Hydrolysed, desalted lipid fractions were examined for the presence of bases by paper chromatography. Their occurrence in the distribution of crude brain lipid in system A (Fig. 4a) is shown in Fig. 5. Glutamic acid,

alanine, threonine (Fig. 5, AA) and serine were identified. Other ninhydrin-staining spots which occurred have not been identified, but they may include histidine, leucine or isoleucine, and valine. During the course of the desalting it was observed that in tubes 0–4 and 18–34 the resistance of the solutions failed to increase, and the solutions became distinctly alkaline. This may be attributed to the presence of choline in tubes 18–24, where it was demonstrated chromatographically. However, no choline was formed in tubes 0–4, and the maintenance of conductivity is unexplained.

Fig. 9. Distribution (19 transfers) of 'sphingosine' base in solvent system of light petroleum:ether:methanol:water (2:2:3:1, v/v) showing weight (—), theoretical curves (---) and the sum of three theoretical curves (.....).

An effort was made to identify sphingosine chromatographically, for which purpose a standard was prepared by the method of Carter, Norris, Glick, Phillips & Harris (1947). The final product was not recrystallized but subjected to a counter-current distribution in a mixture of 25% light petroleum, 25% ether, 37.5% methanol and 12.5% water (all percentages v/v). However, instead of one main component, three were obtained, as shown in Fig. 9. The matter was not pursued further.

Chromatograms stained for phosphorus. Inorganic P and glycerophosphate were detected in all tubes of the distribution of crude brain in system A (Fig. 4a) except in the cholesterol area, and in higher-numbered tubes. In tubes 0–4 inorganic P was much more prominent than glycerophosphate, implying the presence of free phosphate, or of a more acid-labile ester than glycerophosphate. Only glycerophosphate was found in tubes 5–15, while in tubes 16–30 phosphoryl choline or phosphoryl ethanolamine was detected. The greater concentration of choline in this area left little doubt that these spots were due to phosphoryl choline.

DISCUSSION

Our experiments show that much further work on the counter-current distribution of lipid mixtures is necessary to establish the behaviour of pure lipid components and to reveal the differences between fractions that run close to each other. Nevertheless, from the behaviour of lecithin, kephalin, and mixed sphingomyelin and cerebroside, together with the

analyses of fractions obtained in crude brain lipid distributions, a comprehensive picture can be drawn of the relative positions of a number of recognized lipids. These are summarized in Table 2, and the position of their peak tubes in the various solvent systems is estimated.

Table 2. *Estimated position of lipid components in a 49-plate distribution*

	Solvent system		
	A	B	C
(Estimated tube numbers of the peak tubes)			
Lipid components			
Neutral fats	45	—	—
Cholesterol	35	—	36
Cerebroside	29	—	17
Lecithin	27	—	15
Sphingomyelin	21	37	10
Phosphatidyl serine	4-16	10-30	—
Phosphatidyl ethanolamine	4-16	10-30	—
Lecithin	2	—	—
Amino-acids	0	—	—

The most polar tubes (0-5) of the distribution of crude lipid in system *A* (Fig. 4*a*) were remarkable for the low phosphorus and high nitrogen content, the presence of several amino-acids, and of alkali-labile reducing material. The behaviour of these fractions during desalting suggested that a strong base was present, though choline did not appear in the chromatograms. After hydrolysis inorganic phosphate predominated over glycerophosphate. It may be assumed that much of this material is extracted in non-lipid form, though possibly as lipoproteins. Lecithin probably contains much of this diversified material (Fig. 1*a*).

It was anticipated from the distributions of Fig. 1*a* that kephalins would predominate in tubes 5-16 (Fig. 4*a*). The presence of ethanolamine and serine (Fig. 5) confirmed this. The distribution of these bases in the paper chromatograms suggested that in system *A* phosphatidylserine and phosphatidylethanolamine overlapped to a great extent. The position of inositol-containing lipid, possibly diphosphoinositide of Folch (1949), may be inferred from estimations of inositol made on tubes 2, 6, 9 and 14, of the kephalin distribution in system *B* (Fig. 2*a*) by Dr G. H. Sloane-Stanley. All of these tubes contained inositol, tube 6 being the peak. The position of the inositol lipid is thus not dissimilar to that of phosphatidyl ethanolamine and serine. That other materials were present in tubes 5-16 of Fig. 4*a* was demonstrated by the phosphorus analyses (Fig. 7) which showed that phosphatide could account for approximately a third of the material in the polar half of this peak, and most of it in the less polar half. The character of the non-hospholipin material remains undetermined. The

highest iodine numbers occurred in the middle of this curve (Fig. 5).

Approximately half of the non-cholesterol lipid in crude brain is distributed centrally in system *A*. The relative positions of sphingomyelin, lecithin and cerebroside, in order of decreasing polarity, have already been inferred from the nitrogen and phosphorus analyses, and the position of reducing substances. It is not surprising that lecithins are associated with sphingomyelin and cerebroside, since Thannhauser, Benotti & Boncoddio (1946) have demonstrated that ether precipitates saturated lecithin. The iodine numbers of the lipids in this part of the central peak are found to be lower than for the kephalins (tubes 5-16). The occurrence of some ethanolamine in the chromatograms of tubes 28-32 (Fig. 5) and of serine in tube 30 remains unexplained. The only other base found throughout the whole area was choline. The identification of sphingosine was not attempted because of the apparent complexity of the material found in a distribution of one preparation of the base. The remaining material in the central peak is largely cholesterol. Neutral fat is almost absent from brain lipid.

Recently, Lovern (1952) has distributed crude ox-brain glycerophosphatides, and crude phosphatidyl choline, ethanolamine, and serine in a solvent system of aqueous (85-90%) ethanol-petroleum ether. Lovern's system probably resembles our systems *B* or *D*. It would be anticipated then that any material which corresponded with our central peak in system *A* (Fig. 4*a*) would accumulate in the non-polar end of his distributions. Much of this material would have been removed by the ether precipitation to which his phosphatide was submitted. In our experience (see lecithin in system *A*, Fig. 1*a*) some relatively non-polar material (tubes 20-30) escapes this treatment. Lovern's distribution of crude glycerophospholipin has some choline-containing fractions in this region (Lovern, 1952; Fig. 2, fractions 15-20) but this accounts for only 4-5% of the total phosphatide material by weight and a very small fraction of all the choline.

The other place where this material of intermediate polarity would be expected to appear in Lovern's studies is in the non-polar tubes of his lecithin distribution. Although the graph (Lovern, 1952; Fig. 3) is not complete in this region, about 95% of the weight seems to have been accounted for. The impression is left that Lovern's crude lecithin does not contain much material of the type that occurs in the central tubes of our distribution in system *A* (Fig. 1*a*). Most of the choline in Lovern's fractions is distributed toward the polar end of the graph, and most of the weight of his phosphatidylcholine is in this area. In our distributions of

lecithin and of crude brain there are considerable amounts of material (by weight) in this region. Our lecithin fractions have not been examined for choline, and the chromatograms of crude lipid in system A (Fig. 5) do not reveal choline in the very polar region, though from the behaviour during desalting it was concluded that a strong base was present. Choline might have been diluted out by other materials, present in larger amounts, in our crude brain lipid. Nevertheless, the sum of evidence suggests that Lovern's material differs substantially from ours, or alternatively that the lipids behave differently in the two solvent systems.

Regarding ethanolamine and serine phosphatides, Lovern's distributions (Lovern, 1952; Figs. 1 and 6) show, like our own (Fig. 5), that these two kephalins behave very similarly, with much overlapping of lipid fractions.

It was thought at the outset of this work that among the phosphatides the bases would be the main factor determining the distribution between solvent phases. This may be true when phosphatidylcholine is contrasted with phosphatidyl serine and ethanolamine. Among the kephalins, however, other factors such as the degree of unsaturation of the fatty acids, and length of the aliphatic chain may

be equally important. Moreover, we have some evidence that complex formation may occur between one lipid and another when their concentrations are high. In this circumstance two lipids may tend to move together and only separate at greater dilutions.

SUMMARY

1. An emulsion-resistant solvent system of water-methanol-carbon tetrachloride has been devised for the counter-current fractionation of brain lipids.

2. The behaviour of brain kephalin, lecithin, and mixed sphingomyelin and cerebroside has been described.

3. Modifications of the basic solvent system, for the fractionation of more polar lipids and for the separation of cholesterol and neutral fats from other lipids, are presented.

4. The relative positions of various known compounds in the counter-current distribution of crude brain lipid have been indicated.

We are grateful to Dr W. E. Hunt for securing the brain for us. Dr C. E. Dent kindly provided us with a reference sample of phosphoryl serine. Our colleagues, in particular Dr J. Murray, have rendered assistance in more ways than can be specified.

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A Nomogram for Ammonium Sulphate Solutions

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(Received 28 October 1952)

In the purification of enzymes and other proteins it is frequently necessary to fractionate with ammonium sulphate by collecting the fraction which is precipitated on passing from one given percentage saturation to another. One is then faced with the problem of calculating how much solid ammonium

sulphate must be added to a given volume of a solution of a known degree of saturation to bring it to a certain desired degree of saturation.

Such a calculation from the physical tables is distinctly laborious, owing to the form in which the data are given. The main difficulty is caused by the