Metabolism & Physiological Significance of Lipids

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PREFACE

INTEREST IN lipids has expanded greatly during the last fifty years from the study of the technology of oils and fats to embrace almost all branches of the biological sciences. In addition to the triglycerides and the simple phospholipids long known in Nature, modern methods of separation and analysis have revealed the existence of a wide variety of more complex substances containing in their structure long aliphatic chains esterified or bound by another oxygen function and likely to have derived from a fatty acid. The metabolism of these compounds is now under intensive study and much is already known of the details of their biosynthesis and catabolism both in plants and animals.

More enigmatic, at present, is the biochemical or physiological function of these complex lipids. Their occurrence in all living cells and, in many cases, their rapid turnover rate argue a role of importance in the genera metabolism; their amphipathic structure and surface active properties suggest they may play a part in interfacial phenomena such as the solubilization of protein in lipoproteins; their presence as integral and essential elements in membranes is indicative of a more architectural function, for example in the mitochondrial membrane.

In the human body, the implications of this wide diversity of biological function have assumed importance in connection with some diseases. Apart from the nutritional role of fats, the presence of particular types of unsaturation in the fatty acids ingested in the diet is believed to be a major factor in the aetiology of circulatory disease, and various other syndromes have been correlated with disorders in the general fat metabolism or that of specific lipids. The influence of polar lipids on the process of blood coagulation process has also excited interest.

Lipid research gathers, therefore, workers from widely diverse disciplines who rarely have the opportunity to develop their common interest. It appeared desirable, at this period of rapid increase in the tempo of lipid research, to provide this opportunity and, by allowing leading representatives of the workers in various fields to present their latest findings, to attempt to formulate a balanced view of the subject as a whole. This volume is presented in the hope that the deliberations of the Study Course succeeded in attaining these objectives.

January 30, 1964

R.M.C.D.
D.N.R.

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We wish to record the resolution of appreciation and thanks to this body passed by the participants to the Course during the closing session.

EDITORIAL NOTE

VIEWS AND opinions expressed by the authors of papers and contributors to discussions recorded in this volume are those of the individuals concerned. Publication does not imply that the editors necessarily share these views.

The accounts of the discussions have been condensed from tape recordings of the proceedings. It has not proved possible to submit these to the individual participants and, while every care has been taken to ensure accuracy, the editors apologize for any inadvertent errors which may have arisen.

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THE SYNTHESIS OF FATTY ACIDS IN ANIMAL TISSUES

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THE FATTY acids found in animal tissues vary in chain-length from acetic acid (C_2) to lignoceric acid (C_{24}) and include various derivatives and isomers such as unsaturated, hydroxy and branched-chain acids. The most common contain an even number of carbon atoms, and the predominant members have 16 and 18 carbon atoms. The shorter-chain acids (C_4 to C_{12}) are present in relatively low concentrations and are usually synthesized in the mammary gland and excreted in the milk. The fatty acids of longer chain-lengths (C_{14} to C_{24}) are found in most animal tissues, esterified in simple and complex lipids. With the advent of modern techniques, it is now possible to isolate pure fatty acids, determine their structure and to study their biosynthesis. The present report deals with the biosynthesis of fatty acids mainly in animal systems.

The biosynthesis of saturated fatty acids

Palmitic acid

The synthesis of palmitic acid from acetic acid was first demonstrated by Gurin and his colleagues[1–4] in avian liver slices and later in cell-free preparations. A similar system was isolated by Popják and Tietz[5,6] from lactating mammary gland having the same characteristics as that of pigeon liver. Wakil and his co-workers[7–10] have extensively studied the avian liver system. They fractionated the liver extracts into two main protein fractions and established the active components of the system to be acetyl-CoA, HCO_3^-, ATP, Mn^{2+}, and $NADPH_2$.

Acetyl-CoA is carboxylated by one of the enzyme fractions to form malonyl-CoA.[11] The enzyme contains biotin as its prosthetic group

and bicarbonate, ATP and Mn^{2+} are required for the carboxylation reaction as shown by the following reaction:

$$CH_3COSCoA + CO_2 + ATP \overset{Mn^{2+} + \text{biotin enzyme}}{\rightleftharpoons} HOOCCH_2COSCoA + ADP + P_i \quad (1)$$

The enzyme has been named acetyl-CoA carboxylase and specifically requires Mn^{2+}. ATP can be replaced by uridine triphosphate, but only at higher concentration. Biotin participates in the carboxylation of acetyl-CoA as is shown by the ability of avidin to inhibit the reaction and the formation of the CO_2–biotin enzyme complex as an intermediate in the carboxylation reactions.[12–15]

Addition of citrate to preparations of acetyl-CoA carboxylase stimulates malonyl-CoA formation several-fold.[16–18] Citrate can be replaced by other tri- and dicarboxylic acids such as isocitrate, succinate, malonate, α-ketoglutarate, etc. The degree of stimulation varies with the acid; isocitrate and citrate produce the greatest effect. The mechanism of stimulation is not very well understood and evidence presented by Vagelos, Alberts and Martin[19] indicates that addition of citrate results in the formation of an enzyme trimer which has higher activity than the monomer. The physiological significance of citrate stimulation is not clear at present, but indications are that it may exercise a controlling effect on biosynthesis through the regulation of the rate of malonyl-CoA formation.

The second stage in the synthesis of palmitic acid involves the condensation of malonyl-CoA with acetyl-CoA in the presence of $NADPH_2$ and the second enzyme fraction, referred to as fatty acid synthetase. Acetyl-CoA contributes carbons 15 and 16 of the palmitic acid whereas malonyl-CoA contributes carbons 1 to 14, providing the C_2-units necessary for the elongation of the primer acetyl-CoA. CO_2 is released during the reaction and $NADPH_2$ provides the electrons needed for the complete reduction of the carbonyl carbons to methylene. The primary product of this synthesis is palmitic acid, although stearic (C_{18}) and myristic acids (C_{14}) are formed to a limited extent. Since palmitic acid is the major product and this system is the most active fatty acid synthesizing system isolated from animal tissue, it would be expected that the C_{16} fatty acid would predominate in animal tissues. Furthermore, the C_{16} acids (palmitic and palmitoleic acids) would be expected to be important precursors for other fatty acids. Evidence to support this hypothesis will be presented later. On the other hand, the fatty acid synthetase system of plant tissues and many microorganisms appear to yield primarily C_{18} acids (stearic, oleic, linoleic, etc.). The reasons for this variation are not clear at present but can be attributed to the specificity of the fatty acid synthetase systems, which in the case of the animal system releases free palmitic acid to the medium by the action of some deacylating mechanism whereas the plant synthetase releases free stearic acid to the

medium. This appears to be a plausible explanation since the free acids (palmitic or stearic) appear to be the final products of the synthetase reaction and not the coenzyme A derivatives, as was first assumed.[20]

The stoichiometric relationship between the reactants (acetyl-CoA, malonyl-CoA and $NADPH_2$) and the products (palmitic acid, CO_2, CoA, and NADP) is illustrated in the following reaction:

$$CH_3COSCoA + 7HOOCCH_2COSCoA + 14NADPH + 14H^+ \rightarrow CH_3(CH_2)_{14}COOH + 7CO_2 + 8CoASH + 14NADP^+ + 6H_2O \quad (2)$$

The fatty acid synthetase has been prepared from avian liver,[21] rat liver,[22-24] adipose tissue,[25] brain,[26] mammary gland,[27] avocado mesocarp,[28] yeast cells,[29] *C. kluyverii*[30] and *E. coli* cells.[31]

The synthetase preparations from animal tissues have been obtained in soluble form, and present evidence indicates that they are complexes of more than one enzyme and are located in the cytoplasm on sites other than the mitochondria or the microsomes. Attempts to resolve this complex have so far been unsuccessful. In avian liver, six different components have been resolved on starch gel or acrylamide electrophoresis, but they were not active nor did recombination restore the synthetase activity. Bacterial preparations, however, can be fractionated into various active components. Vagelos and his associates[30,31] as well as Bloch and his group[32] and our own laboratory have been able to obtain two or more proteins from *E. coli* that on recombination reconstitute the fatty acid synthetase.

The mechanism involved in the conversion of acetyl-CoA and malonyl-CoA to palmitate is not known with certainty, although several hypotheses have been proposed. The reason for this uncertainty has been the lack of well-defined intermediates in this process. The *E. coli* system has provided us with the best hope of isolating such intermediates because of the response of its synthetase to fractionation into several active proteins. The information available from studies on animal,[21,34,35] yeast[29,36] and *E. coli* synthetases[30,32] can be summarized as follows:

(a) The fatty acid synthetases are SH-enzymes as evidenced by the ability of SH-binding reagents such as *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, iodoacetamide or arsenite[37] to inhibit synthesis. The mercuribenzoate inhibition is reversible whereas the inhibitions by the other reagents are not. Arsenite inhibition appears to be dependent on the presence of a thiol compound such as mercaptoethanol or cysteine. There is no doubt that the fatty acid synthetase is a thiol enzyme but whether it is a dithiol enzyme, where the two SH groups are positioned in such a way as to interact with arsenite is questionable, since arsenite inhibition always requires the presence of a thiol reagent.[31,35,37] The presence of the thiol group on the enzyme and the lack of detectable intermediates in complete or partial reaction

mixtures led Lynen[29] to suggest acyl–S–enzymes as intermediates in fatty acid synthesis.

(b) Acetyl-CoA protects the synthetase against inhibition by SH-binding reagents, suggesting that acetyl-CoA may interact with the protein at the SH group to form acetyl–S–enzyme.[35] Malonyl-CoA on the other hand does not protect the enzyme, indicating that it either does not form a malonyl–S–enzyme complex or that such a complex, if formed, is readily dissociated. Similar conclusions have been reached from studies on the ability of the fatty acid synthetase to effect transacylation of both acetyl-CoA and malonyl-CoA with pantetheine or ^{32}P-coenzyme A.[30,36]

(c) The first step in the synthesis of palmitic acid appears to be the condensation of acetyl-CoA and malonyl-CoA to form a β-keto-acyl derivative with a concomitant release of CO_2. This type of condensation was first proposed by Vagelos,[38] who reported the presence of an acyl-CoA–dependent CO_2–malonyl-CoA exchange reaction in preparations of the fatty acid synthetases from bacteria. A similar exchange reaction was demonstrated for the yeast synthetase.[36] The fatty acid synthetase from animal tissues did not effect this exchange but could catalyze acetyl-CoA–dependent decarboxylation of malonyl-CoA.[21,24,39] The decarboxylation reaction manifested properties similar to that of the fatty acid synthetase reaction.[21] The product of interaction of acetyl-CoA and malonyl-CoA appeared to be a β-keto-acid, but its structure has not yet been determined. Lynen and his co-workers[36] studied this reaction with the yeast synthetase and were able to demonstrate the formation of acetoacetyl–S–enzyme from acetyl-CoA and malonyl-CoA. Similar results were also obtained by Vagelos and his group[30] using the *E. coli* synthetase plus a heat-stable protein fraction. These workers isolated the acetoacetyl–S–protein from the reaction mixture which, in the presence of malonyl-CoA, $NADPH_2$ and the heat-stable fraction, was incorporated into the long-chain fatty acids.[40]

(d) The yeast,[36] avian liver[34] and rat brain[41] preparations of the fatty acid synthetase were shown to catalyze the reduction of acetoacetyl-CoA to D(−)β-hydroxybutyryl-CoA by $NADPH_2$. The enzyme has been named acetoacetyl-CoA reductase. It is not certain that this enzyme is a component of the fatty acid synthetase since the reductase is not an SH-enzyme and its level in the synthetase preparations could be readily reduced to a level well below that of fatty acid synthesis. In such preparations the rate of fatty acid synthesis was 20- to 30-fold higher than that of the reduction of acetoacetyl-CoA. If the reductase was a component of the fatty acid synthetase, then, in such preparations, the reduction of the acetoacetyl group would be a rate-limiting step. Despite these objections, the acetoacetyl-CoA reductase may still be involved in fatty acid synthesis if one assumes: first, that within the synthetase complex, the true substrate for the reductase is acetoacetyl–S–

protein and that acetoacetyl-CoA represents a model compound that the reductase can reduce but at much higher concentrations and lower rates.

(e) In order to form the saturated acyl group, the D(−)β-hydroxyacyl group has to be dehydrated to the αβ-unsaturated derivative which may then be reduced by $NADPH_2$ (possibly via a flavin nucleotide) to the saturated acyl compound. This process would require two additional enzymes which are thought to be part of the synthetase complex.

On the basis of these observations the acyl–S–enzyme hypothesis (Scheme I) appears to be the most probable, though more details are necessary in order to fully understand the mechanism of the total synthesis. The

$$CH_3COSCoA + HS\text{–}E \rightarrow CH_3COS\text{–}E + CoASH \quad (3)$$

$$CH_3COS\text{–}E + HOOCCH_2COSCoA \rightarrow CH_3COCH_2COS\text{–}E + CO_2 + CoASH \quad (4)$$

$$CH_3COCH_2COS\text{–}E + NADPH + H^+ \rightarrow D(-)CH_3CHOHCH_2COS\text{–}E + NADP^+ \quad (5)$$

$$D(-)CH_3CHOHCH_2COS\text{–}E \rightarrow CH_3CH{=}CHCOS\text{–}E + H_2O \quad (6)$$

$$CH_3CH{=}CHCOS\text{–}E + NADPH + H^+ \rightarrow CH_3CH_2CH_2COS\text{–}E + NADP^+ \quad (7)$$

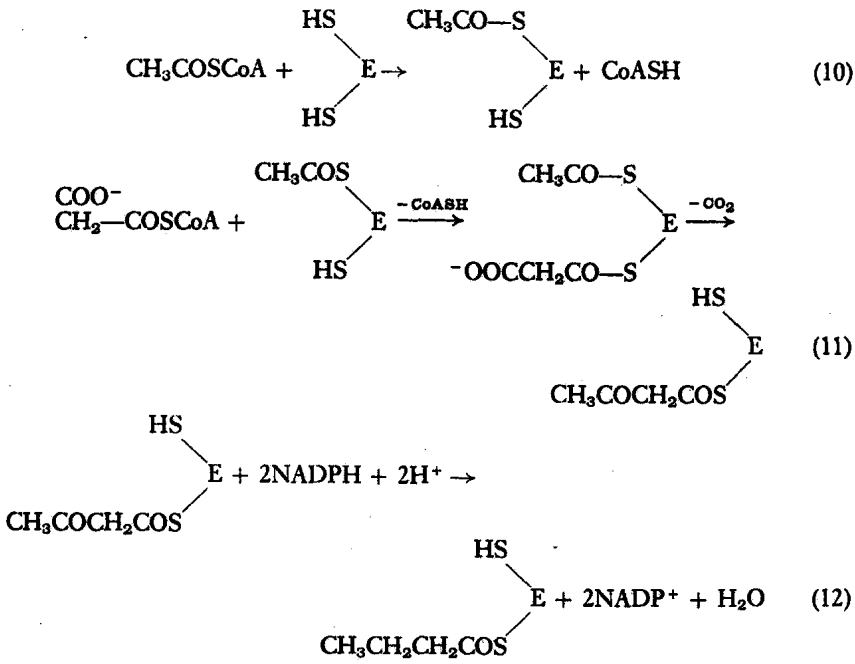
Sum: $$CH_3COSCoA + HS\text{–}E + HOOCCH_2COSCoA + 2NADPH + 2H^+ \rightarrow CH_3CH_2CH_2COS\text{–}E + CO_2 + 2NADP^+ + H_2O + 2CoASH \quad (8)$$

Scheme I

sequence of reactions (3) to (7) can be repeated six more times until palmityl–S–E is synthesized; and this complex is then deacylated perhaps by a specific deacylase to form palmitic acid (9).

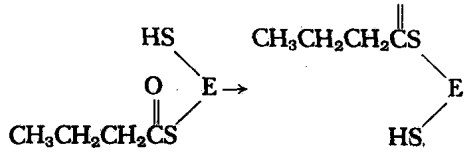
$$CH_3(CH_2)_{14}COS\text{–}E + HOH \rightarrow CH_3(CH_2)_{14}COOH + HS\text{–}E \quad (9)$$

There are still many objections to this hypothesis[42] that remained unresolved. Lynen has recently modified his original hypothesis, suggesting that there are two sulfhydryl groups on the enzyme that bind acetyl-CoA and malonyl-CoA respectively prior to their condensation to form acetoacetyl–S–enzyme. The new hypothesis, illustrated in Scheme II, raises a difficulty concerned with the next elongation step, namely the condensation of butyryl–S–enzyme with malonyl-CoA to form the β-ketohexanoyl–S–enzyme. Since the butyryl group is now attached to the thiol group that was once attached to the malonyl group, the second malonyl-CoA will have to form a malonyl–S–enzyme through the other thiol group that was used for acetyl-CoA. If this is the case, then the two SH groups are equivalent if not identical. This is hardly tenable since it deprives the SH groups of specificity. It is also difficult to see why malonyl-CoA does not protect the



Scheme II

enzyme against SH-inhibitors. There may be a way out of this dilemma if the butyryl group exchanges its position as follows:



thus the formation of butyryl–S–E is made comparable to the acetyl–S–E.

Stearic acid

Stearic acid is the second most abundant saturated fatty acid in animal tissues and is synthesized via two pathways: the *de novo* pathway (the same as that involved in palmitic acid synthesis) and the elongation pathway,[43] which involves the elongation of palmityl-CoA by acetyl-CoA.

Stearic acid usually represents about 10 to 20% of the fatty acids synthesized by the *de novo* synthetase system, the others being palmitic (60 to 80%) and myristic (5 to 10%). Via this pathway, stearic acid derives carbon atoms 1 to 16 from the C_2 units of malonyl-CoA and the last two carbon

atoms (17 and 18) from acetyl-CoA. The mechanism involved is presumed to be the same as that discussed for palmitic acid; some of the palmityl–S–protein complex does not undergo deacylation to palmitic acid but is further elongated by a C_2 unit from malonyl-CoA to form stearyl–S–enzyme. The latter would then be deacylated to form stearic acid and the enzyme–SH. What determines the ratio of stearic to palmitic acid in the products of this system is not known.

The second pathway for the synthesis of stearate is the elongation pathway of palmitic acid by acetyl-CoA. The enzyme system that catalyzes the elongation of the fatty acids is associated with the mitochondria.[43] Evidence

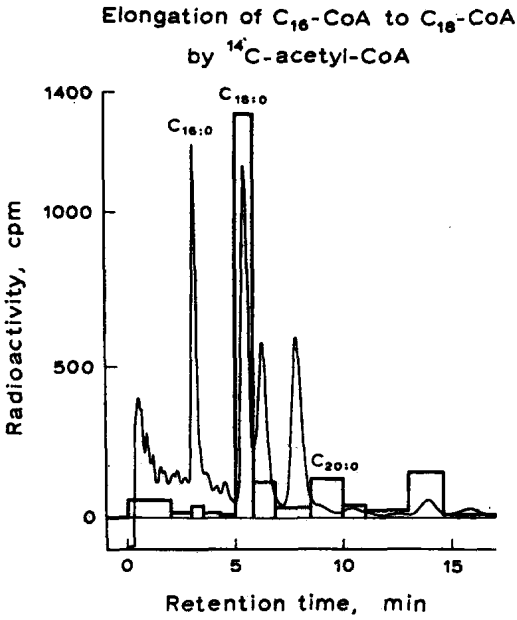


Fig. 1. Gas–liquid chromatographic analysis of the methyl esters of fatty acids synthesized by mitochondria with palmityl-CoA and [1-^{14}C]acetyl-CoA as substrates. Reaction mixture: 40 mμmoles palmityl-CoA, 47 mμmoles [1-^{14}C]acetyl-CoA ($2{\cdot}1 \times 10^5$ cpm), 1·0 μmole $NADH_2$, 1·0 μmole $NADPH_2$, 30 μmoles phosphate buffer pH 6·5, water to 0·5 ml., 0·5 mg mitochondria; incubation at 38°C, 1 hour. The bars represent the radioactivity (cpm) of the effluent gas counted during the time interval covered by the bars.

for the synthesis of stearic acid by the elongation of palmitic acid was obtained as early as 1950 when Zabin found that ^{14}C-stearic acid isolated from rats injected with [1-^{14}C]acetate had significantly more radioactivity in the carboxyl carbon than was calculated on the basis of even distribution of the radioactivity among the odd-numbered carbon atoms of the molecule, whereas the third carbon atoms of stearic acid had the same radioactivity as every other odd-numbered carbon atom of the remaining 16 carbon atoms of the stearic molecule. This finding indicated that carbons 3 to 18 of stearic acid (or the palmitic acid portion) were derived by *de novo* synthesis whereas carbons 1 and 2 were added by a different mechanism. Wakil and