SYMPOSIUM: SPECIAL METHODS IN LIPID CHEMISTRY

conducted by The American Oil Chemist's Society at its 54th Annual Meeting, Atlanta, Georgia April 22-24, 1963

GEORGE ROUSER, PRESIDING
E. J. DROBKA, PROGRAM CHAIRMAN

Fractionation of Lipids by Countercurrent Distribution

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Abstract

The application of countercurrent distribution to the fractionation of lipids, both for analytical and preparative purposes, is presented. The method has been applied to the separation of a wide variety of lipids including: fatty acids, methylesters of fatty acids, glycerides, phospholipids, derivatives of phospholipids, complex lipid mixtures and non-lipid contaminants.

Introduction

ONE OF THE basic problems of the research worker dealing with natural products is that of the isolation and purification of individual compounds. A method which offers a great deal of promise, yet whose possibilities in the field of lipid chemistry have by no means been exploited to the fullest extent, is countercurrent distribution. In countercurrent distribution a mixture of substances is fractionated by repeated partition or distribution between two immiscible liquids. The separation of the mixture into its components depends upon the differences which exist in the differential solubility of the individual chemical compounds when distributed between the immiscible solvents. The law, first clearly realized by Nernst (1), states that a solute dissolved in one phase in equilibrium with another immiscible phase will distribute itself between the two phases so that the ratio of the concentrations in the two phases is a constant at a fixed temperature. This constant is the partition coefficient and is defined by the equation: $C_1/C_2 = K$ —where C_1 and C_2 are the concentrations of the given substance in the upper and lower solvent layers respectively.

A single pure substance behaving ideally in a given system will give a characteristic and reproducible distribution of mass throughout the cells. Since this distribution will depend only on the number of transfers applied and the partition coefficient, the distribution to be expected can be calculated. One of the most important advantages of this technique is that it lends itself to mathematical analysis. This makes it possible to obtain both quantitative and qualitative data from a weight distribution curve. The types of mechanical apparatus used in countercurrent work and the mathematics involved in the analysis of results have been adequately described (2–14).

The application of countercurrent distribution to problems in lipid chemistry is becoming increasingly apparent. Liquid-liquid extractions are relatively mild processes and since they can be easily carried out in an inert atmosphere (under nitrogen) they are quite adaptable to the study of unstable lipids. A second advantage, from a quantitative point of view, is that manipulative losses are negligible since all of the material introduced into the apparatus is recovered. This is a definite advantage over those chromatographic methods using strong adsorbents where irreversible adsorption or chemical change may take place. Reviews have appeared in the past on the application of countercurrent distribution to lipids (15,16). This presentation will emphasize primarily the more recent developments. However, reference to some earlier work will be made in order to place these new applications in the proper perspective.

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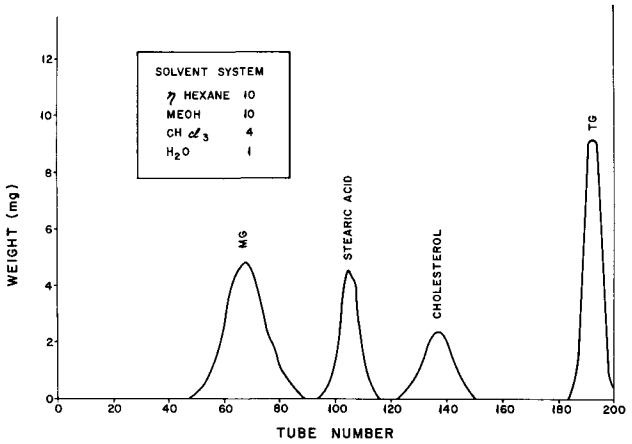

FIG. 1. Countercurrent Distribution of Model Compounds; MG = monoglyceride, TG = triglyceride.

Distribution of Fatty Acids

Countercurrent distribution for the separation of fatty acids was first investigated by Barry, Sato, and Craig (17, 18). As a result of their early work, a solvent system was developed in which the normal saturated fatty acids up to C_{18} had partition coefficients sufficiently different from one another to permit their separation by countercurrent distribution. However, skewing of the various peaks interfered with the quantitative aspects of the distribution. Skewing is a non-ideal behavior which results when the partition coefficient is non-linear. Since higher fatty acids have a considerable tendency to associate and form micelles, the skewing was ascribed to the tendency of the solute to exist in solution as a mixture of associated molecules. In order to prevent associations, Ahrens and Craig later developed a solvent system containing more acidic solvents in which the partition coefficients of the higher fatty acids were largely independent of concentration (19). The two-phase system formed by mixing n-heptane, glacial acetic acid, formamide and methanol (3:1:1:1) was used to quantitatively separate the homologous series, lauric, myristic, palmitic, and stearic acids in 400 transfers. The unsaturated series, oleic, linoleic, and linolenic acids were also separated in 650 transfers. The partition coefficients of fatty acids are influenced by unsaturation as well as by chain length. The effect of increasing the unsaturation by one double bond is nearly equal to decreasing the chain length by two methylene groups. More recently, Will (20) had considerable success in

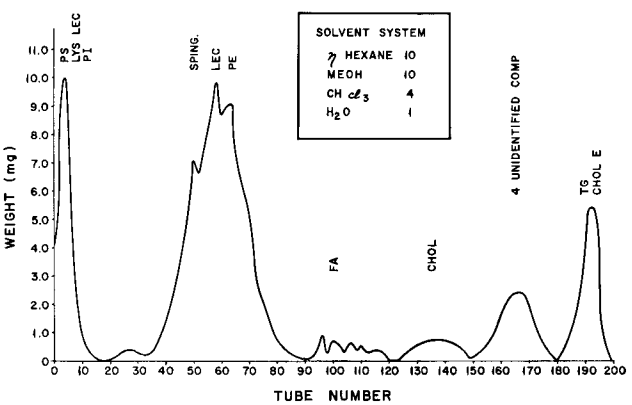

FIG. 2. Countercurrent Distribution of Rat Liver Lipids: PS = phosphatidylserine, Lys Lec = lysolecithin, PI = inositol-phosphatide, Sping = sphingomyelin, Lec = lecithin, PE = phosphatidylethanolamine, FA = fatty acid, Chol = cholesterol, TG = triglyceride, Chol E = cholesterol ester.

the fractionation of fatty acids employing a system consisting of petroleum ether and dimethyl sulfoxide-1-octanol.

Distribution of Methyl Esters of Higher Fatty Acids

Cannon, Zilch, and Dutton reported the use of a nitromethane-nitroethane, pentane-hexane system for the separation of methyl esters of fatty acids (21). They showed that as with fatty acids, the methyl esters can be separated on the basis of chain length or degree of unsaturation. They also demonstrated that the introduction of a hydroxyl or a hydroperoxide group into the chain affected the partition coefficient.

In the isolation and purification of fatty acids and their methyl esters, countercurrent distribution not only has the advantage of high resolving power under extremely mild conditions, but also a comparatively large sample capacity. This technique makes it possible to separate unchanged the naturally occurring isomers of unsaturated acids in quantities sufficiently large for laboratory use. This is in distinction with chromatographic methods which appear to be effective in separating unsaturated fatty acids, but have small capacity. Scholfield, Nowakowska, and Dutton have recently described the use of a system consisting of acetonitril and pentane-hexane for the analytical counter-current distribution of methyl ester mixtures as well as the preparative separation of methyl esters of pure fatty acids (22). Stoffel and Ahrens (23) also made use of the difference in partition coefficients due to unsaturation of fatty acid methyl esters. With a solvent system consisting of n-heptane, methanol, acetonitril, and acetic acid (3:1:1:1), they were able to separate the unsaturated fatty acids in the C_{18} and C_{20} series of menhaden body oils. Sufficiently large amounts of the monoene, diene, triene, tetraene, and pentaene isomers were obtained for complete analyses.

Distribution of Glycerides

Dutton and Cannon (24) found countercurrent distribution to be far more effective than other available methods for the fractionation of triglycerides, particularly the more highly unsaturated oils. When this technique was applied to the glycerides of linseed oil, pure trilinolenin and linoleodilinolenin were isolated. More recently Scholfield et al. have applied the technique successfully to soybean oil (25), safflower oil (26), corn oil (27), and cocoa butter (28).

Zilch and Dutton separated mono from diglycerides by countercurrent distribution between hexane and 80% ethanol (29). Mattson applied this system to the isolation of monoglycerides from the lumen of the intestinal tract of rats (30). Lovern and Olley (31) used a similar system for the separation of phospholipid from neutral lipid of fish. Perry et al. (32) studied the distribution of pure monoglycerides, a diglyceride and a diglycerol monoester and pointed out the usefulness of this technique as an analytical tool and as an aid in characterization of monoglycerides.

Fractionation of Complex Lipid Mixtures

The application of countercurrent distribution to the fractionation of complex lipid mixtures found in total lipid extracts of tissues was investigated in this laboratory. An all glass, Craig 500-cell countercurrent distribution apparatus equipped with a robot for automatic operations, was used. The two-phase solvent system consisted of n-hexane, methanol, chloroform, H_2O in the ratio of 10:10:4:1 by volume. After 200 transfers, the entire content of each tube

was transferred to small aluminum dishes which had been pre-dried to constant weight in a vacuum desiccator. The solvent was evaporated by means of infrared lamps, the dishes placed in a vacuum desiccator and dried to constant weight. The difference in weight is reported as total weight in mg.

Figure 1 presents results obtained with a mixture of triglyceride, monoglyceride, cholesterol, and stearic acid. Every other tube was used for weight determinations. The material in the alternate tubes was examined by silicic acid paper and thin layer chromotography (TLC) to identify the lipid class in the peaks. The triglyceride from peanut oil had the highest partition coefficient, being most soluble in the non-polar phase. Cholesterol, stearic acid, and monoglyceride followed in that order, the monoglyceride being most soluble in the polar phase. Although diglyceride was not examined, it should fall between cholesterol and triglyceride. A total of 200 transfers was sufficient for the complete fractionation of this mixture of neutral compounds. Quantitative recovery of the individual components allows for a complete analysis of such mixtures.

The technique was applied to a total lipid extract from rat liver, Figure 2. Since TLC of the extract and subsequent fractions indicated an absence of monoglyceride, it was not necessary to separate the phospholipid from neutral lipid prior to countercurrent distribution. Analysis of Figures 1 and 2 reveals that monoglyceride and the phospholipids, phosphatidylethanolamine and lecithin have similar partition coefficients in this solvent system and therefore will not be separated in 200 transfer. In a total lipid extract containing monoglyceride, it would be necessary to separate the phospholipid from neutral lipid prior to analysis of the latter fraction by countercurrent distribution.

Analysis of the various peaks in Figure 2 by thin layer and silicic acid paper chromatography indicated that cholesterol esters and triglyceride moved together and were not resolved. These components can easily be separated by silicic acid (33) or Florasil (34) column chromatography. The peak with a maximum at tube 168 is a mixture of four components which have not been identified. Cholesterol occurs in the predicted area as do the free fatty acids. Free fatty acids are spread out a great deal. This may be due to a partial fractionation on the basis of chain length and degree of unsaturation.

All of the material to the right of tube 90 is free of phospholipid and all of the material to the left is free of neutral lipid and thus a complete separation of neutral lipid from phospholipid was obtained. The phospholipid complex was separated into two major peaks, one with maximum at tube 5 containing the more polar phospholipids, the other between tubes 35-90 containing the less polar phospholipids. Two of the components in the peak with maximum at tube 5 have been identified. These are phosphatidylserine and inositolphosphatide. There are other components which have not been identified. One of these behaves as lysolecithin on thin layer chromatograms; however, its identity has not been ascertained. In the major peak between tubes 35-90, chromatographic analysis of each individual tube indicates a partial fractionation of sphingomyelin, lecithin, and phosphatidylethanolamine. These are the only phospholipids present in this peak. However, it seems doubtful that further transfers would achieve their separation, for there are indications that they exist in association with one another.

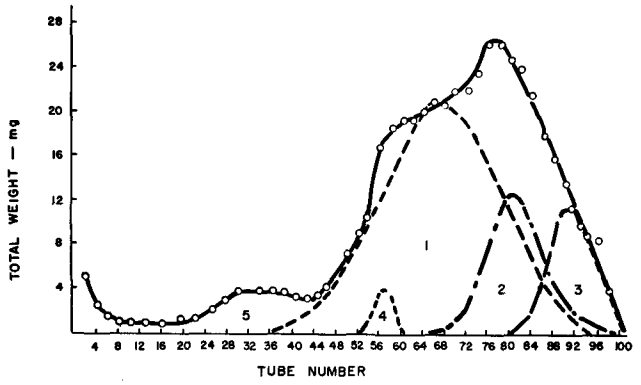

FIG. 3. Countercurrent Distribution of Folch Fraction III: Solid line represents the total dry weight. Areas under the broken lines indicate the calculated amounts of individual components. 1. phosphatidylserine, 2. and 3. inositol phosphatides, 4. unidentified, 5. phosphatidylethanolamine.

Distribution of Phospholipids

The separation of phospholipids presents some special difficulties. First, emulsification problems are pronounced with phospholipids. Secondly, they possess both acidic and basic functional groups and tend to form intermolecular associations. These difficulties have no doubt deterred many laboratories from countercurrent studies of the phospholipids. However, the difficulties are not insurmountable and the technique offers a great deal of promise as a tool either for analysis or isolation of phospholipids.

We have applied the method of countercurrent distribution to the study of phospholipids with some degree of success (35, 36). In an attempt to purify phosphatidylserine from beef brain, a concentrate was prepared by the classical method of Folch (37) and distributed in a solvent system consisting of carbontetrachloride, methanol, and H_2O (62:35:4 by volume). The results of a countercurrent distribution of Folch's fraction III are shown in Figure 3. The points represent the total weight of material present in each tube. The major component, indicated by a broken line, represents phosphatidylserine, but there are other contaminating substances present. By paper

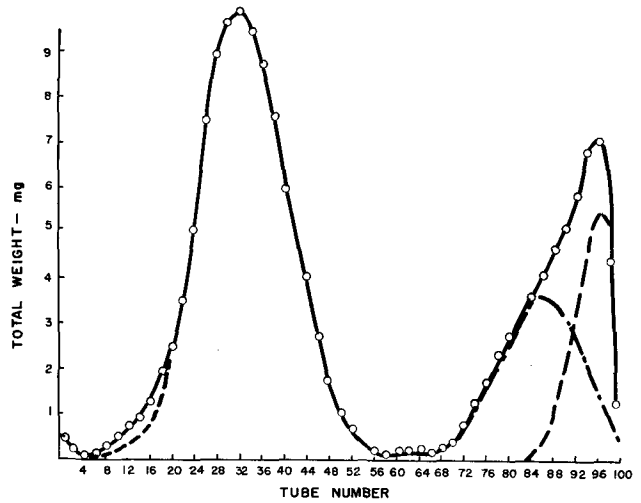

FIG. 4. Countercurrent Distribution of Free Acid Preparation: Solid line represents the total dry weight. Areas under the broken lines indicate calculated amounts of the individual components. Maximum at tube 32, acid form of phosphatidylserine. Maximum at tubes 84 and 95, inositol phosphatides.

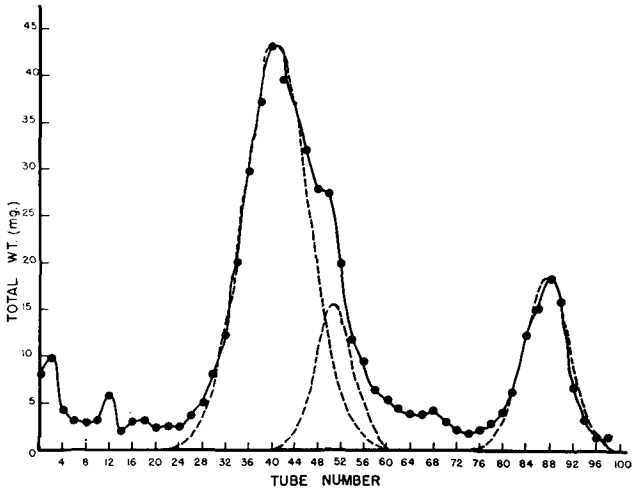

FIG. 5. Countercurrent Distribution of Lecithin Preparation: Solid line represents the total dry weight. Areas under the broken lines indicate the calculated amounts of individual components. Maximum at tube 40, lecithin. Maximum at tube 52, sphingomyelin. Maximum at tube 88, unidentified.

chromatographic analysis of the material in each tube, it is possible to obtain enough information for the Gaussian distribution curves representing the relative inhomogeneity of Fraction III and the degree of separation achieved by countercurrent distribution. These are the curves indicated by the broken lines in Figure 3. The area under curve 1 of Figure 3 is phosphatidylserine, while curves 2 and 3 are thought to be two different inositol phosphatides, possibly mono- and di-phosphoinositide. Curve 4 represents an unidentified component, while curve 5 contains phosphatidylethanolamine.

The system did not seem to offer any promise for the isolation of pure phosphatidylserine. Folch (38) demonstrated that phosphatidylserine, isolated from brain by the use of neutral solvents, was obtained as a mixture of the sodium and potassium salts. He also demonstrated that treatment with 0.05N HCl converted phosphatidylserine to the acid form. The material, rich in phosphatidylserine from tubes 55-100, Figure 3, was precipitated from an aqueous emulsion with dilute acid and dialyzed to remove inorganic ions. This acid-treated material was again fractionated by countercurrent distribution in the manner described above. The distribution curve is shown in Figure 4. Chromatographic analysis of the material contained in the tubes which represent the two major peaks showed that the fractionation of the acid-treated material resulted in the separation of the inositol phosphatides and the unidentified phospholipid from phosphatidylserine. The material from the peak with a maximum at tube 32 was the acid form of phosphatidylserine, chromatographically free of other lipids. Since the salts of phosphatidylserine exhibit solubility characteristics different from those of the acid form, countercurrent distribution offers a convenient method of separating phosphatidylserine from contaminating substances. The partition coefficient of the salt form of phosphatidylserine is high, allowing for the removal of all contaminants of lower partition coefficient. On the other hand, the partition coefficient of the acid form is low, consequently the remaining contaminants of high partition coefficients can be separated.

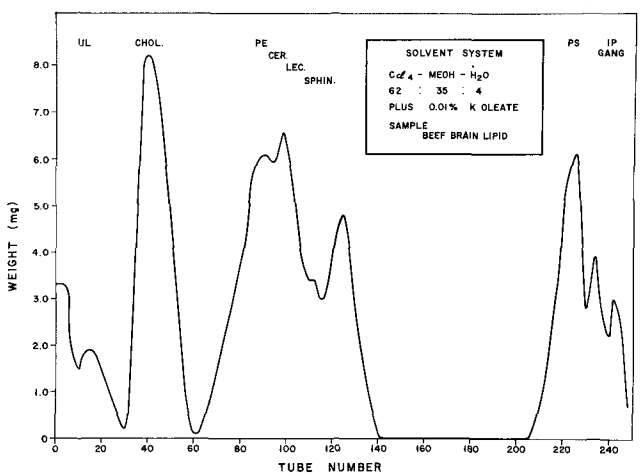

FIG. 6. Countercurrent Distribution of Beef Brain Lipid: UL = unidentified lipid, Chol = cholesterol, PE = phosphatidylethanolamine, Cer = cerebroside, Lec = lecithin, Sphin = sphingomyelin, PS = phosphatidylserine, IP = inositol phosphatide, Gang = gangliosides.

Using the same solvent systems, lecithin prepared by the $CdCl_2$ precipitation method of Pangborn (39) was analyzed. The results are presented in Figure 5. There are at least three substances present in this preparation. The major component reaching a maximum at tube 42 was characterized by paper chromatography as lecithin. The small peak with a maximum at tube 52 was shown to be sphingomyelin. The faster-moving component with a peak at tube 88 has not been identified.

In dealing with phospholipids we were faced with the problem of emulsions. We have found that the addition of 0.01% potassium oleate to the solvent system completely eliminates any tendency of the system to form emulsions. Figure 6 presents the distribution of beef brain lipid after 250 transfers in a system of carbontetrachloride, methanol, and water (62:35:4) containing 0.01% potassium oleate. The phospholipids are separated into two major groups. Those more soluble in the polar phase consisting of phosphatidylserine, inositol phosphatides, and gangliosides, and those more soluble in the non-polar phase, phosphatidylethanolamine, lecithin, sphingomyelin, and cerebroside. Cholesterol was quantitatively isolated free of other substances.

The obvious difficulties encountered due to lipid-lipid association, possibly as a result of the number of basic and acid ionizable groups in phospholipids, could possibly be overcome by forming derivatives which would make them non-ionizable molecules. Collins, et al. (40-43), studied the distribution of such derivatives. The phospholipids were dinitrophenylated using 1- fluoro 2:4 dinitrophenylbenzene followed by methylation using diazomethane, and the resulting phospholipid derivatives are separated by low temperature chromatography on cellulose using acetone, into fractions soluble and insoluble, respectively, in acetone, and then each fraction was further resolved by countercurrent distribution. The major difference in the findings by this method as contrasted with data using chromatographic procedures is in what the authors refer to as "complex" aminophospholipids. These complex aminophospholipids contain ethanolamine, serine, and choline and have a molecular weight at least double that of known phospholipids. Collins suggests that they are triester phospholipids, probably formed by the interaction of phosphatidylethanolamine with lysolecithin or with lecithin through glycerol. The presence of complex aminophospholipids has been indicated not only in

animal tissues, but also in yeast, a virus, and a plant tissue. Collins suggested that chromatography on silicic acid might cause a breakdown of the complex phospholipids which would account for the failure by other investigators to detect these compounds. However, neither Hörhammer and Richter (44), nor ourselves using countercurrent distribution methods on intact lipids, have found evidence for such a complex aminophospholipid.

Separation of Non-Lipid Impurities

Lipid extracts of biological material invariably contain non-lipid contaminants. Lovern and Olley (45) reported as much as 53.5% non-lipid material in ethanol-ether extracts of fish. Rouser, et al. (46), by careful chromatographic techniques, showed that 7% of beef brain lipid consisted of water soluble non-lipid material. This could vary, depending on the method of extraction. The "solubilizing" property of phospholipids due to their polar nature is the main contributing factor responsible for the presence in lipid extracts of non-lipid contaminants. For this reason, ordinary methods such as selective precipitation or repeated extractions are inefficient. One of the simplest and possibly most used methods is the water-solvent partition developed by Folch (47–48). However, a major disadvantage of the method is that there is the selective loss of lipid into the aqueous phase. This, of course, can be extremely critical when quantitative work or radioisotope incorporation studies are carried out.

Figure 7 represents the countercurrent distribution of a lipid fraction in a solvent system consisting of n-butanol, acetic acid, water (4:1:5). A chloroform-methanol (2:1) extract of beef brain was purified by the method of Folch (48) in order to remove non-lipids. The recovered total lipid was then distributed for 250 transfers in a system consisting of carbon-tetrachloride, methanol, water (62:35:4) containing 0.01% potassium oleate (Fig. 6). However, chromatographic analysis indicated that material from tubes 200–250 contained non-lipid material in addition to the more polar phospholipids. It was possible to separate these in a system of n-butanol, acetic acid, water (4:1:5) in as little as 60 transfers. From Figure 7 it can be seen that ca. 15% of the fraction consisted of non-lipid contaminants. The peak with maximum at tube 55 contained phosphatidylserine and inositol phosphatides, the ganglioside having been lost as a result of Folch's partition. It is obvious from these results that in addition to selective lipid losses, the Folch procedure does not remove all non-lipid contaminants.

Conclusion

The application of countercurrent distribution to the fractionation of lipids, both for analytical and preparative purposes, has by no means been fully exploited. From the results thus far obtained, it would seem that the technique offers a great deal of promise. This is especially true where countercurrent distribution can be used for the final purification of a simple mixture, or in the initial fractionation of a complex mixture prior to purification by some other technique such as chromatography. In some instances where a particular separation is difficult if not possible by chromatographic techniques, countercurrent distribution may achieve the separation. It is an excellent complementary method used in conjunction with column chromatography. The development of new solvent systems for use in lipid fractionation will further increase its usefulness.

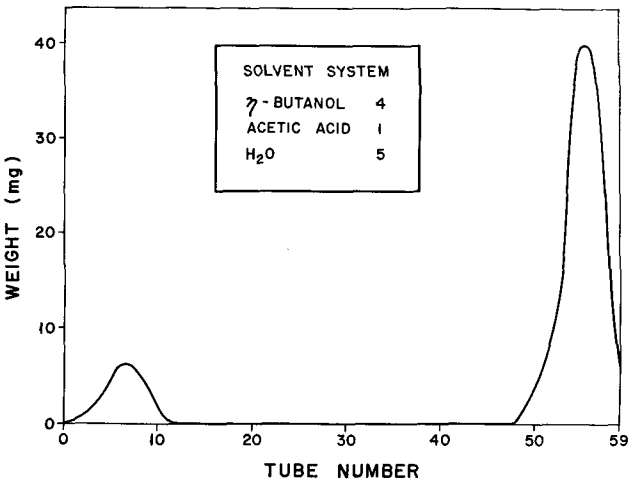

FIG. 7. Countercurrent Distribution of Beef Brain Lipid Subjected to Folch Dialysis to Eliminate Non-Lipids (fraction 200–250 from CCl_4-MEOH-H_2O-Na OLEATE separation). Maximum at tube 7, non-lipid. Maximum at tube 55, inositol phosphatide, phosphatidylserine.

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[Received April 23, 1963—Accepted July 27, 1963]