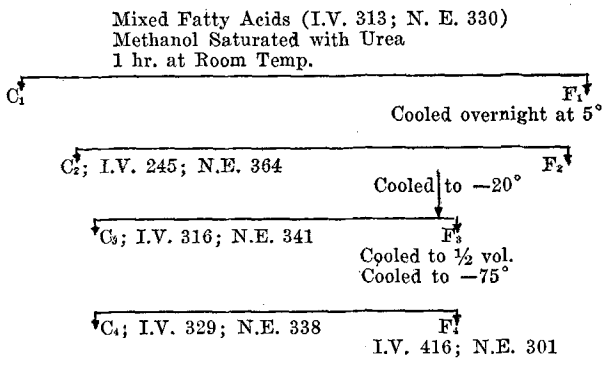
CHART 9

Separation of Polyunsaturated Acids of Bovine Tissue (21)



hydroxamic acids, and to urea inclusion compounds. The urea adducts have been found in recent years to provide several very specific applications. The polyethenoic acid adducts can be crystallized at temperatures above −75° at which temperature the free acids remain liquid in solvent. Some of the mutually intersolubilizing effects observed in direct solvent crystallization of the fatty acids are apparently minimized with the urea adducts.

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The Analysis of Lipids by Countercurrent Distribution

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COUNTERCURRENT DISTRIBUTION is the name given to a particular type of liquid-liquid, multiple-stage extraction. Although this operation can be carried out as a separatory funnel procedure, the ease and labor-saving advantages of Craig's ingenious laboratory instruments makes these devices (6) and the countercurrent-distribution procedure synonymous in practice.

H. J. Dutton

The technique of countercurrent distribution holds particular advantage to the lipid chemist for a number of reasons. First, liquid-liquid extraction processes are inherently mild processes and adaptable to the study of instable lipids. In theory and usually in actual practice all of the material introduced into the fractionating device is recovered. Perhaps a most important advantage of this technique is its mathematical description which permits the prediction of solute behavior and of separability of solutes. Because of this mathematical basis both qualitative and quantitative data may be obtained from a simple weight curve. The partition coefficient which is fundamental to the calculations may be looked upon as being as characteristic a constant of the solutes as is refractive index, boiling point, melting point, and specific rotation. The partition coefficient however has the advantage over the usual constants in suggesting methods of isolation and of indicating structural features of a molecule.

Fundamentals and Useful Calculations of Countercurrent Distribution

Separation by countercurrent distribution depends upon the differences which exist in the differential solubility of individual chemical compounds when these compounds are distributed between two immiscible solvents. This differential solubility is described by the partition coefficient (K) and is defined by the equation

$$C_1/C_2 = K$$

where C_1 and C_2 are the concentrations of the given solute in the lighter upper (hyperphase) and the heavier lower (hypophase) solvent layers, respectively. At high solute concentrations and under other conditions where the ratio of associated and dissociated molecules varies, deviations from this law do occur. In those cases in which the shape of the countercurrent-distribution curve is important, an attempt is made to adjust the concentrations to the range of constancy of partition coefficient. Conditions

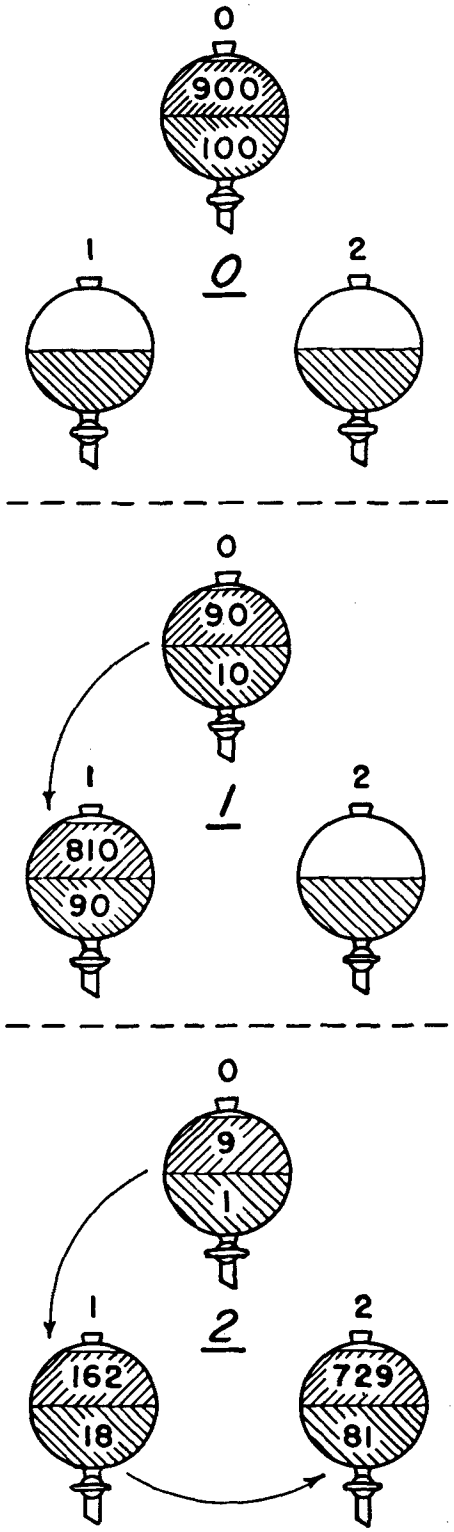

FIG. 1. Countercurrent distribution of solute A($K_A = 9$).

of constancy of partition coefficient are assumed in the subsequent consideration of this section.

The fundamentals of the countercurrent-distribution process and its associated calculations are best illustrated by an example involving the separation of two solutes as a separatory funnel operation (8).

In this instance successive portions of the upper layer are caused to move over and to be equilibrated with equal successive volumes of lower layers. To clarify the relationship of theory to the apparatus actually used for countercurrent distribution, the lower layers will be held stationary. Assume that three separatory funnels, numbered 0, 1, and 2, each contain a given volume of hypophase. To funnel 0 is added an equal volume of hyperphase and 1,000 units each of solutes A and B. Assume further, for illustrative purposes, that solute A is 9 times more soluble in the hyperphase than in the hypophase while solute B is 9 times more soluble in the hypophase than in the hyperphase. The partition coefficient for compound A is therefore 9, and that for B is 0.11.

Considering for the moment only compound A, the distribution of 1,000 units of A in funnel 0 after equilibration and settling is calculated. As shown in Figure 1, 900 units will be found in the hyperphase and 100 in the hypophase after the first equilibration. The hyperphase of funnel 0 is next transferred to funnel 1, and a volume of fresh hyperphase solvent is replaced in funnel 0. After shaking and settling, the distribution of solute A is shown at transfer stage 1. In separatory funnel 0 the 100 parts of A left dissolved in the lower layer are now distributed to give 90 parts in the hyperphase and 10 parts in the hypophase. The 900 units transferred to funnel 1 in the hyperphase are now distributed, 810 parts in the hyperphase and 90 in the hypophase.

In the next operation the hyperphases of funnels 0 and 1 are transferred to funnels 1 and 2, respectively, and fresh hyperphase is again introduced into funnel 0. The distribution after shaking and settling is shown at transfer stage 2 of the figure. The solute A content of each funnel, or the sum of that in the two layers, is 10, 180, and 810 units, respectively, for funnels 0, 1, and 2.

Solute B, by analogous reasoning, will be found to have the reverse distribution. The solute B content for funnels 0, 1, and 2 is 810, 180, and 10 units, respectively. When both solutes are now considered, funnel 0 is found to contain 81% of solute B of 98.8% purity and funnel 3 contains 81% of solute A of 98.8% purity. This two-transfer-stage operation, just described, demonstrates the countercurrent-distribution process and also shows the increased separation of solutes A and B over that for the single equilibration where the corresponding purities of solutes A and B are 90%.

The distribution of solutes in the various stages of a countercurrent distribution can be calculated readily through use of the binomial expansion which describes this process (14)

$$\left[\frac{1}{K+1}+\frac{K}{K+1}\right]^n$$

where n is the number of transfers.

In the example described above where $n = 2$ and $K = 9$, the expansion is:

$$\frac{1}{(K+1)^2}+\frac{2K}{(K+1)^2}+\frac{K^2}{(K+1)^2} \text{ or } 0.01 + 0.18 + 0.81$$

When multiplied by 1,000, the distribution given above is obtained.

A general form of the equation which describes the solute content ($T_{n,r}$) of any given funnel or tube (r) after a given number of transfers (n) is:

$$T_{n,r} = \frac{n!}{r!(n-r)!}\cdot\left(\frac{1}{K+1}\right)^n\cdot K^r$$

Certain shortcuts make the calculation of solute contents in individual tubes, and thus of theoretical distribution curves, quite simple in practice (14).

In the case of automatic countercurrent-distribution operations the number of plates may be hundreds or even thousands. Even the shortcut calculation then becomes laborious. In this instance the normal curve of error becomes applicable and is more readily calculated for specific points (3, 10).

The position of the maximum may be calculated, if one knows the partition coefficient of the solute and the number of plates applied, by the equation (14)

$$N_{max} = n\left(\frac{K}{K+1}\right)$$

By measuring the position of the maximum and knowing the number of plates applied, this equation may be solved for K in terms of N_{max} and n and thus provide a method of determining the partition coefficient.

A problem of frequent occurrence in the practice of countercurrent distribution is the calculation of the width of the band after a given number of plates have been applied. This can readily be calculated from considerations of the standard deviation. Since ± 3 standard deviation units include 99% of the weight curve, the number of tubes enclosing 99% of the weight ($N^{99\%}$) is given by the following (10):

$$N^{99\%} = 6\sqrt{\frac{n\,K}{(K+1)^2}}$$

This equation finds use, for example, in the calculation of how many times one may recycle a given system of solutes before the head of the band catches up and overlaps the tail of the band.

Another problem of frequent occurrence is the determination of how many plates are needed to effect a desired separation. This may be calculated by the use of the two equations just presented and by calculating the position of the maxima for the two solutes under consideration and the width of the band which would include 99% of the weight curve.

For countercurrent distributions involving not over 55 tubes and partition coefficients ranging between 0.25 and 4.0, a nomograph has been published (11) which is reproduced in Figure 2. Given the partition coefficients for solutes A and B, the percentage overlap or impurity of curves A and B is determined for a given number of plates, or conversely, the number of plates required to achieve a given purity may be determined. This nomograph is based upon the equation

$$n = t^2\left[\frac{K_A + K_B + 2K_AK_B}{K_B - K_A}\right]^2$$

where t = is a statistical constant and is related to the percentage impurity (area of overlap) of two curves as follows:

t	Percentage impurity
1	15.9
2	2.27
3	0.13

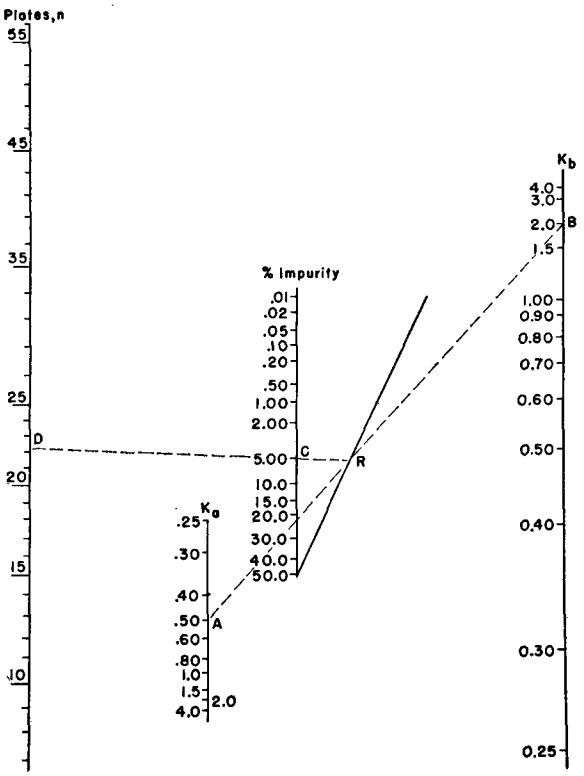

FIG. 2. Nomograph relating plates, n, percentage of impurity, K_A and K_B.

The mathematics of the single withdrawal technique or "elution" technique have not been so completely developed. An equation describing the elution series has been published (3) and is applicable to distribution of a given solute in the tubes of the fraction collector.

The selection of immiscible solvent pairs for countercurrent distribution is unfortunately an empirical process. Particularly with regard to "selectivity" of a solvent system the choice is a matter of cut and try. Wide differences in selectivity between solvent systems do exist, but empirical choice is at present the only approach to this problem. In selecting the solvent systems, one generally searches for those which are temperature insensitive; *i.e.*, the interface between the two layers moves slightly with change in temperature. Solvents with low boiling points are generally desirable in order to reduce the temperature requirements for solvent removal. Particularly in the case where the fundamental technique is employed, solvents are chosen in which partition coefficients for one solute will approach unity. Another desirable characteristic of solvent systems is a wide difference in specific gravity, which tends to speed the rate of settling and minimize emulsification tendencies. Solvents which tend to associate solute molecules are to be avoided.

Apparatus for Countercurrent Distribution

Undoubtedly the countercurrent distribution procedure would never have come to its fruition except for the development of apparatus for rapidly and easily carrying out its many operations. With a modern automatic countercurrent-distribution apparatus (5) as many as 10,000 individual separatory funnel operations are carried out in an hour's time. These operations may be continued night and day with only occasional checking. The conduct of such experiments would therefore be humanly impossible without the development of this automatic equipment.

Historically the operation of countercurrent distribution in separatory funnels was followed by its equivalent in metal apparatus which had 19 to 52 individual tubes (6). This equipment permitted the simultaneous shaking, settling, and transfer operations on the tubes to be performed singly. This metal apparatus now has generally been replaced by apparatus of the glass design. Apart from the obvious advantages of visibility and inertness of glass, this design permits many tubes to be added in a linear arrangement and permits the development of automatic equipment for the introduction of solvent at one end and the automatic collection of samples from the opposite end of the train. A manually operated piece of glass equipment is shown in Figure 3. The lower or hypophases are introduced into each of the individual tubes. The sample and hyperphase are introduced into the one end of the train. After shaking and settling of the phases, this hyperphase is transferred to the next adjacent tube by moving the

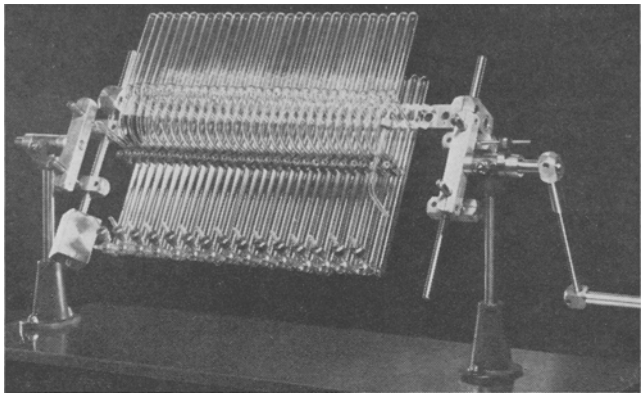

FIG. 3. Glass countercurrent-distribution apparatus.

tubes into the position shown in the photograph. Fresh solvent is then introduced into the starting tube (tube 0). The same process of shaking, settling, transferring, and solvent addition are performed in successive steps until the solvent of tube 0 has passed into the last tube of the train. If the operation is stopped at this point and the contents of the individual tubes are analyzed, it is described as the "fundamental" operation. If, on the other hand, the individual samples are caught as they flow out from the last tube and new solvent continues to be introduced into tube 0 at each stage, this operation is described as the single withdrawal technique. Since this operation is similar in performance to a chromatographic column, this operation is sometimes referred to as "elution."

Automatic countercurrent-distribution equipment provides for the performance of shaking, settling, and transfer operations in accordance with the prescribed program coupled with introduction of fresh solvent at one end of the train and its collection in a fraction collector from the last tube of the train.

The automatic equipment installed at the Northern Utilization Research Branch is shown in Figure 4. Each of the 200 individual tubes has a capacity for 40 ml. of lower layer and 40 ml. of upper layer. This equipment also has provision for recycling the contents of the apparatus. Thus instead of flowing from the last tube into the fraction collector, this and subsequent fractions may be reintroduced into tube 0 of the instrument and once more passed through the 200 fractionating tubes of the instrument. The application of many hundreds of stages is mandatory in problems such as determining the glyceride structure of vegetable oils; however useful separations can be obtained as is shown under the application section by manual apparatus having a limited number of tubes.

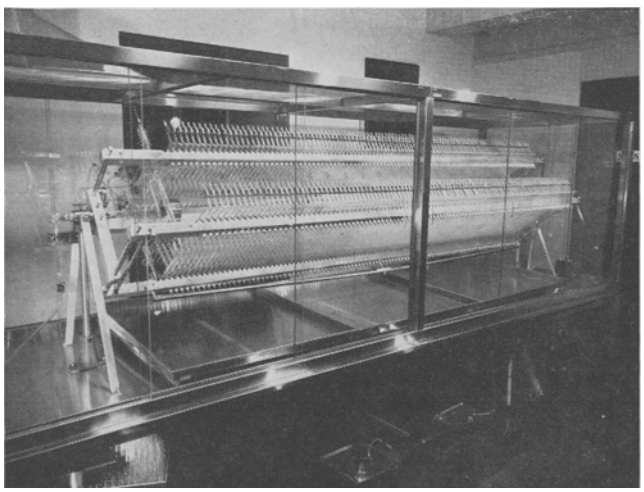

FIG. 4. Completely automatic glass countercurrent-distribution equipment.

Applications of Countercurrent Distribution

The applications of countercurrent distribution to the field of lipids has largely been recorded upon the pages of the Journal of the American Oil Chemists' Society. The compounds studied include fat-soluble pigments, phospholipids, the homologous and isologous series of fat acids and monoesters, fat-oxidation products, and glycerides. The separation of compounds within these general classes will be illustrated by selected examples. Perhaps because of one's ability to see the fractionations performed, distribution between immiscible solvents found its first and early applications in the pigment field, such as in the historic researches of Willstätter and Stoll (15). The data of Figure 5 illustrate the application of

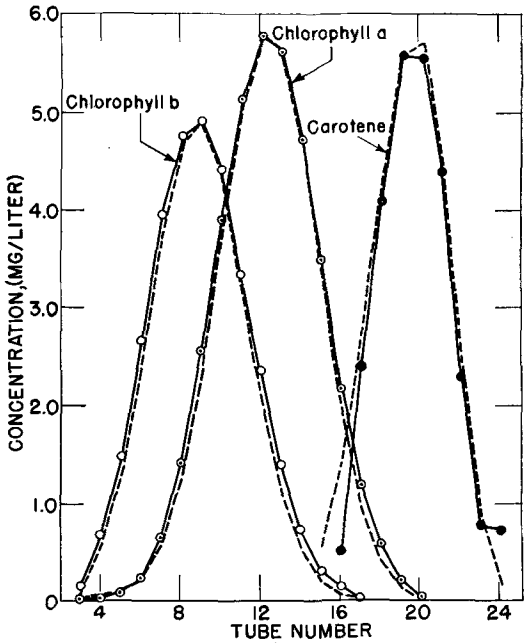

FIG. 5. Countercurrent distribution of chlorophyll a, chlorophyll b, and carotene in the 25-tube Craig distribution apparatus. Solid lines represent experimental data; broken lines show the calculated theoretical distribution.

countercurrent distribution to the separation of chlorophyll A, chlorophyll B, and carotene (11). In this example the immiscible solvent pair was petroleum ether and 90% ethanol. This figure represents the general method of representation of countercurrent-distribution data where the weight concentration, spectral absorption, fluorescence, or radioactivity are plotted on the ordinate and the transfer tube number is plotted on the abscissa. In the 24-tube metal countercurrent-distribution apparatus an incomplete separation of chlorophyll A and chlorophyll B was obtained as shown in the figure. When fractionated in the 200-tube automatic countercurrent-distribution apparatus there were, as anticipated, many clear uncolored tubes between the bands of chlorophyll A,

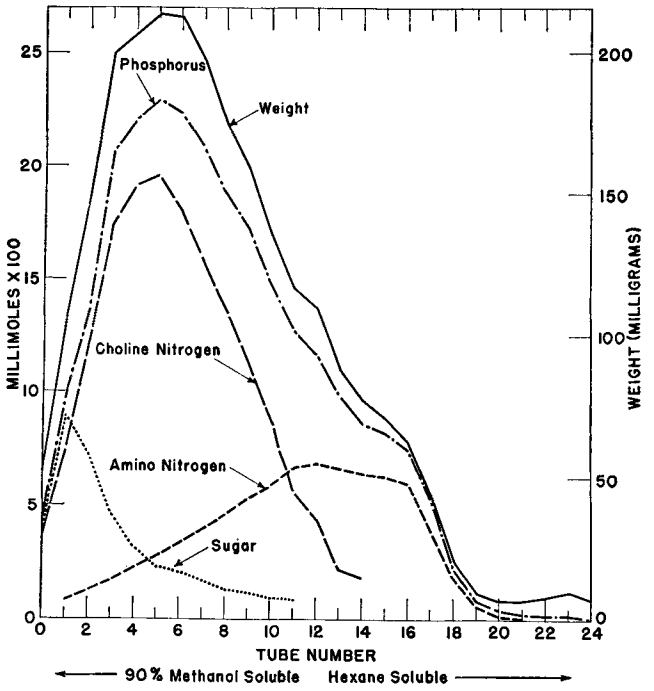

FIG. 6. Countercurrent distribution of the alcohol-soluble fraction of soybean phosphatides.

chlorophyll B, and carotene. The closeness of solid lines and broken lines indicates the closeness of agreement of the experimental data and the theoretically calculated data. In this case the resolution is less than that of the chromatographic column, but countercurrent distribution has the distinct advantage over chromatography of reproducibility and of linear isotherms, and the attendant potentialities of quantitative calculation and predictions.

In recent studies of the carotenoid pigments of citrus fruits both countercurrent distribution and chromatographic separation have been employed to advantage (7). Carotenoid pigments are separated by countercurrent distribution, according to whether they have 0, 1, 2, 3, or 4 hydroxyl groups in the molecule. The separation of carotenes from monohydroxy, dihydroxy, trihydroxy, and tetrahydroxy carotenoids is then followed by chromatographic adsorption and identification of the individual pigments within the class.

Phospholipids

The separation of phospholipids presents unusual difficulties. First, phospholipids are surface-active agents and cause emulsification of solvents with resultant lengthened times of separation of the phases. They possess both acidic and basic functional groups and tend to form intermolecular associations. As extracted from biological tissues, they carry along carbohydrates, sterol glucosides, pigments, etc., but, in addition, have carbohydrates combined in their structures by primary chemical bonds.

Application of a 24-plate instrument to the separation of soybean phosphatides is shown in the next two figures (12). Prior to countercurrent distribution the acetone-precipitated phospholipids were fractionated with absolute ethanol into the alcohol-soluble fraction and the alcohol-insoluble fraction. It is apparent from the distribution curves for these fractions shown in Figures 6 and 7, respectively, that the distributions diverge from theoretical shape for both the phosphatidyl choline and for the phosphatidyl ethanolamine. The associated sugar constituents are separated in the early tubes of the distribution and are followed by the phosphatidyl choline and finally the phosphatidyl ethanolamine. Phosphatidyl ethanolamine (cephalin) according to the previous literature did not occur in the alcohol-soluble (lecithin) fraction. Phosphatidyl ethanolamine was supposed to be the major constituent of the cephalin fraction shown in Figure 7. However, based on this and sub-

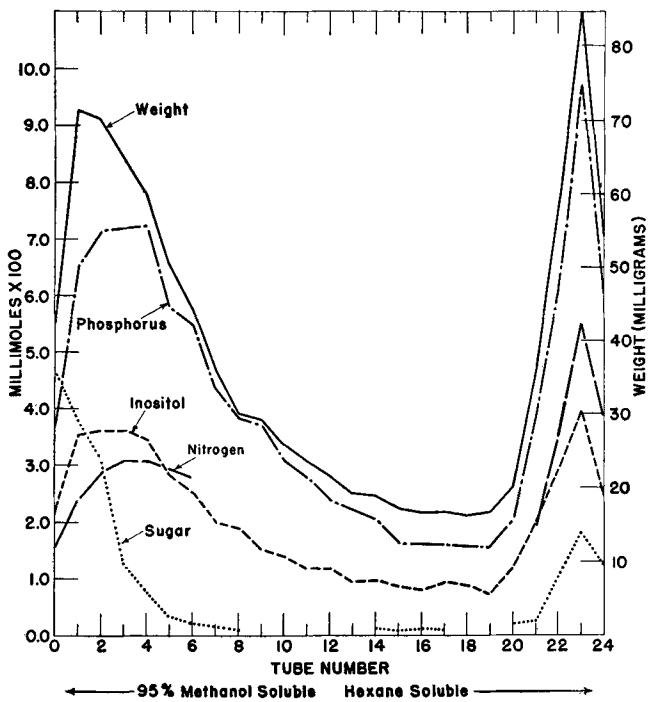

FIG. 7. Countercurrent distribution of the alcohol-insoluble fraction of soybean phosphatides.

sequent studies, it has become apparent that the major constituents of the alcohol-insoluble fraction are not cephalins or phosphatidyl ethanolamines but rather are inositol phospholipids. Moreover, as shown by these data, there are two types of phospholipids, one type of which is more soluble in the hexane solvent, the other of which is more soluble in the 90% methanol solvent. From the shape of the curves it is apparent that association exists between the various phosphatides themselves and between other constituents which distorts the shapes of the curve. Despite this deficiency useful separations are obtained which have permitted the estimation of composition, revealed new phospho-inositides, and suggested new fractionating methods for the study of these compounds.

Fat Acids

The application of the countercurrent-distribution technique to separation of fat acids can be well illustrated by the data of Craig (1). Given in Figure 8 is the separation performed upon the homologous series of fat acids—lauric, myristic, palmitic, and stearic. Four hundred transfer plates were applied to this system in the automatic countercurrent-distribution apparatus. Quantitative separations of these

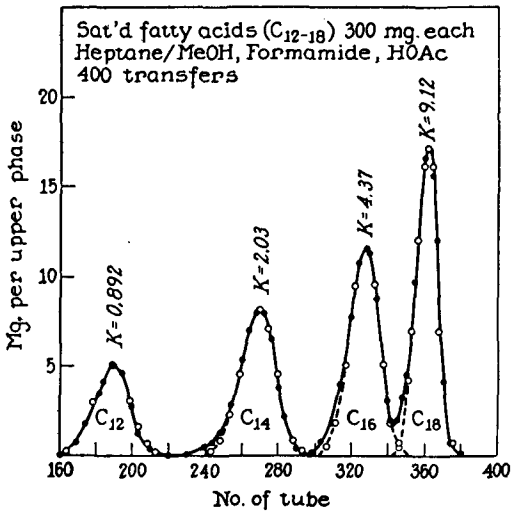

FIG. 8. Countercurrent distribution of the homologous series of higher fat acids.

acids were either obtained or can be accurately calculated by extrapolation. The separation of the isologous series of C_{18} acids is represented in Figure 9 and shows that, by the application of 650 transfers, separation of linolenic, linoleic, and oleic acids is obtained. It may be inferred from the partition coefficients of the previous figure that stearic acid would also be quantitatively separated to complete this isologous series.

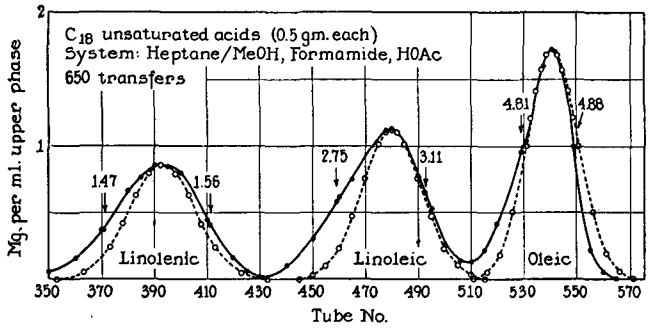

FIG. 9. Countercurrent distribution of the isologous of C_{18} acids.

Methyl Esters of Fat Acids

Methyl esters of fat acids are also amenable to countercurrent distribution but require the development of different solvent systems for their separation. Because the polar carboxyl group of fat acids is replaced by the "more hydrocarbon" ester group, methyl esters therefore have greater solubility in the hydrocarbon hyperphases than the corresponding free acids. The development of hypophase solvents for use with methyl esters therefore required a study of increasing the solubility in the lower layer. A distinct limit exists in this search for solvents in that as one increases the differential solubility for the methyl esters, one also increases their tendency to dissolve and be immiscible with the hydrocarbon layer.

In Table I are given partition coefficients developed for the methyl esters and partition coefficients also for the fatty acids in various solvent systems (8). Calculations of separability made from these partition coefficients indicate that in some cases better separation will be obtained with methyl esters and sometimes better separation will be obtained with the free acids.

TABLE I

Partition Coefficients of Fat Acids and Methyl Esters

	Acids	Methyl esters
Acetic	0.09[a]	
Propionic	0.50[a]	
Butyric	2.24[a]	
Valeric	10.00[a]	
Valeric	0.061[b]	
Hexanoic	0.238[b]	
Heptanoic	1.08[b]	
Octanoic	4.17[b]	
Lauric	0.9[c]	1.3[d]
Myristic	2.0[c]	1.5[d]
Palmitic	4.4[c]	1.9[d]
Stearic	8.9[c]	2.3[d]
Oleic	4.9[c]	1.8[d]
Linoleic	2.9[c]	1.2[d]
Linolenic	1.6[c]	0.94[d]

[a] 2.2 M phosphate buffer at pH 5.7, isopropyl ether.
[b] 1 M phosphate buffer at pH 7.7, isopropyl ether.
[c] Formamide acetic acid, methanol, heptane system.
[d] Nitroethane, nitromethane, pentane-hexane system.

At this point it is pertinent to compare countercurrent distribution and other methods for separation of the fat acid esters. Highly efficient columns are available commercially with a sufficient number of theoretical plates to resolve the homologous series of saturated fat acid esters just presented. However, because of the slight differences of vapor pressures among the isologous series, distillation procedures are quite ineffective. The widely used chemical methods, bromination and debromination, while effective for separating the isologous series, are also known to affect the configuration of the double bond. Urea crystallization can be used to advantage but appears to be limited by cocrystallization (13). By chromatographic absorption small amounts of the unsaturated C_{18} acids can be isolated and constitute the only alternative to countercurrent distribution for the preparation of the natural "unsaturated" acids and esters.

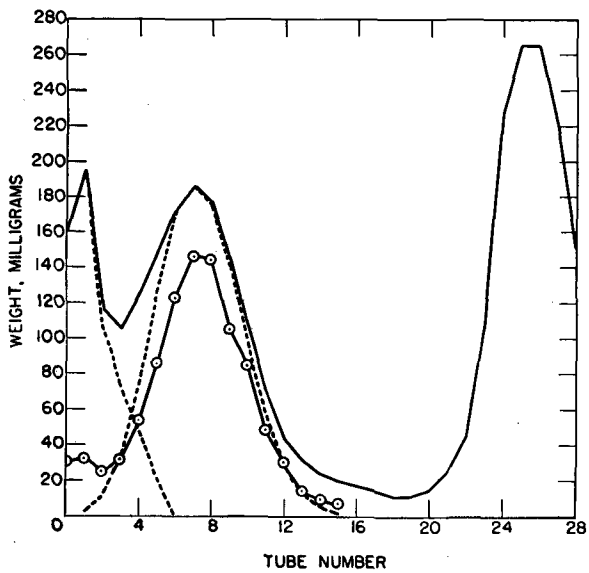

FIG. 10. Weight-distribution curve (solid line) following countercurrent fractionation of autoxidized methyl linoleate (0.62 mole oxygen absorbed per mole ester—light catalyzed). Circles indicate weight of diene-conjugated esters. The broken line at the center is the theoretical curve while the broken line at the left is the difference curve.

Fat Acid Oxidation Products

Countercurrent distribution is particularly adapted to the study of the labile fat oxidation products, for which even chromatography appears to be too drastic. The application of this technique to the field of autoxidation can be illustrated by the countercurrent distribution of methyl linoleate oxidized to the level of 0.62 mole of oxygen per mole of ester (2). The peak on the right-hand side of the weight curve, Figure 10, is that for the unoxidized methyl linoleate. The middle peak is that for the primary oxidation product, methyl linoleate hydroperoxide. The peak on the left-hand side of the graph is that due to secondary oxidation products. At low levels of oxidation this peak for the secondary oxidation products is negligible or non-existent. At lower levels the countercurrent-distribution curves demonstrate that for each molecule of oxygen absorbed one molecule of hydroperoxide is formed. The infrared curve of the hydroperoxide fraction shown in Figure 11-B demonstrates that the hydroperoxide formed under these conditions is almost exclusively the *cis-trans* conjugated hydroperoxide (10.0–10.5 μ region). Measurements of conjugated hydroperoxide illustrated by the circled line show that the purity of this hydroperoxide is 90% or greater. The infrared curve for the secondary oxidation products shown in Figure 11-A shows loss of the conjugated *cis-trans* structure (corroborated also by the ultraviolet measurements) and shows that, in place of the destroyed conjugated double bond system, is left the absorption corresponding to an isolated *trans* bond. Research is continuing to elucidate the nature of the decomposition of the primary oxidation product, linoleate hydroperoxide, through the combined use of countercurrent distribution, infrared, ultraviolet spectrophotometry, and the more usual organic functional group determinations.

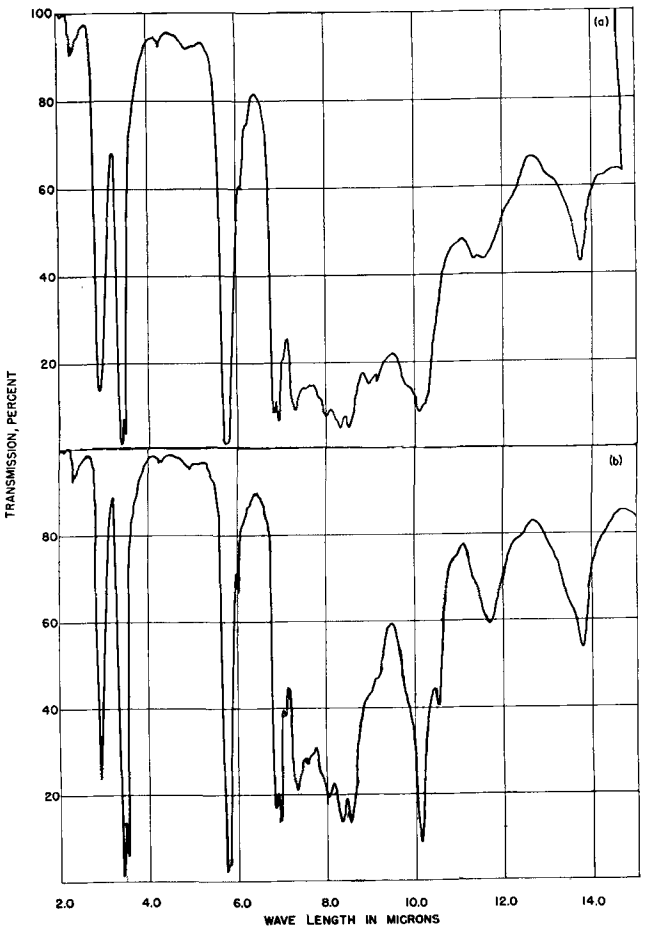

FIG. 11. Infrared absorption spectra of a) the secondary oxidation product (Component III) of methyl linoleate, tube 2-room temperature autoxidation; b) methyl linoleate hydroperoxide, tube 9-room temperature autoxidation.

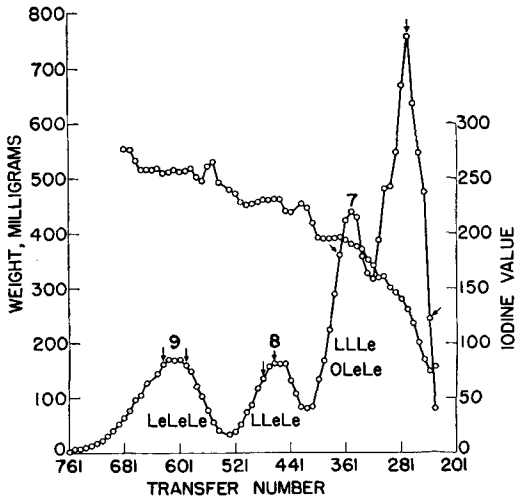

FIG. 12. Countercurrent fractionation of linseed oil glycerides; open circles—weight curve; closed circles—iodine value curve.

Glycerides

One of the most recent and most exciting analytical operations of the countercurrent distribution is in the study of the glyceride structure of vegetable oils. In this area of research the countercurrent-distribution techniques appear to have provided more resolution than has been available through present techniques of chromatography. Perhaps in no field of study have the tools of the lipid chemist been more inadequate for the task presented. Except for a few isolated instances where special conditions were particularly favorable, the fat chemist can point to few examples of the isolation of pure triglycerides from a natural source. When the countercurrent-distribution technique was applied to the study of the glycerides of linseed oil (9), pure trilinolenin and linoleodilinolenin were isolated both qualitatively and quantitatively (Figure 12). This constituted the first time that either of these two glycerides had been isolated in pure form from a natural source, not to mention the quantitative aspects of the isolation. In this instance a triglyceride was separated from its neighboring triglyceride, which differed only by one out of nine double bonds. Two triglycerides are possible having seven double bonds—oleodilinolenin and dilinoleolinolenin. These two glycerides were unresolved, but by spectroanalysis the relative proportions of these two glycerides could be calculated.

It should be apparent that the potentialities of the countercurrent-distribution technique in the field of glyceride structure have only been touched. The same may be said for pigments, phospholipids, fat acids, and other classes of lipids and derivatives. This discussion of the analysis of lipids by countercurrent distribution may appropriately be closed with the

familiar quotation that "every scientific advance is an advance in method" and with the prediction that the countercurrent-distribution method will aid fat chemists of the future in making many scientific advances in their field.

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Microscopy of Fats and Oils

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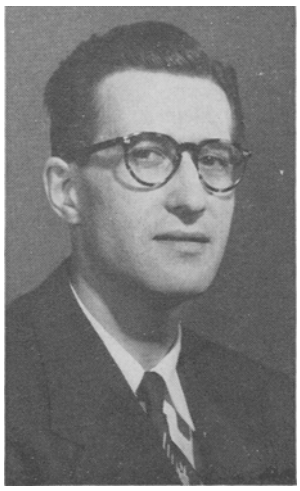
IT HAS BEEN SAID that there is not "a single great industry today where microscopic methods, intelligently applied, will not lead to more or less marked improvements" (1). The fat and oil industry is no exception. With the aid of the microscope much can be learned about the physical states in which fats and oils occur naturally and the various forms in which they are manufactured by man.



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Remarkably few instances of microscopic examination of fats and oils are reported in the literature. The pioneering microscopy of Carlin (2) helped to explain the role of shortenings in air-leavened baked goods. Bailey (3) published a few photomicrographs of various fat crystals. The microscopic investigations of King (4) demonstrated the globular structure of butter. The photomicrographic studies of Quimby (5) have helped to unravel the various polymorphic forms of the triglycerides. The author (6) has utilized polarized light microscopy in characterizing the crystals of lard and rearranged lard. Although these references report applications of microscopy to various phases of fat and oil research, they refer only briefly, if at all, to the techniques and equipment involved.

Of the many books which have been written about the microscope and its uses, either of two relatively simple manuals on photomicrography (7) will acquaint the beginner with the general principles involved. Typical of almost the entire literature on the subject, these books refer to the microscopic examination of biological and mineralogical specimens for it has been in the various fields of biology and geology that the microscope has been most widely utilized. The equipment and techniques used in these areas can however be applied directly to the examination of fats and oils.

Compared with other modern laboratory instrumentation, photomicrographic equipment is relatively simple and need not necessarily be expensive. The optical instrument makers, chiefly Bausch and Lomb Optical Company, E. Leitz Inc., and Carl Zeiss Inc., have developed a wide assortment of microscopes and cameras suitable for fat and oil investigations. Since they have printed many excellent booklets describing the various specifications of their equipment, there is no need for a detailed review here.

The basic instrument for fat and oil applications is a microscope with appropriate lens combinations which possess good resolution at magnifications of 50–250 X. Suitable illumination must, of course, be provided. It is virtually essential that the microscope be provided with light polarizing equipment. All modern polarizing microscopes are equipped with a pair of nicol prisms, one of which is capable of being rotated 90° with respect to the other. A practical necessity with polarizing equipment is a revolving stage capable of being rotated 360°.

For purposes of recording observations, a suitable camera must be provided. Although some 35 mm. cameras can be adapted to the microscope, this negative size has not generally been considered satisfactory for the purposes which will be discussed. Most photomicrographic cameras utilize 3¼ x 4¼, 4 x 5, or 5 x 7-in. films. The first two sizes are most commonly used. Any of the relatively fine-grained films, such as one of the contrast process films, is satisfactory. Extremely fast shutter speeds are unnecessary since exposures of the order of several seconds are generally of little consequence in this work.

For purposes of accurately determining the dimensions of physical structures, such as crystals, water droplets, air bubbles, etc., some form of measuring device must be provided. In general use is the ocular net reticule consisting of a glass disc on which is ruled a rectilinear grid of accurately-spaced fine lines. This disc is placed at the appropriate position between the ocular lens components. The grid can be calibrated by means of a stage micrometer consisting of a glass slide on which is ruled an accurate scale usually 2 mm. in length, graduated in 0.01-mm. intervals. By viewing the stage micrometer through the ocular reticule, the apparent spacings of the grid lines can readily be determined at various magnifications.

There are no hard-and-fast rules regarding the preparation and viewing of fat and oil samples. Except for certain exceptional instances, such as investi-