

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

Displacement Analysis of Lipids. V. Separation of Substances Analogous to Fatty Acids¹

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In an effort to find suitable carrier systems of homologous substances for the carrier displacement separation of fatty acids, several homologous series of normal aliphatic substances have been studied. The displacement separation of members of the homologous series of alkyl halides, alcohols and ethyl esters of fatty acids is presented. These may find use as carrier substances in displacement separations. A series of corresponding normal C₁₈ compounds has been arranged in order of decreasing adsorbability as determined by actual displacement separations. The series is as follows: iodide > bromide? > chloride? > mercaptan > hydrocarbon > unsaturated hydrocarbon > nitrile? > amide? > alcohol > ethyl ester. Examples are given of the use of an analogous substance of greater solubility for the displacement of a homologous series and the identification of zones in a chromatogram by "step height."

Displacement chromatography has been successfully applied to the separation of the homologous series of saturated fatty acids^{2,3,4} and carrier displacement separation introduced by Tiselius and Hagdahl⁵ has been found to be applicable to the separation of small quantities (5–15 mg.) of the saturated fatty acids.⁶ In the course of the latter investigation, several series of homologous substances were tested as possible carriers for fatty acid separations, and the displacement separation of various types of C₁₈ normal aliphatic substances was investigated. The results of these investigations are presented as basic information for separation of these and related compounds by displacement chromatography.

Experimental

The apparatus used in these experiments was a modification⁷ of the Tiselius–Claesson interferometric adsorption analysis apparatus.⁸ All experiments were performed using the Hagdahl coupled filters⁹ and interferometric measurements were made at 37° in a 76 mm. cuvette. The adsorbent used in all experiments was one part Darco G-60 charcoal mixed with 2 parts Hyflo, and the solvent was ethanol. The detailed operations performed in making displacement or frontal analysis experiments have been described elsewhere.⁷

The substances used in these investigations were Eastman or Matheson products and were found to be homogeneous by single displacement experiments. Several methods were used to determine sequence of adsorbed zones, and chemical identification was made whenever possible. For example, in experiments involving acids, amines or unsaturated substances, titration of small fractions with base, acid or bromine solution was used to identify zones. When components differed widely in melting points, a few drops from representative fractions were dried and melting points were taken on the residue. When these techniques were not applicable, identification was made from the refractive index levels determined on single displacement experiments performed using the same displacer.

The retention (or retardation) volumes of a series of C₁₈ compounds were measured on 1.0% solutions in absolute ethanol using a standard filter column of 15.3-cc. volume, built from 6.3, 4.4, 2.5, 1.2, 0.6 and 0.3 cc. filters. Using the relative adsorbabilities derived in this manner¹⁰ as a guide, experiments were planned to determine the actual displacement sequence of these compounds.

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

Homologous Series.—In the search for carrier systems suitable for separation of saturated fatty acids, three homologous series were investigated. Conditions were found for the separation of alkyl bromides from hexyl bromide through octadecyl bromide in two groups. The separation of hexyl, octyl and decyl bromides was accomplished in absolute methanol solution using a coupled filter of 27, cc. total volume and 1.2% dodecyl bromide as displacer. The results of this successful experiment are shown in Fig. 1. It will be noticed that hexyl bromide emerges in such extremely low concentration as to be virtually eluted.

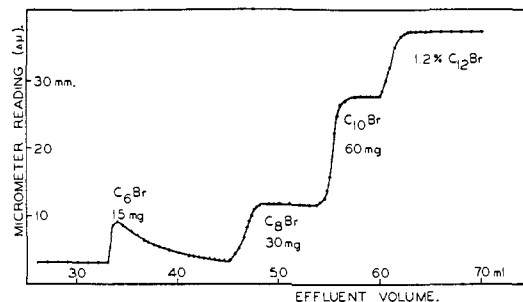


Fig. 1.—Displacement separation of hexyl, octyl and decyl bromides using a 27 cc. coupled filter, methanol as solvent, and 1.2% dodecyl bromide as displacer.

The higher members of the alkyl bromide series, C₁₂–C₁₈ were separated using a 15.3-cc. coupled filter, absolute ethanol as solvent and 1.0% octadecyl bromide as displacer. This separation is shown in Fig. 2. This easily separable homologous series, however, did not prove to be entirely satisfactory as a carrier system for saturated fatty acids. Although octadecyl bromide was found able to displace docosanoic acid, and hexadecyl bromide displaced eicosanoic acid, carrier separation of the acids by means of the alkyl bromides was found unsatisfactory.

The separation of ethyl esters by displacement chromatography was easily accomplished in 95% ethanol on a 56 cc. coupled filter using 1.0% ethyl stearate as displacer (Fig. 3). However, this homologous series also failed as a carrier system for saturated fatty acids, for although an acid is displaced by its ethyl ester, it emerges mixed with the ethyl ester of its next lower even-numbered homolog. From the results with bromides and esters it

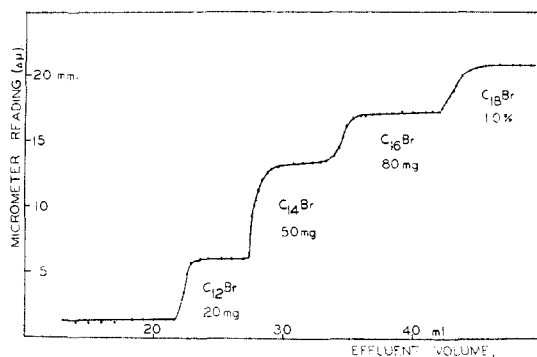


Fig. 2.—Displacement separation of dodecyl, tetradecyl and hexadecyl bromides using a 15.3 cc. coupled filter, absolute ethanol as solvent, and 1.0% octadecyl bromide as displacer.

appeared as though the adsorptive characteristics of members of these homologous series differed greatly enough to be easily separable, but that members of these series are so similar to members of the fatty acid series in adsorptive properties, that in a carrier system the small quantities of the acids emerge *within* rather than *between* the zones of the carriers. Fundamentally this is still carrier separation, but it is difficult in practice to demonstrate separation of several acids in such a system.

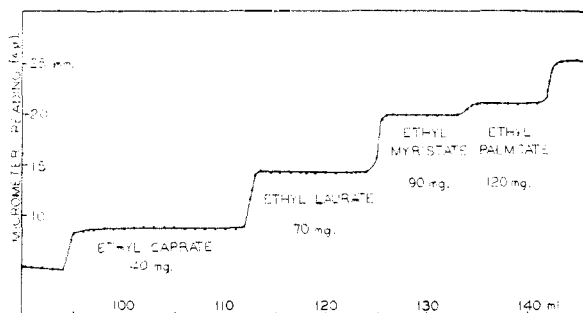


Fig. 3.—Displacement separation of ethyl caprate, ethyl laurate, ethyl myristate and ethyl palmitate on a 56 cc. coupled filter in 95% ethanol using 1.0% ethyl stearate as displacer.

A successful separation of four homologous long chain alcohols is illustrated in Fig. 4. The separation was made in absolute ethanol on an 86 cc.

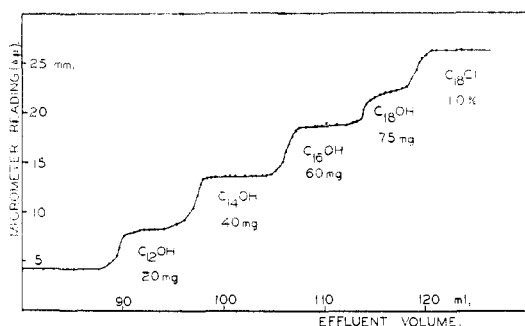


Fig. 4.—Displacement separation of dodecyl, tetradecyl, hexadecyl and octadecyl alcohols on an 86 cc. coupled filter in absolute ethanol using 1.0% octadecyl chloride as displacer.

coupled filter. Because of the very limited solubility of long chain alcohols in absolute ethanol, it is not feasible to use a long chain alcohol as displacer. However, the long chain alkyl halides are more soluble than the alcohols, and yet easily displace the alcohols of equal chain length. The experiment shown in Fig. 4 illustrates the usefulness of selecting a suitable analogous substance as displacer when selection of a higher homolog is not feasible.

Series of C₁₈ Analogs.—With the aim of testing various substances analogous to the fatty acids for use as displacers, the adsorbabilities of a series of normal C₁₈ compounds were measured by frontal analysis.¹⁰ The retention volume, resulting from these experiments, shown in Table I, indicate that octadecyl iodide is most strongly adsorbed and that octadecyl alcohol least adsorbed from 1.0% solution. Stearic acid is relatively weakly adsorbed compared to its analogous alkyl halides.

TABLE I

ADSORBABILITIES OF NORMAL C₁₈ COMPOUNDS ON DARCO G-60 FROM 1.0% SOLUTIONS IN ABSOLUTE ETHANOL

| Substance | M.p., °C. | Mol. wt. | Retention volume 15.3 cc. filter |
|---------------------|-----------|----------|----------------------------------|
| Octadecyl iodide | 32 | 380 | 38.6 |
| Octadecyl bromide | 27 | 333 | 30.0 |
| Octadecyl chloride | | 288.5 | 28.6 |
| Octadecyl mercaptan | 25 | 286 | 27.4 |
| Octadecene-1 | | 252 | 25.0 |
| Octadecane | 28 | 254 | 23.6 |
| Stearic acid | 70 | 286 | 22.2 |
| Octadecyl nitrile | 41 | 268 | 20.8 |
| Ethyl palmitate | 25 | 286 | 20.8 |
| Octadecyl alcohol | 59 | 270 | 19.6 |

To determine by actual displacement the order of adsorbability of this series of compounds, displacement experiments were performed on pairs or triplets of these substances using 22.0 cc. coupled filter, ethanol as solvent and 1.0% octadecyl iodide as displacer. Whenever possible identification of zones was verified chemically. Table II comprises a list of these experiments in which definite separations were observed and from which the adsorbability series is deduced. The order of displacement is roughly as follows: iodide > bromide? > chloride? > mercaptan > octadecane > octadecene > amide? > nitrile? > acid > alcohol > ethyl palmitate. Question marks indicate that exact positions in series had not been determined. In comparison

TABLE II

DISPLACEMENT EXPERIMENTS EXHIBITING SEPARATIONS IN THE NORMAL C₁₈ SERIES

| | | | | |
|------------|-----------|--------------|-----------|-----------------|
| Iodide | Displaces | Mercaptan | Displaces | Octadecene-1 |
| Iodide | Displaces | Mercaptan | Displaces | Stearic acid |
| Iodide | Displaces | Mercaptan | Displaces | Alcohol |
| Iodide | Displaces | Octadecane | Displaces | Acid |
| Iodide | Displaces | Octadecene-1 | Displaces | Nitrile |
| Iodide | Displaces | Octadecene-1 | Displaces | Amide |
| Iodide | Displaces | Octadecene-1 | Displaces | Acid |
| Iodide | Displaces | Octadecene-1 | Displaces | Alcohol |
| Iodide | Displaces | Octadecene-1 | Displaces | Ethyl palmitate |
| Iodide | Displaces | Nitrile | Displaces | Acid |
| Iodide | Displaces | Acid | Displaces | Ethyl palmitate |
| Iodide | Displaces | Amine | Displaces | Alcohol |
| Iodide | Displaces | Alcohol | Displaces | Ethyl palmitate |
| Octadecane | Displaces | Octadecene-1 | Displaces | Acid |

with the series of retention volumes, a few minor discrepancies in adsorbability order are apparent. They occur with pairs whose adsorbabilities are quite similar as judged by retention volume. It is possible that the reversals in order may be due to crossed adsorption isotherms. In the frontal studies, all concentrations were 1.0%, and in the displacement studies concentrations were often considerably lower. If the adsorption isotherms of the two substances crossed between the concentrations established in the displacement experiment and 1.0%, reversals in order would take place. However, the data indicate the general usefulness of the simple frontal experiments in predicting relative adsorbabilities.

To illustrate the separations made in establishing this series a few examples are shown. In Fig. 5 the displacement of octadecyl alcohol and stearic acid by 1.0% octadecyl iodide is shown. The second

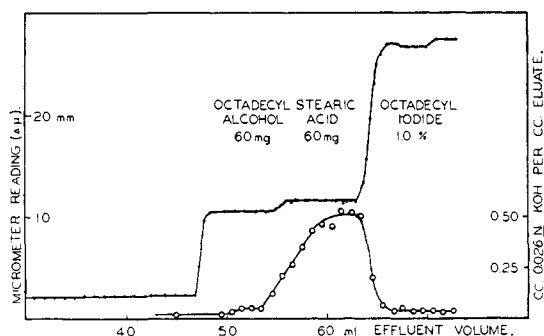


Fig. 5.—Displacement separation of octadecyl alcohol and stearic acid on a 26.1 cc. coupled filter in absolute ethanol using 1.0% octadecyl iodide as displacer.

zone was identified as stearic acid by titration with alkali (open circles). In Fig. 6 the displacement of stearic acid and octadecene by octadecane is shown. Curve A represents the titration of alternate 1.0-cc. fractions with 0.025 *N* alkali, and Curve B represents titration of alternate samples with a dilute bromine solution. From these two experiments it is demonstrated that octadecane > octadecene > stearic acid > octadecyl alcohol.

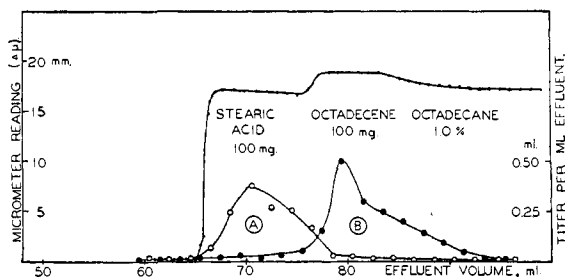


Fig. 6.—Displacement separation of stearic acid and octadecene-1 on a 36.8 cc. coupled filter in absolute ethanol using 1.0% octadecane as displacer: curve A, titration of alternate 1.0-cc. samples with 0.025 *N* alkali; curve B, titration of alternate 1.0-cc. samples with dilute bromine solution in methanol.

In Fig. 7 the sequence of displacement of octadecyl alcohol and octadecyl mercaptan is shown. No

simple chemical method was available for identification of the alcohol or mercaptan, so identification was made by "step height." Single displacements of 60 mg. of octadecyl alcohol (curve A) and 100 mg. of octadecyl mercaptan (curve B) were made using 1.0% octadecyl iodide as displacer. A double displacement of the same quantities of these substances (curve C) shows that the alcohol is displaced by the mercaptan which in turn is displaced by the iodide. This order was verified by evaporating samples from the two zones to yield a solid from the first zone and a liquid from the second.

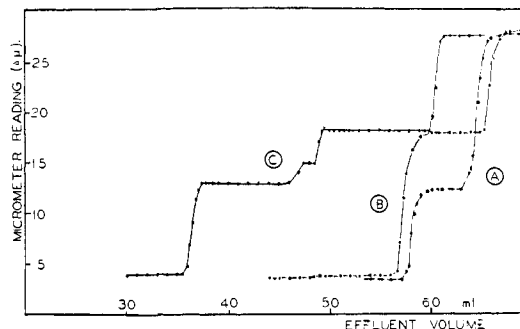


Fig. 7.—Displacement separation of octadecyl alcohol and octadecyl mercaptan on a 19.1 cc. coupled filter in absolute ethanol using 1.0% octadecyl iodide as displacer: curve A, 60 mg. of octadecyl alcohol displaced by octadecyl iodide; curve B, 100 mg. octadecyl mercaptan displaced by octadecyl iodide; curve C, 60 mg. of alcohol and 100 mg. of mercaptan displaced by octadecyl iodide.

Discussion

From the experiments presented it is apparent that several types of substances other than acids are available as effective displacers for the fatty acids and esters. Some of these offer the distinct advantages of greater solubility than the corresponding acids and the ability to displace acids of considerably greater chain length. Thus, it is possible to extend the range of displacement separations of fatty acids in ethanol beyond the limit at C_{18} or C_{20} set by the diminishing solubility of the saturated acids. The substances investigated have the practical advantage also of being readily available commercially, freeing the investigator of the necessity of isolating or preparing special substances as displacers. The alkyl bromide homologs, the alcohol series and the ester series are also readily available in sufficient purity for possible use as carrier systems for separations on a micro scale, and may find application in separations of certain acid mixtures of other substances.

The adsorbability of a substance bears little obvious relation to its common physical properties. Although the adsorbability increases with molecular weight, and consequently melting point in homologous series, no such relationship holds within the series of C_{18} analogs (see Table I). The polarity of the molecule likewise does not seem to parallel its adsorbability, for octadecane, the least polar substance in the group, falls in the middle of the adsorbability series. However, within the alkyl halide series, the adsorbability is parallel to molecular weight and melting point. Likewise, with the

strict analogs, octadecyl alcohol and octadecyl mercaptan, the same is true. Molecular weight seems to be of less importance than structure for with the two isomers stearic acid and ethyl palmitate, considerable differences in adsorption were apparent. Moreover, octadecene, the lowest molecular weight substance of the group was able to displace several substances of higher molecular weight.

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man Fund of the Research Corporation, and a contract between the Office of Naval Research and the Texas A. and M. Research Foundation. The generosity of the Armour Research Laboratory in supplying octadecyl nitrile, octadecyl amide and octadecyl amine is deeply appreciated. The author is indebted to Monette B. Bird and Betty Gibson for their competent technical assistance.

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The Isolation and Purification of Morin on an Ion-Exchange Resin^{1,2}

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Amberlite IRC-50(H) synthetic cation exchange resin has been utilized for the isolation and purification of morin (2',3,4',5,7-pentahydroxyflavone) from aqueous extracts of the heartwood of *Chlorophora tinctoria*. The morin is further purified, after elution from the column with ethyl alcohol, by recrystallization from acetic acid, conversion to the potassium salt and re-adsorption on a fresh column of Amberlite IRC-50(H). The morin, thus prepared, compares favorably in its properties with authentic morin obtained by the more involved classical procedures. The method offers a practical low-cost procedure for the preparation of morin or other flavonoid pigments from plant extracts.

Introduction

Rekers and Fields³ recently reported the successful use of morin, 2',3,4',5,7-pentahydroxyflavone, in the prevention of mortality in dogs from mid-lethal doses of total-body X-radiation. Analyses elsewhere⁴ and in this Laboratory have revealed that the morin content of the Eastman Technical grade used by Rekers and Field was not in excess of two per cent. This has led to efforts to obtain pure morin in order to evaluate properly its protective effect in radiation sickness.

Bonner⁴ and Haley and Bassin⁵ have recently reported methods for the isolation and purification of morin. These methods involve either vacuum sublimation or the evaporation of considerable quantities of water in the isolation procedure.

For the preparation of morin in quantities of 50–100 g., sublimation or evaporation at reduced temperature and pressure requires a considerable investment in time and equipment. Furthermore, in the purification of morin obtained by aqueous extraction of *Chlorophora tinctoria*⁶ wood chips followed by evaporation of the extract, we have found that a significant quantity of the product was in the form of the potassium and calcium salts.

The procedure to be described in this paper does not require the use of vacuum sublimation, and, by passage through a cation exchange resin bed, sharply reduces the amount of contamination due to metallic ions. This is the first reported instance of the use of ion exchange resins for the isolation, purification and recovery of morin.

(1) This investigation was supported in part by grants-in-aid from the Atomic Energy Commission (Project AT-(40-1)-235) and from the Office of Naval Research (Project NR 059 226).

(2) Presented before the Sixth Southwest Regional Meeting of the American Chemical Society, December 7–9, 1950, San Antonio, Texas.

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(5) Thomas J. Haley and Murray Bassin, Report UCLA-73, University of California at Los Angeles Atomic Energy Project, P. O. Box 31, Beverly Hills, California.

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Experimental

One kilogram of ground heartwood of the *Chlorophora tinctoria* tree was extracted four times with 12-gal. portions of distilled water. Each extract was boiled for 2 hr. and then filtered through flannel bags. The cooled filtrate was then passed through 2 in. × 48 in. Pyrex columns packed with Amberlite IRC-50(H) cation exchange resin. Four such columns were used. The resin bed had been previously washed with 0.2 *N* hydrochloric acid, backwashed with distilled water and downwashed with additional distilled water until the washings were neutral.

Approximately 12 gal. of the red-brown extract was passed through each column. The filtrate from the columns was only slightly less colored. The resin bed gradually assumed a yellow-brown tint as the solution passed through. The columns were next washed with distilled water until the filtrate was clear (3–4 l.). The adsorbed material, including morin and maclurin, was then eluted with 95% ethyl alcohol. Each column required about 1 l. of alcohol. The alcohol wash was followed by distilled water in order to flush the last of the alcoholic solution through the resin bed.

Some yellow-brown material moved down the column just in advance of the alcohol-water interface. This material precipitated on leaving the column. It was discarded since it gave negative tests for flavonoid material. The alcoholic filtrates from the four columns were combined and concentrated to approximately 250–300 ml. Considerable yellow-brown solid material precipitated during the concentration. An equal volume of water was added to the concentrate and the solution set in the refrigerator overnight to allow further precipitation to take place; yield 26.4 g. or 2.64% of crude morin. Most of the maclurin remained in solution.

The crude morin was recrystallized from 900 ml. of 60% acetic acid solution; yield 12 g. or 1.2%. The last traces of maclurin were removed at this point. The dried morin was dissolved in a minimum quantity of 95% ethyl alcohol and 15 g. of solid potassium acetate added. A bright yellow precipitate of the potassium salt of morin separated at once. The potassium salt was suspended in approximately 20 l. of distilled water and a few drops of potassium hydroxide solution were added to complete solution. The pH of the resulting solution was approximately neutral.

The solution of the potassium salt was then passed through two fresh columns of Amberlite IRC-50(H) resin in order to decompose the complex. The potassium was exchanged for hydrogen ion and the adsorbed morin was then eluted with ethyl alcohol. Concentration of the alcoholic solution at reduced pressure and subsequent addition of water yielded pale yellow morin; yield 9.5 g. or 0.95%.