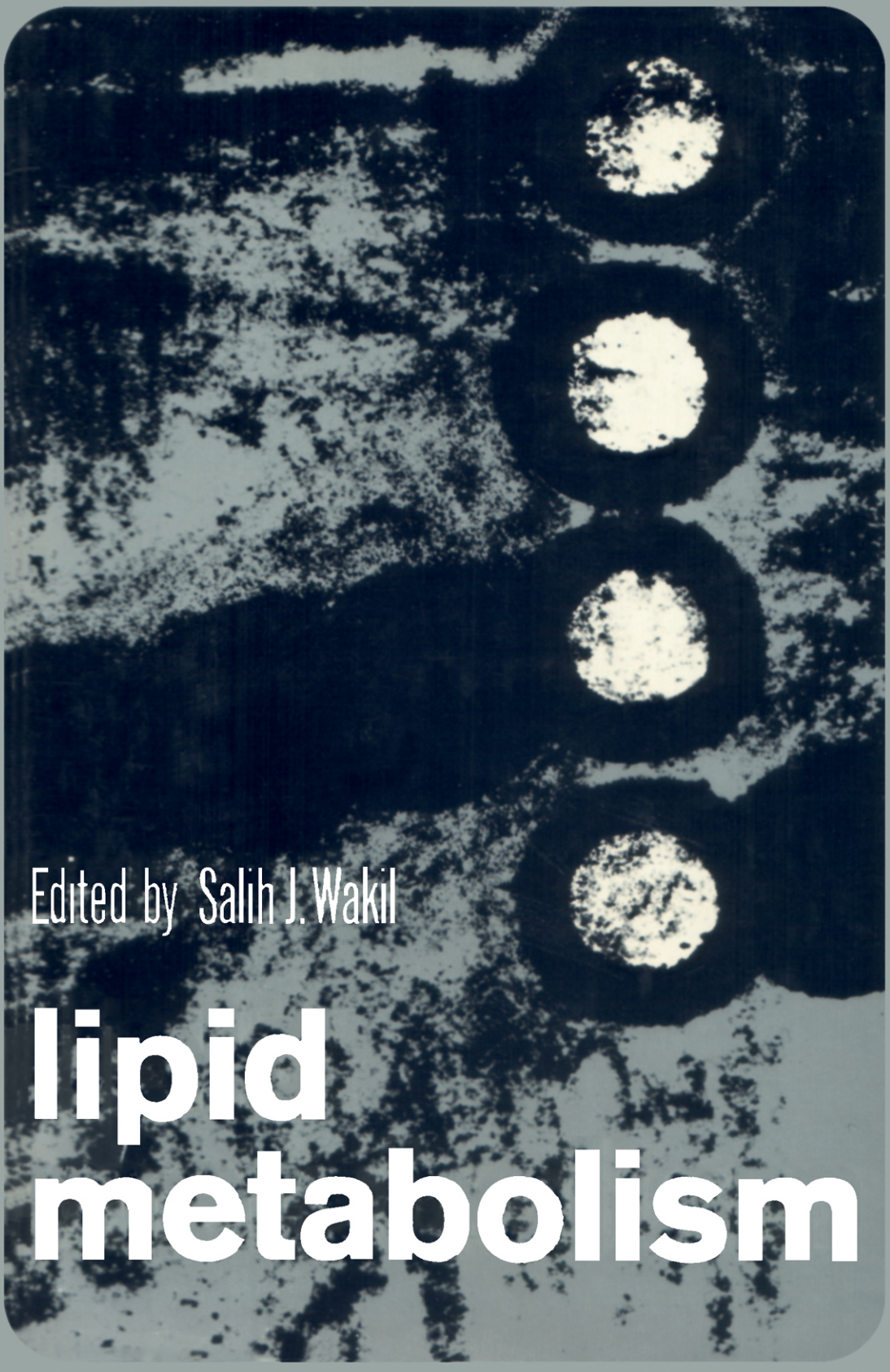

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lipid metabolism

LIPID METABOLISM

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Edited by

Salih J. Wakil

Department of Biochemistry
Duke University Medical Center
Durham, North Carolina

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Contents

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List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

RONALD BENTLEY (481), Department of Biochemistry and Nutrition, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania

RUBIN BRESSLER* (49), Departments of Medicine, Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina

JOHN W. CORCORAN (431), Department of Biochemistry, Northwestern University Medical School, Chicago, Illinois

F. J. DARBY (431), Medical Genetics, The University of Liverpool, Liverpool, England

EDWARD E. HILL (185), Department of Biochemistry, West Virginia University School of Medicine, Morgantown, West Virginia

P. W. HOLLOWAY (371), Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia

G. HÜBSCHER (279), Department of Biochemistry, University of Nottingham, Nottingham, England

WILLIAM E. M. LANDS (185), Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan

W. J. LENNARZ (155), Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland

BENGT SAMUELSSON (107), Department of Medical Chemistry, Royal Veterinary College, Stockholm, Sweden

P. K. STUMPF (79), Department of Biochemistry and Biophysics, University of California, Davis, California

SALIH J. WAKIL (1), Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

*Present address: Department of Pharmacology, College of Medicine, University of Arizona, Tucson, Arizona.

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Preface

Some of the most active areas in biochemical research today involve lipids. Their presence in cell membranes, for example, involve them in the problems of oxidative phosphorylation, active transport, mitosis, chromosomal replication, and cellular compartmentalization. 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. As a result, there is a pressing demand from investigators, teachers, and students for a comprehensive account of the major achievements and trends in this field. This treatise on the regulation and metabolism of lipids was conceived to help satisfy this need.

The present volume deals with fatty acids, prostaglandins, glycerides, glycerophosphates, cholesterol, isoprenoids, and aromatic compounds. When future developments warrant another volume, it will cover other areas of lipid metabolism, including lipid–protein interactions and the physical and chemical properties of lipids.

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LIPID METABOLISM

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Chapter I Fatty Acid Metabolism

Salih J. Wakil

I. Introduction

Fatty acids are commonly found in all types of cells, usually bound in an ester linkage to various cellular lipids. They vary in chain length from acetic acid (C_2) to lignoceric acid (C_{24}) and include various derivatives and isomers, such as saturated, unsaturated, hydroxy, and branched-chain acids. Although the

most common fatty acids contain 16 or 18 carbon atoms, fatty acids of different chain lengths (both even and odd) also exist but at lower levels than the C_{16} and C_{18} fatty acids.

The presence of unsaturated fatty acids is important to the structure and function of various lipids. Generally, all unsaturated fatty acids have a cis configuration of the olefinic bond. In polyunsaturated acids, the double bonds exist in polyallyl forms separated by a CH_2 group. The cellular distribution of these unsaturated fatty acids varies from one cell type to another. Animal and plant cells usually contain mono- and polyunsaturated fatty acids, whereas most bacteria contain monounsaturated fatty acids or their cyclopropane derivatives. The present chapter surveys the overall knowledge of fatty acid metabolism and its controlling factors.

II. Fatty Acid Oxidation

A. β-Oxidation of Saturated Acids

The β-oxidation sequence of fatty acids, first formulated by Knoop in 1904, is the universal pathway by which fatty acids are successively oxidized to acetate units (C_2) (Green, 1954; Lynen, 1954). The various steps involved in this sequence of reactions are: (*1*) Formation of the coenzyme A thioester; (*2*) α,β-dehydrogenation of the acyl-CoA; (*3*) hydration of α,β-unsaturated fatty acyl-CoA; (*4*) oxidation of the β-hydroxyacyl-CoA; and (*5*) thiolytic cleavage of the β-ketoacyl-CoA.

1. Formation of the Coenzyme A Thioester

Fatty acids are usually activated to the corresponding thioester derivatives of coenzyme A as an initial step in their metabolism. This activation reaction is catalyzed by the acyl-CoA synthetases according to the following general reaction:

$$\text{RCOOH} + \text{CoASH} + \text{ATP} \underset{\text{Mg}^{2+}}{\overset{\text{Acyl-CoA synthetase}}{\rightleftharpoons}} \text{RCOSCoA} + \text{AMP} + \text{PPi}$$

Three different synthetases are known, and they vary according to the chain length of the fatty acids. The acetyl-CoA synthetase [acetate: CoA ligase (AMP), EC 6.2.1.1] activates acetate, propionate and acrylate in a decreasing order. Crystalline preparations of the enzyme obtained from bovine heart mitochondria were found to have an average molecular weight of 35,200 (Webster, 1965a). The enzyme binds one mole of acetyladenylate per mole and requires both monovalent ions (K^+) and divalent ions (Mg^{2+}) for optimal activity (Webster, 1965b, 1966). The cations appear to be essential for the

formation of the acetyladenylate–enzyme intermediate during acetyl-CoA synthesis, according to the following partial reactions:

$$\text{Acetate} + \text{ATP} + \text{E} \rightleftharpoons \text{E-(acetyladenylate)} + \text{PPi} \quad (1)$$

$$\text{E-(acetyladenylate)} + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{AMP} + \text{E} \quad (2)$$

The sum of these two reactions is:

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{AMP} + \text{PPi} \quad (3)$$

A second acyl-CoA synthetase [acid: CoA ligase (AMP), EC 6.2.1.2] isolated from beef liver mitochondria was found to be specific for medium-chain fatty acids from C_4 to C_{11} and their corresponding 3-hydroxy- and 2,3- or 3,4-unsaturated acids (Mahler *et al.*, 1953). Another acyl-CoA synthetase [acid: CoA ligase (AMP), EC 6.2.1.3] is present in microsomes and acts on fatty acids of chain length C_{10} to C_{20} (Kornberg and Pricer, 1953).

Recently, a fatty acid-activating enzyme has been isolated from a mitochondrial membrane requiring GTP rather than ATP for its activity. Here, orthophosphate is the product of phosphorolysis rather than pyrophosphate, as shown in the following reaction:

$$\text{RCOOH} + \text{GTP} + \text{CoASH} \rightleftharpoons \text{RCOSCoA} + \text{GDP} + \text{Pi} \quad (4)$$

The enzyme acts on long-chain fatty acids and requires phospholipids for its activity (Galzigna *et al.*, 1967). It is inhibited by orthophosphate and fluoride.

Another mechanism by which acyl-CoA is formed is a transacylation reaction catalyzed by thiophorase:

$$\text{Succinyl-CoA} + \text{RCOOH} \rightleftharpoons \text{succinate} + \text{RCOSCoA} \quad (5)$$

The fatty acid is usually a short-chain fatty acid (C_4–C_6) or its β-keto derivative (Stern *et al.*, 1956). The enzyme is absent from liver; it is found only in extrahepatic tissues.

2. The α,β-Dehydrogenation of the Acyl-CoA

This reaction is catalyzed by the acyl-CoA dehydrogenase as shown below:

$$\text{RCH}_2\text{CH}_2\text{COSCoA} + \text{acceptor} \rightarrow \textit{trans}\text{-RCH=CHCOSCoA} + \text{reduced acceptor} \quad (6)$$

Three enzymes with different chain-length specificity have been isolated from pig liver mitochondria (Crane *et al.*, 1955, 1956; Crane and Beinert, 1956; Hauge *et al.*, 1956): a butyryl-CoA dehydrogenase [butyryl-CoA: (acceptor) oxidoreductase, EC 1.3.99.2] and two acyl-CoA dehydrogenases [acyl-CoA: (acceptor) oxidoreductase, EC 1.3.99.3]. The relative specificity of each of these enzymes is illustrated in Fig. 1. These data make it apparent that fatty acyl-CoA derivatives of chain lengths C_4–C_{20} are readily oxidized to their corresponding α,β-unsaturated derivatives.

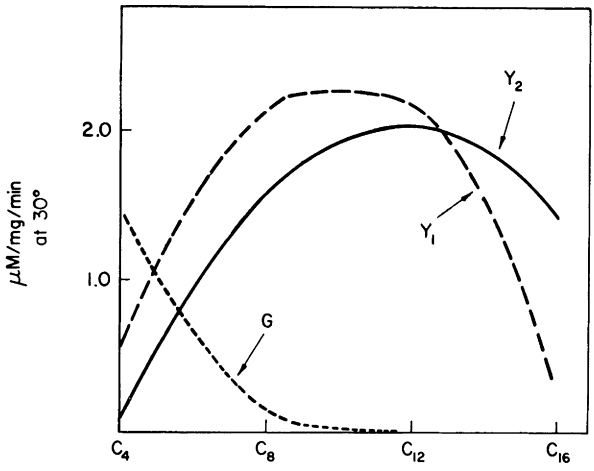


Fig. 1. Specificity of fatty acyl-CoA dehydrogenases for substrates of different chain length. G, Butyryl-CoA dehydrogenase; Y_1 and Y_2, acyl-CoA dehydrogenases (*Crane* et al., *1955*).

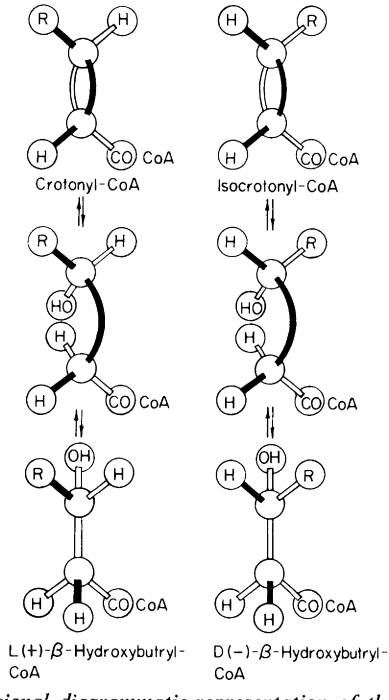


Fig. 2. A three-dimensional diagrammatic representation of the reaction of unsaturated acyl-CoA hydrase on crotonyl-CoA and isocrotonyl-CoA (*Wakil, 1956*).

The acyl-CoA dehydrogenases are flavoproteins containing flavine adenine dinucleotides (FAD) as prosthetic groups. The electrons extracted from the substrate reduce the FAD of the dehydrogenase to the $FADH_2$ and are then transferred to another FAD-containing flavoprotein, the electron-transferring flavoprotein, which is directly linked to cytochrome b of the mitochondrial electron transport system (Crane and Beinert, 1956; Garland *et al.*, 1967).

3. The Hydration of α,β-Unsaturated Fatty Acyl-CoA

The trans α,β-unsaturated acyl-CoA esters from the previous reaction are subsequently hydrated by enoyl-CoA hydrase (L-3-hydroxyacyl-CoA hydrolyase, EC 4.2.1.17) to the β-hydroxyacyl-CoA derivatives (Wakil and Mahler, 1954; Stern and Del Campillo, 1956) as shown in the following equation:

$$\textit{trans}\text{-RCH=CHCOSCoA} + H_2O \rightleftharpoons \text{L-RCHOHCH}_2\text{COSCoA} \qquad (7)$$

The enzyme has a broad specificity and is active on both trans and cis α,β-unsaturated fatty acyl-CoA derivatives (Wakil, 1956). The product of hydration of the trans isomer is the L(+)-β-hydroxyacyl-CoA, whereas the product of hydration of the cis derivative is the D(−) antipode. Figure 2 illustrates how the hydration of trans and cis α,β-unsaturated acyl-CoA brings about formation of L and D enantiomeric forms. Postulation of this mechanism was based on the assumptions that a three-point attachment (Ogston, 1948) occurs between the enzyme and substrate, that the addition of the elements of water always occurs from the same side [in this case the attack leads to the opening of the lower ("light") bond, Fig. 2], and that in the reverse direction the same hydrogen of the α-carbon is removed as H_2O. Similar postulates have been advanced to account for the hydration of fumarate (Krebs, 1948), dehydration of citrate (Potter and Heidelberger, 1949), and the oxidation of NAD^+ by alcohol dehydrogenase (Fischer *et al.*, 1953).

4. Oxidation of the β-Hydroxyacyl-CoA

The L-β-hydroxyacyl-CoA obtained from the preceding step is oxidized by NAD^+ in the presence of the L-β-hydroxyacyl-CoA dehydrogenase (L-3-hydroxyacyl-CoA: NAD oxidoreductase, EC 1.1.1.35) to the corresponding β-ketoacyl-CoA derivative, according to the following reaction:

$$\text{L-RCHOHCH}_2\text{COSCoA} + NAD^+ \rightleftharpoons \underset{\underset{\text{O}}{\|}}{\text{RCCH}_2\text{COSCoA}} + NADH + H^+ \qquad (8)$$

The enzyme is specific for the L form, and is active on the various chain-length fatty acyl derivatives (Wakil *et al.*, 1954; Stern, 1957).

5. Thiolytic Cleavage of the β-Ketoacyl-CoA

The last step in the β-oxidation of fatty acyl-CoA derivatives is the thiolytic cleavage of the β-ketoacyl-CoA, as shown in the following reaction:

$$\underset{\underset{\text{O}}{\|}}{\text{RCCH}_2\text{COSCoA}} + \text{CoASH} \rightleftharpoons \text{RCOSCoA} + \text{CH}_3\text{COSCoA} \qquad (9)$$

Crystalline thiolase (acyl-CoA: acetyl-CoA C-acyltransferase, EC 2.3.1.16) prepared from ox liver (Seubert *et al.*, 1968) has been found to have broad substrate specificity. The enzyme cleaves β-ketoacyl-CoA derivatives of C_4–C_{16} chain lengths. The relative activities of thiolase with the various β-ketoacyl-CoA derivatives are as follows: $C_4:C_6:C_8:C_{10}:C_{12}:C_{16} = 1:5.1:4.3:3.8:3.6:3.6$. The enzyme has an active thiol group which is involved in the formation of an acyl-S-enzyme as an intermediate in the overall cleavage process, as shown in the following two reactions:

$$\text{RCOCH}_2\text{COSCoA} + \text{HS-E} \rightleftharpoons \text{RCOS-E} + \text{CH}_3\text{COSCoA} \qquad (10)$$

$$\text{RCOS-E} + \text{CoA-SH} \rightleftharpoons \text{RCOS-CoA} + \text{HS-E} \qquad (11)$$

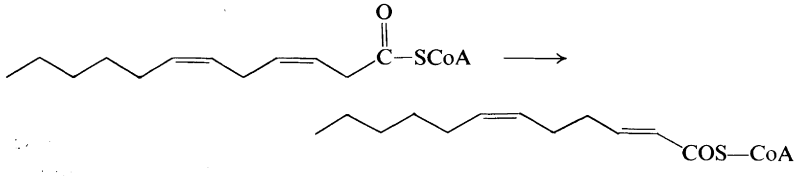
Although the thiolase reaction is reversible, the equilibrium greatly favors acetyl-CoA formation from β-ketoacyl-CoA (the equilibrium constant for acetyl-CoA formation from acetoacetyl-CoA equals 6×10^4).

B. β-Oxidation of Unsaturated Fatty Acids

As stated earlier, several chemical features characterize the naturally occurring unsaturated fatty acids. Important among these is the presence of the cis configuration in all the double bonds and, in the case of polyenoic acids, their separation by one methylene group. The position of the double bonds within the molecule is usually several carbons removed from either the carboxyl or the methyl end. In biological systems, it is more convenient to count the position of the double bond from the ω-CH_3 carbons of the fatty acids. Consequently, unsaturated fatty acids are classified in four different groups; the palmitoleic, oleic, linoleic, and linolenic series.

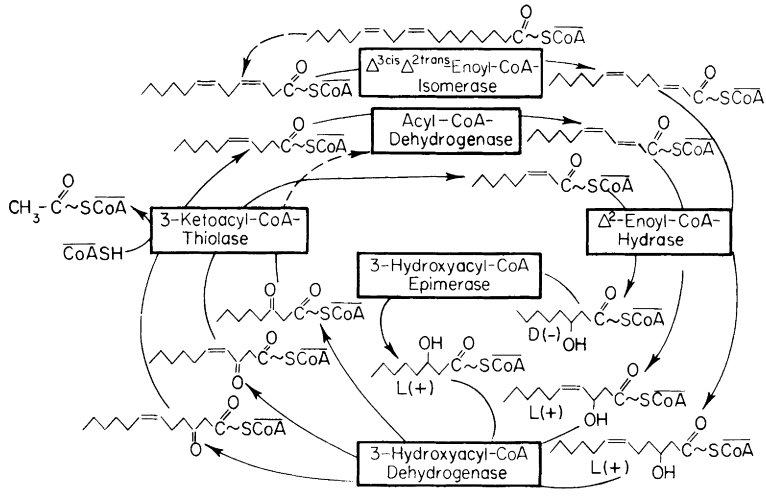
In oxidizing any of these unsaturated fatty acids, one encounters some serious problems, all of which can be resolved by supplementing the enzymic steps needed for the oxidation of saturated acids by the addition of at least two different enzymes. The oxidization of linoleic acid, for example, involves activation of the molecule to the CoA derivative, then oxidation to the α,β-unsaturated derivative, hydration to the L-β-hydroxyacyl-CoA, oxidation to the β-ketoacyl-CoA, and cleavage to acetyl-CoA and *all-cis*-hexadec-7,10-dienoyl-CoA. These reactions are catalyzed by the same enzymes needed for the oxidation of saturated long-chain fatty acids. The sequence of β-oxidation is

then repeated two more times, leading to the cleavage of two acetyl-CoA molecules and the formation of *all-cis*-$\Delta^{3,6}$-C_{12}-CoA. The latter compound is not a substrate for the acyl-CoA dehydrogenase or for enoyl-CoA hydrase. However, it is a substrate for an isomerase which catalyzes the conversion of the cis Δ^3 isomer to the trans Δ^2-enoyl derivative, as shown in the following reaction:



(12)

The enzyme has been isolated from rat liver mitochondria and named Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (Stoffel *et al.*, 1964). The product of the isomerase is hydrated by the enoyl-CoA hydrase to the L-β-hydroxyacyl-CoA, which is then oxidized to the β-ketoacyl-CoA and cleaved to acetyl-CoA and *cis*-Δ^4-decenoyl-CoA. The latter derivative undergoes another cycle of β-oxidation, yielding *cis*-Δ^2-octenoyl-CoA. This is then hydrated by enoyl-CoA hydrase to the corresponding D-β-hydroxyacyl-CoA derivative according to the mechanism outlined previously for hydrase (Wakil, 1956). The D-isomer is then epimerized by the D(−)-β-hydroxyacyl-CoA epimerase (Stoffel *et al.*, 1964; Stern *et al.*, 1955) to the L(+) antipode, which is the substrate for the



Scheme I. Sequence of reactions by which unsaturated fatty acids are oxidized to acetyl-CoA (Stoffel and Caesar, 1965).

L(+)-β-hydroxyacyl-CoA dehydrogenase. Thus, with the aid of the isomerase and the epimerase, and because of the ability of the hydrase to hydrate both trans and cis α,β-unsaturated acyl-CoA derivatives, it is possible to oxidize polyunsaturated fatty acids to acetyl-CoA via the β-oxidation cycle, as shown in Scheme I. All these enzymes are located in the mitochondria where the rates of oxidation of both saturated (palmitic acid) and polyunsaturated fatty acids are found to be the same (Stoffel and Scheefer, 1965).

C. Oxidation of Odd-Chain Fatty Acids

Although odd-chain fatty acids exist in relatively low concentrations, most cells do contain appreciable amounts of C_{15} and C_{17} fatty acids. These acids are oxidized by the β-oxidation pathway to acetyl-CoA and one equivalent of propionyl-CoA. The latter compound can also be formed by direct activation of propionic acid by the acetyl-CoA synthetase or from the degradation of branched-chain amino acids. In animal tissues, the propionyl-CoA thus formed is then converted to succinyl-CoA, according to the following reaction sequence:

$$CH_3CH_2COSCoA + HCO_3^- + ATP \rightleftharpoons \underset{\text{(S)-Methylmalonyl-CoA}}{H_3C{-}\overset{\overset{COSCoA}{|}}{C}{-}H}{\scriptstyle\overset{|}{COOH}} + ADP + Pi \qquad (13)$$

$$\underset{\text{(S)-Methylmalonyl-CoA}}{H_3C{-}\overset{\overset{COSCoA}{|}}{C}{-}H}{\scriptstyle\overset{|}{COOH}} \rightleftharpoons \underset{\text{(R)-Methylmalonyl-CoA}}{H_3C{-}\overset{\overset{COOH}{|}}{C}{-}H}{\scriptstyle\overset{|}{COSCoA}} \qquad (14)$$

$$\underset{\text{(R)-Methylmalonyl-CoA}}{H_3\overset{*}{C}{-}\overset{\overset{COOH}{|}}{C}{-}H}{\scriptstyle\overset{|}{COSCoA}} \rightleftharpoons \underset{\text{Succinyl-CoA}}{\overset{\overset{COOH}{|}}{CH_2}{-}\overset{|}{*CH_2}}{\scriptstyle\overset{|}{COSCoA}} \qquad (15)$$

Propionyl-CoA is carboxylated to methylmalonyl-CoA by propionyl-CoA carboxylase [propionyl-CoA: carbon dioxide ligase (ADP), EC 6.4.1.3]. Crystalline carboxylase (Kaziro *et al.*, 1961) has been prepared from pig heart with a molecular weight estimated to be 700,000. It contains 4 moles of biotin per mole of enzyme, suggesting that the enzyme consists of four subunits of molecular weight 175,000. The role of biotin in this enzyme is the same as that

in other biotin enzymes; i.e., it forms the carboxylated intermediate (carboxybiotin–enzyme) which is the CO_2 donor to the propionyl-CoA (this mechanism will be further discussed in Section III,B).

The methylmalonyl-CoA formed by the carboxylase has the (S) configuration which is the wrong isomer for the mutase reaction (Specher *et al.*, 1966). The (S)-methylmalonyl-CoA is racemized by methylmalonyl-CoA racemase (Mazumder *et al.*, 1962) to the (R)-methylmalonyl-CoA, which is then converted to the succinyl-CoA by the methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carboxylmutase, EC 5.4.99.2). This enzyme (Lengyel *et al.*, 1960; Mazumder *et al.*, 1963) contains coenzyme B_{12} which is involved in the intramolecular rearrangement of the molecule (Kellermeyer and Wood, 1962). During the rearrangement, a shift of the CoA–carboxyl unit to the methyl group occurs (Eggerer *et al.*, 1960), as shown in reaction (15). It is of interest to note that all of these reactions occur within the mitochondria.

III. Fatty Acid Biosynthesis

A. Introduction

When the mechanism of the β-oxidation of fatty acid was elucidated, it was assumed that fatty acid synthesis occurred by the reversal of the β-oxidation pathway. The first indication that the pathway of fatty acid synthesis differed from the β-oxidation pathway was the observation that fatty acid synthesis in partially purified extracts of avian liver required ATP and bicarbonate (Gibson *et al.*, 1958a), two components which could not be accounted for by the scheme of β-oxidation. ATP undergoes phosphorolysis to ADP and Pi. The bicarbonate, though absolutely required for fatty acid synthesis, did not incorporate into the final long-chain fatty acid synthesized. It is required for the carboxylation of acetyl-CoA to malonyl-CoA (Wakil, 1958). The latter is the C_2 donor for the chain elongation resulting in the synthesis of palmitic acid. The overall reactions involved in the synthesis of long-chain fatty acids from acetyl-CoA are as follows (Bressler and Wakil, 1961; Wakil, 1961, 1963; Lynen, 1961; Vagelos, 1964):

$$CH_3COSCoA + HCO_3^- + ATP \rightleftharpoons HOOCCH_2COSCoA + ADP + Pi \quad (16)$$

$$CH_3COSCoA + 7\ HOOCCH_2COSCoA + 14\ TPNH + 14\ H^+ \rightarrow$$

$$CH_3CH_2(CH_2CH_2)_6CH_2COOH + 7\ CO_2 + 14\ TPN^+ + 8\ CoA + 6\ H_2O \quad (17)$$

The first reaction is catalyzed by a biotin enzyme named acetyl-CoA carboxylase (Wakil and Gibson, 1960), whereas the second is catalyzed by a group of enzymes, often referred to as fatty acid synthetase. These enzymes can exist either in a tight complex, such as that found in animal tissues (Bressler and Wakil, 1961; Hsu *et al.*, 1965) or yeast cells (Lynen, 1967b), or in a readily

dissociable form such as that found in bacteria (Lennarz *et al.*, 1962; Alberts *et al.*, 1963; Wakil *et al.*, 1964) or plant tissues (Simoni *et al.*, 1967).

For the conversion of malonyl-CoA to long-chain fatty acids, a short-chain acyl primer is required (Wakil and Ganguly, 1959). Always, the "primer" becomes the methyl terminus of the fatty acid, and malonyl-CoA denotes the remaining carbons of the molecule. Acetyl-CoA is the preferred "primer" but other normal or branched short-chain acyl derivatives may also be used (Bressler and Wakil, 1961; Horning *et al.*, 1961). If the primer is propionyl-CoA or isopropionyl-CoA, the fatty acid synthesized will be an odd or a branched-chain fatty acid, respectively.

B. Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase [acetyl-CoA: CO_2 ligase (ADP), EC 6.4.1.2] was the first biotin–enzyme recognized of a class of enzymes that were later to be known as biotin enzymes. Biotin is covalently bound to the protein and is concentrated throughout the purification of the enzyme. Avidin, an egg-white protein which binds biotin very tightly, inhibited the reaction completely by binding the enzyme-bound biotin (Wakil and Gibson, 1960). Many biotin–enzymes have since been isolated and their mechanism of action found to be a general one, applicable to all members of the group. Essentially, two steps are involved in the overall carboxylation of acetyl-CoA:

$$\text{Biotin–protein} + \text{ATP} + HCO_3^- \rightleftharpoons CO_2 \sim \text{biotin–protein} + \text{ADP} + \text{Pi} \quad (18)$$

$$CO_2 \sim \text{biotin–protein} + CH_3COSCoA \rightleftharpoons \text{biotin–protein} + {}^-OOCCH_2COSCoA \quad (19)$$

The sum of these reactions is:

$$\text{ATP} + HCO_3^- + CH_3COSCoA \rightleftharpoons \text{ADP} + \text{Pi} + {}^-OOCCH_2COSCoA \quad (20)$$

When the biotin–enzyme is incubated with ATP and $H^{14}CO_3^-$, $^{14}CO_2 \sim$ biotin–enzyme is formed. If this complex is isolated from the reaction mixture and incubated with acetyl-CoA, the $^{14}CO_2$ is transferred to acetyl-CoA to form malonyl-CoA. The nature of the carboxybiotinyl–enzyme has been investigated and found to be 1′-*N*-carboxybiotinyl enzyme (illustrated in Fig. 3).

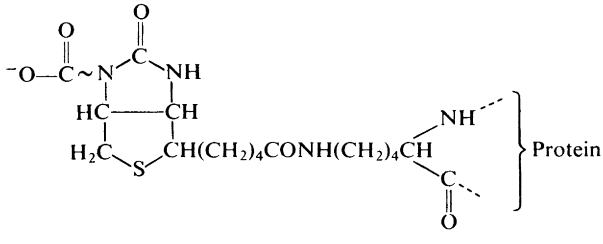
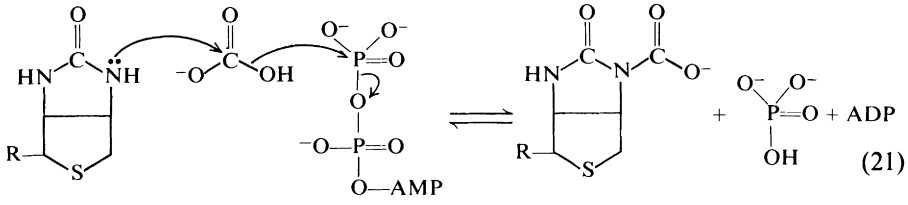


Fig. 3. 1′-N-*Carboxybiotinyl*-L-*lysyl-protein.*

The biotin is bound to the enzyme through the ϵ-amino groups of lysine in the same manner as that found in biocytin (Wright *et al.*, 1952).

The structure diagrammed in Fig.3 was found to be the same for all biotin enzymes (Wood *et al.*, 1963; Lane and Lynen, 1963; Numa *et al.*, 1964; Scrutton *et al.*, 1965; Waite and Wakil, 1966). The chemical mechanism by which the carboxybiotinyl–enzyme is formed is still unclear, although available evidence (Kaziro *et al.*, 1962) indicates that bicarbonate rather than free carbon dioxide is the reactive species in the carboxylation reaction. Whether the carboxylation step occurs by a concerted mechanism such as that shown in Eq. (21) or by a stepwise mechanism involving the formation of a phosphorylated intermediate [Eqs. (22) and (23)] remains to be determined (Lynen, 1967a). The fact that ADP–ATP exchange was noted in the presence of pyruvate carboxylase (Scrutton and Utter, 1965) would indicate that the stepwise mechanism is more likely.



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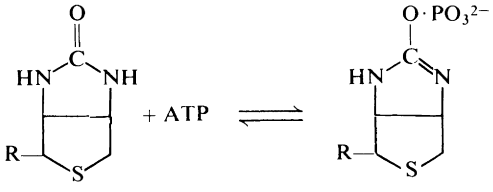

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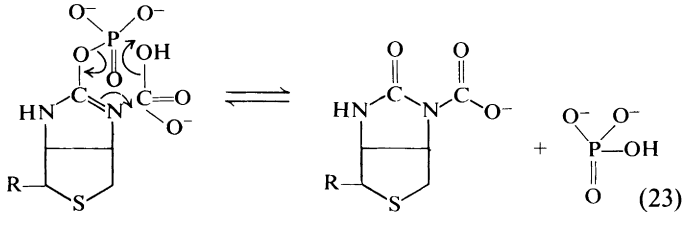
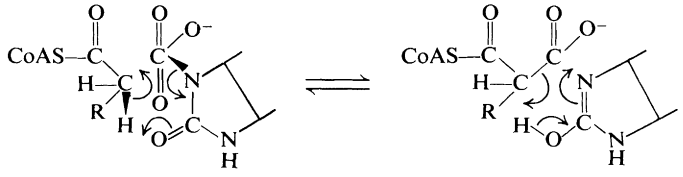


(23)

The acetyl-CoA carboxylase of *Escherichia coli* has recently been fractionated into three proteins (Alberts and Vagelos, 1968): a biotin-containing protein and two proteins that do not contain biotin. The biotin protein is of low molecular weight and has no apparent enzymatic activity. Of the two remaining proteins, one is a biotinyl carboxylase which catalyzes the formation of

$CO_2 \sim$ biotinyl protein [reaction (18)]; the second catalyzes the transcarboxylation from the carboxybiotinyl–protein to the acceptor molecule acetyl-CoA [reaction (19)]. This finding suggests that one biotinyl protein may be common to all carboxylases and that the specificity of the carboxylases is due to the transferring protein, but more information is required to confirm this.

The transfer of the carboxyl group from carboxybiotin–protein to the acceptor molecule is stereospecific, as shown in reaction (24), which means that the entering carboxyl group occupies the same configuration as the departing hydrogen atom (Lynen, 1967a). This mechanism would explain the stereospecific formation of (S)-methylmalonyl-CoA by the propionyl-CoA carboxylase (Specher *et al.*, 1966).



(24)

The carboxybiotinyl–protein is relatively unstable, the half-life of the complex in the absence of substrate being about 10 minutes. The half-life is shorter in the presence of substrates, allosteric affectors (citrate or isocitrate) or both (Kaziro and Ochoa, 1961; Knappe *et al.*, 1963; Ryder *et al.*, 1967). The free energy change of the cleavage of the decarboxylation reaction shown below:

$$CO_2 \sim \text{biotinyl–protein} + H^+ \rightleftharpoons CO_2 + \text{biotinyl–protein} \qquad (25)$$

was estimated to be −4.7 kcal/mole at pH 7.0 (Wood *et al.*, 1963). This value is thermodynamically sufficient to permit the carboxylation of the acceptor molecules.

The initial observations that citrate or isocitrate-stimulated fatty acid synthesis in cell-free extracts of pigeon liver were explained by the ability of these acids to exert positive allosteric control on acetyl-CoA carboxylase. In the presence of isocitrate or citrate, acetyl-CoA carboxylase was stimulated 15- to 16-fold. Whereas the incompletely activated enzyme appeared to be the "pace-setter" of fatty acid biosynthesis, the citrate-activated enzyme generated malonyl-CoA at a rate equal to the capacity of the fatty acid synthetase to convert malonyl-CoA to fatty acid (Chang *et al.*, 1967). The stimulation of this enzyme by citrate or isocitrate may be physiologically important in the regulation of fatty acid synthesis in the cell.

The mechanism of citrate stimulation has been elucidated by studies of the acetyl-CoA carboxylase of rat adipose and liver tissues and of chicken liver

(Martin and Vagelos, 1962; Vagelos *et al.*, 1963; Lynen *et al.*, 1964; Gregolin *et al.*, 1968a). It was found that citrate or isocitrate directly activates the carboxylase by increasing the V_{max} of the reaction but not the K_m for the substrates. Citrate is tightly bound to the carboxylase (dissociation constant = $2\text{–}3 \times 10^{-6}$) and it induces polymerization of the enzyme. These phenomena have been studied extensively with homogeneous preparations of acetyl-CoA carboxylase from chicken liver (Gregolin *et al.*, 1968a,b). The enzyme was isolated in the aggregated active form and found to exist in either protomeric or polymeric forms which are reversibly interconvertible. Polymer formation is promoted by certain anions (citrate, isocitrate, malonate, tricarballylate, sulfate, and orthophosphate), acetyl-CoA, high protein concentration, and pH 6 to 7. Dissociation of the polymeric form is induced by Cl^-, pH values greater than 7.5, and formation of the carboxybiotinyl–enzyme. Citrate and isocitrate, but not tricarballylate or Pi, promote polymer formation of carboxybiotinyl–enzyme. They also cause a 15- to 16-fold stimulation of the catalytic activity of the enzyme; tricarballylate and Pi do not stimulate enzyme activity. The polymeric form of the enzyme has a molecular weight of 4×10^6 to 8×10^6, whereas the protomeric form has a molecular weight of 410,000. There is one biotinyl prosthetic group per protomeric molecule (410,000) (Wakil, 1961; Gregolin *et al.*, 1966) and one acetyl-CoA and one citrate binding site per molecule of protomer. In the presence of dodecyl sulfate, the protomeric form of acetyl-CoA carboxylase dissociates into subunits with a sedimentation coefficient ($s_{20,w}$) of 4.3 S or an estimated molecular weight of 110,000. Thus, it appears that the protomeric form of the acetyl-CoA carboxylase consists of four subunits having a molecular weight of 100,000 each. One of these subunits contains biotin and forms the carboxybiotin–protein intermediate; another may catalyze the carboxylation of the biotin–protein and the transfer of the carboxyl group from biotin to the acceptor molecule. The remaining two subunits may be involved in the control of the carboxylase and contain the binding sites of malonyl-CoA and acetyl-CoA, respectively.

Electron microscopy of the acetyl-CoA carboxylase has shown a remarkable correlation between its structure, its aggregation, and its state of activation (Gregolin *et al.*, 1966). The protomer, which is the catalytically inactive enzyme, is present as a small particle having a minimum dimension of 70–156 Å and a maximum dimension of 100–300 Å. In the presence of isocitrate, the polymeric form is obtained, which is composed of ten to twenty protomeric molecules assembled in a linear fashion and appearing as filaments 70–100 Å wide and up to 4000 Å long.

C. Fatty Acid Synthetase

As stated earlier, the second step in fatty acid synthesis is the conversion of malonyl-CoA to palmitate. This reaction requires acetyl-CoA and TPNH

[reaction (17)] and is catalyzed by a complex of enzymes referred to as fatty acid synthetase. This complex has been isolated in homogeneous form from both animal livers and yeast and its component proteins fractionated as a single multienzyme unit (Bressler and Wakil, 1961; Lynen, 1961; Hsu *et al.*, 1965). Since no free intermediates have been found to accumulate during the synthesis of fatty acids, and since the enzyme complex is stimulated by thiols and inhibited by sulfhydryl binding agents, protein-bound acyl derivatives have been postulated as intermediates in the synthesis of fatty acids (Lynen, 1961).

In contrast to the yeast and animal enzyme complexes, the fatty acid-synthesizing system of *E. coli* was readily dissociated into its component enzymes (Lennarz *et al.*, 1962; Alberts *et al.*, 1963; Wakil *et al.*, 1964). The acyl intermediates are bound as thioesters to a low molecular weight protein named acyl carrier protein (ACP) (Wakil *et al.*, 1964; Majerus *et al.*, 1964). This protein contains 4′-phosphopantetheine as a prosthetic group, onto which the various intermediates of fatty acid synthesis are attached by a thioester linkage (Pugh and Wakil, 1965; Majerus *et al.*, 1965b). Although ACP is a protein, its role in fatty acid synthesis is that of a coenzyme analogous to CoA in the β-oxidation of fatty acids. The acyl intermediates in both pathways are bound to the thiol group of 4′-phosphopantetheine. In fatty acid synthesis, acetyl and malonyl groups are initially bound to coenzyme A and subsequently transferred to ACP before their conversion to fatty acids. All ensuing acyl intermediates remain bound to the ACP molecule. The following equations summarize the sequence of reactions involved in the synthesis of fatty acids from acetyl-CoA and malonyl-CoA (Wakil *et al.*, 1964; Majerus *et al.*, 1964):

$$CH_3COS\text{-}CoA + ACP\text{-}SH \rightleftharpoons CH_3COS\text{-}ACP + CoA\text{-}SH \quad (26)$$

$$HOOCCH_2COS\text{-}CoA + ACP\text{-}SH \rightleftharpoons HOOCCH_2COS\text{-}ACP + CoA\text{-}SH \quad (27)$$

$$CH_3COS\text{-}ACP + HOOCCH_2COS\text{-}ACP \rightarrow CH_3COCH_2COS\text{-}ACP + CO_2 + ACP\text{-}SH \quad (28)$$

$$CH_3COCH_2COS\text{-}ACP + NADPH + H^+ \rightleftharpoons D(-)CH_3CHOHCH_2COS\text{-}ACP + NADP^+ \quad (29)$$

$$D(-)CH_3CHOHCH_2COS\text{-}ACP \rightleftharpoons CH_3CH{=}CHCOS\text{-}ACP + H_2O \quad (30)$$

$$CH_3CH{=}CHCOS\text{-}ACP + NADPH + H^+ \rightarrow CH_3CH_2CH_2COS\text{-}ACP + NADP^+ \quad (31)$$

The acyl groups of both acetyl-CoA and malonyl-CoA are transferred to ACP by their respective transacylase enzymes, acetyl-CoA–ACP transacylase and malonyl-CoA–ACP transacylase [reactions (26) and (27)]. Acetyl-ACP and malonyl-ACP are converted to acetoacetyl-ACP by the acyl–malonyl-

ACP-condensing enzyme [reaction (28)]. Acetoacetyl-ACP is then reduced to β-hydroxybutyryl-ACP, dehydrated to crotonyl-ACP, and finally reduced to butyryl-ACP [reactions (29)–(31)]. Butyryl-ACP is elongated to β-ketohexanoyl-ACP by condensation with malonyl-ACP, according to reaction (28). Reduction of β-ketohexanoyl-ACP, followed by dehydration and further reduction, yields hexanoyl-ACP [reactions (29)–(31)]. This sequence of reactions is repeated five more times until palmityl-ACP is formed. The latter is then hydrolyzed by a specific thioesterase to palmitic acid and ACP, according to the following reaction:

$$CH_3(CH_2)_{14}COS\text{-}ACP + H_2O \rightarrow CH_3(CH_2)_{14}COOH + ACP\text{-}SH \quad (32)$$

With some minor variations, these general reactions appear common to all fatty acid-synthesizing systems.

1. Acyl Carrier Protein

Acyl carrier protein (ACP) has been purified from *E. coli* to a state of homogeneity and its physical and chemical properties have been studied extensively (Vanaman *et al.*, 1968a,b). The protein has a sedimentation

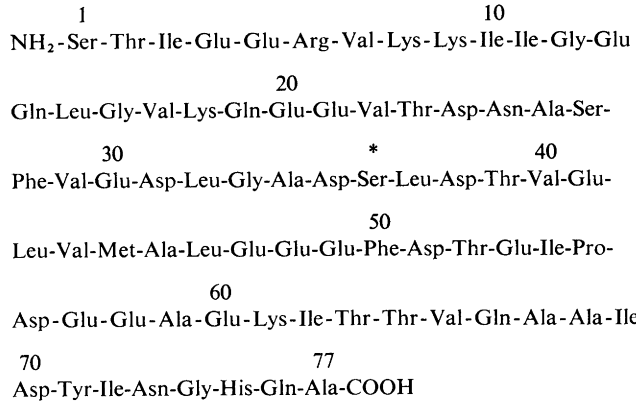
Table I. Amino Acid Composition of ACP

Amino acid	Assumed no. of residues[a] (residues/molecule)
Lysine	4
Histidine	1
Arginine	1
Aspartic acid	9
Threonine	6
Serine	3
Glutamic acid	18
Proline	1
Glycine	4
Alanine	7
Valine	7
Methionine	1
Isoleucine	7
Leucine	5
Tyrosine	1
Phenylalanine	2
β-Alanine[b]	1
Cysteamine[b,c]	1

[a] The total number of residues was 77.
[b] Not included in the totals.
[c] Measured as taurine.

coefficient $s_{20,w} = 1.34$ S and low frictional coefficient, indicating that ACP in aqueous solution is a typical globular protein, compactly folded, and sparingly hydrated (Takagi and Tanford, 1968). The optical rotatory dispersion measurements of the native protein are the same as those of typical globular proteins with an ordered structure. In the presence of 6 *M* guanidine hydrochloride, native ACP is completely and reversibly denaturated (Takagi and Tanford, 1968).

Structural studies have shown that ACP is composed of a single polypeptide chain with NH_2-terminal serine and COOH-terminal alanine. The protein is devoid of cysteine or cystine but contains a single thiol group to which a variety of acyl groups are attached during fatty acid synthesis. This thiol group is identified as that of cysteamine, which is derived from the 4′-phosphopantetheine prosthetic group. The latter group is linked covalently to the



Scheme II. The complete amino acid sequence of acyl carrier protein from E. coli.

polypeptide of ACP through a phosphodiester linkage to one of the seryl residues of the molecule (Pugh and Wakil, 1965; Majerus *et al.*, 1965b).

The amino acid composition of *E. coli* ACP shown in Table I differs slightly from those reported previously, possibly because of impurities in the earlier preparations (Majerus *et al.*, 1964; Sauer *et al.*, 1964). The composition reported in Table I agrees exactly with the complete amino acid sequence of ACP shown in Scheme II. This sequence was based on the sequential analyses of the various peptides obtained after partial chemical or enzymic hydrolysis of the ACP molecule (Vanaman *et al.*, 1968a,b).

Several interesting properties can be deduced from this sequence. Acyl carrier protein has a molecular weight of 8847. It contains fourteen residues of glutamic and seven of aspartic acid, but only four residues of glutamine and

two of asparagine. The isoelectric point is estimated to be about pH 4.2, which is close to the pH where ACP is least soluble. The basic amino acids, though few in number, are clustered mainly near the NH_2- and COOH-terminal regions of the polypeptide chain. The acidic residues, on the other hand, are found throughout the sequence and in some regions occur with great frequency, as in residues 47–49 and 56–58, where Glu-Glu-Glu and Asp-Glu-Glu occur, respectively. The frequency of acidic amino acids is also high between residues 47 and 60, in which nine of fourteen residues are either glutamic or aspartic acids. There are no extensive regions of the polypeptide that are rich in hydrophobic residues, with the exception of residues 62–72 which contain only one glutamic residue. Finally, the prosthetic group 4′-phosphopantetheine is covalently linked through a phosphodiester bond to the hydroxyl group of serine 36, indicating that the fatty acyl thioesters are located near the center of the polypeptide chain.

Knowledge of the sequence of ACP and the finding that ACP behaves in solution as a typical globular protein led to the suggestion that native ACP may have an unusually high density of charged sidechains on its surface. The importance of these surface structures to the functions of ACP as a coenzyme in fatty acid synthesis remains to be determined.

The ability of subfractions of ACP or its derivatives to substitute native ACP in fatty acid synthesis has been studied. Acetylated ACP is fully active but removal of the first six residues from its NH_2 terminus yields an inactive polypeptide (Majerus, 1968). Removal of the three residues from the COOH terminus of ACP with carboxypeptidase A yields a product which is fully active. Clearly, more information is needed in order to fully appreciate the unique role of ACP and its derivatives in fatty acid synthesis.

a. Biosynthesis of Acyl Carrier Protein. Recent studies (Vagelos and Larabee, 1967) show that extracts of *E. coli* contain an enzyme which hydrolyzes the prosthetic group from ACP according to the following reaction:

$$\text{ACP} \xrightarrow{Mn^{2+}} \text{apo-ACP} + \text{4′-phosphopantetheine} \qquad (33)$$

The enzyme has been named ACP hydrolase (ACPase), which is a phosphodiesterase specific for ACP. It requires Mn^{2+} (or other divalent cations) for its activity and is stimulated by sulfhydryl compounds, such as dithiothreitol or 2-mercaptoethanol. The role of this enzyme in the metabolism of ACP is unclear at present but it may possibly play an important part in controlling the cellular levels of holo-ACP.

The synthesis of holo-ACP was recently demonstrated in extracts of *E. coli* (Elovson and Vagelos, 1968). An enzyme, holo-ACP synthetase, has been isolated from these extracts. It catalyzes the reversible transfer of 4′-phosphopantetheine from reduced coenzyme A to ACP-apoprotein (apo-ACP)