

Q55  
0461

# **ADVANCES IN ENZYMOLOGY**

**AND RELATED AREAS OF MOLECULAR BIOLOGY**

**Founded by F. F. NORD**

**Edited by ALTON MEISTER**

**VOLUME 45**

# **ADVANCES IN ENZYMOLOGY**

**AND RELATED AREAS OF MOLECULAR BIOLOGY**

**Founded by F. F. NORD**

**Edited by ALTON MEISTER**

**CORNELL UNIVERSITY MEDICAL COLLEGE,  
NEW YORK, NEW YORK**

**VOLUME 45**

**1977**

**AN INTERSCIENCE® PUBLICATION**

**JOHN WILEY & SONS  
New York · London · Sydney · Toronto**

## CONTRIBUTORS TO VOLUME 45

- KONRAD BLOCH, *James Bryant Conant Laboratory, Harvard University, Cambridge, Massachusetts 02138*
- W. WALLACE CLELAND, *Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706*
- PIERRE DOUZOU, *Institut National de la Santé et de la Recherche Médicale, and Institut de Biologie physico-chimique, Paris, France.*
- PETER E. DUNN, *Department of Biochemistry, University of Chicago, Chicago, Illinois 60637*
- KARL J. KRAMER, *U.S. Grain Marketing Research Center Agricultural Research Service, U.S. Department of Agriculture, Manhattan, Kansas 66502*
- JOHN H. LAW, *Department of Biochemistry, University of Chicago, Chicago, Illinois 60637*
- TING-KAI LI, *Departments of Medicine and Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana 46202*
- GEORGE MICHAELS, *Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106*
- WILLIAM E. O'BRIEN, *Department of Pediatrics, Division of Human Genetics, School of Medicine, Case Western Reserve University, Cleveland Metropolitan General Hospital, Cleveland, Ohio 44109*
- HARLAND G. WOOD, *Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106*

An Interscience® Publication.

Copyright © 1977, by John Wiley & Sons, Inc.

All rights reserved. Published simultaneously in Canada.

No part of this book may be reproduced by any means, nor transmitted, nor translated into a machine language without the written permission of the publisher.

Library of Congress Catalog Card Number: 41-9213

ISBN 0-471-02726-X

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

## CONTENTS

Control Mechanisms for Fatty Acid Synthesis in <i>Mycobacterium smegmatis</i>	
By Konrad Bloch . . . . .	1
Properties of Carboxytransphosphorylase; Pyruvate, Phosphate Dikinase; Pyrophosphate-phosphofructokinase and Pyrophosphate-acetate Kinase and Their Roles in the Metabolism of Inorganic Pyrophosphate	
By Harland G. Wood, William E. O'Brien, and George Michaels . . . . .	85
Enzymology at Subzero Temperatures	
By Pierre Douzou . . . . .	157
Determining the Chemical Mechanisms of Enzyme-Catalyzed Reactions by Kinetic Studies	
By W. Wallace Cleland . . . . .	273
Insect Proteases and Peptidases	
By John H. Law, Peter E. Dunn, and Karl J. Kramer . . . . .	389
Enzymology of Human Alcohol Metabolism	
By Ting-Kai Li . . . . .	427
Author Index . . . . .	485
Subject Index . . . . .	505

# CONTROL MECHANISMS FOR FATTY ACID SYNTHESIS IN *MYCOBACTERIUM SMEGMATIS*

By KONRAD BLOCH, *Cambridge, Massachusetts*

## CONTENTS

I. Introduction: Comparative Aspects of Fatty Acid Synthesis	2
A. Identity of Enzyme Source	8
II. <i>M. smegmatis</i> Acetyl-CoA Carboxylase	9
III. <i>M. smegmatis</i> Fatty Acid Synthetase (Type I)	11
A. Isolation and Purification of the <i>M. smegmatis</i> FAS Multienzyme Complex	11
1. Ionic Strength	13
B. Acetyl-CoA Concentrations	13
1. Malonyl-CoA	14
2. Coenzyme A	14
3. Pyridine Nucleotides	15
4. FMN	15
C. Kinetics of Fatty Acid Synthetase (Type I)	16
1. The Initial Burst	16
2. Effect of Acetyl-CoA Concentrations	21
D. Fatty Acid Patterns	23
1. Bimodal Fatty Acid Pattern of <i>M. smegmatis</i>	24
2. Effect of Substrate Concentrations on Fatty Acid Spectra	25
3. Effects of Polysaccharide on Chain Length	27
4. Effect of BSA on Chain Length	30
5. Liposomes	31
6. Palmitoyl-CoA Thioesterase	32
7. Temperature Effects	33
IV. Partial Reactions Catalyzed by <i>M. smegmatis</i> FAS	34
A. Acetyl Transacylase	36
B. Malonyl Transacylase	36
C. Evidence for Separate Acetyl and Malonyl Transacylase Sites	36
D. Long-Chain Transacylase	38
E. Transacylation and Bimodal Product Distribution	42
F. Condensation Reaction	44
G. Other Partial Reactions	44

H. $\beta$ -Ketoacyl Reduction	45
I. $\beta$ -Hydroxyacyl Dehydration	46
J. $\alpha,\beta$ -Enoylacyl Reduction	46
V. <i>M. smegmatis</i> Synthetase Catalyzed Palmitoyl-CoA Elongation	48
A. Distribution Pattern of Elongation Products	52
VI. Regulation of <i>M. smegmatis</i> Synthetase by End Product	53
A. Sites of Control	54
B. Control by Palmitoyl-CoA	55
C. Control by Polysaccharide	58
VII. Subunit Structure of Fatty Acid Synthetases	58
VIII. The Polysaccharides of <i>M. smegmatis</i>	62
IX. The ACP-Dependent <i>M. smegmatis</i> Fatty Acid Synthetase (Type II)	75
X. Summary	78
Abbreviations	80
Acknowledgments	80
References	80

## I. Introduction: Comparative Aspects of Fatty Acid Synthesis

Enzyme systems for synthesizing long-chain fatty acids occur in the vast majority of cells. They provide the paraffinic moieties of phospholipids and related amphipathic molecules that in turn are requisite components of the universal plasma membrane and the membrane envelopes of organelles. There are few exceptions to this generalization. Some mycoplasma strains, bacterial mutants that occur naturally, have lost the biosynthetic machinery for fatty acid synthesis. However, such organisms will not grow in the absence of added fatty acids (1). In some of the highly specialized halobacteria the phospholipids contain the branched-chain phytyl group instead of straight acyl chains. In these bacteria the enzyme system for long-chain fatty acid synthesis may still be present, but it is possible that they are normally repressed (2).

Wherever fatty acid synthesis occurs, the operating chemical mechanism is always the same (3). Acetyl and malonyl groups activated in the form of thioesters of acyl carrier protein (ACP) condense to  $\beta$ -ketoacyl thioester in a concerted process driven by decarboxylation of the free malonyl carboxyl group (4). Three subsequent reactions serve to convert the  $\beta$ -carbonyl oxygen to methylene by way of  $\beta$ -hydroxyacyl- and  $\alpha,\beta$ -enoyl intermediates. This sequence (Table I, reactions 1-7) affords the elongated saturated product with retention of the thioester function. Repeti-

TABLE I

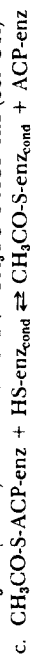
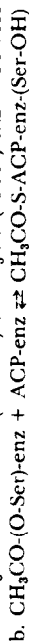
## Reaction Sequence for Fatty Acid Synthesis in Multienzyme Complexes

Reaction 1. Acyl transacylation consists of three experimentally separable events: (a) acyl transfer to a serine residue, (b) acyl transfer to 4-phosphopantetheine-SH of the "ACP" region; and (c) acyl transfer to a SH group of condensing enzyme. Reaction c is usually written as a component of reaction 3.

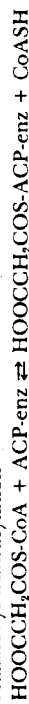
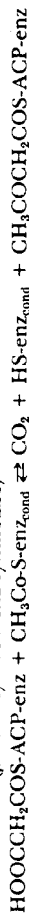
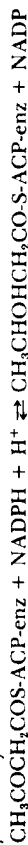
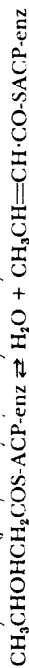
Reaction 6. In most synthetases NADPH is the specific electron donor for both  $\beta$ -ketoacyl- and  $\alpha,\beta$ -enoyl reduction. In some instances, NADH is the more effective or specific reductant for  $\alpha,\beta$ -enoyl reduction.

Reaction 7. In all animal tissue synthetases the synthetic process is terminated by "deacylases" which transfer completed acyl chains to water, while in microbial synthetases acyl chains are transferred to CoASH. In some microbial synthetases, the catalytic regions for malonyl transacylation (reaction 2) and long-chain transacylation (reaction 7b) are the same, while in animal synthetases malonyl transacylation and deacylation are distinct.

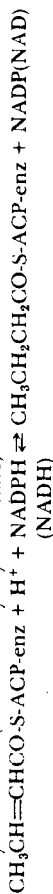
## 1. Acyl transacylation



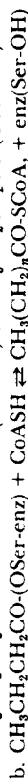
## 2. Malonyl transacylation

3. Condensation ( $\beta$ -ketoacyl-ACP-enz synthetase)4.  $\beta$ -Ketoacyl reduction5. Dehydration ( $\beta$ -hydroxyacyl dehydratase)

## 6. Enoyl reduction (2,3-trans-enoylacyl reductase)



## 7. Long-chain transacylase or deacylase



tion of the same set of reactions six or seven more times yields palmitate and stearate, respectively. At the end of each round (condensation, first reduction, dehydration, and second reduction) the resulting saturated acyl chain returns from protein-linked 4-phosphopantetheine to condensing enzyme ( $\beta$ -ketoacyl-ACP synthetase) for addition of another malonyl unit. In this manner the activated carboxyl function at the growing end of the acyl chain is preserved until the chain reaches the appropriate length. In all nonaggregated or type II fatty acid synthetase systems (see below), fatty acid synthesis ends at this stage, the long acyl chains remaining attached to ACP. The ACP derivatives can then enter directly into lipid formation by transacylation (5). On the other hand, synthetases that are multienzyme complexes (type I) discharge completed acyl chains by transacylation either to water (6, 7) or to coenzyme A (8). Such transacylase activities are integral parts of multienzyme complexes. Apart from these differences in the termination events, the sources of reductant may also vary. Some synthetases use NADPH effectively for both the first and the second reduction step; in others only the  $\beta$ -ketoacyl thioester reduction is NADPH specific, NADH serving more effectively in the enoyl thioester reduction (9). Only the yeast synthetase is known to employ reduced FMN as a direct electron donor for enoyl reduction (8).

While the covalent chemistry of fatty acid synthesis appears essentially invariant for all forms of life—the differences noted are mechanistically trivial—this uniformity does not extend to the macromolecular structure and organization of the participating enzyme activities. There are two fundamentally different types of enzyme system that carry out fatty acid synthesis. In the more conventional systems, the various steps are catalyzed by separable enzymes that act individually, but constitute a single functional system. Such nonaggregated or type II synthetases occur in many bacteria and plants, but apparently not in animal tissues. Since in type II systems ACP is also a separate entity (10–12) synthetases in this category consist of a total of seven individual proteins. By contrast, the catalysts for fatty acid synthesis in all animal tissues, in many eukaryotic microorganisms, and in some bacteria are polyfunctional entities known as multienzyme complexes (type I). This terminology was introduced after the successful isolation of a



fatty acid synthetase from yeast that could be purified to homogeneity without loss of any of the seven partial activities needed for the *de novo* synthesis (13). The cohesive properties of this complex justifiably led to the assumption that in the yeast system the seven enzyme components were held together by sufficiently strong protein-protein interactions to allow the isolation of a single physical entity. The precedents for the existence of multienzyme aggregates were the pyruvate dehydrogenase complexes of *E. coli* and muscle (14,15). While more recent genetic evidence requires a redefinition of the term multienzyme complex as applied to fatty acid synthetases, the distinction between aggregated, or type I, and nonaggregated, or type II, synthetases remains operationally valid.

The natural distribution of the two synthetase prototypes is shown in Table II. No phylogenetic pattern or trend is discernible except perhaps for the beginning and the end of the evolutionary tree. The structurally primitive eubacteria and a representative blue-green alga (*Phormidium lunidum*) (16) contain type II synthetases, while in animal species, invertebrates (17) and vertebrates (nonmammalian and mammalian), the synthetases are without exception multienzyme complexes. It may be assumed that multienzyme complexes are more efficient and therefore more advanced. However, since type II synthetases occur in plants and multienzyme complexes occur in certain bacteria and since bacterial phylogeny is uncertain, the notion that the function of these enzymes improved in the course of evolution remains to be verified.

In nonaggregated (more primitive?) type II systems, which lack a higher level of organization, each intermediate is released into solution so that the further progress of the reaction sequence depends on collision between substrate and enzyme at each step. Conceivably, the component enzymes of type II systems are not entirely disorganized but are loosely associated in the membrane space. However, no such evidence exists except for the suggestive observation that the bulk of *E. coli* ACP as seen by radioautography is localized in the bacterial membrane (18).

By contrast, all intermediates generated by a multienzyme complex remain enzyme bound throughout the synthetic process. Because it remains covalently linked, the *enzyme-bound* product of

TABLE II

Occurrence of Mutienzyme Complex (Type I) and Nonaggregated (Type II) Fatty Acid Synthetases

Only relatively well-characterized fatty acid synthetases known to be type I or type

II systems have been included. The synthetases from vertebrate sources are thoroughly reviewed by Volpe and Vagekos (3). The recently isolated synthetase from goose uropygial gland (24a) forms branched acids from acetyl-CoA and methylmalonyl-CoA. The type II *B. subtilis* system (31) produces long-chain iso and ante-iso acids.

Type I		Type II
A. Animal tissues (3)		A. Procaryotic
1. Mammalian	Rat liver (19)	<i>Escherichia coli</i> (10, 11, 29)
	Adipose tissue	<i>Clostridium butyricum</i> (11)
	Mouse brain (24)	<i>Pseudomonas aeruginosa</i> (30)
	Mammary gland (22, 23)	<i>Bacillus subtilis</i> (31)
2. Avian	Chicken (21) and pigeon (20)	<i>Mycobacterium smegmatis</i> * (32)
	Liver	<i>Phormidium lunidum</i> (16) (blue-green alga)
	Goose uropygial gland (24a)	
3. Invertebrate	<i>Ceratitis capitata</i> (17) (insect)	B. Eucaryotic
		<i>Euglena gracilis</i> (33, 34) (photoauxotrophic)
		<i>Chlamydomonas reinhardtii</i> (35)
B. Microbial		Avocado mesocarp (36)
1. Eucaryotic	<i>Saccharomyces cerevisiae</i> (13)	Lettuce chloroplasts (37)
	<i>Neurospora crassa</i> (25)	Spinach chloroplasts (38)
	<i>Euglena gracilis</i> (26)	
2. Procaryotic	<i>Corynebacterium diphtheriae</i> (27)	
	<i>Mycobacterium smegmatis</i> (28)	

\* Palmitoyl-CoA elongating system.

the first partial reaction is directly transferred to the second catalytic site, which necessarily must be in close spatial proximity to the donor site and in turn adjacent to the center that catalyzes the following step. Such successive transfers of covalently bound intermediates between neighboring sites are possible only if the topological ordering of catalytic centers corresponds to the sequence of chemical events. Kinetically, the important property of the multienzyme complex is the absence of free intermediates. The rate of the overall reaction is determined only by the rate of transfer between sites, not by the  $K_m$  of the individual intermediates.

Evolutionary considerations prompted the decision in this laboratory to investigate fatty acid biosynthesis in a species of mycobacteria, the subject under review. It was thought that multienzyme complexes might have evolved in stages from nonaggregated systems as a result of modifications in protein structure, amino acid substitutions in individual enzymes producing mutual affinities until protein-protein interactions become sufficiently strong to stabilize the fatty acid synthetase system in the form of a single multifunctional complex. The hypothesis predicted the possible existence of transitional fatty acid synthetases that lacked the tight organizational features of the known type I systems but showed some tendency to form loose aggregates. It was further felt that the isolation of aggregates of limited stability might yield valuable information on developmental aspects of FAS multienzyme complexes and, more generally, on protein-protein interactions between functionally related enzymes. For this purpose *Mycobacterium smegmatis* was selected as a source (28) because mycobacteria are relatively advanced procaryotes. The initial results obtained with the mycobacterial synthetase seemed to support the working hypothesis. First of all, the ACP-independence of the system established it as a type I synthetase, the first demonstration that FAS multienzyme complexes are not restricted to eucaryotic cells. Also, the enzyme system was much more labile under normal assay conditions than known type I synthetases, but could be stabilized in an environment of high ionic strength. Later, it became increasingly doubtful, however, that the *M. smegmatis* synthetase represented a loose

complex or aggregate of individual enzymes. The instability in solutions of low ionic strength was not due to weak interactions between monofunctional entities, but was caused by the facile dissociation of the complex into subunits of molecular weight 200–250,000 (39). Presumably, these subunits are polyfunctional polypeptide chains. It now appears that all type I fatty acid synthetases are susceptible to dissociation into subunits and that they are always of similar size. However, the ease of the ionic-strength-dependent dissociation differs from case to case. At low ionic strength the *M. smegmatis* synthetase is exceptionally labile, the animal tissue synthetases are moderately stable (40), and the yeast synthetase retains its native structure even in distilled water. To effect the dissociation of the yeast complex requires 1–2 *M* salt solutions (41). The stabilizing noncovalent interactions in the various multienzyme complexes must therefore differ not only in strength, but also in kind.

Up to the time of this writing the original hypothesis postulating the existence of labile, transitional fatty acid synthetase complexes has remained without experimental support. In fact, the protein structure and organization of the procaryotic *M. smegmatis* synthetase does not appear to differ in principle from others in this category. However, various properties of this enzyme system proved to be sufficiently unique to warrant further investigation. Most prominent among these properties are (1) a bimodal product pattern that includes fatty acyl chains of unusual length, (2) the extraordinarily high  $K_m$  for acetyl-CoA, (3) stimulation by certain mycobacterial polysaccharides, and (4) catalysis of palmitoyl-CoA elongation, as well as of *de novo* synthesis.

In addition to the multienzyme FAS complex, mycobacterial extracts contain a second, ACP-dependent, type II synthetase that extends but does not start long carbon chains. Systems of this type have not been found elsewhere.

#### A. IDENTITY OF ENZYME SOURCE

For the investigations described in this chapter the bacterial strain listed as ATCC 356, *Mycobacterium phlei*, was used throughout in this laboratory. From information now available it is clear that ATCC 356 is in fact a strain of *M. smegmatis* and not of *M.*

*phlei*. This culture had earlier been mislabeled accidentally (42). The revised designation of ATCC 356 as *M. smegmatis* is based, *inter alia*, on the observation that this strain produces mycobactin S rather than mycobactin P, and on several other diagnostic tests (42).

## II. *M. smegmatis* Acetyl-CoA Carboxylase

Acetyl-CoA carboxylases, though separate enzymes, function in concert with fatty acid synthetase systems. They catalyze the formation of malonyl-CoA, the first specific event in lipogenesis. In most instances this is also the committed step because malonyl-CoA is not used elsewhere in metabolism.

In analogy with the existence of two FAS prototypes, acetyl-CoA carboxylases also occur as aggregated and nonaggregated enzyme systems (3). From sources such as animal tissues and yeast the carboxylases can be isolated as single physical entities of high molecular weight, whereas in *E. coli* three separable proteins, biotin-carrier protein (BCP), biotin carboxylase, and biotin trans-carboxylase, catalyze the overall conversion of acetyl-CoA to malonyl-CoA (43). One of the plant carboxylases (from wheat germ) seems to be intermediate in molecular properties between the animal-yeast and bacterial systems (44), whereas the spinach chloroplast enzyme system can be resolved into three active components analogous to those obtained from *E. coli* (44). Since fatty acid synthesis in *M. smegmatis* conforms with the "animal-yeast" pattern rather than the "bacterial" pattern\* a characterization and classification of the mycobacterial acetyl-CoA carboxylase seemed of interest. This enzyme appears to be of the aggregated type (45). However, the possibility that *M. smegmatis* contains two distinct carboxylating systems has not been ruled out.

Conventional fractionation yielded a 35- to 60-fold enriched fraction that catalyzed the overall conversion of acetyl-CoA to malonyl-CoA. Under conditions that allow separation of the *E. coli* carboxylase system into three components, the *M. smegmatis* enzyme remained an intact entity.

\* This statement applies only to *de novo* fatty acid synthesis. The second enzyme, the nonaggregated, palmitoyl-CoA elongating system of *M. smegmatis* requires external ACP and on this basis is to be classified as "bacterial."

The most notable property of the *M. smegmatis* carboxylase is that it catalyzes the carboxylation of both propionyl-CoA and acetyl-CoA. Both reactions are affected by inorganic sulfate. The respective kinetic parameters are, for acetyl-CoA,  $V_{\max} = 0.79$  mM in the absence of  $\text{SO}_4^{2-}$  and 1.35 mM in the presence of  $\text{SO}_4^{2-}$ ; and  $K_m = 0.30$  in the presence and 0.22 in the absence of  $\text{SO}_4^{2-}$ ; for propionyl CoA,  $\text{SO}_4^{2-}$  changes  $V_{\max}$  from 1.28 to 1.48, but leaves the  $K_m$  unchanged (at 0.1 mM). Thus the enzyme carboxylates propionyl-CoA somewhat more effectively than acetyl-CoA. All the data indicate that these two activities reside in a single catalytic entity. However, since sulfate, regardless of the nature of the counteranion, raises the activity for acetyl-CoA carboxylation substantially and of propionyl-CoA carboxylation only slightly, it is clear that the relative specificity of the enzyme for the two substrates can be modulated. The sulfate effect does not relate to ionic strength; it is not duplicated by  $\text{Cl}^-$  or  $\text{HPO}_4^{2-}$ .

Differential effects on the two activities are also shown by palmitoyl-CoA. Even at the highest concentrations tested (250  $\mu\text{M}$ ), palmitoyl-CoA inhibited propionyl-CoA carboxylation by less than 50%, while acetyl-CoA carboxylase activity was reduced to 50% at 30–40  $\mu\text{M}$  palmitoyl-CoA. Interestingly, 0.75 *M*  $(\text{NH}_4)_2\text{SO}_4$  completely relieved palmitoyl-CoA inhibition of both substrates. Since acetyl-CoA carboxylation is far more sensitive to palmitoyl-CoA than the carboxylation of propionyl-CoA, this response may be related to end-product inhibition of straight-chain fatty acid synthesis. It should be noted, however, that the effective palmitoyl-CoA concentrations are very much higher than those needed to inhibit yeast or liver acetyl-CoA carboxylase (43). For the latter enzymes the regulatory role of palmitoyl-CoA is well established.

If the dual substrate specificity should persist on further purification of the *M. smegmatis* carboxylase, then the reactions that the enzyme catalyzes would not be the "committed steps" as usually defined for pathways that utilize malonyl-CoA and methylmalonyl-CoA. Methylmalonyl-CoA is not a substrate for chain elongation by the *M. smegmatis* fatty acid synthetase (45) in accord with the absence of branched-chain acids (mycocerosic or phthienoic acids) in this particular mycobacterial strain (46). Methylmalonyl-CoA mutase has been detected in *M. smegmatis*

(47), but the metabolic importance of this conversion (propionyl-CoA to succinyl-CoA) is not known.

The further possibility exists that malonyl-CoA itself may serve as precursor for products other than straight-chain fatty acids. 6-Methylsalicylic acid derived from malonyl-CoA is a major extracellular product of *M. phlei* and is used as a marker for this mycobacterial strain. *M. smegmatis* produces salicylic acid by the skikimic acid pathway (46).

In summary, arguments both for and against a regulatory role of the *M. smegmatis* acetylpropionyl-CoA carboxylase can be adduced. The differential effects of sulfate and palmitoyl-CoA on carboxylation rates of acetyl- and propionyl-CoA suggest that the enzyme can be manipulated to accelerate one pathway in preference to the other. On the other hand, the physiological significance of sulfate as a metabolic regulator is questionable and the palmitoyl-CoA effects occur only at concentrations that may be outside the physiological range. Since later discussion shows that there are other effective mechanisms for regulating the *M. smegmatis* synthetase proper, the carboxylase activity is probably not a major factor in the control of mycobacterial fatty acid synthesis.

The principal point of comparative interest is that the *M. smegmatis* acetyl-CoA carboxylase seems structurally related to the carboxylase multienzyme complexes of yeast and animal tissue and not to the nonaggregated *E. coli* system.

### III. *M. smegmatis* Fatty Acid Synthetase (Type I)

#### A. ISOLATION AND PURIFICATION OF THE *M. SMEGMATIS* FAS MULTIENZYME COMPLEX

For enzyme isolation *M. smegmatis* cells (ATCC 356) are grown on a medium containing glucose and Tween-80 and are harvested in the stationary phase (48 hr.). The cells are suspended in 0.1 M phosphate buffer, pH 7.0, and are ruptured in the French press (39). Fractionation of the 105,000g supernatant with  $(\text{NH}_4)_2\text{SO}_4$  yields two synthetic activities, the type I synthetase at 35–55% saturation and an ACP-dependent palmitoyl-CoA elongating system in the supernatant. Chromatography on Sephadex G-150 (32)

TABLE III

Purification of FAS Complex from *M. smegmatis*

This procedure is essentially that published (39) except that cells were harvested in the mid-log phase instead of the stationary phase. Such "exponential" cells yield enzyme with a tenfold greater initial specific activity. The specific activity of the homogeneous enzyme obtained in a yield of about 5 mg/100 g cells is the highest of any synthetase described so far (58a).

Steps	Specific activity (nmoles of [ <sup>14</sup> C]malonyl-CoA incorporated per min per mg of protein	Fold purification	Yield, %
Broken cells	14.6	1	100
S <sub>105</sub>	21.2	1.5	73
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitate (35-55%)	84.6	5.8	118
DEAE-cellulose column	439	30.1	39
Biogel A-5 m column	2183	150	25

or Bio-gel (62) also separates the two synthetases (see Section IX). Table III gives the subsequent steps that ultimately yield a 150-fold purified synthetase with a specific activity of about 2200 (nanomoles of malonyl-CoA incorporated per milligram of protein per minute). The S<sub>20</sub> value of the enzyme is 23.6 and the estimated molecular weight is  $1.4 \times 10^6$  (28,39). At the ammonium sulfate step and from thereon, synthetase I activity is assayed in 0.1 M phosphate buffer, pH 7.0 in the presence of 300  $\mu$ M acetyl-CoA, 20  $\mu$ M [1-<sup>14</sup>C]malonyl-CoA, 30  $\mu$ M NADPH, 30  $\mu$ M NADH, 1 mM DTT, and one of the purified mycobacterial polysaccharides. For these last compounds the abbreviations MMP (methylmannose-containing polysaccharide) and MGLP (methylglucose-containing polysaccharide) are used throughout.

The activity of the enzyme and the end-product pattern depend critically on a large number of variables, including ionic strength of the medium, concentration of substrates and coenzymes, and the presence and concentrations of MMP or MGLP. Since some of these requirements differ substantially from those of other type I



fatty acid synthetases and besides are critical to the discussion of control mechanisms they are considered here in some detail. A beginning has been made in unraveling the exceedingly complex kinetics of the type I mycobacterial FAS. Three stages have been reorganized, a brief "initial burst," a somewhat longer steady state, and a post-steady state period.

### 1. Ionic Strength (39)

To preserve the native molecular size and full *de novo* synthetase activity, the enzyme is kept in 0.5 M phosphate buffer, pH 7. As the ionic strength is lowered, enzyme activity declines at a rate that appears to parallel dissociation of the complex to subunits (mol. wt. 200–250,000). The half-time for decay in 0.005 M phosphate at 4°C is about 30 min. On return to solutions of high ionic strength (0.5 M phosphate buffer) the enzyme regains about 40% of the original activity in the course of 2 hr. Since inactivation and reactivation are time dependent, the primary ionic strength effect is on the quaternary structure, which in turn determines the catalytic activity. All type I fatty acid synthetases are sensitive to ionic strength, but this does not necessarily involve time-dependent changes in quaternary structure. For example, the FAS complex of *Euglena gracilis* responds instantaneously and reversibly to changes in phosphate buffer concentration (26).

An ionic strength corresponding to 0.5 M K-phosphate is far in excess of what might be expected to exist in the bacterial cytoplasm. Conceivably, the intact mycobacterial cell provides some other molecules that can stabilize the complex under physiological conditions. However, such stabilizing factors have not been found. It is also conceivable, and not without precedent, that after cell breakage, proteolytic enzymes partially degrade the native synthetase. In this event the homogenous enzyme as isolated eventually may be an artifact, much more labile in a low-ionic-strength environment. Finally, the possibility cannot be excluded that in the intact cell the synthetase is loosely associated with the bacterial membrane.

### B. ACETYL-CoA CONCENTRATIONS

$K_m$  values for acetyl-CoA reported for the various type I FAS systems range from 20 to 50  $\mu\text{M}$ . For the *M. smegmatis* synthetase