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

Displacement Analysis of Lipids. IV. Carrier Displacement Separation of Saturated Fatty Acids[1]

BY RALPH T. HOLMAN

In an effort to develop a milligram scale separation of natural fatty acids, experiments in carrier displacement separation have been attempted. The most satisfactory system of carriers has been found to be the methyl esters of the fatty acids themselves. With a dilute solution of the displacer substance as solvent for all operations, it has been found that recoveries of 5–15 milligram quantities of lauric, myristic, palmitic and stearic acids approach 100%. This type of chromatographic separation thus offers the possibility of a clear cut separation and determination of small quantities of these acids, and it seems likely that carrier displacement can be applied to other substances.

Introduction

The technique of displacement chromatography has proven to be valuable in the separation of the saturated fatty acids,[2,3,4] sugars[5] and amino acids.[6] Recent experiments have shown that displacement separation is easily performed in other homologous series such as alkyl bromides, alcohols and esters.[7] A new technique of separation of small quantities of substances by means of displacement has been introduced by Tiselius and Hagdahl.[8] In their method of displacement separation called "carrier displacement" convenient quantities of a series of easily separable carriers are added to the small sample to be separated. If the series of carriers is properly selected, the components of the sample arrange themselves between the broad zones of the carrier substances. In this manner the components wanted are actually separated by zones of the carrier, and are themselves found in the fronts of the carrier zones. Thus, minute quantities of substances can be separated and recovered by carrier displacement, whereas much greater quantities of sample would be required to observe the zones of each of them in an ordinary displacement system. The application of this technique to separation of fatty acids is herein reported.

In the search for compounds suitable for use as displacers for fatty acids it was found that the alkyl bromides are easily separable by displacement chromatography. Likewise, the two series, normal alcohols and ethyl esters, are easily separable by the routine techniques.[7] These homologous series offer themselves then as possible carrier systems for fatty acid separations. Attempts have been made with alkyl bromide or ethyl ester carrier systems, but the results were not wholly satisfactory. Losses were high and the acids were found throughout the carrier zones rather than between them. The use of methyl esters of fatty acids as carrier systems is far more promising and will be described in detail.

Experimental

The fatty acids used in these investigations were Eastman Kodak Co. products and were found of suitable purity by single displacement experiments. The methyl esters used as carriers were prepared from these acids by esterification in the usual manner. The last traces of acid were removed from the products by passing them through columns of activated alumina.

The apparatus used in these investigations was a modified Tiselius–Claesson adsorption analysis apparatus, the design and use of which is described elsewhere.[9] In the experiments to be described, the adsorbent used was one part Darco G.60 charcoal plus 2 parts Hyflo Supercel. The solvent used was 95% ethanol and the column built of coupled filters[10] totalled 40 cc. volume. The quantities of carrier substances in all experiments were 50 mg. of methyl laurate, 80 mg. of methyl myristate and 120 mg. of methyl palmitate. The displacer was 1.0% methyl stearate. The cuvette was 76 mm. long and all readings were made at 37°. When the esters began emerging from the column, 1-cc. fractions of effluent were collected and titrated with standardized base.

Results and Conclusions

The displacement separation of methyl laurate, methyl myristate and methyl palmitate by methyl stearate is quite conveniently performed. The refractive index levels characteristic of each are sufficiently different to make the zones of these compounds in the displacement diagram easily discernible. When a few milligrams of myristic acid are added to the three esters, the acid is found between the zones of methyl laurate and methyl myristate (Fig. 1). When palmitic acid is added to the sample it appears between the zones of methyl myristate and methyl palmitate. When stearic acid is added it appears between the zones of methyl palmitate and the methyl stearate displacer. Thus, an acid is displaced by its corresponding methyl ester.

When all three acids are included in the sample, they appear in the displacement diagram at their predicted positions (Fig. 1). If fractions were cut at the positions indicated by the dotted lines, the acids would then be isolated from each other. Recovery of the acids and their measurement would then be a simple matter.

Determination of the recoveries of lauric, myristic, palmitic and stearic acids has been made in several chromatograms such as described above. Rather than taking 1-cc. fractions to locate the acid zones, larger fractions were cut at the points on the interferometric displacement diagram when the micrometer readings had been constant for 4 cc. after a step on the curve, points which correspond to minima in the acid titration curves. The recoveries of each acid and of total acid are listed in Table I.

(1) Presented at the 118th Meeting of the A. C. S., Chicago, September, 1950.

(2) Holman and Hagdahl, *Arch. Biochem.*, **17**, 301 (1948).

(3) Hagdahl and Holman, THIS JOURNAL, **72**, 701 (1950).

(4) Holman and Hagdahl, *J. Biol. Chem.*, **182**, 421 (1950).

(5) Tiselius and Hahn, *Kolloid Z.*, **105**, 177 (1943).

(6) Tiselius, "The Svedberg," Almquist and Wiksells, Stockholm (1944).

(7) Holman, Abstracts 117th A. C. S. Meeting, Houston, March, 1950, p. 6G (THIS JOURNAL, in press).

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

(9) Holman, *Anal. Chem.* in press.

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

TABLE I

RECOVERIES OF INDIVIDUAL AND TOTAL ACIDS IN CARRIER SEPARATIONS, SOLVENT 95% ALCOHOL

Acid		Expt. 1	Expt. 2	Expt. 3
Lauric	Sample, mg.	4.9	6.5	1.95
	Recovery, %	88	82	65
Myristic	Sample, mg.	10.9	11.1	19.35
	Recovery, %	90.5	107.0	85
Palmitic	Sample, mg.	15.4	16.0	8.4
	Recovery, %	107.7	92.5	118.0
Stearic	Sample, mg.	16.8	16.3	15.1
	Recovery, %	72.7	85.9	71
Total recovery, %		89.7	97.0	88.5

The recoveries of the acids, although not quantitative were encouraging. To minimize losses two devices were attempted. A column which had been used for one experiment was washed with 95% ethanol until no detectable methyl stearate was present in the eluate in the hope that charcoal which had been saturated with displacer would retain less of the sample acids. Unfortunately this device failed, for the shape of the curve of the next experiment was distorted.

By treating the charcoal beforehand with 0.05% methyl stearate, by washing the column with this dilute solution of the displacer, and by dissolving the sample in this solution, the charcoal would always be exposed to a low displacer concentration, and the irreversible adsorption of the acids might be decreased. When this device was tried, the recoveries were considerably better. The results of such experiments are shown in Table II. It is clear that the total recovery approaches 100% by the use of a low concentration of displacer in the wash and sample of the chromatogram.

TABLE II

RECOVERIES OF INDIVIDUAL AND TOTAL ACIDS IN CARRIER SEPARATIONS, SOLVENT 0.05% METHYL STEARATE

Acid		Expt. 1	Expt. 2
Lauric	Sample, mg.	4.75	3.85
	Recovery, %	100	93.5
Myristic	Sample, mg.	10.2	9.6
	Recovery, %	100.4	100.1
Palmitic	Sample, mg.	14.0	14.5
	Recovery, %	100.2	98.5
Stearic	Sample, mg.	14.4	14.8
	Recovery, %	98	100.3
Total recovery, %		100.1	100.1

By inspection of the curves shown in Fig. 1 it will be seen that the acid largely occurs in the chromatogram just ahead of its methyl ester. However, the acid reaches into the zone of the lower methyl ester. When the acid is increased in relation to the amount of esters used, it builds up in concentration throughout the zone of methyl ester preceding it. Thus it becomes clear, that the ratio of esters to acids must remain high, probably in the order of 10/1 for best results.

It is to be noticed that where zones of acids occur in these chromatograms, slight irregularities appear in the interferometric displacement diagrams. These serve as qualitative indicators of which acids are present. The acids are effectively displaced by their methyl esters, and within a few cc. after the point where the displacement diagram shows

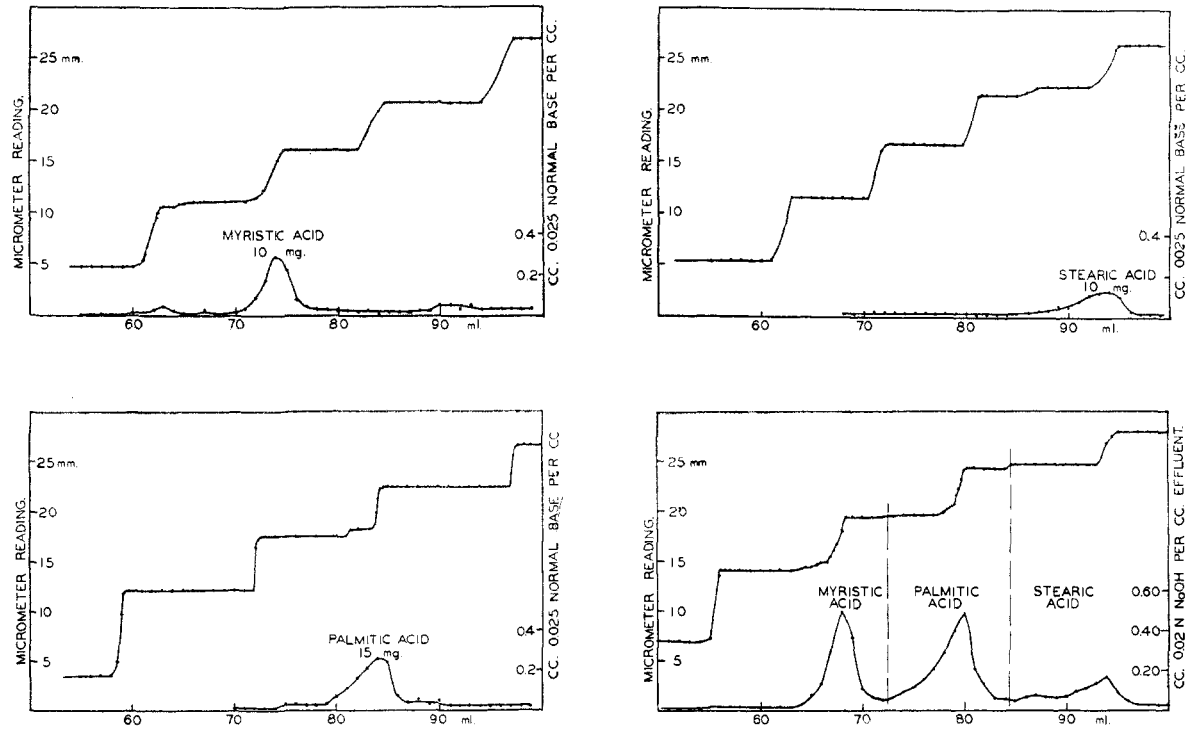

Fig. 1.—Carrier displacement separation of myristic, palmitic and stearic acids in a carrier system of methyl esters of these acids: filter column capacity 40 cc.; solvent 95% ethanol; carriers 50 mg. methyl laurate, 80 mg. methyl myristate, 120 mg. methyl palmitate; displacer 1.0% methyl stearate. Acid quantities indicated on curves.

a rise, virtually all of the acid has passed. Thus it is suggested that cuts be made when each step has remained constant for 4 cc.

With this technique of carrier separation a micro method is available for fatty acid separations and analyses. It appears that if the methyl esters of the acids in question are separable by displacement chromatography, the acids themselves can be measured by using their esters as carriers. This arrangement would appear to be preferable, for interposing small quantities of unsaturated acids in the saturated methyl ester series showed that oleic acid appeared in the methyl myristate zone, and linoleic acid appeared between methyl laurate, and methyl myristate. If a system can be worked out for displacement of both saturated and unsaturated methyl esters, the analysis of naturally occurring mixtures of saturated and unsaturated fatty acids should be possible. This technique of carrier displacement also provides a means of identifying small amounts of fatty acids or other types of compounds.

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The Cleavage of Cholesteryl and 7-Dehydrochloesteryl Ethers[1a]

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A study has been made of the cleavage of cholesteryl and 7-dehydrocholesteryl ethers to the free sterols by means of sodium alkyls. In addition to sterols, unsaturated hydrocarbons are formed. Cholesteryl ethers give 3,5-cholestadiene. 7-Dehydrocholesteryl ethers give a new triene whose probable structure has been shown to be 3,5,7-cholestatriene, the configuration assigned by Eckhardt to his compound which, however, is probably the 2,4,6-cholestatriene. A mechanism is proposed for the reaction involving attack on a free β-hydrogen which accounts for the formation of the products of the reaction and the failure of methyl ethers to give the free sterols.

The methyl, ethyl and isopropyl ethers of 7-dehydrocholesterol have been prepared from the corresponding cholesteryl ethers.[2,3] Since on irradiation they gave products which were considerably less active than vitamin D_3, an investigation of their cleavage to the free sterol was undertaken. As 7-dehydrocholesterol is sensitive to strong acids, the method of cleavage was limited to basic reagents.[3] Of these the metal alkyls appeared most promising.

The cleavage of ethers by metal alkyls was first reported by Schorigin.[4] Ziegler[5] and also Gilman[6] have shown that various organoalkali metal compounds react with ethyl ether. Hückel and Bretschneider[7] cleaved alicylic ethers such as *l*-menthyl ethyl ether with ethylsodium, and obtained a 70% yield of *l*-menthol along with a hydrocarbon.

A consideration of the literature[8,9] led us to choose *n*-amylsodium, prepared from *n*-amyl chloride and sodium[10] although a number of other metal alkyls were also investigated. This reagent was successfully used to cleave the model cholesteryl ethers, as well as the 7-dehydrocholesteryl ethers to the free sterols.

To a hexane solution of the cholesteryl ether was added sodium sand and then *n*-amyl chloride. The formation of the reagent took place in the presence of the ether and the products of the reaction were separated chromatographically into a sterol and an ether–hydrocarbon fraction. The sterol fraction was essentially pure cholesterol. The other fraction contained unreacted ether together with a hydrocarbon shown to be 3,5-cholestadiene (XII, $\Delta^{3,5}$). In certain cases these were separated quantitatively by further chromatography; in others the estimation depended on the absorption spectra of the diene. The

TABLE I

CLEAVAGE OF CHOLESTERYL ETHERS WITH *n*-AMYLSODIUM

Ether	Benzene–methanol eluate, %[a]	Sterol, %[b]	Benzene eluate: Unreacted ether, %[c]	Benzene eluate: Diene, %[c]
Methyl	3[d]	Trace	46[e]	51[e]
Ethyl	74.1	100	15	10
Isopropyl	69.2	96.1	21	8
t-Butyl	32.2	99.7	61.3	3
Benzyl	Trace	Nil	51[e]	45[e]

[a] This chromatograph fraction contains crystalline sterol or tails of early fractions. [b] By digitonide determination on (*a*). [c] The 3,5-cholestadiene is usually determined by its absorption spectrum and the ether by difference. [d] This small oily fraction was probably a residue of methyl ether on the column. The methyl ether gives a positive digitonide. [e] Isolated by chromatography. See Experimental.

(1) (a) Presented before the Organic Division of the American Chemical Society at New York City, September, 1947. (b) Schering Corporation, Bloomfield, N. J. (c) Department of Microbiology, Rutgers University.

(2) W. L. Ruigh, D. H. Gould, H. Urist and K. H. Schaaf, to be published.

(3) H. Rosenberg and S. G. Turnbull [U. S. Patent 2,386,636] cleaved the trityl ether of 7-dehydrocholesterol with acetic acid, and also prepared the methyl and ethyl ethers. These ethers were prepared, however, only from 7-keto cholesterol. In general cholesteryl ethers cleaved by mild acids, *e.g.*, trityl, vinyl, etc., cannot be oxidized satisfactorily in the Windaus process.

(4) P. Schorigin, *Ber.*, **43**, 1931 (1910).

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(9) J. Schmidt, "Organometallverbindungen," Vol. II, Wissenschaftliche Verlagsgesellschaft m. b. H., Stuttgart, 1934, p. 10.

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