Advances in LIPID RESEARCH

EDITED BY

Rodolfo Paoletti and David Kritchevsky

VOLUME I

ACADEMIC PRESS New York · London

Advances in Lipid Research

Volume 1

Edited by

Rodolfo Paoletti
Institute of Pharmacology
Milan, Italy

David Kritchevsky
The Wistar Institute
Philadelphia, Pennsylvania

1963

ACADEMIC PRESS • New York and London

ACADEMIC PRESS INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS INC. (LONDON) LTD.
Berkeley Square House, London W.1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 63-22330

PRINTED IN THE UNITED STATES OF AMERICA

CONTRIBUTORS

Numbers in parentheses indicate the page on which the author's contribution begins.

ROSLYN B. ALFIN-SLATER, *Division of Nutritional Science, School of Public Health, University of California, Los Angeles, California (183)*

A. D. BANGHAM, *Department of Physiology, A. R. C. Institute of Animal Physiology, Babraham, Cambridge, England (65)*

A. A. BENSON, *Department of Marine Biology, Scripps Institution of Oceanography, University of California, La Jolla, California (387)*

THOMAS B. CLARKSON, *Vivarium, Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, North Carolina (211)*

M. H. COLEMAN, *Unilever Research Laboratory, Sharnbrook, Bedford, England (1)*

HENRY DANIELSSON, *Department of Chemistry, Karolinska Institutet, Stockholm, Sweden (335)*

IRVING B. FRITZ, *Department of Physiology, University of Michigan, Ann Arbor, Michigan (285)*

JOHN M. JOHNSTON, *Department of Biochemistry, University of Texas, Southwestern Medical School, Dallas, Texas (105)*

ROSEMARY SHULL MORRIS, *Division of Nutritional Science, School of Public Health, University of California, Los Angeles, California (183)*

M. PASCAUD, *Centre de Recherches sur la Cellule Normale et Cancéreuse, Villejuif (Seine), France (253)*

D. S. ROBINSON, *External Staff of the Medical Research Council, Sir William Dunn School of Pathology, Oxford, England (133)*

PREFACE

Lipid research is a many-faceted field. It ranges widely between chemistry and biology. This is to be expected since the lipids themselves are a widely disparate group of substances grouped together mainly through solubility characteristics.

In recent years the broadening of interest in many aspects of lipid research has brought many new workers into this field. It is patently impossible for any person to maintain an interest in the entire area of study. This situation is due not only to a lack of sufficient time for the extensive reading necessary, but also to the obstacles imposed by the difficulty of evaluating results obtained by unfamiliar techniques. However, new insights may be obtained into one's own work by reading concise, critical expositions of advances in areas of tangential interest. This series has been brought into being with the idea that it may serve as an instrument for cross-fertilization within the broad area of lipid research.

It is our purpose to bring together a series of chapters written by recognized authorities in the various subclassifications within the area of lipid research. The volumes will be interdisciplinary, as is lipid research itself. We encourage reports that are critical evaluations of specific subjects, speculation, or work-in-progress. These can be of greater value than general summaries. We hope that this and subsequent volumes in this series will stimulate scientists familiar with the topics under discussion and instruct those who are not.

The material covered in the first volume of this series ranges from chemical and physicochemical discussions of the structure and behavior of lipids through one of the newer areas in lipid research, sulfolipids. The most recent work on fat absorption and transport is presented by experts in the various special phases of this work. Other chapters cover the latest developments in cholesterol metabolism, Vitamin E as it affects lipid metabolism, and a treatment on atherosclerosis. All areas of lipid research ranging from physical chemistry to physiology and pathology are touched upon in this volume.

October, 1963

RODOLFO PAOLETTI
DAVID KRITCHEVSKY

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M. H. COLEMAN

Unilever Research Laboratory, Sharnbrook, Bedford, England

"Therefore by this *Magistery,* We must necessarily come to the determinate *Separation* of all the *Elements,* of every *Vegetable Thing,* and of that which from *Vegetable* proceeds to a *Being,* and of every like *Thing*: but by that, which is made by *Descent,* We may attain the *Oyl* of every *Thing* determinately."

Jabir ibn Hayyan, 8th Century, A.D.,
"The Works of Geber," trans. by R. Russell, London, 1678.

I. Introduction

A. Historical Review

Although natural fats have always been an important constituent of human diet, and have been used from early times for the manufacture of soap, and in spite of the importance fats have recently assumed as industrial raw materials, there are still serious gaps in our knowledge of their formation and constitution.

The chemical nature of fats, as esters of glycerol, was recognized early in the nineteenth century by Chevreul. But although Berthelot drew attention to the possible existence of mixed triglycerides in 1860, it was tacitly assumed throughout most of the last century that natural fats consisted of mixtures of simple triglycerides.

It was not until the end of the nineteenth century that Heise (1897) demonstrated the presence of a mixed glyceride, oleodistearin, in the fat from a member of the genus *Allanblackia*. In the early years of the present century Bömer and his collaborators adduced much evidence for the essentially mixed character of the glycerides of natural fats (Bömer *et al.*, 1907; Bömer, 1909). Most of the early work depended on separating the component glycerides of natural fats by fractional crystallization from suitable solvents; but as early as 1903 Krafft had employed fractional distillation under reduced pressure as a means of isolating trilaurin from laurel kernel fat and trimyristin from nutmeg butter (Krafft, 1903).

After the first World War fractional crystallization was also applied to natural fats which had previously been subjected to hydrogenation (Amberger, 1920). Another approach used the same technique for the separation of the products formed by bromination of fats (Eibner *et al.*, 1927).

Most of these early investigations were purely qualitative and, although the data served to confirm that fats are largely mixtures of mixed triglycerides, no really quantitative conclusions could be drawn from them. In 1927 Hilditch and his collaborators embarked on their extensive series of investigations. Their methods included the determination of the trisaturated glyceride content, by the oxidation of the remaining glycerides, to facilitate their removal. They also employed fractional crystallization, chiefly from acetone, at around 0°C for the more saturated fats, and down to —70°C for the liquid oils. These investigations have continued down to comparatively recent years, and have furnished us with the largest single body of data currently available.

A number of new lines of attack on the problem of the constitution of natural fats has developed in recent years, mostly since the second

World War. Thus the advent of gas chromatography (James and Martin, 1952) has greatly simplified the analysis of mixtures of fatty acids, an essential prerequisite in any investigation of mixtures of glycerides. Countercurrent distribution between solvents has assumed considerable importance as a method for separating fats into their component glycerides, and the various chromatographic techniques—paper, column, thin-layer, and gas-liquid chromatography—have all now been applied both to the separation of fatty acids and to the glycerides themselves. Another promising method of investigation has developed from a study of the products of hydrolysis of fats, using the enzyme, pancreatic lipase, as a catalyst. The application of two or more of these techniques in concert promises to yield a complete picture of the composition of any mixture of glycerides, natural or synthetic.

B. Conventions

To date however, most of the data on the glyceride composition of fats have been obtained in the form of the proportions of the four main triglyceride classes, trisaturated (S_3), disaturated (S_2U), monosaturated (SU_2), and triunsaturated (U_3). In more recent work the isomeric forms of the two mixed glyceride classes have sometimes been distinguished; i.e., S_2U has been separated into the symmetrical SUS and the unsymmetrical SSU glyceride classes, while SU_2 has been separated into SUU and USU. In some cases it has been possible to distinguish glycerides with a particular fatty acid composition. Where positional isomers are distinguished the convention, e.g., POP and PPO, will be used for the symmetrical and unsymmetrical oleodipalmitins respectively; when the isomers are not distinguished this type of triglyceride will be represented by P_2O. For triacid triglycerides brackets will indicate the sequence is not significant; thus POS represents 2-oleopalmitostearin, whereas [POS] merely represents oleopalmitostearin.

II. Determination of Fatty Acid Composition

A. Preparation of Free Acids and Esters

Most methods for the analysis of glyceride mixtures require the determination of the fatty acid composition of the whole mixture and, usually, of its component fractions. The fatty acid analysis of individual glycerides, or of mixtures, has commonly been carried out, not with the free acids but with their methyl (or ethyl) esters. The esters may be prepared directly by interesterification of the fat with methanol and a suitable catalyst, or indirectly by first preparing the free acids and subsequently esterifying them.

In the former case the fat is conveniently methylated by dissolving

in hot methanol, and then refluxing gently for about 3 hours with 0.5% of sodium methoxide.

In the latter case, the free fatty acids may be prepared by saponifying the fat with five times its own weight of 6% w/v alcoholic potassium hydroxide for 3 hours. The alcohol is then evaporated (preferably under reduced pressure) and the resultant soaps dissolved in water. This is a convenient point to remove any unsaponifiable matter by extraction with ether. The fatty acids are then liberated by the addition of a slight excess of mineral acid; it may be desirable to carry out this acidification under an inert atmosphere, if appreciable amounts of unsaturated acids are present. The free acids may then be taken up in ether and, after drying the solvent with anhydrous sodium sulfate, the ether removed under reduced pressure.

The methyl esters may then be prepared by refluxing the fatty acid mixture in four times its own weight of methanol, with the addition of 1% of concentrated sulfuric acid. Any free fatty acids remaining may be removed by washing an ethereal solution of the esters with dilute potassium carbonate solution.

Where only small quantities of methyl esters are required for subsequent analysis (e.g., by gas chromatography) various alternative methods of methylation may be employed. A very convenient method uses diazomethane, which may be prepared in the way described by DeBoer and Backer (1954), but experience in the author's laboratory suggests that it is advisable to distil between 60° and 65°C, instead of the recommended temperature. This reagent is added dropwise to a solution of the fatty acids in ether, until a slight excess is present. Schlenk and Gellerman (1960) have described a simple apparatus in which the preparation of diazomethane and the methylation reaction may be carried out, obviating the necessity of storing this dangerous reagent. They report that methylation is not complete in ethereal solution unless 10% of methanol is present. Stoffel *et al.* (1959) have described a methylation procedure using "super-dry" methanol, followed by a sublimation procedure for purifying the methyl esters. Experience in the author's laboratory suggests that the sublimation procedure may lead to fractionation of the esters, and for this reason should be used with care. Metcalfe (1961) has described the use of borontrifluoride-methanol for methylation. Methylation of fatty acids has also been discussed by Vorbeck (1961) and Luddy *et al.* (1960).

B. Ester Distillation

Fractional distillation of the methyl (or ethyl) esters was, until recently, the most widely used method of analyzing a mixture of the

higher fatty acids. Some preliminary resolution of the mixture is desirable, and particularly so when lower fatty acids are present. In the latter case the more volatile acids may be removed by steam distillation. Thereafter it is convenient to separate the higher fatty acids, into saturated and unsaturated classes, either by utilizing the difference in solubility of their lead soaps (Bertram, 1925) or, perhaps more satisfactorily, by fractional crystallization at low temperatures (Brown, 1931). Low temperature crystallization of the fatty acids (or their methyl esters) has the advantage of separating poly- from monounsaturated acids, in addition to the separation of saturated from unsaturated. For fats containing much saturated acid, this is best removed by a preliminary crystallization from methanol at —20°C (Gunstone and Paton, 1953) before proceeding to the fractional crystallization of the remaining acids.

The actual distillation of the methyl esters is usually carried out in an electrically heated fractionating column, filled with a suitable packing material. Longenecker (1937) has described a suitable column with which he has fractionated the acids of beef and butter fat and groundnut oil. Norris and Terry (1945) have compared various packing materials, and have recommended the "Heli-Grid" of Podbielnak (1933) as being the most efficient. Wyman and Barkenbus (1940) have shown that mixtures of methyl esters of the saturated fatty acids from caprylic to stearic may be separated satisfactorily in comparatively small quantities using the "spinning band" column of Lesesne and Lochte (1938). Birch *et al.* (1947) have also used this type of column very successfully for fractionating fatty acid esters, and they have described a convenient manostat for pressure control and a suitable device for collecting fractions. Horn and Hougen (1953) have described various modifications for the improvement of a commercially available spinning band column.

The technique of amplified distillation introduced by Weitkamp (1945), which involves the addition of, say, a suitable mixture of hydrocarbons to the mixture of methyl esters, permits quite small quantities of the esters to be separated very effectively. Hatt and Szumer (1953) have also used this method.

Another interesting variation is the technique of molecular distillation, applied by Farmer and Van den Heuvel (1938) for the fractionation of the fatty acids of cod liver oil.

The work of Norris *et al.* (1941, 1943) recently confirmed by Privett *et al.* (1959) suggests that fractional distillation of the methyl esters is suitable for fatty acids containing up to three double bonds; more unsaturated acids, however, undergo appreciable change when subjected to this process. The subject of fractional distillation has been reviewed by Murray (1955), who concludes that it still provides a valuable tool

in fatty acid analysis. Its limitations include the need for an efficient column with all the ancillary equipment, the danger of altering highly unsaturated materials, and the need for appreciable quantities of the mixture under investigation.

C. Spectrophotometry

The application of spectroscopic methods to fatty acid analysis stems from the observation of Dann and Moore (1933) that the spectral absorption of the fatty acids of cod liver oil is greatly intensified when they are heated with alkali. Moore (1937) suggested that this increase resulted from conjugation of previously isolated double bonds. Mitchell *et al.* (1943) first described a method for estimating the linoleic and linolenic acids of fats and oils spectroscopically. Their method, which with some modifications has been widely used, consists of first heating 100 mg of the fat in 1.3 *N* KOH in ethylene glycol solution for 25 minutes at 180°C. The absorption at 234 and 268 mμ is then determined, and from absorption constants determined on the pure acids the proportions of linoleic and linolenic acids may be calculated. By determining the iodine value of the fat, the oleic content may be calculated, and hence the proportion of saturated acids, by difference.

Hilditch *et al.* (1945) have suggested that different isomerization conditions are desirable for the two acids: 60 minutes at 180°C for linoleic, and 15 minutes at 170° C for linolenic. Hilditch and his collaborators (1951) have also suggested that the absorption constants determined on acids isolated from natural fats by purely physical methods are to be preferred to those of the chemically isolated materials. Herb and Riemenschneider (1953) have described a modification of the method which permits analysis of as little as 1 mg of fat, while Collins and Sedgwick (1956) have described a rapid method for the analysis of soybean oil. Herb (1955) has reviewed the various modifications, and has emphasized the necessity for taking the absorptions of all the acids present into account when making calculations from spectral data. This author has also drawn attention to the difficulties associated with this method when it is applied to fish oils and to tung and castor oils.

O'Connor *et al.* (1947) have described a spectrophotometric method of determining the proportions of α- and β-eleostearic acids in tung oil.

Privett *et al.* (1959) have recently redetermined the absorption constants for methyl arachidonate after alkali isomerization. These authors and others (Tuna *et al.*, 1958; Craig and Murty, 1959; Gracian *et al.*, 1959; Herb *et al.*, 1960) have published comparisons of alkali isomerization with gas-liquid chromatography and other methods. In general this method gives comparable results with others, although Craig and

Murty have reported lower values for linoleate and higher values for linolenate than those obtained by gas chromatography.

D. Chromatography

1. *Paper*

A number of reversed phase paper chromatographic systems for the analysis of fatty acid mixtures have been reported. Wagner *et al.* (1955) have used a hydrocarbon-impregnated paper, the fatty acid spots being detected as copper-ferrocyanide complexes, and estimated by absorption measurements in a densitometer.

Perilä (1956) has also used a hydrocarbon-impregnated paper, with development in an aqueous acetic acid solvent. After soaking in a 1.5% silver nitrate solution, and spraying with 0.05% *p*-diethylaminobenzylidenerhodanine, the silver was estimated colorimetrically by measuring the absorption at 6250Å.

Gellerman and Schlenk (1956) using siliconed paper (Mangold *et al.*, 1955) have developed mixtures of free fatty acids and methyl esters with a solvent consisting of equal volumes of 88% formic and 85% acetic acids. Simple triglyceride mixtures were developed with chloroform-methanol (3:1). Spots were visualized the α-cyclodextrin and iodine vapor (Mangold *et al.*, 1955), and measured in a densitometer. Satisfactory analyses of mixtures of known composition were obtained. In further work, Schlenk *et al.* (1957) draw attention to the need to run suitable standards with the unknown mixture. Leibnitz *et al.* (1957) have described the estimation of acids from acetic to pentacosanoic, using a recording photometer for density measurements. Ballance and Crombie (1958) have used paraffin-impregnated paper developed with aqueous acetic acid for mixtures of saturated and unsaturated acids. Quantitative measurements were made by converting the fatty acids to the corresponding copper soaps, complexing these with dithiooxamide, and reading the densities at 1-mm intervals in an "Eel" densitometer. "Critical pairs" of acids may be estimated by a comparison of chromatograms of the original mixture, the mixture after hydrogenation, and the mixture after permanganate oxidation. Hydroxy acids were run on paper impregnated with castor oil.

Mangold *et al.* (1958) have shown that the addition of peracetic acid, or hydrogen peroxide, to the developing solvent causes unsaturated acids to run much faster than the saturated acids. Using C^{14}-labeled diazomethane, they were able to estimate the fatty acids as their labeled methyl esters. Skipski *et al.* (1960) have also used hydrogen peroxide to separate unsaturated acids from their saturated "critical partners."

The spots were visualized here by treating with bismuth subnitrate solution, followed by ammonium sulfide.

Hansen (1961) has reported that varying spot size does not preclude quantitative estimation of labeled fatty acids, separated by reversed phase paper chromatography, but recommends that both sides of the chromatogram should be scanned. Abdel-Wahab and El-Kinawi (1961) have separated the fatty acids of linseed oil on siliconed paper, and estimated them by measuring the activity of the spots after treatment with I^{131}.

Kaufmann and his collaborators, in an extensive series of investigations, have reported a variety of separations and techniques for the quantitative estimation of fatty acids in mixtures. In 1956 Kaufmann discussed and illustrated a number of methods (Kaufmann, 1956), including radio-assay as Co^{60} soaps or addition compounds of I^{131}, ultraviolet (UV) absorption, and the polarographic determination of Cu or Hg from the Cu soaps or mercuriacetate compounds, respectively. Details of the polarographic determination of Cu from the Cu soaps of corn oil and rapeseed oil are given by Kaufmann and Deshpande (1958), a method also employed by Seher (1956). Soybean and linseed oil fatty acids have been estimated, after chromatographic separation, by reaction with mercuric sulfide and copper acetate (Kaufmann and Schnurbusch, 1958). Kaufmann and Karabatur (1958) have analyzed various synthetic mixtures, and the fatty acids of rapeseed oil, before and after hydrogenation, while Kaufmann and Makus (1959b) have described a technique for the saponification of fats on the paper, in which the free acids are liberated with HCl gas, and applied this technique to the analysis of cottonseed, soybean, almond, and plum kernel oils. Various animal fats have been analyzed by Kaufmann *et al.* (1960) using a photometric measurement of the dithiooxamide derivative of the Cu soaps for the estimation. This contribution includes the separation of "critical pairs" of fatty acids by rechromatographing after hydrogenation. Kaufmann and Makus (1960a) have reviewed the application of paper chromatography in fatty acid analysis, with particular emphasis on this problem of "critical pairs" of acids, and its solution by two dimensional chromatography, with hydrogenation on the chromatogram, before the second development.

An interesting application of the paper chromatographic technique is that described by Kaufmann and Sud (1960) for the analysis of oils containing conjugated acids. They have found that parinaric acid may be chromatographed in an atmosphere of CO_2, and the analysis of *Impatiens* oil so performed agrees with that determined spectrophotometrically.