to be of great value in separations of saturated and unsaturated acids. The customary elution separation has been used by numerous investigators with success for the separation and isolation of both saturated and unsaturated fatty acids. The present investigation has indicated the usefulness of Tiselius' displacement technique[19] in separating unsaturated acids from 4 to 18 carbon atoms in length. Thus displacement chromatography can be used for separation of saturated and unsaturated acids and for separation of acids differing in degree and kind of unsaturation whenever the advantages of this type of chromatography make it desirable.

From the experiments shown and from other experience gained in this type of adsorption work, it can be concluded that within a family of acids of equal chain length, increasing unsaturation decreases the adsorbability on Darco G 60 charcoal if the unsaturation is non-conjugated. On the other hand, if the double bonds are conjugated either with the carboxyl group or with themselves, the adsorbability is increased. Increasing conjugated unsaturation increases adsorption.

The usefulness of displacement chromatography in separation of fatty acids on the basis of kind and degree of unsaturation has been demonstrated. The experiments presented here also indicate the usefulness of the technique in the detection of impurities. In many cases, preparations thought to be pure were found to contain impurities detectable by displacement separation. This is most striking in the case of the *n*-caproic acid which had been subjected to three fractional distillations prior to use, yet still contained an unsaturated impurity. The detection of small amounts of unexpected chromophoric substances in the chromatograms involving the long chain polyunsaturated acids suggests the possible usefulness of this method in their purification and in studies of their oxidation products.

(19) Tiselius, *Arkiv Kemi Mineral. Geol.*, **16A**, 18 (1943).

COLLEGE STATION, TEXAS RECEIVED MAY 3, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, TEXAS AGRICULTURAL AND MECHANICAL COLLEGE SYSTEM, COLLEGE STATION]

Displacement Analysis of Lipids. VII. Carrier Separation of Unsaturated Fatty Acids[1a]

BY RALPH T. HOLMAN[1b]

The positions of a series of single unsaturated fatty acids in a carrier displacement chromatogram have been determined using methyl esters of even saturated acids in one series and methyl esters of odd saturated acids in another using Darco G 60 as adsorbent and 95% ethanol as solvent. It was found that increasing the number of isolated double bonds decreases the adsorption, changing a saturated acid to an unsaturated acid to an acetylenic acid decreases adsorption, conjugation of double bonds increases adsorption. Differences in adsorbability of *cis*- and *trans*-isomers are slight. One isolated double bond in the molecule decreases adsorption roughly equivalent to 2 less carbon atoms. Separation of stearic and linoleic acids and of linoleic acid and its conjugated isomer by carrier displacement are demonstrated.

Carrier displacement chromatography introduced by Tiselius and Hagdahl[2] makes use of relatively large quantities of substances whose adsorption isotherms lie between those of the components of a sample. When the sample plus these additional substances are displaced, the constituents arrange themselves in order of adsorbability, and the components of the sample are sandwiched between much larger zones of the added substances. Thus. small quantities of sample constituents travel at the fronts of the zones of the more abundant "carriers," and are, under ideal conditions, completely separated from each other. Such a technique has been found applicable to separations of amino acids and peptides[2] and separations of saturated fatty acids.[3] The study of fatty acids has now been extended to a series of unsaturated fatty acids to obtain qualitative information on the influence of kind and degree of unsaturation upon the position occupied by an acid within a carrier system of methyl esters of saturated fatty acids.

Experimental

The apparatus used in this investigation was a modification of the Tiselius–Claesson adsorption–analysis apparatus[4] equipped with Hagdahl coupled filters.[5] The apparatus and its use is described elsewhere.[6] The interferometric observations were made with a 76-mm. cuvette at 37°. The adsorbent was 1 part Darco G 60 and 2 parts Hyflo Supercel. The carrier systems were similar to those used in the study of carrier separation of saturated acids.[3] The acids were chromatographed in two carrier systems. One system consisted of approximately 120 mg. of methyl palmitate, 95 mg. of methyl myristate and 60 mg. of methyl laurate displaced by 1.0% methyl stearate. The other system consisted of approximately 150 mg. of methyl heptadecanoate, 130 mg. of methyl pentadecanoate, and 75 mg. of methyl tridecanoate displaced by 1.0% methyl stearate. The solvent, unless otherwise specified, was 95% ethanol. The esters used were prepared from commercially available acids and last traces of acid were removed from the ester by treatment with aluminum oxide. All esters were sufficiently pure for the present use as determined by single displacement experiments. The unsaturated fatty acids were prepared by standard procedures.[7]

In the simple carrier experiments, the acid component was located by titration of 1.0-ml. fractions with approximately 0.02 *N* KOH. In the experiments involving linoleic acid, its concentration was determined in the 1.0-ml. frac-

(1) (a) Presented before the 12th International Congress of Pure and Applied Chemistry, New York, September, 1951. Supported in part by grants from the Research Corporation of New York, the National Dairy Council on behalf of the American Dairy Association, and by a contract between the Office of Naval Research and the Texas A. and M. Research Foundation. (b) Hormel Institute, Austin, Minn.

(2) Tiselius and Hagdahl, *Acta Chemica Scand.*, **4**, 394 (1950).

(3) Holman, THIS JOURNAL, **73**, 1261 (1951).

(4) Tiselius and Claesson, *Arkiv for Kemi Mineral. Geol.*, **15B**, 18 (1942).

(5) Hagdahl, *Acta Chemica Scand.*, **2**, 574 (1948).

(6) Holman and Hagdahl, *Anal. Chem.*, **23**, 794 (1951).

(7) Ralston, "Fatty Acids and Their Derivatives," John Wiley and Sons, Inc., New York, N. Y., 1948.

tions by measurement of its enzymatic oxidation product.[8] Conjugated linoleic acid was determined by means of its light absorption at 2340 Å. The unsaturated acids were prepared by conventional methods and were found to be of sufficient purity for this use by the experiments themselves. In a few preparations minor impurities were detected in the carrier displacement diagrams (for example, arachidonic acid).

Results and Discussion

Qualitative experiments were performed with one unsaturated fatty acid at a time in each of the two (even and odd) carrier systems. With a given carrier system it was found that some acids were located *between* zones of carrier substances, whereas others were located *within* zones of carrier substances. By combining the data obtained with both carrier systems (methyl esters of even and odd acids), it was possible to arrange the unsaturated acids in an adsorbability series using the carrier methyl esters as reference substances. The data are shown in Table I. It should be mentioned that the

TABLE I

ADSORBABILITIES OF FATTY ACIDS WITH REFERENCE TO METHYL ESTERS OF EVEN AND ODD FATTY ACIDS. DECREASING ADSORBABILITY DOWNWARD

Even carriers	Acids	Odd carriers
Methyl stearate		(Methyl stearate)
	Brassidic	
	Erucic	Methyl heptadecanoate
	Stearic Gadelaidic	
Methyl palmitate		
	10,12-Linoleic	
	Palmitic Arachidonic	Methyl pentadecanoate
	conj.-Linoleic (8°)	
	Petroselaidic	
	Vaccenic	
Methyl myristate	Petroselinic	
	Elaidic	
	Oleic	
	Linoleic	
	Linolenic	
	Stearolic	Methyl tridecanoate
	Myristic	
	Palmitoleic	
	11-Octadecynoic	
Methyl laurate		

position of an acid in this table indicates its relative adsorbability in this chromatographic system *only*. Position of an acid within a group should not be interpreted as indicating its adsorbability with reference to other acids in that group, but only in reference to acids in other groups. Thus far, such fine differences in adsorbability have been worked out in only a few cases.

As examples of the data obtained in this manner, single carrier displacement diagrams are shown for oleic acid (Fig. 1), palmitoleic acid (Fig. 2) and linolenic acid (Fig. 3) using the even acid methyl ester carriers. It will be noticed that oleic and linolenic acids appear within the methyl myristate zone, whereas palmitoleic acid appears between methyl laurate and methyl myristate.

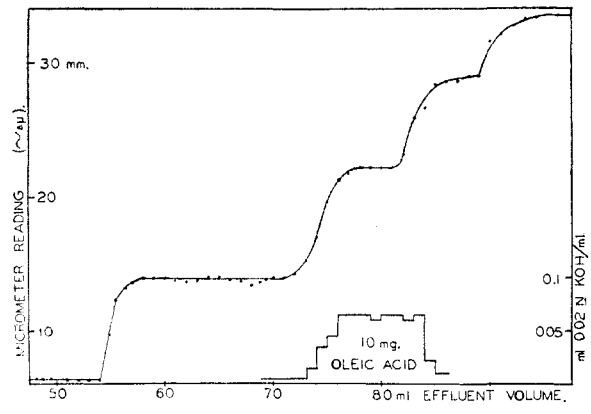

Fig. 1.—Position of oleic acid in carrier system of methyl laurate, myristate and palmitate.

(8) Holman, unpublished data.

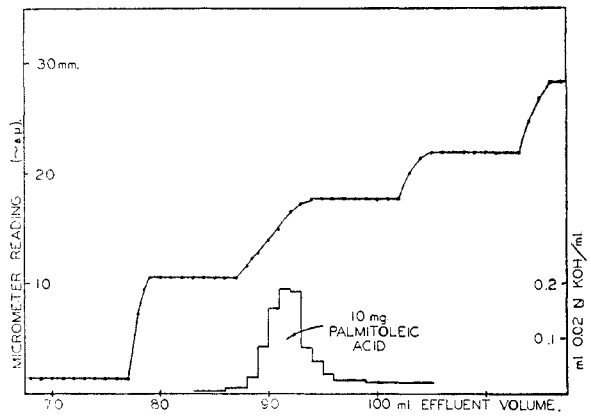

Fig. 2.—Position of palmitoleic acid in carrier system of methyl laurate, myristate and palmitate.

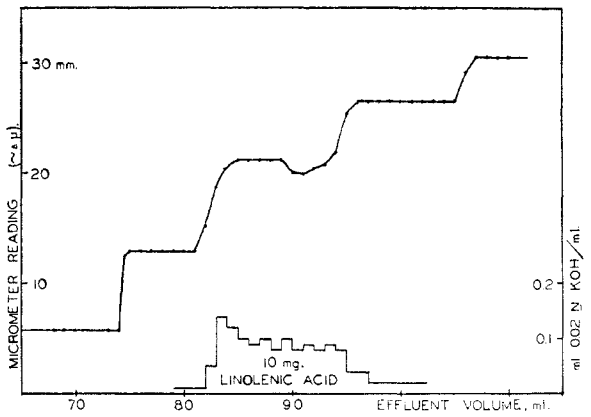

Fig. 3.—Position of linolenic acid in carrier system of methyl laurate, myristate and palmitate.

Separation of 5.0 mg. of linoleic acid from 15.0 mg. of stearic acid by carrier displacement is shown in Fig. 4. The carriers were 49 mg. of methyl laurate, 155 mg. of methyl myristate and 102 mg. of methyl palmitate in 0.05% methyl stearate in 95% ethanol. The displacer was 1.0% methyl stearate. The column had a total capacity of 40.0 ml. One-ml. fractions were collected, of which 0.20 ml. was used for the determination of linoleic acid. The remainder was titrated with 0.0087 *N* KOH. To each of the 0.20-ml. aliquots from each of the portions was added 0.05 ml. of 0.5 *N* KOH and the samples were placed in an oven at 100° until the samples were nearly dry. To each of the saponified samples was added a lipoxidase preparation to cause the oxidation of the linoleic

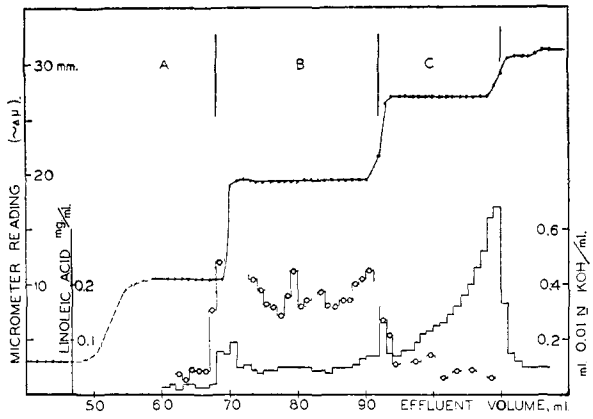

Fig. 4.—Carrier displacement separation of 5.0 mg. of linoleic acid and 15.0 mg. of stearic acid in a carrier system of 49 mg. of methyl laurate, 155 mg. of methyl myristate and 102 mg. of methyl palmitate in 0.05% methyl stearate in 95% ethanol with 1.0% methyl stearate as displacer; filter 40.0 ml.

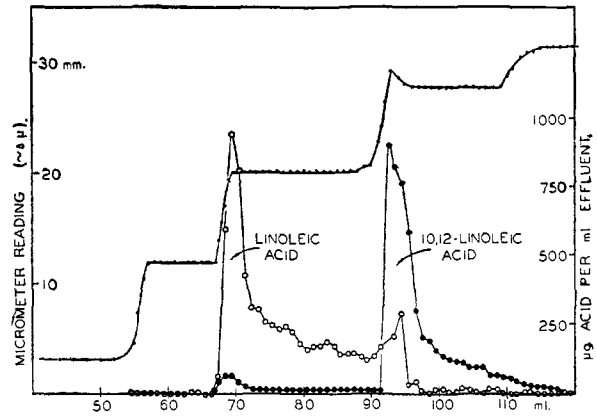

Fig. 5.—Carrier displacement separation of 9.7 mg. of linoleic acid and 10.5 mg. of 10,12-linoleic acid in a carrier system of 50 mg. of methyl laurate, 154 mg. of methyl myristate and 102 mg. of methyl palmitate in 0.05% methyl stearate in 95% ethanol with 1.0% methyl stearate as displacer; filter 40.0 ml.

acid, thereby inducing conjugation of its double bonds. Light absorption at 2340 Å. was measured and the amount of linoleic acid calculated. The experiment indicates that linoleic acid appears at the junction of methyl myristate and methyl laurate and is present throughout the methyl myristate step. Stearic acid appears largely at the junction between methyl palmitate and methyl stearate but extends into the palmitate step. Under these conditions the linoleic and stearic acids were separated. Unfortunately several fractions during the experiment were spoiled, but even so, the estimated linoleic acid accounted for 80% of the 5.0-mg. sample. By titration 0.069 meq. of acid was found (*ca.* 19.5 mg. acids). Stearic acid found in fractions subsequent to #92 totalled 14.7 mg. Thus it is demonstrated that the losses are not excessive, and it is quite possible that precautions against oxidation of the unsaturated acid would increase its recovery.

The carrier displacement of linoleic acid and its conjugated (10,12) isomer is shown in Fig. 5. The sample contained 9.7 mg. of linoleic acid and 10.5 mg. of 10,12-linoleic acid and the carrier system consisted of 49.6 mg. of methyl laurate, 154 mg. of methyl myristate and 102 mg. of methyl palmitate in 0.05% methyl stearate. The column had a volume of 40.0 ml. and the displacer was 1.0% methyl stearate in 95% ethanol. One-ml. fractions were cut and the light absorption at 2340 Å. was determined on each. Then 0.20 ml. of each fraction was heated with 0.05 ml. of 0.5 *N* KOH at 100° to saponify the esters. The samples were then treated with the lipoxidase preparation to cause oxidation of the linoleic acid. Following dilution, optical density at 2340 Å. was again measured, and the increase over the previous value was related to linoleic acid content by use of a standard curve. Inasmuch as the purpose of the experiment was to determine qualitatively if separation had taken place, no precautions against oxidation of the unsaturated components were made. Nevertheless, determinations of linoleic acid showed a recovery of 7.5 mg. and although determination of 10,12-linoleic acid was only semi-quantitative, 5.30 mg. of it was found in the latter samples. These recoveries could undoubtedly be improved by precautions against oxidation and more quantitative technique. However, qualitative separation of the two isomers is amply demonstrated.

From the data gained in these and similar carrier experiments and in displacement separation of unsaturated acids,[9] the following generalizations can be made concerning the effects of unsaturation upon adsorbability in the systems used: (a) With acids of equal chain length, increasing the number of double bonds decreases adsorbability (stearic acid > oleic acid > linoleic acid > linolenic acid). (b) Changing a single bond to a double bond to a triple bond decreases adsorbability (behenic acid > brassidic acid, erucic acid > behenolic acid; stearic acid > vaccenic acid > 11-octadecynoic acid; stearic acid > elaidic acid, oleic acid > stearolic acid). (c) Conjugation of isolated double bonds increases adsorption (conj. linoleic acid > linoleic acid; β-eleostearic acid > linolenic acid). (d) Unsaturation conjugated with the carboxyl group increases adsorbability.[9] (e) Differences in adsorbability between *cis-* and *trans-*isomers are slight though detectable (petroselaidic acid > petroselinic acid). (f) One isolated double bond in the acid reduces adsorbability approximately as much as shortening the chain 2 carbon atoms. (g) One triple bond in the acid reduces adsorbability approximately as much as shortening the chain 4 carbon atoms.

Carrier displacement separation of unsaturated acid has certain limitations. Many of the acids which are of biological importance and with which the analyst is concerned, fall in the same or neighboring groups, and cannot therefore be distinguished in simple systems such as used here. Possibly the use of more discriminating systems would solve this problem. However, the method does offer possibility of separation of many mix-

(9) Holman and Williams, THIS JOURNAL, 73, 5285 (1951).

tures of fatty acids of interest and offers the advantage that small samples can be used. The usefulness of the method is dependent upon the carriers employed. Only greatly differing substances can be separated if the carriers differ greatly in adsorption. With a system of carriers containing numerous components differing only slightly in adsorbability, the resolution is greater. The carrier hydrocarbon system used by Weitkamp[10] in amplified distillation is an example of the latter. Unfortunately, the hydrocarbon system was unsuitable for the present use. However, it seems probable that some highly complex natural or synthetic mixture having suitable solubility and adsorbability characteristics could be found for use as a more adequate carrier system.

The suggestion of group separation of saturated and unsaturated fatty acids by displacement from silica gel has been made by Claesson.[11] Unfortunately the experiments reported here cannot be closely compared to Claesson's, for the chromatographic systems were dissimilar, the effect of increasing unsaturation generally increasing adsorption on silica gel. However, it seems unlikely that all saturated acids can be separated groupwise from all unsaturated acids. It is more probable that the "group separation" reported was the segregation of saturated from unsaturated acids of similar chain lengths which was observed in the present investigation.

Acknowledgment.—Opportunity is taken to express gratitude to Betty Gibson and Myrtis Schrode for valuable technical assistance.

(10) Weitkamp, *J. Am. Oil Chem. Soc.*, **24**, 236 (1947).

(11) Claesson, *Rec. trav. chim.*, **T65**, 9 (1946).

COLLEGE STATION, TEXAS RECEIVED MAY 3, 1951

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY, DEPARTMENT OF SURGERY OF BETH ISRAEL HOSPITAL AND HARVARD MEDICAL SCHOOL]

Half-Salts of Naphthyl Acid Phosphates[1]

BY ORRIE M. FRIEDMAN AND ARNOLD M. SELIGMAN

A half-salt of β-naphthyl dihydrogen phosphate has been obtained (1) by treatment of an alkaline solution of the dihydro acid with excess mineral acid, (2) by precipitation of the acid from solution with sodium chloride or sodium sulfate and (3) by coprecipitation of the acid with its mono sodium salt. The precipitated complex has been shown to be a combination of the dihydrophosphoric acid ester and the mono sodium salt in equimolar amount. Half-salts of α-naphthyl dihydrogen phosphate and 6-bromo-2-naphthyl dihydrogen phosphate have also been obtained. Previous formulation of half-salts of this type as eight-membered ring structures is discussed and an alternate six-membered ring structure suggested.

When β-naphthyl dihydrogen phosphate,[2] m.p. 178–179°, dissolved in aqueous sodium hydroxide, was treated with an excess of mineral acid, the expected β-naphthyl dihydrogen phosphate as such was not recovered, but there was obtained a substance of higher melting point. The same higher melting substance was apparently also obtained when β-naphthyl dihydrogen phosphate was crystallized from aqueous sodium chloride or aqueous sodium sulfate. The substance melted at 203–205°, partially resolidified almost immediately and remelted completely at 250°. The potentiometric titration curve (Fig. 1) suggested that this material was an unsymmetrical dimer with a molecular weight of about 470. Although the melting point of this substance remained practically unchanged after repeated recrystallization from water–ethanol, the potentiometric titration curve of the recrystallized product showed a significant change in the relative position of the breaks in the titration curve, indicating that the dimeric product consisted of two substances. This was in fact proven to be the case by the isolation of β-naphthyl dihydrogen phosphate and sodium β-naphthyl monohydrogen phosphate in apparently equimolar amounts by extraction of the complex with boiling ethyl acetate–alcohol. Since β-naphthyl dihydrogen phosphate is a weaker acid than hydrochloric, the removal of sodium ion from sodium chloride in solution is apparently a solubility phenomenon.

The addition of separate solutions of equimolar amounts of the free acid and of the monosodium salt to one another gave an immediate precipitate of the identical complex. The same solubility phenomenon was observed when a relatively concentrated solution of β-naphthyl dihydrogen phosphate was titrated with alkali. Just before the half-molar point in the titration a precipitate appeared and then disappeared slowly with further addition of alkali.

Methylation of the complex with diazomethane gave in equimolar amounts a white, crystalline, ether-insoluble solid, which proved to be sodium methyl β-naphthyl phosphate, and an ether-soluble oil, which proved to be dimethyl β-naphthyl phosphate. The identical solid was obtained by treatment of the monosodium salt of β-naphthyl dihydrogen phosphate with diazomethane. The identical oil was obtained both by treatment of β-naphthyl dihydrogen phosphate with diazomethane and by reaction of β-naphthyl phosphoryl dichloride with methanol.

Comparison of infrared absorption spectrum of the complex with that of a finely ground mixture of equimolar portions of the free acid and of the monosodium salt in suspension in mineral oil revealed differences in the 3.0, 6.4, 9.3–9.6, 11.2, 11.5 and 15.5 micron regions (Fig. 2). The specific significance of these differences is obscure but the

(1) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

(2) O. M. Friedman and A. M. Seligman, THIS JOURNAL, **72**, 624 (1950).