

[CONTRIBUTION FROM DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY AND THE HORMEL INSTITUTE, UNIVERSITY OF MINNESOTA]

## Displacement Analysis of Lipids. X. Model Mixtures of Glycerides<sup>1</sup>

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Displacement chromatography was successfully applied to the separation of model mixtures of glycerides. The adsorbabilities of glycerides from ethanol onto a charcoal form the following series: (1) trilaurin > tricaprins > tricaprins > tricaprins > tributyrin; (2) monostearin > monopalmitin and monoolein > monolaurin; (3) triglyceride > diglyceride > monoglyceride. The adsorbabilities from benzene form the following series: (1) tristearin > palmitidistearin > tripalmitin > lauryldipalmitin > trimyristin or triolein > trilinolein > trilaurin; (2) monoglyceride > diglyceride > triglyceride. In a preliminary experiment beef tallow was separated into fractions having widely different melting points.

**Introduction.**—Displacement chromatography has been applied to lipids for the separation of saturated fatty acids,<sup>3-5</sup> unsaturated fatty acids,<sup>6</sup> substances analogous to fatty acids,<sup>7</sup> oxidation products of fatty acids,<sup>8</sup> and steroids.<sup>9</sup> Its use in lipid separations has been recently reviewed.<sup>10</sup> This investigation was undertaken to assess the applicability of this method for the separation of glycerides. In the field of natural products glycerides have proven difficult to separate because of the extremely complex mixture of glycerides present in most lipids. Chromatography has been extensively used for separating natural products, but has not been widely used for separating glycerides. Elution chromatography has been used with some success for separation of glycerides of natural oils.

Walker and Mills<sup>11-13</sup> dissolved linseed oil in *n*-hexane and passed the solution through a column of alumina. By a complex and time-consuming procedure, they were able to separate the glycerides into fractions containing from 4 to 9 double bonds per molecule. Reinbold and Dutton,<sup>14</sup> using alumina as an adsorbent and diethyl ether as an eluant, segregated soybean oil into fractions having a difference in iodine value of 69 units. They believed that chromatography compared favorably with countercurrent extraction and low temperature crystallization as a means of separation of glycerides.

By comparing the retention volumes obtained from single frontal analyses on a charcoal of tripalmitin, trimyristin, triolein and trilaurin in benzene,

(1) From a dissertation submitted by James G. Hamilton to the University of Minnesota in partial fulfillment of the requirements for the degree of doctor of philosophy. Supported in part by a contract with the Office of Naval Research. Hormel Institute publication no. 105. Presented at meeting of the American Oil Chemists' Society, San Antonio, April, 1954.

(2) Hormel Institute, Austin, Minnesota.

(3) R. T. Holman and L. Hagdahl, *Arch. Biochem.*, **17**, 301 (1948).

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chloroform and ether, Claesson<sup>15</sup> found the following adsorbability series: tripalmitin > trimyristin > triolein > trilaurin. His attempts to separate these compounds by displacement analysis were unsuccessful. Because newer techniques of displacement chromatography have been applied with such success to other lipid separations, it seemed likely that glycerides now could be separated by this means.

**Experimental.**—The instrument<sup>16</sup> used in this investigation was a modification of the original instrument developed by Tiselius and Claesson.<sup>17</sup> During the course of this investigation it became necessary to make further modifications of the apparatus to allow the use of non-polar solvents for analysis of the long chain triglycerides. Because the large gasket in the cuvette of the instrument was adversely affected by non-polar solvents, it was replaced by 1.5-mm. stainless steel tubing, and the two halves of the cuvette were soldered together. Rubber gaskets in the driving piston were also swollen by non-polar solvents. Those gaskets were replaced by special machined Teflon gaskets that seemingly were not affected by the solvent employed, and had the advantage of requiring no lubricant.

The adsorbent used was a mixture of 1 part Darco G-60 charcoal and 2 parts Hyflo Super-Cel, and the column capacities varied according to need. Ethanol was used for separation of the monoglycerides, and the triglycerides up to and including trilaurin. Benzene was used for the separation of the higher triglycerides.

Prior to the changes in design of the instrument, attempts were made to separate the long chain triglycerides in other adsorbent and solvent systems. Petroleum ether, b.p. 85-100° (Skellysolve C) was found to be adequate as a solvent but all attempts at displacement analysis on Darco G-60 met with failure. Alumina and silica gel were tried as adsorbents with this solvent. There was little difference in adsorbabilities of trilaurin, tripalmitin and tristearin on alumina. On silica gel, trilaurin was more strongly adsorbed than tripalmitin or tristearin, but displacement was incomplete.

Because chemical or physical identification of small quantities of glycerides is such a difficult matter, identification of individual glycerides in a mixture was made by comparison with frontal analyses of pure compounds, single displacements, and by separations of all possible combinations of a given mixture.

Trilinolein used in these experiments was prepared in this Laboratory. Synthetic 1-monopalmitin, 1,3-dipalmitin and 2-lauryl-1,3-dipalmitin were obtained from Dr. Hermann Schlenk and tricaprins and 2-palmityl-1,3-distearin were obtained from Dr. W. O. Lundberg. Tristearin, trilaurin, triolein, 1-monostearin, 1-monolaurin and 1-monoolein were obtained from Dr. Fred Mattson of the Procter & Gamble Company. Other compounds used in this investigation were commercially available products.

**Experimental Results.**—Examples of separations of saturated triglycerides in the benzene system are shown in Fig. 1. Curve I demonstrates

(15) S. Claesson, *Arkiv Kemi, Min. Geol.*, **15A**, No. 9 (1942).

(16) R. T. Holman and L. Hagdahl, *Anal. Chem.*, **23**, 794 (1951).

(17) S. Claesson, *Arkiv Kemi, Min. Geol.*, **23A**, No. 1 (1946).

the separation of three simple monoacid triglycerides. Curve II shows the separation of a mixed acid triglyceride from its related monoacid triglyceride. In both experiments the displacer was 0.5% tristearin in benzene. Trilaurin is so weakly adsorbed in this system that it was eluted, that is, it appeared at the end of dead volume as a spike. The boundary between lauryl dipalmitin and tripalmitin is not sharp, and it cannot be stated whether this is due to impurities present in the preparations or to incomplete separation. When triolein and trilinolein were displaced by 0.5% tristearin in benzene, these unsaturated triglycerides were very poorly adsorbed, and trilinolein was eluted. However, these glycerides were separated. From a consideration of the adsorbability of each of the two compounds in various experiments, triolein was found to be more strongly adsorbed than trilinolein. Triolein has approximately the same adsorbability characteristics as trimyristin.

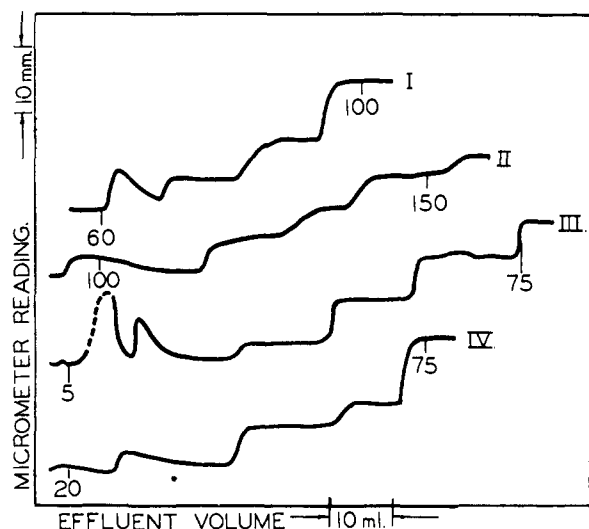


Fig. 1.—I, displacement separation of 15 mg. of trilaurin, 25 mg. of trimyristin and 50 mg. of tripalmitin; column 64.8 ml., solvent benzene, displacer 0.5% trimyristin. II, separation of 15 mg. of trilaurin, 30 mg. of lauryl-dipalmitin, 50 mg. of tripalmitin and 72 mg. of palmityl-distearin; column 95 ml., solvent benzene, displacer 0.5% tristearin; III, separation of 5 mg. of tributyrin, 11 mg. of tricaproin, 39 mg. of tricapyrylin and 65 mg. of tricaprinn using 0.4% trilaurin in absolute ethanol as displacer. Column 14.1 ml.; IV, separation of 11 mg. of monolaurin, 31 mg. of monopalmitin and 40 mg. of monostearin in absolute ethanol using 0.4% trilaurin as displacer; column 14.1 ml.

Because the lower triglycerides are soluble in ethanol, it was used for their separations. An example is shown in Fig. 1-III, in which separation of tributyrin, tricaproin, tricapyrylin and tricaprinn was accomplished. In a number of experiments an eluted impurity appeared in those including tricapyrylin. The more strongly adsorbed (second) peak is believed to be tributyrin and the larger and less strongly adsorbed substance is the impurity associated with tricapyrylin.

Fig. 1-IV illustrates the separation of saturated monoglycerides using trilaurin in absolute ethanol as displacer. Monolaurin was very weakly ad-

sorbed and emerged in a long low step with a hint of an elution peak. In similar experiments, monostearin was shown to be more strongly adsorbed than monoolein and separable from it by displacement. Attempts to separate monopalmitin from monoolein were not successful because their adsorbabilities were too similar.

Adsorbabilities determined via retention volumes in single frontal analyses demonstrated that a triglyceride is more strongly adsorbed from ethanol solution than is a monoglyceride (Table I). Thus from ethanol, the adsorbability series is triglyceride > diglyceride > monoglyceride.

TABLE I  
ADSORBABILITIES OF GLYCERIDES AS DETERMINED BY  
FRONTAL ANALYSES EXPRESSED AS MG./G. CHARCOAL

	In ethanol		In benzene	
	0.1% soln.	0.4% soln.	0.25% soln.	0.5% soln.
Tributyrin	..	14	..	..
Tricaproin	..	60	..	..
Tricaprylin	..	100	..	..
Tricaprin	..	130	..	..
Trilaurin	135	156	..	3.0
Trimyristin	..	..	..	9.5
Tripalmitin	..	..	16.8	15.5
Tristearin	..	..	23.3	25.5
Palmitodistearin	..	..	..	25.5
Triolein	..	..	..	10.0
Monolaurin	..	40	..	..
Monopalmitin	46	88	30.5	..
Monostearin	..	104	35.7	..
Monoolein	..	78	..	..
Dipalmitin	120	..	..	..

On the other hand frontal analyses of these glycerides indicated that the adsorbability series is reversed in the case of adsorption from benzene (Table I). Single displacement experiments verified the reversal of adsorbabilities dependent upon solvents. Thus in ethanol, trilaurin displaces monostearin (Fig. 2A), and in benzene monostearin is adsorbed more strongly than trilaurin (Fig. 2B). Tristearin was also readily displaced by 0.25% monostearin in benzene.

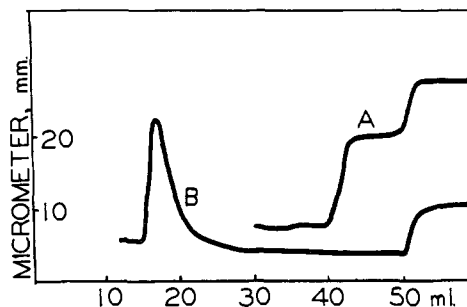


Fig. 2.—A, single displacement of 30 mg. of monostearin on a 9.1-ml. column using 0.4% trilaurin in absolute ethanol as displacer; B, single displacement of 29 mg. of trilaurin on a 19.2-ml. column using 0.25% monostearin in benzene as displacer.

**Discussion.**—The adsorbabilities of homologous glycerides on Darco G-60 charcoal increase with molecular weight and the consequent decrease in solubility. This is true whether the solvent be

ethanol or benzene. This is a direct parallel with the case of saturated fatty acids and esters, and suggests that the fatty acid components of glycerides determine their adsorptive properties.

The order of adsorbability of the mono-, di- and triglycerides depends upon the polarity of the solvent. In the polar solvent, ethanol, the least polar glyceride (triglyceride) is adsorbed most strongly. In the non-polar solvent, benzene, the most polar glyceride (monoglyceride) is adsorbed most strongly. It should be emphasized that within a homologous glyceride series, increased molecular weight causes increased adsorption, irrespective of solvent.

The possible usefulness of displacement chromatography as a method of separation of glycerides was demonstrated by experiments with model mixtures. It appears that with the systems thus far used, saturated glycerides are more easily handled than are the unsaturated glycerides. Thus, application of these methods to separation of oils containing large proportions of tri-unsaturated glycerides will be limited by the fact that the latter are poorly adsorbed.

The method has been applied to a natural fat in one preliminary experiment. Definitive means of identification of mg. quantities of a series of closely related triglycerides do not exist, but segregation of component triglycerides was demonstrated by means of melting point. Thus beef tallow was displaced by 0.5% tristearin in benzene and the effluent cut into 14 arbitrary portions. The first five fractions were liquid at room temperature, suggesting that they were unsaturated triglycerides. The latter nine portions varied in melting point from 19 to 60° indicating fractionation of less unsaturated glycerides. The analysis was performed on 800 mg. of fat, took 12 hours, and showed the presence of several distinct components. No other method known to the authors could be applied to the analysis of such a small sample of glyceride to yield such information. It is hoped that further study of the method will reduce it to a more routine procedure, and that it can be applied to the characterization of natural glyceride mixtures.

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## Syntheses of DL- $\alpha$ -Lipoic Acid

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DL- $\alpha$ -Lipoic acid has been synthesized and its structure confirmed by three new procedures. Tosylation of  $\eta$ -benzylmercapto- $\epsilon$ -hydroxycaprylic acid (IV) followed by replacement of the tosyloxy group by the benzylmercapto group yielded  $\epsilon,\eta$ -dibenzylmercaptocaprylic acid. This substance was reduced and the resulting dimercaptan oxidized to DL- $\alpha$ -lipoic acid. Reduction of the precursors of IV, ethyl 6-oxo-7-octenoate or  $\eta$ -benzylmercapto- $\epsilon$ -oxocaprylic acid by means of hydrogen and hydrogen sulfide in the presence of a cobalt sulfide catalyst also gave the desired product. The third process involved a novel reduction of  $\epsilon,\epsilon,\eta$ -tribenzylmercaptocaprylic acid followed by oxidation yielding the cyclic disulfide of  $\epsilon,\eta$ -dimercaptocaprylic acid. This type of reaction has been shown to yield cyclohexanethiol from cyclohexane dibenzylmercaptol in 18% yield.

DL- $\alpha$ -Lipoic acid has been reported to be synthesized in poor yields<sup>1</sup> as a rearrangement product. In the other reported instance in which this substance is described as 6-thioctic acid,<sup>2</sup> the yields have not been indicated.

It is the purpose of this paper to describe several other methods of synthesis and by these methods also show that DL- $\alpha$ -lipoic acid is the cyclic disulfide derived from  $\epsilon,\eta$ -dimercaptocaprylic acid. The various routes which are followed are shown in the accompanying diagram

By following the synthetic route from I through VI it was hoped that the rearrangements<sup>1,2</sup> met in the other procedures could be avoided. This idea was based on the reports that the replacement of the *p*-toluenesulfonyl group proceeds without rearrangement<sup>3</sup> and that this group can be used as an intermediate in the synthesis of mercaptans.<sup>4</sup> The unsaturated ester I was easily converted to ethyl

$\eta$ -benzylmercapto- $\epsilon$ -oxocaprylate (II) in excellent yields. This ester could not be purified but was identified by oxidation to the sulfone and by hydrolysis to the keto acid III. This acid also yielded a sulfone after oxidation with hydrogen peroxide. Reduction of this keto acid yielded  $\eta$ -benzylmercapto- $\epsilon$ -hydroxycaprylic acid (IV). The *p*-toluenesulfonyl ester of this hydroxy acid was synthesized to be converted to  $\epsilon,\eta$ -dibenzylmercaptocaprylic acid (V). This tosyl ester was never isolated in pure form but was converted directly into the acid V. From this substance DL- $\alpha$ -lipoic acid (VI) was obtained by using the ordinary sodium-liquid ammonia reduction procedure followed by oxidation with iodine to close the disulfide ring.

By using the procedure of Farlow, Lazier and Signaigo<sup>5</sup> ethyl 6-oxo-7-octenoate (I) or ethyl  $\eta$ -benzylmercapto- $\epsilon$ -oxocaprylate (II) in the presence of hydrogen, hydrogen sulfide and cobalt sulfide at about 200° and 2000 pounds pressure could be converted to DL- $\alpha$ -lipoic acid after working up in the usual manner. This product was contaminated with a polymeric substance from which it was difficult to separate crystalline material.

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