cation of the relative strength of the bonds involved in dimer formation.

That the frequency of the single band formed in hydrogen-bonding solvents appears to move toward lower frequencies with increasing hydrogen bonding ability of the solvent and that the appearance of the single band in hydrogen bonding solvents is concentration dependent (see Fig. 2) indicates a dissolution of the dimer and the formation of two molecules of the ester, each associated through hydrogen bonding with a solvent molecule.

The bonding which results in dimer formation, as postulated, must be very weak since molecular weight determinations by freezing point depression, as determined in benzene or cyclohexane, give the normal value for a monomeric unit.

It is of interest to note that Schotte[6] has recently recorded the infrared spectra of the diastereomers of $HO_2CCHEtCHMeCO_2H$, m.p. 177–178° (I) and 100–101° (II) and of $HO_2CCHEtCH_2CHMeCO_2H$, m.p. 104.5–5.5 (III) and 83.5–84.5° (IV), and has noted that II and III show a split peak for the carbonyl stretching frequency. He assumed these to have the racemoid configuration. I and IV showed no such split and were presumed to be the mesoid isomers.

Since the esters herein reported are all racemic mixtures, it may be that the proposed dimer is a racemic pair. Molecular models of such a racemic pair and of a pair having a single configuration indicate that the racemic pair may allow a closer approach of the ring oxygens to the carbonyl carbons because of less interference by the alkyl substituents.

The observations might be interpreted in a different way by assuming that two rotational isomers are present in which the carbonyl oxygen is on the same side of the molecule as the ring oxygen in one case and away from the ring oxygen in the other. Since this would represent an equilibrium mixture and since the extinction coefficients of the two observed bands are approximately equal in every case, the unlikely assumption must be made that equal numbers of molecules exist in each of the states for each of the esters. Since resonance splitting does seem to be indicated, we believe that dimer formation offers a better explanation of the data than the assumption of rotational isomerism.

(6) L. Schotte, *Arkiv. Kemi*, **9**, 397 (1956).

Experimental

The infrared spectra were measured with a Baird Associates model 4–55 recording infrared spectrometer employing sodium chloride optics. The same 0.029-mm. cell was used for all determinations. No reference cell was used. The accuracy of the wave length measurement is estimated to be ± 5 cm.$^{-1}$ in the region discussed. Polystyrene bands at 1602.5 and 1594.7 cm.$^{-1}$ were recorded on each spectrum for calibration purposes.

Compounds XV, XVI, XVIII and XIX may contain small amounts of impurities. The other esters are considered to be quite pure (see reference 2).

Acknowledgment.—We wish to express our great appreciation for the financial aid and encouragement given us through a Cottrell grant from Research Corporation.

ORONO, MAINE

[CONTRIBUTION FROM THE FISHERY TECHNOLOGICAL LABORATORY[1]]

Liquid–Solid Countercurrent Distribution of Fatty Acids with Urea

BY W. N. SUMERWELL

RECEIVED FEBRUARY 7, 1957

The reaction of fatty acids with urea to form inclusion adducts has been used as the basis of a liquid–solid countercurrent distribution method for separating mixtures of fatty acids. The fatty acids and urea, dissolved in an appropriate solvent, served as the moving liquid phase while the precipitated reaction products served as the stationary solid phase. The character of the distribution curve obtained for a given mixture of fatty acids depends on the differences in the distribution coefficients for the individual fatty acids when they are distributed between solid inclusion adducts and organic solvent. The method was found to be effective in the separation of mixtures of fatty acids such as arachidic, stearic, palmitic and oleic acid as well as in the separation of a mixture of the *cis–trans* isomers, oleic and elaidic acids. The method also was applied to a mixture of highly unsaturated salmon egg oil fatty acids (iodine value 350). The results indicate that urea distribution may be a valuable tool in the separation of such unstable compounds.

Introduction

Studies on the formation of urea inclusion adducts as a means of fractionating marine oil fatty acids and their derivatives were begun in this Laboratory several years ago. A preliminary report of this work has been published.[2] The present paper describes the development of a liquid–solid countercurrent distribution procedure based on the differential binding of fatty acids by urea and its application to some synthetic and natural mixtures of fatty acids.

Subsequent to Craig's description of his two-phase liquid–liquid countercurrent distribution technique[3] and its use in the separation of organic compounds, he published a report on a liquid–solid countercurrent system.[4] By this technique, he distributed chrysene and anthracene between cyclohexane and alumina. He defined a coefficient A as the ratio of the weight of solute dissolved in the liquid phase to the weight of solute adsorbed on the solid phase. The position of the maximum concentration of a compound in the distribution system was then calculated by substituting the value of A

(1) A laboratory of the Branch of Commercial Fisheries, U. S. Fish and Wildlife Service, Seattle, Washington.

(2) C. Domart, D. T. Miyauchi and W. N. Sumerwell, *J. Am. Oil Chemists' Soc.*, **32**, 481 (1955).

(3) L. C. Craig, *J. Biol. Chem.*, **155**, 519 (1944).

(4) L. C. Craig, C. Golumbic, H. Mighton and E. Titus, *Science*, **103**, 587 (1946).

in the equation $N - n(A)/(A + 1)$ in the same way that the distribution coefficient is used to calculate the position of maximum concentration in a liquid–liquid countercurrent system.

A different approach to liquid–solid countercurrent distribution can be made if, instead of an adsorbent being used as the solid phase of the system, a precipitated reaction product, produced by a reaction involving the compounds to be distributed, is used. This precipitation reaction, however, must be of a special type in that the relative amounts of any compound in equilibrium between the liquid and solid phases of the system must be governed by a distribution coefficient instead of the solubility product which generally governs precipitation reactions of inorganic compounds. In other words, the solid phase of the system, made up of precipitated reaction products, must act as a solid solution. The requirement would most likely be met by products which are bound by only secondary bonds of the van der Waals and hydrogen bonding variety. Such products could be expected to resemble solutions in their behavior.

Consider a mixture of compounds A_1, A_2, A_3. .A_j, each of which reacts with a compound B to form the solid reaction products (bound by secondary bonds) P_{A_1B}, P_{A_2B}, P_{A_3B}. . .P_{AjB}. These reactions are represented by the general equation $A_i + B \rightarrow P_{AiB}$.

In this system, A_i and B are present in solution in an appropriate solvent and as such constitute the liquid phase. The precipitated reaction products P_{AiB} constitute the solid phase. From this reaction, distribution coefficients K_1, K_2, K_3 . . . K_j are defined by the general equation

$$K_i = \frac{[A_i \text{ liquid}]}{[A_i \text{ solid}]}$$

where the terms in brackets are the concentrations of A_i in liquid and solid phase, respectively.

It is evident that this equation will hold strictly true only when the volume of the liquid phase and the concentration of B in this phase are held constant. If the values of K_1, K_2, etc., differ sufficiently, then countercurrent distribution should effectively separate A_1, A_2, etc.

Since the binding forces existing between aliphatic compounds and urea are presumably van der Waals forces and in some cases hydrogen bonds, one might expect their adducts to act as solid solutions and to exhibit distribution coefficients. If such is the case, then it should be possible to develop a liquid–solid countercurrent method for the separation of fatty acids using urea, the fatty acids and a suitable solvent.

Preliminary Considerations

The reaction of a fatty acid with urea is represented by the equation xF.A. + yUrea $\rightleftarrows$ F.A.$_x$·Urea$_y$.[5]

In terms of the liquid–solid countercurrent distribution system, the inclusion adducts are the stationary solid phase, whereas the supernatant liquid is the moving phase.

(5) See H. Schlenk, "Progress in the Chemistry of Fats and Other Lipids," Vol. 2, Academic Press, Inc., New York, N. Y., 1952, p. 243, for a review of the urea inclusion compounds of fatty acids.

To determine whether the amounts of fatty acid in equilibrium between the liquid and solid phases are governed by a distribution coefficient, quantitative measurements using pure palmitic acid as a reactant were made. The reactions were carried out at 30° at three different concentrations of palmitic acid, each in 100 ml. of methanol. In each solution, 19.2 g. of urea was dissolved, which is the amount required to saturate 100 ml. of methanol at 30°. Enough additional urea also was added to each solution to react with the weight of palmitic acid present at a ratio of 12.8 moles of urea per mole of fatty acid.[5] The solutions were held at 30° for 6 hr. Aliquots of the supernatant liquid were then removed and the palmitic acid in solution was extracted and weighed. The data and results of these experiments are given in Table I. It is evident that the palmitic acid does have a definite distribution coefficient in this system.[6]

TABLE I

DISTRIBUTION OF PALMITIC ACID IN A TWO-PHASE METHANOL–UREA SYSTEM

Wt. of palmitic acid, g.	Wt. of urea, g.	Distribution coefficient $K = C$ liquid
1.4200	23.45	0.013
0.6931	21.27	.014
0.3189	20.16	.012

To determine whether sufficient difference exists between the distribution coefficients of long-chain fatty acids to warrant investigation of the urea reaction as a countercurrent method, quantitative measurements employing palmitic, stearic and arachidic acids were made. The reactions were each carried out at 20° in a urea-saturated solution consisting of 70% methanol and 30% ethyl acetate. The K-values obtained were 0.016 for arachidic acid, 0.037 for stearic acid and 0.091 for palmitic acid. Although these values are small, they differ considerably in magnitude and, for this reason, studies on a countercurrent separation were begun.

The concentration of urea in solution at equilibrium should be constant throughout the countercurrent system. This condition can be met through the use of solvents that remain saturated with urea when the reaction has reached equilibrium. In addition, the distribution of different mixtures of fatty acids will, in general, require different concentrations of urea, from one system to another, depending on the chain length and degree of unsaturation of the component fatty acids. This requirement can be met by using a series of solvents with varying urea solubility characteristics.

In the separation of saturated fatty acids at or near room temperature, the solubility of urea can be effectively varied by the use of mixtures of methanol and ethyl acetate. Methyl alcohol when saturated with urea at 20° contains 16.5 g.

(6) The distribution of a fatty acid between solid urea and supernatant solution appears to be similar to the distribution of a compound between an ion exchange resin and supernatant solution. Helferich, THIS JOURNAL, **76**, 5567 (1954), has defined the distribution coefficient of a substance in contact with resin as $\bar{c}/c$ where $\bar{c}$ is the concentration of substance in the pore liquid of the resin and c is the concentration in the supernatant solution.

of urea per 100 ml. of solution. Ethyl acetate at 20° contains 0.35 g. of urea per 100 ml. of solution. By varying the proportions of methanol and ethyl acetate, it is possible to prepare saturated urea solutions of any desired concentration between the two limits. A solubility curve for urea in mixtures of methanol and ethyl acetate is shown in Fig. 1.

In the separation of unsaturated fatty acids at lower temperatures, methanol is used as the solvent although the solubility of urea in methanol in the temperature range of −10 to −30° is less than is desirable. Efforts are being made to find a better solvent for work in this range.

It has been found that the solubility of urea in methanol varies linearly with temperature in the range from 20 to −30°. The solubility equation is: $x = 10 + 0.32T$, where T is the centigrade temperature and x is the solubility (g. per 100 ml. of solution) of urea in methanol at that temperature. This equation is useful for computing the concentration of urea in methanol solution at any temperature at which a separation is to be made.

Experimental

Procedure.—Solutions of urea in methanol or a mixture of methanol and ethyl acetate, sufficient in volume for the number of stages (usually, 12 to 30) in the distribution system, were prepared so that the concentration of urea would be the same as that of a saturated solution at the temperature at which the distribution was to be performed.

The distribution was carried out in a series of 500-ml. erlenmeyer flasks, each fitted with a cork stopper holding a short air condenser. Each flask in the series was charged with 10 g. of dry urea (to maintain the solvent at saturation after complex formation). The sample of fatty acids to be distributed, weighing from 5 to 15 g., was placed in the first (0) flask. A 250-ml. volume of the urea solution was added and the mixture was heated until complete solution was obtained. The flask was then cooled to the desired temperature, either in a water-bath or in a refrigerated chest, and allowed to stand until the precipitation of the adducts was complete. The cooling of the reaction mixture should be carried out gradually. Rapid cooling results in less satisfactory separations.

After the reaction in flask 0 has reached equilibrium (about 2 hr.), the supernatant liquid was transferred into flask 1. In most cases, the adduct crystals were well-formed and the liquid layer was decanted easily. If the crystals were small, however, the liquid layer was filtered off with suction. In order that this operation could be accomplished without removing the solid phase from the reaction flask, a rubber stopper was inserted inside the filtering section of a No. 1 Coors büchner funnel. The stopper was ground so that it fitted snugly against the perforated face of the funnel and was bored so that the neck of an erlenmeyer flask fitted into it tightly. A very narrow slit was made on the inside of the stopper so that air could pass into the flask during the filtration. In operation, the stopper was fitted over the flask. The funnel, fitted with filter paper, was placed over the stopper. This assembly was then inverted and attached to a suction flask. In this way, the liquid layer was removed quickly, while the solid phase remained in the flask. Another 250-ml. volume of urea solution was added to flask 0. Flasks 0 and 1 were heated until complete solution was achieved, and they were then cooled to the desired temperature and held until the reaction had reached equilibrium.

The liquid layer in flask 1 was then transferred to flask 2, and the liquid layer in flask 0 was transferred to flask 1. Angther 250-ml. volume of urea solution was added to flask 0, and the flasks were heated and then cooled.

This procedure was repeated until the fatty acids in the sample were distributed over the total number of flasks in the system.

To extract the fatty acids from the fractions, each fraction was transferred to a separatory funnel, and 300 ml. of warm water and 10 ml. of concd. HCl were added. The fatty acids were then extracted with 1:1 mixed ether and petroleum ether. The ether extract was dried over magnesium sulfate after which the ether was removed by distillation. Traces of solvent were removed by heating the residual fatty acid fractions in a vacuum oven at 40° for 30 min. The solid fatty acids were crystallized from acetone prior to the determination of their physical constants.

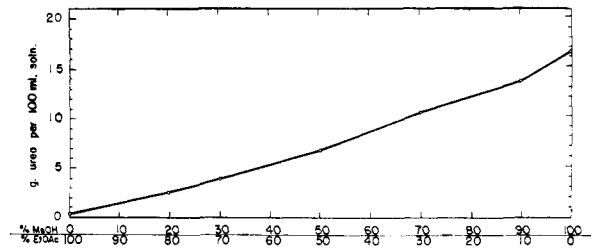

Fig. 1.—Solubility of urea in mixtures of methanol and ethyl acetate at 20°.

Results

1. Distribution of Mixed Stearic, Palmitic and Oleic Acids.—A mixture consisting of 5 g. each of technical-grade stearic, palmitic and oleic acid was distributed over 24 stages at 20° in a urea-saturated solvent made up of 70% methanol and 30% ethyl acetate. The results are shown in Fig. 2.

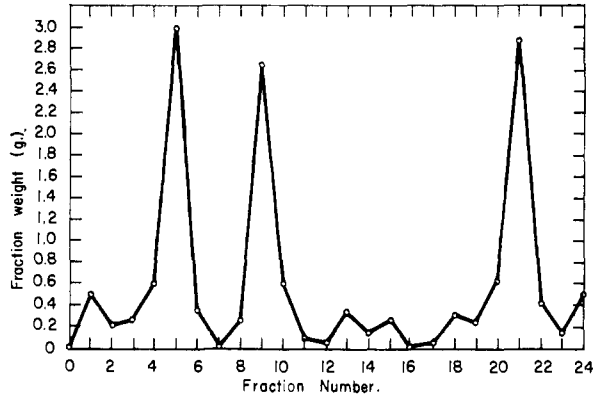

Fig. 2.—Distribution of a mixture of stearic, palmitic and oleic acids (5 g. each).

Fractions 0 through 15 were solids and the remaining fractions were oils. Stearic acid reached a maximum concentration in fraction 5; the physical constants of this fraction were m.p. 69.5°, I value 0.0 and $n^{72}D$ 1.4329. Palmitic acid was concentrated in fraction 9; the constants were m.p. 62.0°, I value 0.6 and $n^{70}D$ 1.4315. Oleic acid was concentrated in fraction 21; the constants were I value 89.5 and $n^{35}D$ 1.4545.

In addition to the major components, arachidic acid was concentrated in fraction 1; the constants of this fraction were m.p. 75.0°, I value 0.0, and $n^{80}D$ 1.4327. Fraction 13 apparently consists of myristic acid contaminated with an unsaturated fatty acid; the constants of this fraction were m.p. 50.5°, I value 2.3 and $n^{70}D$ 1.4305. Fractions 23 and 24 were considered to be largely dienoic fatty acid, owing to their respective refractive index values, $n^{35}D$ 1.4619 and $n^{35}D$ 1.4621.

One of the problems encountered in this separa-

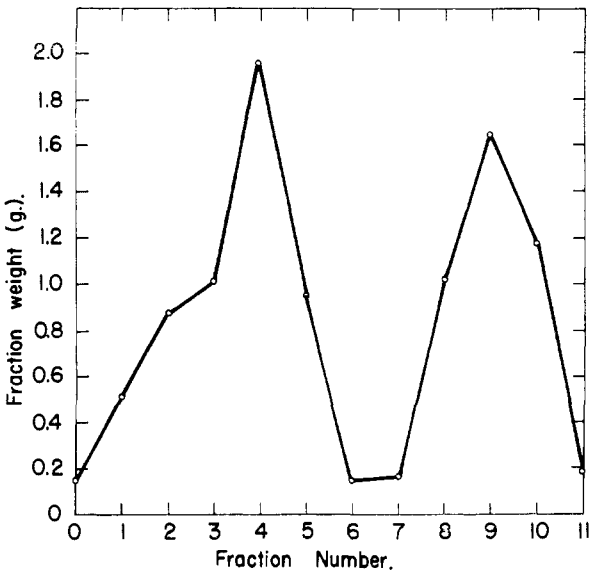

Fig. 3.—Distribution of a mixture of oleic and elaidic acids (5 g. each).

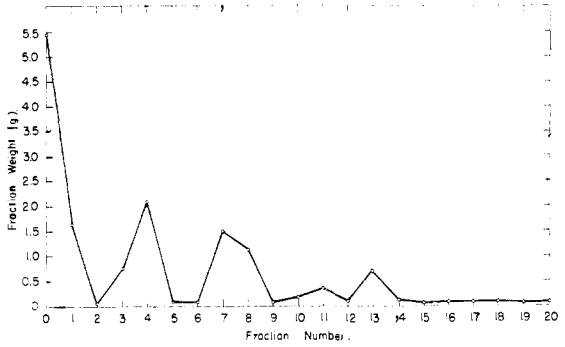

Fig. 4.—Distribution of hydrogenated salmon-egg oil fatty acids (15-g. sample).

tion as well as in other fatty acid separations is the apparent failure of the equation

$$N = n\frac{(K)}{K+1} \qquad (1)$$

to predict the point where the maximum of the distribution curve of a particular fatty acid will occur. In this equation, K is the distribution coefficient, N is equal to the number of flasks the maximal concentration has migrated from flask 0, and n is the total number of flasks used in the system. If $K/(K+1)$ is multiplied by 3.5, then the calculated values of N are in fair agreement with the experimental results. Possible explanations for this unexpected behavior might be: (1) large errors in the determined values of K, (2) failure of the reactions to completely reach equilibrium in the time allowed for each crystallization, (3) interaction between mixed fatty acids in the solution has a marked effect on the distribution coefficient of each individual fatty acid. It is felt that explanations 1 and 2 are unlikely to account for such a large discrepancy. Explanation 3, although of unknown significance, probably is the most likely one. The question is likely to remain unanswered until more detailed studies can be carried out. Some light may be thrown on the problem, by further experiments using pure fatty acids and their esters to avoid effects of interaction.

It is of interest to compare the results of urea countercurrent distribution of fatty acids with the results obtained by Ahrens and Craig[7] using a liquid–liquid system containing heptane, methanol, formamide and acetic acid. One difference is in the number of stages required to obtain a satisfactory separation. The liquid–liquid system required from 400 to 600 stages, depending on the nature of the fatty acid mixture, as compared to the 25 stages used for the urea system. The urea system permits larger amounts of fatty acids to be used per distribution as compared with either liquid–liquid countercurrent distribution or the various chromatographic methods which have been used.[8] It also does not require special equipment. However, operation of the urea system is very tedious and time consuming.

Distribution of the *cis–trans* Isomers, Oleic and Elaidic Acids.—It is a well-established fact that the formation of urea–fatty acid complexes is hindered by any distortion in the carbon chain of the fatty acid. It follows that the *cis* isomer of an unsaturated fatty acid should fit less readily in the urea crystal than the *trans* isomer. Thus, the urea countercurrent distribution technique may serve to separate *cis–trans* isomers, such as oleic and elaidic acids.

Elaidic acid was prepared from purified oleic acid by treatment with nitrous acid. The product was crystallized twice from petroleum ether.

A mixture made up of 5 g. each of oleic and elaidic acid was distributed over 12 stages at 20° in urea-saturated methanol. The results are shown in Fig. 3.

Fractions 0 through 5 were solid and the remaining fractions were liquid. Fraction 4 contained the maximum concentration of elaidic acid. The physical constants of this fraction were m.p. 45.5° and $n^{70}D$ 1.4401. Fraction 9 contained the maximum concentration of oleic acid. The physical constants of this fraction were I value 89.3 and $n^{35}D$ 1.4552. The elaidic acid distribution curve was skewed perhaps owing to the stearic acid present as an impurity in the system.

It appears from these results that urea countercurrent distribution may be an efficient method for separating a mixture of *cis–trans* isomers of the long-chain alkene type.

3. Distribution of Hydrogenated Salmon-Egg Oil Fatty Acids.[9]—Salmon-egg oil is characterized by a high content of poly-unsaturated C_{20}-, C_{22}- and C_{24}-fatty acids. Consequently, hydrogenated salmon-egg oil fatty acids should offer suitable raw material for the separation of C_{20} to C_{24} saturated fatty acids.

The hydrogenated sample (I value 6.7) was first separated into saturated and unsaturated fractions by treatment with acetone at −30°. Fifteen

(7) E. H. Ahrens, Jr., and L. C. Craig, *J. Biol. Chem.*, **195**, 299 (1952).

(8) R. T. Holman, "Progress in the Chemistry of Fats and Other Lipids," Vol. I, Academic Press, Inc., New York, N. Y., 1952, p. 104.

(9) Kindly furnished by Mr. Robert Kyte of the Fishery Products Laboratory, Ketchikan, Alaska.

grams of the saturated fraction was then distributed over 21 stages at 18° in a urea-saturated solvent consisting of equal volumes of methanol and ethyl acetate. The results are shown in Fig. 4. All of the fractions obtained from the distribution were solids except for the very small residues in fractions 17 through 20. Over one-third of the sample was found in fraction 0; the physical constants of this fraction were m.p. 73.8°, *I* value 0.0 and $n^{75}D$ 1.4341. Fraction 4, $n^{75}D$ 1.4322, was stearic acid; fraction 7, $n^{75}D$ 1.4302, was palmitic acid; and fraction 13, $n^{75}D$ 1.4285, was mainly myristic acid. Fraction 11, $n^{75}D$ 1.4292, was unidentified.

The results of this experiment suggest that the distribution of saturated fatty acids with more than 18 carbons could be done more successfully at temperatures above room temperature, probably in the range 30° to 35°.

4. Distribution of Poly-unsaturated Fatty Acids.—One of the difficult problems in oil chemistry is the separation of natural mixtures of unsaturated fatty acids containing 2 to 6 double bonds, such as those commonly found in marine oils. The preparation of highly unsaturated fatty acid concentrates from marine oils by the urea inclusion method has been reported.[1,10] The mild conditions inherent in the urea procedure are ideally suited for use in the separation of poly-unsaturated fatty acids, although the presence of double bonds in the chain greatly hinders the formation of urea–fatty acid adducts.

To determine whether poly-unsaturated fatty acids could be separated by the urea countercurrent technique, a 15-g. sample of unsaturated salmon-egg oil fatty acid (*I* value 350) was distributed over 18 stages at −30° in urea-saturated methanol. This experiment was exploratory and no attempt was made to characterize the products completely. The results are shown in Fig. 5. It appears from the curve that four fatty acids have been concentrated, one being in each of the fractions 7, 10, 12 and 14. Another fatty acid has perhaps been concentrated in fraction 9 but, if so, it is overshadowed by the large amount of material in fraction 10. The refractive indices of the fractions from this separation are given in Table II, together with the iodine numbers of the principal fractions. Fraction 15, after standing in a vacuum desiccator, was found to contain very fine, silky crystals, melting between 30–32°, in addition to oil. The crystals dissolved readily in the usual organic solvents.

TABLE II

REFRACTIVE INDEX

Fraction	Wt., g.	$n^{30}D$	*I* value	Fraction	Wt., g.	$n^{30}D$	*I* value
0	0.000	....		9	1.503	1.4762	302
1	.020	....		10	1.771	1.4835	341
2	.030	....		11	0.961	1.4865	
3	.021	....		12	2.143	1.4874	364
4	.035	1.4667		13	1.514	1.4890	
5	.096	1.4573		14	2.018	1.4890	358
6	.219	1.4588		15	1.078	1.4912	
7	.747	1.4632	206	16	0.518	1.4924	
8	.531	1.4702		17	0.124	1.4928	

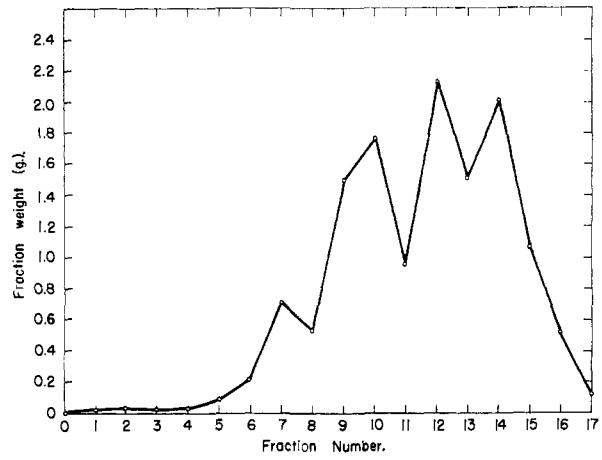

Fig. 5.—Distribution of unsaturated fatty acids from salmon-egg oil (15-g. sample).

The results of this distribution suggest that a larger number of stages should be used in future experiments. Although a considerable amount of work remains to be done toward improving the separation and also in characterizing the distribution products, the urea countercurrent method appears to offer promise for the separation of poly-unsaturated fatty acids.

The question still remains as to whether poly-unsaturated fatty acids form true inclusion adducts in the sense that the more saturated acids do. It is conceivable that the poly-unsaturated acids form only a loose type of adduct that does not require the creation of a crystalline urea lattice along the entire length of the fatty acid molecule.

Experiments are being carried out to develop a continuous type of separation based on urea countercurrent distribution. A continuous process would be greatly superior to the discontinuous system described in this paper from the standpoint of convenience of operation and time required. The experimental technique being used at present employs a 5.5 cm. × 45 cm. column packed with urea and maintained at a temperature suitable for the particular separation under study. The fatty acid sample dissolved in a suitable solvent is placed on the column, and the complexed fatty acids are then eluted with a non-polar solvent, such as hexane. The elution can be facilitated by raising the temperature of the column above the temperature used for forming the complexes.

In conclusion, it should be noted that the phenomenon of inclusion compound formation may become increasingly important in the future in the fields of organic chemistry and biochemistry.[7] In particular, the urea countercurrent technique could be applied to the problem of separating mixtures of petroleum hydrocarbons of the longer chain lengths as well as other types of aliphatic compounds.

Acknowledgment.—The author expresses his grateful appreciation to Mrs. Harriet Starr and Mr. Dave Wieg for their assistance in this work.

WASHINGTON, D. C.

(10) A. M. Abu-Nasr, W. M. Potts and R. T. Holman, *J. Am. Oil Chemists' Soc.*, **31**, 16 (1954)