The Biochemistry of Plants

A COMPREHENSIVE TREATISE

P. K. Stumpf and E. E. Conn

EDITORS-IN-CHIEF

Volume 9

Lipids: Structure and Function

P. K. Stumpf

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Lipids: Structure and Function

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General Preface

In 1950, a new book entitled "Plant Biochemistry" was authored by James Bonner and published by Academic Press. It contained 490 pages, and much of the information described therein referred to animal or bacterial systems. This book had two subsequent editions, in 1965 and 1976.

In 1980, our eight-volume series entitled "The Biochemistry of Plants: A Comprehensive Treatise" was published by Academic Press; this multivolume, multiauthored treatise contained 4670 pages.

Since 1980, the subject of plant biochemistry has expanded into a vigorous discipline that penetrates all aspects of agricultural research. Recently a large number of research-oriented companies have been formed to explore and exploit the discipline of plant biochemistry, and older established chemical companies have also become heavily involved in plant-oriented research. With this in mind, Academic Press and the editors-in-chief of the treatise felt it imperative to update these volumes. Rather than have each chapter completely rewritten, it was decided to employ the approach used so successfully by the editors of *Methods in Enzymology,* in which contributors are invited to update those areas of research that are most rapidly expanding. In this way, the 1980 treatise constitutes a set of eight volumes with much background information, while the new volumes both update subjects that are rapidly developing and discuss some wholly new areas. The editors-in-chief have therefore invited the editors of the 1980 volumes to proceed on the basis of this concept. As a result, new volumes are forthcoming on lipids; general metabolism, including respiration; carbohydrates; amino acids; molecular biology; and photosynthesis. Additional volumes will be added as the need arises.

Once again we thank our editorial colleagues for accepting the important task of selecting authors to update chapters for their volumes and bringing their

volumes promptly to completion. And once again we thank Mrs. Billie Gabriel and Academic Press for their assistance in this project.

P. K. Stumpf
E. E. Conn

Preface to Volume 9

Since the publication in 1980 of "Lipids: Structure and Function," Volume 4 of "The Biochemistry of Plants," so much progress has been made that the original volume no longer serves as the source of current information on plant lipid biochemistry. As a result, this new volume is designed to update Volume 4; 12 chapters have been written to cover the impressive progress made since 1980 in each of the 12 areas of plant lipid research.

The development of recent physical techniques employed in lipid research is covered in Chapter 1, and the increased knowledge on ß-oxidation systems in higher plants and the oxidative modifications of unsaturated fatty acids are discussed in Chapters 2 and 3, respectively. An entire chapter is devoted to lipases (Chapter 4). The resolution of the enzymology of the plant fatty acid synthetases is covered in Chapter 5, and the remarkable progress in understanding the function of acyl carrier protein is discussed in Chapter 6. New information on desaturases is reviewed in Chapter 7. The biochemistry of the complex lipids such as triacylglycerols, galactolipids, and sulfolipids is covered in Chapters 8, 9, and 10, respectively. Chapter 11 brings together the new information about surface waxes and their participation in the defense mechanisms of plants, and finally Chapter 12 reviews the new developments in the lipids of the blue-green algae.

A number of chapters in Volume 4 were not revised and included in this new volume. These will be updated in future volumes.

Once again I extend my warm appreciation to all the contributors of this volume who kept to their deadlines and submitted chapters of high caliber. And once again I thank Mrs. Billie Gabriel for her usual excellent secretarial assistance.

P. K. Stumpf

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1

Analysis and Structure Determination of Acyl Lipids

MICHAEL R. POLLARD

I. INTRODUCTION

It is a statement of the obvious that the development of plant lipid biochemistry depends on our knowledge of the structures of the lipid molecules and on the methods available to separate and quantitate them. It is the purpose of this chapter to foster an awareness of the chemists' tools to perform the tasks of structure determination and quantitative analysis. Historically, organic chemists isolated and identified the major components in fats and oils using methods such as distillation, fractional crystallization, and chemical degradations (Gunstone, 1978). Fats were assayed by such indices as saponification, iodine, and hydroxyl numbers (Rossell, 1986). The early pioneers did an excellent job, but only abundantly occurring natural products were accessible to these time-consuming methods. With the development of chromatography and spectroscopy it has become possible to detect and characterize more complex molecules in nature in ever-smaller amounts, and to

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increase the speed, sensitivity, and resolution of quantitative assays, thus increasing our knowledge of biochemical processes. This chapter discusses the analysis and structure determination of lipids, especially acyl lipids, with emphasis on the more recent advances. Where possible, examples are chosen from the plant literature. However, the purpose of this chapter is not to review all classes of plant lipids but to show the approaches and limitations of various methods to problems of a chemical nature.

There could be many reasons for initiating a lipid analysis and structure determination. Perhaps a taxonomic study is being undertaken, or a plant oil exhibits interesting bioactive properties, or a membrane fraction needs characterization, or a tracer study has revealed interesting intermediates. Whatever the reason, the first step will almost certainly involve extraction with organic solvents. The lipid mixture will then be separated into its individual components, probably by a combination of chromatographic methods. And finally, the compounds will need identification. Physicochemical methods will be used, especially mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy. With the lipid structure defined, the scientist can further develop methods of quantitative assay. It should be noted that "separation" and "identification" are interdependent processes. The chromatographic behavior of a molecule is a function of its molecular structure. Thus elution behavior will give clues to the structure of the molecule. Conversely, the definition of the structure or a partial structure of a molecule may suggest improved purification or assay techniques. At the end of the chapter a worked example of a simple isolation and structure determination recently performed in our laboratory will be described.

II. EXTRACTION

Extraction is the first step in purification. It is almost always accomplished by the use of organic solvents. Common methods include extraction with chloroform–methanol (Folch *et al.*, 1957; Bligh and Dyer, 1959) or hexane–isopropanol (Hara and Radin, 1978) followed by addition of a salt solution to result in partition between an aqueous and an organic phase. These may be regarded as general methods for the extraction of both neutral and polar lipids, but there are a variety of more specialized extraction methods. Steam distillation is used to produce the volatile essential oils, in which mono- and sesquiterpenes dominate, while continuous extraction with hot solvents is accomplished by soxhlet extraction. A very simple extraction method is the short-duration dip in organic solvents for epicuticular lipids. In some cases a separation of lipid classes can be accomplished by partition during an extraction procedure. A case in point is the separation of neutral and polar lipids from very polar lipids and acyl-CoAs from acyl-ACPs in the procedure devised by Mancha *et al.* (1975). Sometimes "extraction" can involve a chemical treatment. The depolymerization of cutin and suberin layers is required before the monomers are soluble in organic solvents (Kolattukudy, 1980).

There are several points to consider when designing a new extraction or utilizing an existing one. These include problems of incomplete extraction, the release of lipolytic enzymes, and oxidative and other chemical stabilities of the extracted compounds. Taking this list in order, an extraction method should always be standardized for exhaustive extraction of the lipids and, where possible, with regrinding of tissue. Losses in partition methods may include highly polar lipids which partition into the aqueous phase or to insoluble material at the interface. In this respect cereal grains require forcing conditions of extraction of certain lipids. Sphingolipids (Fujino and Ohnishi, 1976) and lysophospholipids (Fishwick and Wright, 1977) require direct extraction with water-saturated *n*-butanol. Also, acyl-CoAs are notorious for sticking to interfaces or binding to proteinaceous materials at the interface, so care in quantitation in tracer experiments is essential. Homogenization of plant tissues will release lipolytic enzymes. Even if the tissue is being homogenized in organic solvents, problems can arise. In particular, phospholipase D is active in organic solvents, and can give rise to phosphatidic acid. In the presence of alcohols a transphosphatidylase reaction also occurs, giving rise to phosphatidyl alcohols. A short heat treatment of the tissue is usually used to inactivate the phospholipase D and other lipolytic enzymes prior to homogenization of the tissue in organic solvent (Colborne and Laidman, 1975; Phillips and Privett, 1979). A greater problem can exist if the researcher wishes to isolate a particular membrane fraction from a tissue and then perform a membrane analysis on it. Moreau (1984) has documented the extreme variability of phospholipase activity in a range of plant tissues. In potato tubers, which have been most extensively characterized, there is extensive phospholipase and galactosidase activity (Galliard, 1970). However, even within the potato there are large varietal differences (Moreau, 1985). A final problem when considering extractions is that of chemical stability. Oxidation can be a problem, particularly with polyunsaturated fatty acids (Holman, 1967). Precautions to minimize oxidation include the use of peroxide-free solvents, the addition of antioxidants such as ethoxyquin or butylated hydroxytoluene (although the natural antioxidants in the extract will often be sufficient), shielding from strong light in order to prevent photooxidation and photoisomerization, and the use of nitrogen or argon to evaporate and degas solvents. Stability to acidic and basic conditions is also a prime consideration. At the end of a process of extraction, purification, and identification the researcher should always ask whether any compounds isolated could be artifacts arising from incorrect extraction and handling techniques.

III. CHROMATOGRAPHIC METHODS OF SEPARATION

Chromatography is the term used to describe the separation of components in a mixture based on sample partitioning between a mobile (gas or liquid) phase and a stationary (liquid or solid) phase. Its discovery is usually credited to Tswett (1906), who first described the separation of leaf pigments by passage of petroleum ether

down a calcium carbonate column. The theoretical principles of chromatography were first described by Martin and Synge (1941). Since then, the practice of chromatography has expanded tremendously, to take a central position in separation sciences with techniques like thin-layer chromatography (TLC), column chromatography, gas–liquid chromatography (GLC), and most recently, high-performance liquid chromatography (HPLC). A description of modern chromatographic practices, including simple theoretical treatments, is given by Williard *et al.* (1981) and by Poole and Schuette (1984). In this section we will confine discussion to fatty acids and acyl lipids, but the principles are general. Separation and analysis can therefore be classified as the definition of lipid classes, of the acyl composition, and of the molecular species of lipids. A comprehensive compendium of the chromatography of lipids has been edited by Mangold (1984). Books by Christie (1982a) and Kates (1972) are excellent laboratory guides to the earlier, and still highly relevant techniques of lipid analysis.

A. Gas–Liquid Chromatography

In GLC the mobile phase is an inert gas while the stationary phase is usually a liquid coating, either a capillary wall or an inert column packing. With a nonpolar stationary phase, separation is based largely on the difference in boiling points, while with increasingly polar stationary phases interactions between polarized (and polarizable) groups on the solute molecules and polar groups on the stationary phase become increasingly important. Thus more polar solute molecules are retained longer on the column. Detection and quantitation of the components eluting off the GC column can be by a variety of devices. These include thermal conductivity and electron-capture detectors, which are nondestructive, and flame-photometric, flame ionization, and alkali flame ionization (N,P) detectors. GC-MS, which will be considered in Section IV,A, can be used as a universal detector (total ion current monitoring) or as a tuneable, specific detector (selective-ion monitoring). The flame ionization detector is the workhorse detector for lipid analysis, as it is a nonspecific carbon detector. Its sensitivity can be 100 pg/sec, which means that 1–10 ng of sample is detectable. Generally, however, sample loadings of 0.01–0.1 μg (capillary column GC) or 1–10 μg (packed-column GC) per component are used. The sample is injected in a small volume of organic solvent. For a homologous series of compounds the flame ionization detector response is often proportional to percentage carbon, so that over a limited range within the series peak areas in the chromatogram approximate to masses. However, for precise quantitation and for comparisons between different classes of lipid molecules, calibration of the flame ionization detector response is required. The major developments in GC in the last decade have been in the automation of data processing, the widespread use of capillary columns, new stationary phases, and new derivatization methods. The latter three will be considered in relation to the analysis of fatty acids and lipids. The basic principles of GC have been described by McNair and Bonelli (1969).