"STUDIES OF LIPID METABOLISM IN THE AQUATIC FUNGI *PYTHIUM DEBARYANUM* AND *ACHLYA SP.*.

by

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TO

MY MOTHER, SISTERS, BROTHERS

AND

MY LATE FATHER

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ABSTRACT

The presence of a fatty acid synthetase, capable of de-novo synthesis of long chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH has been demonstrated in the simple fungus Pythium debaryanum (ATCC 9998), a member of the class Oömycetes. The enzyme is found in the supernatant fraction after centrifugation at 123,000 g for 35 min.

The fatty acid synthetase was demonstrated to be of the multienzyme complex type, showing no dependence on acyl carrier protein for activity. The fatty acid products were identified as principally a mixture of free and esterified palmitic and stearic acids by gas-radiochromatography, and were shown to be synthesized de-novo by the ratio of incorporation of acetyl-and malonyl-CoA, the Schmidt decarboxylation, and the lack of activity of medium and long chain acyl-CoA derivatives as acceptors of two carbon units in an elongation reaction. The pH optimum for the reaction was 6.8 and the K_m for acetyl-CoA was 3.3 μM.

The molecular weight of the fatty acid synthetase was estimated by gel filtration on Sepharose-4B as being of the order of 4×10^6. Attempts to reduce the apparent size of the enzyme by treatment with detergents and various enzymes were unsuccessful.

Some aspects of lipid metabolism were studied in another closely related aquatic fungus, Achlya sp. (1969). This organism has a life cycle involving germination of spores to form coenocytic somatic hyphae, followed by vegetative mycelial growth and ultimately differentiation of hyphal tips into sporangia. The respective stages of development can be delineated on a time scale, showing a certain degree of synchrony within the Achlya life cycle.

Total lipid made up 10% of dry weight in the spore. After germination total lipid fell to 6% of dry weight in 8-10 hours, then rose to about 8% of dry weight at the time of sporangium formation. Half of this loss took place within 2 hours of germination. Total lipid was fractionated on silicic acid, which revealed that in the spore, total lipid was composed of 62% neutral lipid, 13% phospholipid and 25% glycolipid. After germination, the proportion of neutral lipid rose slightly after 2 hours, then fell sharply to 10% after 8 hours, whereupon it rose to 55% of total lipid after 30 hours. Conversely, phospholipid rose to 77% of total lipid after 8 hours and then declined to 40% after 30 hours. Glycolipid

was constant at between 10 and 20% of total lipid throughout. Fractionation showed that triglycerides made up 20% of neutral lipid in spores and fatty acids made up 50%. During growth, triglycerides fell to 3% after 10 hours then rose to 37% after 30 hours. Fatty acids increased to 65% of neutral lipid after 8 hour, then declined to 20% after 24-30 hour.

The biosynthesis of lipid by Achlya throughout its life cycle was studied by pulsing whole cells at various stages of the life cycle with 1-^{14}C-acetate. Total lipid was extracted and it was shown the ^{14}C-acetate incorporation varied over the life cycle, peaking at about the mature sporangial stage. Analysis of total lipid by thin layer chromatography revealed that radioactivity was located principally in the glyceride fraction at all times in the life cycle except the period of active mycelial growth which showed considerable radioactivity in the phospholipid fraction.

Fatty acid synthetase activity was assayed in microsomal supernatant preparations of Achlya by measuring the incorporation of 1-^{14}C-acetyl CoA into long chain fatty acids. The activity was low in the spore and throughout mycelial development. It then increased rapidly, peaking at the time of sporangium maturation. The formation of $^{14}CO_2$ from 1-^{14}C palmitic or 1-^{14}C-

hexanoic acids by whole cells of Achlya was used as a measure of β-oxidation activity throughout the life cycle. This activity was also found to peak at sporangial developmental phase. Cyclic AMP and dibutyrl cyclic AMP were found to delay and induce abnormal sporulation respectively, in Achlya. The endogenous cyclic AMP level was highest during Achlya spore germination and sporulation.

TABLE OF CONTENTS

Table of Contents Continued PAGE

Table of Contents Continued PAGE

Table of Contents Continued PAGE

LIST OF TABLES

List of Tables Continued

LIST OF FIGURES

List of Figures Continued

List of Figures Continued

List of Figures Continued

List of Figures Continued

List of Figures Continued

ABBREVIATIONS

ACP	-	Acyl carrier protein
BSA	-	Bovine serum albumin
CoA	-	Coenzyme A
CD-medium	-	Czapek-Dox medium
cAMP	-	Cyclic 3'5'adenosine monophosphate
cGMP	-	Cyclic 3'5'guanosine monophosphate
DEAE	-	Diethylaminoethyl cellulose
dbcAMP	-	Dibutyryl 3'5'cyclic adenosine monophosphate
DTNB	-	5,5'-Dithio(bis)-2-nitrobenzoic acid
DTT	-	Dithiothreitol
EDTA	-	(Ethylene dinitrilo) - tetracetic acid
EF	-	Elongation factor
EMP	-	Embden-Meyerhof pathway
FAD	-	Flavin adenine dinucleotide
FAS	-	Fatty acid synthetase
FFA	-	Free fatty acids
FMN	-	Flavin mononucleotide
G_2Y	-	medium : Glucose yeast extract medium
GMP	-	Guanosine 5'-monophosphate
HMP	-	Hexose monophosphate pathway
MGLP	-	6-0-methyl glucose containing polysaccharide
MMP	-	3-0-methyl mannose containing polysaccharide

MNNG	-	(N-methyl-N'-nitro-N-nitrosoguanidine)
NADH	-	Nicotinamide adenine dinucleotide (reduced)
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced)
NL	-	Neutral lipid
NLMF	-	Neutral lipid minus free fatty acids
PL	-	Phospholipids
4'-PP	-	4'-phosphopantetheine
TCA	-	Trichloroacetic acid
TL	-	Total lipid

Introduction

Some of the most active areas in biochemical research today involve lipids. Their presence in cell membranes for example, involves them in the problems of oxidative phosphorylation, active transport, mitosis, chromosomal replication and cellular compartmentation. A deep knowledge of the biochemistry and physical chemistry of lipids is central to progress in such problems. Lipids are water insoluble and difficult to handle, and in the past this has discouraged many investigators from studying them. Even today, it is often difficult and tedious to obtain a lipid or lipoprotein in a high state of purity. Nevertheless, the involvement of lipids in so many processes of importance has led to an upsurge in the amount of research being performed on them.

Recent advances in methodology and instrumentation have made more impact on the field of lipids than on any other area of biochemistry. It is obvious that lipid biochemistry will continue to be an area of fundamental interest and importance in the future development of biological research.

It is now well established that enzyme systems capable of de novo synthesis of long chain fatty acids are of two general types. Fatty acid synthetases isolated

from bacteria and higher plants exist, in cell-free extracts, as mixtures of invidual and separable proteins which exhibit the various enzymatic activities of the fatty acid synthetic pathway. The activity of these fatty acid synthetases is absolutely dependent on the presence of acyl carrier protein (ACP), which contains 4'-phosphopantetheine as prosthetic group. In contrast, the fatty acid synthetases of yeast and all vertebrates examined have been isolated as tightly bound multienzyme complexes. Several of these complexes have been shown to contain protein bound 4'-phosphopantetheine. However, a few bacterial species particularly that of Mycobacterium smegmatis (formerly M. phlei) and a phyto-flagellate, Euglena gracilis have been found to possess fatty acid synthetases of both types. Therefore, the value of fatty acid synthetase as a criteria to show phyllogenetic relationship among living organisms is questionable.

However, on closer examination, one would realize that all bacterial species that have been found possessing a multienzyme complex belong to the order Actinomycetes. It is known that these bacterial organisms possess many characteristics that are unique for fungal cells. Since it is well established that fatty acid synthetase in yeast is a multienzyme complex, as is the case with another fungus Penicillium patulum, I would like to propose that those bacterial organisms which possess both types of

fatty acid synthetases may represent a transitional stage between bacteria and fungi in the evolutionary process. This statement may be pre-mature since very little is known about the fatty acid synthetases of other fungi. It is one of the purposes of this dissertation therefore, to study the fatty acid synthetase of an aquatic fungus Pythium debaryanum, to add to our presently scanty knowledge of fatty acid synthetases in fungi. Like many other multi-enzyme complexes that have been identified, fatty acid synthetase complexes offer the cell with a new dimension of enzyme regulation. A study of Pythium debaryanum fatty acid synthetase may help us to elucidate many long standing problems in de-novo synthesis of long chain fatty acids.

As a result of rapid advances in methodology and instrumentation, accurate and comprehensive survey of lipid composition, structure, etc. in various organisms, organs and tissues are now possible. Surprising contributions can be rendered by these studies. For instance, it was found that in leaf tissue, which is responsible for the light-dependent fixation of carbon dioxide, four complex lipids are always present: monogalactosyldiglycerides, digalactosyldiglycerides, sulfoquinovosyl-diglycerides and phosphatidylglycerol (Roughan and Batt 1969; Nichols and James1968). Moreover, these lipids have α-linolenic acid as the predominant esterified polyunsaturated fatty acid.

This constancy of lipid composition in important plant organelles must relate to the membrane structures that must have an architecture carefully designed for optimal functional capacity. Thus, their lipoidal composition cannot vary according to the lipids characteristic of a given species. For example, to insert ricinoleic acid into the fatty acid moiety of either a phospholipid or a galactolipid in the mitochondrial membranes of seed cells of Ricinus communis would probably lead to serious changes in the physical structure of the membranes with a concomitant alteration in functional effectiveness. Moreover, the endosperm of the developing seed of Ricinus communis synthesizes large quantities of ricinoleic acid which is found esterified in triglycerides present in discrete oil droplets in the cell cytoplasm. The mitochondria of the endosperm, however, contain phospholipids and galactolipids with fatty acid composition devoid of ricinoleic acid but very similar to the mitochondrial lipids of other higher plants. Furthermore, the leaf lipids of Ricinus are not only free of ricinoleic acid but also contain fatty acids closely resembling those found in leaves of other plants.

Thus, plants contain a complement of fatty acids and complex lipids quite similar in compositional patterns. Superimposed on this normal pattern, may be lipids characteristically associated with specialized tissues of

that species. Evidently, higher plants allocate to the neutral lipids, namely the triglycerides, the repository for bizarre fatty acids. In general, seed lipids contain fatty acids characteristic of a given plant species (Wolff 1966). Although lipids have been studied quite extensively in plant, bacteria and animals, very little is known about their composition or their involvement and fate during fungal cell development. A lot of research has been centered around the possibility of using fungi as potential oil producers from industrial wastes. However, many of these studies are searching for optimal conditions for oil accumulation by fungi; these involved parameters such as substrates, pH, temperature, aeration and strain selection. Although, a few fungal species have been chosen as models for cellular development research, the possibility of lipid involvement in these processes has not been considered until very recently. Achlya sp. was chosen for this investigation because it has a relatively short life-cycle, and it has defined morphologically distinguishable stages of development. Efforts are made in this thesis to correlate results of Achlya lipid metabolism and morphogenesis. This investigation was by no means exhaustive, but, hopefully it will serve the role of initiating interest in this promising area of research.

H I S T O R I C A L

Early Development on the Biosynthesis of Fatty Acids

Studies on the formation of fatty acids were initiated during the classical work of Rittenberg and Bloch (1945) who showed that both isotopes of doubly-labelled sodium acetate ($CD_3{}^{13}COONa$) were incorporated into fatty acids and cholesterol in rats and mice. Degradation of the fatty acids indicated that the labelled atoms were distributed at alternate positions along the chain. Further work indicated that fatty acids were also derived from acetate in yeast (White and Werkman, 1947) and the mold Neurospora (Ottke et al., 1951). Similarly plant systems incorporated acetate into long-chain fatty acids and these were isolated after conversion into lipid (Stumpf and Barker, 1957). This established the involvement of C_2 units derived from acetate in the biosynthesis of the long-chain fatty acids in various mammalian tissues, microorganisms and plants. This involvement of C_2 units prompted the belief (Lynen, 1951; Lynen and Ochoa, 1953a) that the synthesis of long chain fatty acids is operated by a reversal of the β-oxidation pathway, which was originally postulated by the brilliant and classical studies of Knoop (1904) and its individual steps substantiated some 50 years