The Partial Purification and Characterization of a Bacterial Fatty Acyl Coenzyme A Synthetase*

Edward J. Massaro† and William J. Lennarz‡

ABSTRACT: The presence of a long-chain fatty acyl CoA ester synthetase has been demonstrated in extracts of *Bacillus megaterium*, strain M. The enzyme is localized in the cytoplasmic portion of the cell and has been purified over 20-fold by $(NH_4)_2SO_4$ fractionation, adsorption on alumina gel, and DEAE-cellulose chromatography. The purified enzyme requires ATP and Mg^{2+}, and manifests maximal activity toward dodecanoic acid. The branched-chain fatty acid, 12-methyltetradecanoic acid, which is the major fatty acid present in the lipids of this organism, is a relatively poor substrate for the fatty acyl CoA ester synthetase.

During the past several years the results of numerous studies concerned with the biosynthesis of fatty acids in eubacteria have been published. Up to the present time there is no evidence to indicate that fatty acyl CoA esters are the end products of fatty acid synthesis; rather it appears, at least in the case of *Escherichia coli* (Goldman *et al.*, 1963) and *Bacillus megaterium* (W. J. Lennarz, unpublished observations), that the final products are the free fatty acids. Little is yet known of the mechanism by which these fatty acids are incorporated into complex lipids in the eubacteria, but it is reasonable to assume that the process requires an activated form of the fatty acid.[1] In connection with a study of some aspects of phospholipid biosynthesis in *B. megaterium*, strain M, we have tested the ability of extracts of this organism to catalyze the activation of long-chain fatty acids. The presence of a fatty acyl CoA ester synthetase, having optimal activity for dodecanoic acid, has been demonstrated; the enzyme has been partially purified. Preliminary reports on some of this work have been published (Lennarz, 1963; Massaro and Lennarz, 1964).

Materials

Coenzyme A was obtained from the Pabst Laboratories, Milwaukee, Wis. Stearoylhydroxamic and palmitoylhydroxamic acids, kindly provided by Mr. G. Hensley, were recrystallized from ethanol. Purified cetyltrimethylammonium bromide was a gift of Dr. A. Chung. Total lipids of *B. megaterium* were obtained by extraction of whole cells with chloroform-methanol. Egg yolk lecithin was the gift of Mr. C. Huang. All other chemicals were obtained from commercial sources. The French pressure cell was purchased from American Instrument Co., Silver Spring, Md.

The 12-methyltetradecanoic acid was isolated from the lipids of *B. megaterium* by standard methods (Lennarz *et al.*, 1962); after removal of the unsaturated fatty acids by the mercuric acetate procedure (Goldfine and Bloch, 1961), the branched-chain acid was purified by gas-liquid chromatography. The purified material was at least 95% C_{15} branched-chain fatty acid, of which greater than 85% was the 12-methyl isomer. Straight-chain fatty acids were purchased from Applied Science Laboratories, State College, Pa., and were at least 99% pure. Suspensions of the fatty acids in the form of potassium salts, *p*H 7.5–8.5, at a concentration of 0.03 *M*, were warmed prior to addition to incubation mixtures.

Methods

Protein was measured by standard biuret methods (Gornall *et al.*, 1949; Zamenhof, 1957).

Cells of *B. megaterium*, strain M, grown as previously described (Lennarz, 1963), were harvested in the late log phase. All subsequent operations were carried out at 0–5°. The cells were washed with 0.01 M K_3PO_4 (potassium phosphate), 10^{-3} M mercaptoethanol, *p*H 8.0, suspended in 1.4 volumes of the same buffer, and disrupted by extrusion through a French pressure cell. The supernatant obtained after centrifugation of the resulting suspension at 15,000 × *g* for 15 minutes was centrifuged at 78,000 × *g* for 60 minutes. The resulting supernatant (crude extract) had a specific activity of 0.05–0.08 μmole hydroxamate formed/mg protein/90 minutes (*vide infra*).

The crude extract (containing 20–25 mg protein/ml) was adjusted to *p*H 6.3 with cold 1 M acetic acid. A solution of protamine sulfate (10 mg/ml, *p*H 5.0), was added so that the bacterial protein–protamine ratio was

* From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Md. *Received August 18, 1964.* Supported by a research grant (AI-04948) from the U.S. Public Health Service.

† U.S. Public Health Service, National Cancer Institute, Postdoctoral Fellow. Present address: Mergenthaler Laboratory for Biology, Johns Hopkins University, Baltimore, Md.

‡ Clayton Scholar.

[1] Dr. R. Pieringer, Temple University, has recently informed us in a private communication that he has demonstrated the enzymatic formation of phosphatidic acid from L-α-glycerophosphate and palmitoyl CoA in crude extracts of *E. coli* B.

20:1. The resulting solution was stirred for 10 minutes and then insoluble material was removed by centrifugation at 15,000 × *g* for 12 minutes. To the supernatant, 0.1 volume of 1 M K_3PO_4, *p*H 8.0, was added. The extract was then treated with $(NH_4)_2SO_4$ in order to obtain the protein fraction precipitating between 40 and 65% $(NH_4)_2SO_4$ saturation (Green and Hughes, 1955). This fraction contained 60–70% of the original total enzyme activity, and had a specific activity of 0.13–0.18. The precipitated protein was dissolved in 0.05 M K_3PO_4, *p*H 7.5, to give a protein concentration of 20–30 mg/ml and then dialyzed for 14–20 hours against 300–500 volumes of 0.01 M K_3PO_4, 10^{-3} M mercaptoethanol, 10^{-4} M EDTA, *p*H 7.0. After dialysis, insoluble material was removed by centrifugation at 15,000 × *g* for 15 minutes. The supernatant was then mixed with alumina Cγ gel (gel-to-protein ratio, 1.0:0.62). The pellet obtained upon centrifugation of this mixture was washed with 0.01 M K_3PO_4, *p*H 7.0, and extracted several times with 0.1 M K_3PO_4, *p*H 7.0. The combined extracts were dialyzed against 0.01 M K_3PO_4, 0.01 M mercaptoethanol, 10^{-4} M EDTA, *p*H 7.5, for 11 hours. At this point the over-all recovery of the original enzyme activity was 30–40%. The specific activity of the preparation was 0.30–0.35.

The dialyzed solution, generally containing 200–500 mg of protein in 25–75 ml of buffer, was chromatographed on a DEAE-cellulose column (3 × 25 cm) using a linear gradient apparatus containing 1 liter of 0.01 M K_3PO_4, 0.001 M mercaptoethanol, *p*H 7.5, in the mixing chamber and 1 liter of 0.8 M K_3PO_4, 0.001 M mercaptoethanol, *p*H 7.5, in the reservoir. Enzyme activity was found in the fractions collected between 250 and 350 ml of effluent. The over-all recovery of enzyme activity was 15–20%. The pooled fractions generally had a specific activity of 1.4–1.6, while the value for the original crude extract was *ca.* 0.07; thus the over-all purification was approximately 20-fold. The partially purified enzyme was stored at 0°; approximately 50% of the activity was lost after 7–10 days.

CoA ester formation was determined by a modification of the hydroxamate method of Kornberg and Pricer (1953). Enzymic incubations (final volume 1.0 ml) were terminated by heating at 65° for 3 minutes. After addition of 0.12 ml of 5 N NH_2OH (*p*H 7.0) the mixture was maintained for 30 minutes at 30° to ensure complete conversion of the CoA ester to the hydroxamic acid. Then 0.7 ml of 7% perchloric acid and 0.25 ml of bovine serum albumin (10 mg/ml) were added and the resultant precipitate was collected by centrifugation and washed once with 1.3 ml of 3.5% perchloric acid. The washed pellet was extracted with 1.5 ml of absolute ethanol at 65° for 5 minutes. The ethanol extract obtained after centrifugation of the mixture was treated with 0.03 ml of the concentrated $FeCl_3$ reagent of Hill (1947). Optical density at 520 mμ was measured in a Beckman DU spectrophotometer. The recovery of added stearoylhydroxamic acid, used as a standard, was at least 80%.

Under these assay conditions, the hydroxamates of decanoate and dodecanoate are not completely precipitated upon addition of perchloric acid. The amount of these compounds remaining in solution was determined by the method utilized by Kornberg and Pricer (1953) for the hydroxamates of short-chain fatty acids.

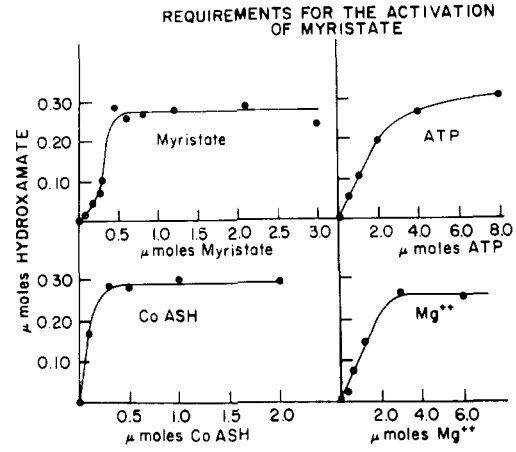

FIGURE 1: Requirements for CoA ester formation. Incubation mixtures for optimal CoA ester formation contained (in μmoles): K^+ myristate, 2.1; ATP, 4.0; CoASH, 0.5; $MgCl_2$, 3.0; mercaptoethanol, 55; K_3PO_4, 35, *p*H 7.5; Tris-HCl, 70, *p*H 8.55; 150 μg protein in a final volume of 1.0 ml. Final *p*H was 8.5. Reaction was initiated by the addition of myristate after preincubation of the mixture for 3 minutes. After incubation with shaking at 30° for 90 minutes the reaction was terminated and assayed as indicated in the text.

Results

Requirements for the Formation of Fatty Acyl CoA Esters. The results in Figure 1 illustrate the requirements for the formation of myristyl (tetradecanoyl) CoA. The bacterial enzyme requires ATP and Mg^{2+}, as do most of the mammalian acyl CoA synthetases. The reaction appears to be specific for ATP; when tested with crude extracts, GTP, UTP, and CTP have less than 15% of the activity of ATP. Mn^{2+} can substitute for Mg^{2+}. Cysteamine is an ineffective substitute for CoASH; reactivity toward other sulfhydryl-containing compounds has not been studied. Estimation of the K_m of the various components in the system from the data in Figure 1 suggests that the K_m of CoASH is almost an order of magnitude less than that of Mg^{2+} and ATP. Estimates of the K_m of the fatty acid are difficult to make since it is evident that the enzyme saturation curve is anomalous. The same sigmoidal curve is observed with the two other fatty acids studied in detail, namely, dodecanoic and pentadecanoic acid (*vide infra*).

To demonstrate conclusively that the hydroxamate formed from the CoA ester was, in fact, the hydroxamate of the particular long-chain fatty acid added to the incubation mixture, an experiment utilizing [1-^{14}C] palmitic acid was carried out. The ^{14}C-labeled hydroxamate isolated from the ethanol extract (see Methods)

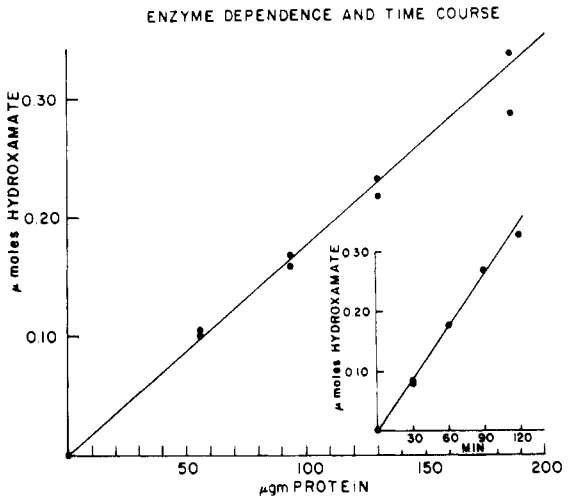

FIGURE 2: CoA ester formation as a function of time and of enzyme concentration. Conditions as in Figure 1 except for indicated variations in time and enzyme concentration.

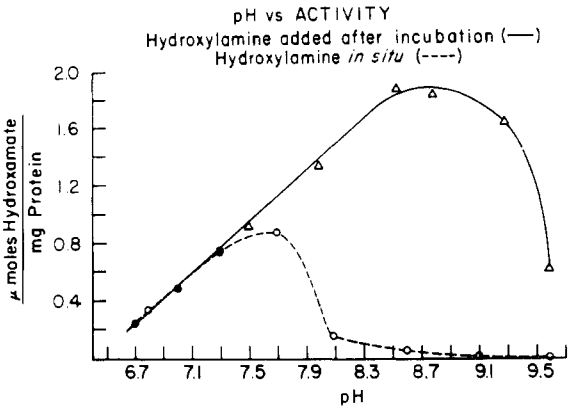

FIGURE 3: The effect of hydroxylamine on CoA ester formation. Conditions for incubation and assay when NH_2OH was added after termination of reaction (———) were as in Figure 1 except that the *p*H of the incubation mixture was varied by altering the *p*H of the K_3PO_4 and Tris-HCl buffers. For incubations with NH_2OH *in situ* (---------), 600 μmoles of NH_2OH were added and the Tris-HCl was deleted. The *p*H of the mixture was varied by altering the *p*H of the NH_2OH. Reaction was terminated by addition of perchloric acid and the mixture was assayed as indicated in the text.

was diluted with carrier palmitoyl hydroxamate. By means of silicic acid–column and thin-layer chromatography, as well as recrystallization to constant specific activity, the product was identified as [^{14}C]palmitoyl hydroxamate.

With the optimal concentrations of the various components indicated in Figure 1, fatty acyl CoA ester formation proceeds at an essentially linear rate for 90 minutes (Figure 2). The reaction exhibits linear dependence on enzyme concentration under these conditions.

In order to obtain information on the cellular site of the fatty acyl CoA synthetase, cytoplasmic and membrane fractions of *B. megaterium* were isolated by the protoplast technique as described by Weibull *et al.* (1959). Fatty acyl CoA synthetase activity was demonstrated in the cytoplasmic fraction, but was not detectable in the membrane fraction. Furthermore, all the activity in the cytoplasmic fraction was retained in the supernatant when this fraction was centrifuged at 150,000 × *g* for 60 minutes.

*p*H *Optimum: Effect of Hydroxylamine.* In crude preparations of the enzyme it is not possible to obtain an accurate measure of CoA ester formation by converting the CoA esters to hydroxamates subsequent to completion of incubation. Presumably this is owing to the action of deacylases present in the extract. For this reason the *in situ* hydroxylamine-trapping method was used during the early phases of purification. It is evident, however, that the presence of hydroxylamine in incubation mixtures with the purified enzyme results in a decrease in the specific activity of the enzyme and a shift in the apparent *p*H optimum (Figure 3).

Acyl CoA Synthetase Activity as a Function of Fatty Acid Chain Length. In order to study the rate of acyl CoA ester formation as a function of the fatty acid chain length, a survey was made of the activation of various

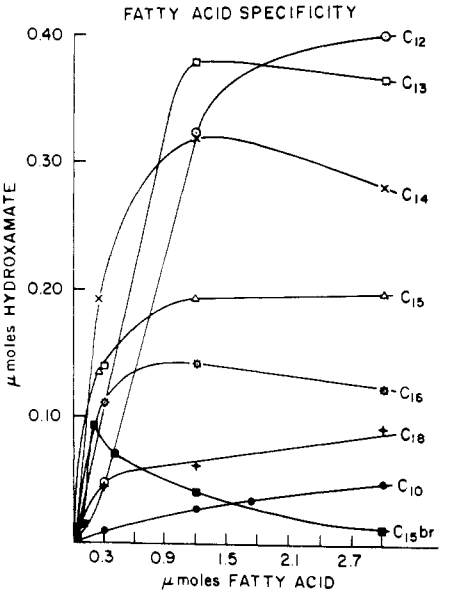

FIGURE 4: CoA ester formation as a function of fatty acid chain length and concentration. Conditions as in Figure 1 except that 187 μg protein was used. C_{15}br represents 12-methyltetradecanoic acid. Chain length of the straight-chain acids is indicated by subscript.

fatty acids at several concentration levels. From the results shown in Figure 4 it is evident that half-saturation of the straight-chain acids between C_{12} and C_{16} is obtained at a concentration of 2–6 × 10^{-4} M. Furthermore

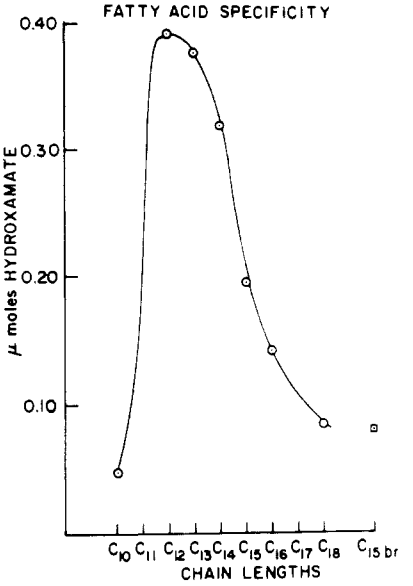

FIGURE 5: CoA ester formation as a function of fatty acid chain length.

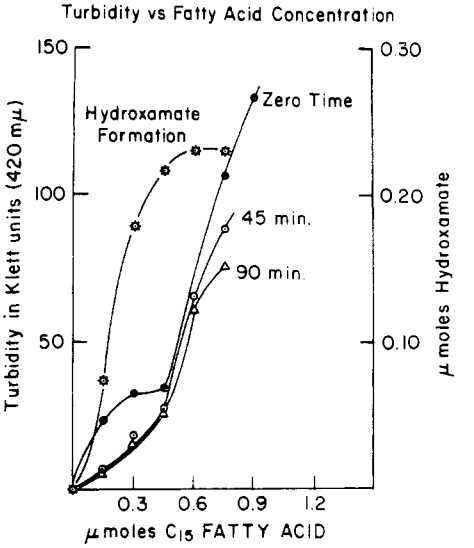

FIGURE 6: Relationship between fatty acid concentration, turbidity of the incubation mixture, and fatty acyl CoA ester formation. General conditions as in Figure 1 except that the amount of all components was increased 2-fold and the amount of protein was 374 μg. Incubations were carried out in 2.0 ml micro-Klett colorimeter tubes. At 0, 45, and 90 minutes the turbidity of the incubation mixtures was determined at 420 mμ. At 90 minutes all tubes were assayed for acyl CoA ester.

it is clear that dodecanoate and tridecanoate show the maximum rate of activation. In contrast the branched-chain acid, 12-methyltetradecanoate, manifests both a lower half-saturation level (approximately 1×10^{-4} M) and a lower apparent maximal rate of activation. However, due to the low level of 12-methyltetradecanoyl CoA ester formation and the relative insensitivity of the assay method, only a limited study of the kinetics of the activation of 12-methyltetradecanoate has been made. For this reason the possibility that the factor controlling the extent of CoA ester formation with this compound is one of equilibrium rather than kinetics cannot definitely be excluded. The rate of acyl CoA formation as a function of chain length at optimum fatty acid concentration is summarized in Figure 5.

The salts of the fatty acids with chain lengths greater than ten carbon atoms are relatively insoluble. For this reason the question of the relationship between the solubility of the substrate and the enzyme saturation curve is one of particular interest. The results of an experiment in which both the solubility of the fatty acid (as determined by turbidity measurements) and the extent of acyl CoA ester formation were studied as a function of fatty acid concentration are presented in Figure 6. It is evident that, at the lowest levels of fatty acid tested, the substrate is not in true solution (i.e., causes measurable turbidity), although the enzyme is not saturated with fatty acid at these low levels. This situation persists throughout the period of incubation; however some decrease in the turbidity of samples containing the lower levels of fatty acids does occur. This is understandable since at these low levels an appreciable percentage of the fatty acid is converted to the more soluble fatty acyl CoA ester. The reason for the apparent "plateau" region in turbidity observed at low fatty acid concentrations at zero time is not known.

In order to study the effect of various lipids, detergents, and other lipophilic compounds on the rate of acyl CoA ester formation, incubations were carried out under standard conditions (legend, Figure 1) in the presence of the following compounds: cetyltrimethylammonium bromide, sodium lauryl sulfate, Triton A-20 (all tested at 10^{-4} to 10^{-2} M); ethanol, 2-butanol, octanol, dimethylformamide, toluene, diethyl ether (tested at 2% v/v); egg yolk lecithin, total *B. megaterium* phospholipids, cardiolipin (tested at 10^{-4} to 2×10^{-3} M). None of these compounds had any stimulatory effect on acyl CoA ester formation. Sodium lauryl sulfate inhibited the reaction 90% at 10^{-3} M; cetyltrimethylammonium bromide had no effect at this level but became inhibitory at higher concentrations. Of the various organic solvents tested, 2-butanol and octanol were the most inhibitory, causing 75% inhibition at 2% (v/v). None of the lipids affected the reaction at 2×10^{-3} M, although lecithin caused 60% inhibition at 10^{-2} M.

Discussion

Since the discovery and characterization of long-chain fatty acyl CoA synthetase in guinea pig liver preparations by Kornberg and Pricer (1953), several studies on the long-chain fatty acid activating enzymes from various mammalian sources have been reported (Senior and Isselbacher, 1960; Steinberg *et al.*, 1960; Creasey, 1962;

Ailhaud *et al.*, 1963). All the enzymes studied require a divalent cation (Mg^{2+} or Mn^{2+}) and utilize ATP in the process of forming the thioester. Another feature common to the various synthetases is their cellular localization; with one exception (Kornberg and Pricer, 1953) all the mammalian long-chain fatty acyl CoA synthetases studied in any detail have been found to be localized in the particulate cell fractions. The bacterial enzyme characterized in this study also requires ATP and Mg^{2+}, but, in contrast to the mammalian enzymes, it is not found in the membrane-containing fractions of the cell but in the cytoplasm. Of interest in this connection is the fact that the fatty acid synthetase of *B. megaterium*, strain M, is also a cytoplasmic enzyme (W. J. Lennarz, unpublished observations). Thus two of the early enzymic steps leading ultimately to the formation of neutral and phospholipids occur in the cytoplasm. In view of the fact that at least 55–75% of the cellular lipids of *B. megaterium*, strain M, are found in the cell membranes (Weibull, 1957), it will be of great interest to determine the cellular site of the enzymes involved in the later stages of phospholipid synthesis.

Since fatty acyl CoA synthetases are often assayed by the hydroxamate method, our findings on the effects of hydroxylamine may be of general interest. The inhibitory effect of hydroxylamine on some enzymic reactions is well known (Green and Wakil, 1960). Such an effect on the long-chain fatty acyl CoA synthetase is evident from the data presented in Figure 3. Moreover, it is clear that hydroxylamine becomes increasingly inhibitory at higher *p*H values. As a consequence of this phenomenon, the apparent *p*H optimum, when the assay is carried out by the *in situ* hydroxylamine method, is 1.0–1.2 *p*H units lower than the true *p*H optimum of the enzyme. Also noteworthy is the fact that the rate of CoA ester formation, under standard assay conditions, is linearly dependent on the *p*H in the range *p*H 7.6–8.4. Similar observations have been made by Mahler *et al.* (1953) on a mammalian acyl CoA synthetase, and it has been postulated that the rate law describing the reaction involves a hydroxyl ion term.

The availability of a soluble partially purified acyl CoA synthetase afforded an opportunity to examine one aspect of the thiokinase reaction not readily studied with the particulate mammalian enzymes, namely, the relationship between the physical state of the fatty acid and its interaction with the enzyme. We have investigated two questions bearing on this point. (1) Is the apparent enzyme saturation curve, with respect to fatty acid, directly related to the solubility of the fatty acid? Two lines of evidence suggest that this is not the case. First, it is clear that the concentration of the straight-chain acids between C_{10} and C_{18} required to saturate the enzyme is approximately the same, despite the fact that the solubility of the fatty acids decreases 50-fold between C_{10} and C_{18} (Figure 4).[2] Second, it is evident, at least in the case of pentadecanoic acid, that the solution is saturated (in the sense of reaching the point at which the amount of fatty acid in true solution is maximal) at a level far below the point at which the enzyme becomes saturated with fatty acid (Figure 6). These results, although not conclusive, do suggest that the enzyme can act upon fatty acid molecules that are not in true solution; whether the fatty acid is in the form of micelles or larger molecular aggregates is not known.

(2) The second approach used to investigate the effect of the physical state of the substrate on the enzymic reaction was a study of the effect of various lipids, detergents, and organic solvents on the rate of thioester formation. It seemed possible that any alterations in the physical state of the fatty acid owing to interaction with these compounds might result in an alteration in the rate of thioester formation. Several publications have reported studies in which detergents or other surface-active compounds were found to have a pronounced effect on an enzymic reaction involving a lipid substrate (Kanfer and Kennedy, 1964; Chung and Law, 1964). However, in the case of the reaction catalyzed by acyl CoA synthetase, no noteworthy effects on the reaction by various lipids or detergents were observed.

Approximately 70% of the fatty acids of *B. megaterium* are C_{15} branched-chain fatty acids, the predominant isomer being the *anteiso*, 12-methyltetradecanoic acid. One question that becomes obvious as a result of the studies on the chain-length specificity is: What is the physiological function of this enzyme? Clearly, 12-methyltetradecanoic acid is a poor substrate in terms of the rate of CoA ester formation. On the other hand the enzyme saturation level of the branched-chain acid is at least five times lower than that of the straight-chain fatty acids. In addition the optimum concentration range is quite narrow, higher levels of this fatty acid being strongly inhibitory.

It has been reported previously that the specific activity of this fatty acyl CoA synthetase in crude cell extracts increases 20-fold as the cells progress through the logarithmic growth phase into the stationary phase (Lennarz, 1963). A number of *in vivo* studies have revealed that significant alterations in the fatty acid composition of microorganisms can occur during the later stages of growth (Croom and McNeill, 1961; Lennarz *et al.*, 1962). However, in the case of *B. megaterium*, only small changes in the amount of total bound fatty acids and free fatty acids occur throughout the growth phase; the proportions of the individual fatty acids are also essentially constant (W. J. Lennarz, unpublished observations). Furthermore, unpublished preliminary studies in this laboratory indicate that there is little if any turnover of the fatty acid moieties of the lipids in this organism during logarithmic growth. In view of these findings it is clear that further studies, probably at the *in vivo* level, will be necessary to gain an understanding of the role of this enzyme in the over-all lipid metabolism of the cell.

[2] This 50-fold difference is based on the solubility of the fatty acids in water (Deuel, 1951). Under the conditions that obtain in the incubation mixture the fatty acids must exist in the salt form. However, the presence of Na^+, K^+, Mg^{2+}, and protein makes it very difficult to deduce the nature and the solubility of the predominant salt form of the fatty acid.

Acknowledgment

It is a pleasure to acknowledge the technical assistance of Mrs. Karen McCauley and Mr. Norman Argue.

References

Ailhaud, G., Samuel, D., and Desnuelle, P. (1963), *Biochim. Biophys. Acta 67*, 150.
Chung, A., and Law, J. H. (1964), *Biochemistry 3*, 967.
Creasey, W. A., (1962), *Biochim. Biophys. Acta 64*, 559.
Croom, J. A., and McNeill, J. J. (1961), Bacteriological Proceedings, Abstracts of the 61st Annual Meeting, Baltimore, p. 170.
Deuel, H. J. (1951), *in* The Lipids, New York, Interscience, p. 58.
Goldfine, H., and Bloch, K. (1961), *J. Biol. Chem. 236*, 2596.
Goldman, P., Alberts, A. W., and Vagelos, P. R. (1963), *J. Biol. Chem. 238*, 3579.
Gornall, A. G., Bardawill, C. J., and David, M. (1949), *J. Biol. Chem. 177*, 751.
Green, A. A., and Hughes, W. L. (1955), *Methods Enzymol. 3*, 76.
Green, D. E., and Wakil, S. J. (1960), *in* Lipide Metabolism, Bloch, K. ed., New York, Wiley, p. 10.
Hill, U T. (1947), *Anal. Chem. 19*, 932.
Kanfer, J., and Kennedy, E. P. (1964), *J. Biol. Chem. 239*, 1720.
Kornberg, A., and Pricer, W. E. (1953), *J. Biol. Chem. 204*, 329.
Lennarz, W. J. (1963), *Biochim. Biophys. Acta 73*, 335.
Lennarz, W. J., Scheuerbrandt, G., and Bloch, K. (1962), *J. Biol. Chem. 237*, 664.
Mahler, H. R., Wakil, S. J., and Bock, R. M. (1953), *J. Biol. Chem. 204*, 453.
Massaro, E. J., and Lennarz, W. J. (1964), *Federation Proc. 23*, 269.
Senior, J. R., and Isselbacher, K. J. (1960), *Biochim. Biophys. Acta 44*, 399.
Steinberg, D., Vaughan, M., Margolis, S., and Karmen, A. (1960), *Federation Proc. 19*, 227.
Weibull, C. (1957), *Acta Chem. Scand. 11*, 881.
Weibull, C., Beckman, H., and Bergström, L. J. (1959), *J. Gen. Microbiol. 20*, 519.
Zamenhof, S. (1957), *Methods Enzymol. 3*, 702.

Reaction of Ficin with Diisopropylphosphorofluoridate. Evidence for a Contaminating Inhibitor*

Norman R. Gould† and Irvin E. Liener

ABSTRACT: Commercial samples of diisopropylphosphorofluoridate (DFP) which inhibit ficin were found to contain a small amount of impurity which combines irreversibly with the SH groups of ficin, cysteine, and glutathione. The inhibition of ficin could be prevented by prior activation of the enzyme with cysteine; the latter presumably exerts this protective effect by reacting preferentially with the inhibitor. By means of fractional distillation DFP could be obtained free of the inhibitor which remained in the nondistillable residue. DFP which had been purified in this manner was capable of phosphorylating ficin without affecting its activity. The inhibitor has been further purified by thin-layer chromatography, but its chemical identity remains unknown

The inhibition of animal proteases and esterases by the organophosphorus compound, DFP, has provided an extremely useful technique for elucidating the chemical nature of the active site of these enzymes (see, for example, the review by Koshland, 1963). A decided lack of agreement exists in the literature, however, regarding the inhibitory effect of DFP on the proteases of plant origin, most of which are considered SH enzymes. Jansen *et al.* (1948) and Kimmel and Smith (1954) were unable to inhibit papain with DFP, contrary to the observations of Masuda (1959), Ebata *et al.* (1962), and Heinicke and Mori (1959), all of whom reported inhibition. Several papers (Heinicke and Mori, 1959; Ota *et al.*, 1961; Ebata *et al.*, 1962) have reported the

*From the Department of Biochemistry, University of Minnesota, St. Paul. *Received September 4, 1964.* Paper No. 5510, Scientific Journal Series, Minnesota Agricultural Experiment Station, University of Minnesota, St. Paul. This investigation was supported by research grants from the National Institutes of Health, U.S. Public Health Service (GM 04616) and from the National Science Foundation (G 13965).

† This report is based on a dissertation presented by one of us (N. R. G) in partial fulfillment for the degree of Doctor of Philosophy in the Department of Biochemistry, Institute of Agriculture, University of Minnesota.