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Acyl-Carrier Protein. II. Intermediary Reactions of Fatty Acid Synthesis

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The fatty acid synthetase of *Escherichia coli* has been fractionated and several enzymatic activities have been partially purified. Two of these, acetyl transacylase and malonyl transacylase, catalyze the transfer of acetyl and malonyl groups, respectively, from CoA (coenzyme A) to ACP (the acyl-carrier protein). The condensation between acetyl-ACP and malonyl-ACP to form acetoacetyl-ACP is catalyzed by an enzyme which purifies along with the malonyl transacylase fraction. The reduction of acetoacetyl-ACP to form β-hydroxybutyryl-ACP is catalyzed by β-ketoacyl-ACP reductase. This enzyme is relatively specific for acyl-ACP compounds, but it also catalyzes the reduction of acetoacetyl-CoA and acetoacetyl pantetheine. The reaction is readily reversible, and study of the oxidation of stereoisomers of β-hydroxybutyryl thiolesters has established that the enzyme specifically reacts with the D(−) isomer. The conversion of crotonyl-ACP to a product tentatively identified as β-hydroxybutyryl-ACP is catalyzed by enoyl-ACP hydrase which has been partially purified from the *E. coli* system. Two mammalian enzyme systems, fatty acid synthetase of rat adipose tissue and pig heart β-hydroxyacyl-CoA dehydrogenase, catalyze the reduction of *E. coli* acetoacetyl-ACP.

The discovery by Lynen of protein-bound acetoacetate in a yeast fatty acid–synthesizing system led to the proposal that all the intermediates in long-chain fatty acid biosynthesis might be similarly protein bound (Lynen, 1961, 1962). Fractionation of an *Escherichia coli* fatty acid synthetase has allowed the demonstration of a unique acyl-carrier protein (ACP),[1] formerly designated as Enzyme II (Alberts *et al.*, 1963; Goldman *et al.*, 1963a,b). Acyl compounds which are involved in fatty acid biosynthesis are bound through thiolester linkage to the sulfhydryl group of ACP. ACP has been isolated and has been shown to contain a single sulfhydryl group (Majerus *et al.*, 1964). Acetyl-ACP, malonyl-ACP (Majerus *et al.*, 1964), acetoacetyl-ACP (Goldman *et al.*, 1963b; Goldman, 1964), and butyryl-ACP (Goldman, 1964) have been isolated, and the latter two have been shown to be intermediates in fatty acid synthesis (Goldman *et al.*, 1963b; Goldman, 1964). Chemical synthesis of various acyl-ACP derivatives has allowed study of the intermediate steps in fatty acid biosynthesis. The following reactions have been proposed:

$$\text{acetyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{acetyl-S-ACP} + \text{CoA-SH} \quad (1)$$

$$\text{malonyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{malonyl-S-ACP} + \text{CoA-SH} \quad (2)$$

$$\text{acetyl-S-ACP} + \text{malonyl-S-ACP} \rightleftharpoons \text{acetoacetyl-S-ACP} + \text{ACP-SH} + CO_2 \quad (3)$$

$$\text{acetoacetyl-S-ACP} + \text{NADPH} + H^+ \rightleftharpoons \text{D(}-\text{)-}\beta\text{-hydroxybutyryl-S-ACP} + \text{NADP}^+ \quad (4)$$

$$\text{D(}-\text{)-}\beta\text{-hydroxybutyryl-S-ACP} \rightleftharpoons \text{crotonyl-S-ACP} + H_2O \quad (5)$$

$$\text{crotonyl-S-ACP} + \text{NADPH} + H^+ \rightleftharpoons \text{butyryl-S-ACP} + \text{NADP}^+ \quad (6)$$

The purification of acetyl transacylase, malonyl transacylase, condensing enzyme, and β-ketoacyl-ACP reductase, which catalyze reactions 1, 2, 3, and 4, respectively, in the *E. coli* system, will be reported in this paper. Evidence that reaction 5 is catalyzed by a partially purified protein fraction of *E. coli* will be presented. In addition, experiments which show that *E. coli* acyl-ACP derivatives are metabolized by mammalian enzyme systems will be reported.

[1] Abbreviations used in this work: ACP, acyl-carrier protein; this name has been agreed upon by Drs. K. Bloch, P. Stumpf, and S. J. Wakil.

Methods

Preparation of Acetyl and Malonyl Transacylases.—Nine g of lyophilized cells of *E. coli* (strain K-12, Hfr 132) was suspended in 90 ml of 0.01 M triethanolamine-HCl, *p*H 7.5, containing 0.01 M 2-mercaptoethanol. The cells were ruptured in a French pressure cell at 9000 psi and the extract was diluted to 250 ml with buffer. The particulate matter was removed by centrifugation at 37,500 × *g* for 30 minutes. The supernatant solution was adjusted to 15 mg protein per ml with the same buffer, and 0.3 volume of 5% streptomycin sulfate was added with stirring. This suspension was immediately centrifuged for 15 minutes at 37,500 × *g*, and the precipitate was discarded. The supernatant solution (350 ml) was diluted 3-fold with water and was applied to a 2.5 × 20–cm DEAE-cellulose column. The column had been equilibrated with, and all subsequent solutions contained, 0.01 M potassium phosphate, *p*H 7.0, and 0.01 M 2-mercaptoethanol. The column then was washed with 500 ml of 0.1 M LiCl. The enzymes were eluted with a 3-liter linear gradient of LiCl between 0.1 and 0.5 M, and 200 fractions were collected. The acetyl transacylase (fractions 50–64) was diluted 3-fold and applied to a DEAE-cellulose column (1.8 × 12 cm) equilibrated as before. The column was washed with 100 ml of 0.1 M LiCl and eluted with a 1500-ml linear gradient of LiCl between 0.1 and 0.25 M. Two hundred fractions were collected. The malonyl transacylase from the first DEAE-cellulose column (fractions 65–85) was treated in a similar manner except that a LiCl gradient between 0.1 and 0.3 M was utilized. The fractions containing acetyl transacylase and malonyl transacylase activity from these column steps were stored at 4°.

Preparation of β-Ketoacyl-ACP Reductase.—This enzyme was purified from *E. coli* fraction A which was prepared from 35 g of dried cells as previously described (Goldman *et al.*, 1963a), except that the protein precipitating between 45 and 95% ammonium sulfate saturation was dissolved in 0.1 M potassium phosphate, *p*H 7.0 (Table V). The resulting solution was dialyzed for 7 hours against 6 liters of 0.01 M potassium phosphate, *p*H 7, with two changes of the dialysis medium. The enzyme solution (4820 mg protein) was adjusted to a final concentration of 22 mg protein per ml. The *p*H was adjusted to 6.5 by the addition of 0.1 M acetic acid. Calcium phosphate gel was added in the ratio of 3.76 mg gel/4.46 mg protein, and the suspension was stirred for 2 minutes. The gel with the adsorbed enzyme was collected by centrifugation, suspended in 0.1 M potassium phosphate, *p*H 8.0, and stirred for 15 minutes to elute the enzyme. This elution procedure was repeated twice, and the resulting supernatant solutions were combined. This solution (100 ml) was diluted 10-fold with cold water and applied to a DEAE cellulose column (2.5 × 26 cm, 40–100 mesh) which had been equilibrated with 0.01 M potassium phosphate, *p*H 7.0. The column was washed with 300 ml of the same buffer and then eluted with a linear gradient between 0 and 0.3 M LiCl in 0.01 M potassium phosphate, *p*H 7.0. One hundred 10-ml fractions were collected. The tubes containing reductase activity were pooled and stored at −20°.

Enoyl-ACP Hydrase.—A crude extract of *E. coli*, prepared as previously described, was brought to 65% ammonium sulfate saturation. The resulting precipitate was separated by centrifugation and discarded. The supernatant solution was adjusted to 90% ammonium sulfate saturation, and the precipitated protein was dissolved in a minimal volume of 0.05 M potassium phosphate, *p*H 7.0. This preparation was stored in liquid nitrogen and desalted by gel filtration over Sephadex G-25 before use.

ACP was purified through the second DEAE-cellulose step and reduced prior to use as previously described (Majerus *et al.*, 1964). Fatty acid synthetase from rat epididymal adipose tissue was purified through the calcium phosphate gel step as previously described (Martin *et al.*, 1961).

Assay for Acetyl Transacylase and Malonyl Transacylase.—The malonyl and acetyl transacylases were assayed by measuring the transfer of the respective acyl groups from [2-^{14}C]malonyl-CoA or [1-^{14}C]- or [^{3}H]acetyl-CoA to ACP. Assay mixtures for acetyl transacylase contained 10 μmoles of imidazole-HCl, *p*H 6.1, 1–10 units of acetyl transacylase, 8 mμmoles of ACP (measured as sulfhydryl groups), and either 0.01 μmole of [^{3}H]acetyl-CoA (10 μc/μmole), or 0.01 μmole of [1-^{14}C]acetyl-CoA (0.5 μc/μmole) in a volume of 0.1 ml. Reaction mixtures for the assay of malonyl transacylase were similar except for the substitution of 1–10 units of malonyl transacylase and 0.01 μmole of [2-^{14}C]malonyl-CoA (0.5 μc/μmole) for the acetyl transacylase and acetyl-CoA. The incubations were carried out at 30° for 1 minute. The reactions were stopped with 0.4 ml of 5% perchloric acid and cooled in an ice bath for 15 minutes. The resulting precipitates were separated from the supernatant solutions by filtration on 25-mm Millipore filters having a pore size of 0.45 μ. After two washes with 5 ml of perchloric acid, the filters were transferred to scintillation vials containing 0.5 ml of 0.1 M NaOH and 10 ml of Bray's solution (Bray, 1960). The filters dissolved in this mixture which was counted in a Packard liquid-scintillation spectrometer. An enzyme unit is defined as the amount required to catalyze the formation of 1 mμmole of acyl-ACP per minute under these experimental conditions.

Assay for Condensation Reaction.—The condensation reaction was assayed by measuring the formation of acetoacetyl-ACP. Reaction mixtures contained 50 μmoles of imidazole-HCl, *p*H 6.1, 0.05 μmole acetyl-CoA, 0.05 μmole malonyl-CoA, 8 mμmoles of ACP, 15 units of malonyl transacylase containing condensing activity, and 2 units of acetyl transacylase in a volume of 0.5 ml. After incubation at 30° for 15 minutes, the reactions were terminated by boiling for 1 minute, followed by precipitation of the protein-bound products with 0.1 volume of 1 N HCl. The precipitates were collected by centrifugation and dissolved in 0.5 ml of 0.2 M Tris-HCl buffer, *p*H 8.5, containing 0.5 M $MgCl_2$. The optical densities were determined at 303 mμ and redetermined after the addition of 0.02 ml of neutral hydroxylamine which cleaves the thiolester bond. The decrease in optical density that results is proportional to the amount of acetoacetyl-ACP. The molar extinction coefficient for β-ketothiolesters under these conditions is 3 × 10^4 (Lynen *et al.*, 1952; Stern *et al.*, 1953).

Assay of β-Ketoacyl-ACP Reductase.—The reduction of acetoacetyl thiolesters by NADPH was measured by following the decrease in absorbancy at 340 mμ due to the oxidation of the NADPH. Reaction mixtures contained 50 μmoles of potassium phosphate, *p*H 7.0, 0.2 μmole NADPH, and either 0.2 μmole acetoacetyl pantetheine or 0.2 μmole acetoacetyl-CoA in a total volume of 1.0 ml. Reactions were started by the addition of 0.001–0.01 unit of enzyme and absorbancies were recorded every 30 seconds. Under these conditions the assay was linear for several minutes. A control to correct for NADPH oxidase activity lacked acetoacetyl thiolester. This control was omitted after the calcium phosphate gel step which eliminated the

TABLE I
PURIFICATION OF ACETYL TRANSACYLASE AND MALONYL TRANSACYLASE

Fraction	Protein (mg)	Total Units AT[a]	Total Units MT[b]	Specific Activity AT (units/mg)	Specific Activity MT (units/mg)	Purification AT (-fold)	Purification MT (-fold)
Crude	4,620	5,260	66,300	1.14	14.3		
DEAE-cellulose column							
Fx 50–64	61.0	2,190	12,000	35.9	164	31.5	11.5
Fx 65–85	99.0	1,450	35,200	14.7	355	12.8	24.8
DEAE-cellulose column of pooled Fx 50–64	10.8	1,200	1,500	111	117	98.0	8.2
DEAE-cellulose column of pooled Fx 65–85	32.0	700	25,300	21.9	782	19.2	55.0

[a] AT refers to acetyl transacylase. [b] MT refers to malonyl transacylase.

NADPH oxidase activity. An enzyme unit is defined as the amount required to catalyze the acetoacetyl pantetheine–dependent oxidation of 1 μmole NADPH per minute under these experimental conditions. Alternatively, reductase activity was tested using acetoacetyl-ACP as substrate. In these experiments 15 mμmoles of acetoacetyl-ACP, 0.1 μmole of NADPH, and 0.1–1.0 $\times 10^{-4}$ unit of enzyme were incubated in a volume of 0.5 ml.

Sulfhydryl determinations were by the method of Ellman (1959) and protein determinations by the method of Lowry *et al.* (1951). Thiolesters were measured quantitatively by the hydroxamate method (Stadtman, 1957) unless otherwise specified. β-Hydroxybutyryl hydroxamate was identified by paper chromatography in two solvent systems: 1-butanol-water (100:18) (R_F 0.48) and secondary butanol–formic acid–water (75:13:12) (R_F 0.57). β-Hydroxybutyric acid was chromatographed in the solvent system, 95% ethanol–ammonia (100:1), where it has an R_F of 0.38.

MATERIALS

Acetyl-CoA was prepared by the method of Simon and Shemin (1953), malonyl-CoA by the method of Trams and Brady (1960); acetoacetyl-CoA and acetoacetyl pantetheine by the method of Lynen and Wieland (1955); D(−)-, L(+)-, and racemic β-hydroxybutyryl pantetheine by the method of Wieland and Köppe (1953); and crotonyl pantetheine and crotonyl *N*-acetyl cysteamine by the method of Wieland and Rueff (1953). Acetyl-CoA and malonyl-CoA were purified on DEAE-cellulose columns by the method of Moffatt and Khorana (1961).

[^{14}C]CoA was synthesized enzymatically from 4-phosphopantetheine, which was prepared by the method of Moffatt and Khorana (1961), and [8-^{14}C]ATP was synthesized using a partially purified hog liver phosphopantetheine pyrophosphorylase (Novelli, 1955). At the end of the reaction an excess of acetic anhydride was added to the reaction mixture to acetylate the CoA formed, and the acetyl-[^{14}C]CoA was chromatographed on DEAE-cellulose.

Acyl-ACP derivatives were synthesized by the following general procedure. ACP was incubated with 2-mercaptoethanol as described previously to reduce fully the sulfhydryl groups (Majerus *et al.*, 1964). Reduced ACP was adjusted to *p*H 6.2 and poured onto a 1 × 2-cm DEAE-cellulose column which had been equilibrated with 0.01 M potassium phosphate, *p*H 6.2. The column was washed with buffer until free of 2-mercaptoethanol. ACP was then eluted with 0.5 M LiCl containing 0.01 M potassium phosphate, *p*H 6.2, and the sulfhydryl content was assayed. Aliquots were adjusted to *p*H 8 by the addition of $KHCO_3$ and converted to [^{3}H]acetyl-ACP, [2-^{14}C]malonyl-ACP, acetoacetyl-ACP, or crotonyl-ACP by the addition of the appropriate acylating agent (as described for the synthesis of the CoA or panthetheine thiolesters) in at least 100-fold excess. The acylations were carried out at 0° under a stream of helium and allowed to proceed for 5 minutes. Hydrochloric acid was then added to a final concentration of 0.1 N to precipitate the acyl-ACP. The precipitated acyl-ACP was washed four times with 0.1 N HCl and then dissolved in 0.2 M imidazole-HCl buffer, *p*H 6.1. [^{3}H]Acetyl-ACP and [2-^{14}C]malonyl-ACP were assayed by counting aliquots in a scintillation spectrometer; acetoacetyl-ACP was assayed by its absorption at 303 mμ at *p*H 8.5 in the presence of 0.5 M $MgCl_2$, and crotonyl-ACP was assayed by its absorption at 263 mμ.

D(−)- and L(+)-β-Hydroxybutyric acids were the generous gift of Dr. G. D. Greville. These acids had $[\alpha]_D^{20} = -24.7°$ and $+21.0°$, respectively.[2] [^{3}H]-Acetic anhydride, [1-^{14}C]acetic anhydride, [2-^{14}C]-malonate, and [8-^{14}C]ATP were purchased from New England Nuclear Corp.; CoA from Pabst Laboratories; NADPH, $NADP^+$, and NADH from Sigma Chemical Corp.; Sephadex G-100 from Pharmacia Corp.; and DEAE-cellulose from Brown Paper Co. Pantethine, purchased from Calbiochem, was reduced to pantetheine with borohydride at *p*H 9.0. Crystalline pig heart β-hydroxyacyl-CoA dehydrogenase was obtained from Calbiochem.

RESULTS

Acetyl and Malonyl Transacylases.—Acetyl transacylase and malonyl transacylase catalyze the transfer of acetyl and malonyl groups, respectively, from CoA to ACP (reactions 1 and 2). As reported earlier, each of these acyl groups is transferred to the sulfhydryl group of ACP and is linked to ACP through thiolester linkage (Majerus *et al.*, 1964). The purification procedure and results of a typical preparation are summarized in Table I. Although the acetyl transacylase was purified 98-fold by this procedure, this purified preparation was not resolved of malonyl transacylase activity. These two activities were further resolved by filtration of the acetyl transacylase preparation on Sephadex G-100. The pooled fractions containing acetyl transacylase activity from the second DEAE-cellulose column were concentrated by precipitation at 95% ammonium sulfate saturation prior to gel filtration. Figure 1 indicates that this procedure yielded acetyl transacylase (tubes 50–65) essentially free of malonyl transacylase activity. Malonyl transacylase activity was purified 55-fold (Table I), but still con-

[2] We wish to thank Mrs. Katherine Warren for the rotary dispersion data.

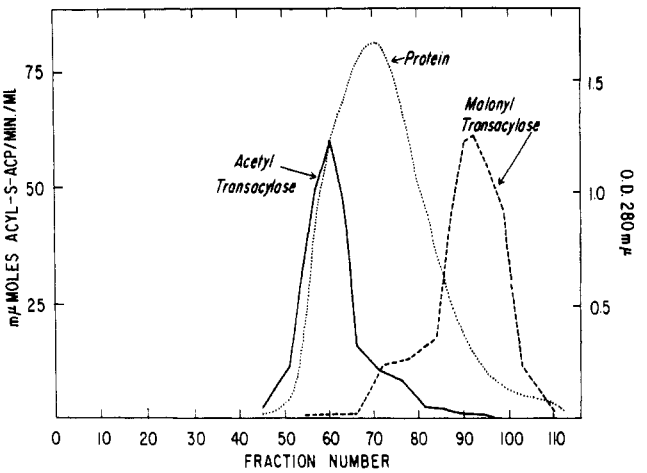

FIG. 1.—Gel filtration of acetyl transacylase. Acetyl transacylase from the second DEAE-cellulose column was precipitated with 95% saturated ammonium sulfate. The resulting precipitate was dissolved in 4 ml of 0.1 M potassium phosphate buffer, *p*H 7.0, containing 0.01 M 2-mercaptoethanol and applied to the top of a 4.5 × 30-cm Sephadex G-100 column which had been equilibrated with 0.01 M potassium phosphate buffer, *p*H 7.0, containing 0.01 M 2-mercaptoethanol. The protein was then eluted with the latter buffer. One hundred twenty 3-ml fractions were collected and assayed for transacylase activities as described under Methods.

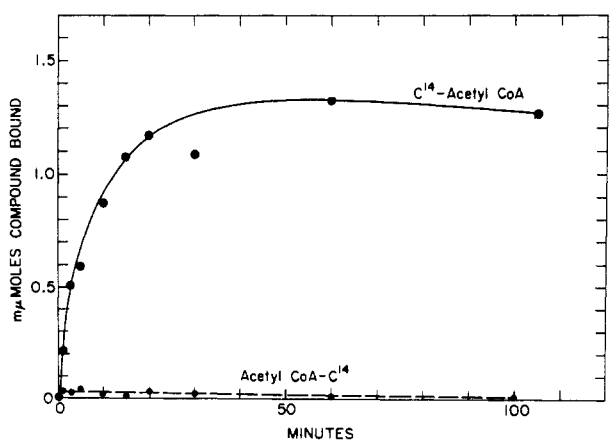

FIG. 2.—Transfer of acetate from acetyl-CoA to ACP. The reaction mixture (1.0 ml) contained 0.1 μmole (0.5 μc/μmole) of [1-^{14}C]acetyl-CoA (solid line) or 0.1 μmole (0.5 μc/μmole) of acetyl-[^{14}C]CoA (broken line), 100 μmoles of imidazole-HCl buffer, *p*H 6.1, 2.5 mμmoles of ACP, and 2 units of acetyl transacylase. At the times indicated, 0.1-ml aliquots were removed from the incubation mixture and pipetted into 0.5 ml of 5% perchloric acid. The resulting precipitates were assayed as described under Methods. "Compound bound" refers only to the radioactive component of either [^{14}C]acetyl-CoA or acetyl-[^{14}C]CoA.

tained significant acetyl transacylase activity until it was subjected to gel filtration similar to that described for the acetyl transacylase. Although not shown here, condensing activity (reaction 3) purifies in parallel with malonyl transacylase activity through DEAE-cellulose column chromatography.

The formation of acetyl-ACP and malonyl-ACP, using the purified enzyme preparations, are demonstrated in Figures 2 and 3, respectively. The activities were measured as the transfer of [^{14}C]acyl group from [^{14}C]acyl-CoA to ACP. Figure 2 demonstrates that only the acetyl moiety of acetyl-CoA is transferred to ACP. In the lower curve, where acetyl-[^{14}C]CoA was

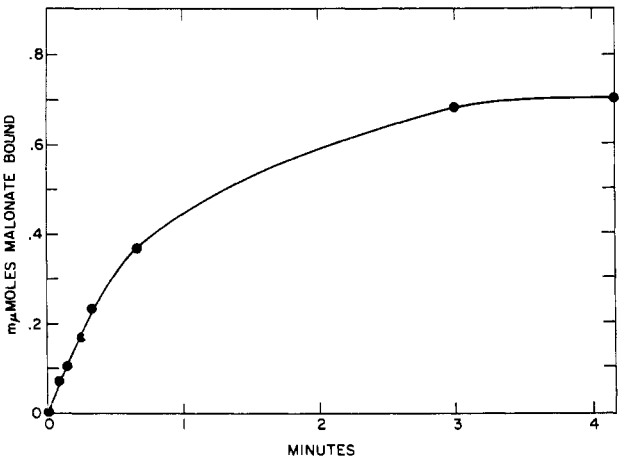

FIG. 3.—Transfer of malonate from malonyl-CoA to ACP. The reaction mixture (1.0 ml) contained 0.1 μmole (0.5 μc/μmole) of [2-^{14}C]malonyl-CoA, 100 μmoles of imidazole-HCl buffer, *p*H 6.1, 2.5 mμmoles of ACP, and 6 units of malonyl transacylase. At the times indicated, 0.1-ml aliquots were removed from the incubation mixture and transferred to 0.5 ml of 5% perchloric acid. The resulting precipitates were assayed as described under Methods.

utilized as substrate instead of [^{14}C]acetyl-CoA, none of the [^{14}C]CoA was bound to ACP. Thus the acyl groups are transferred to ACP and CoA is released in the transacylation reactions. From this point in metabolism, acyl-ACP derivatives are the active intermediates in fatty acid biosynthesis.

Condensation Reaction.—The product of the condensation of acetyl-CoA and malonyl-CoA is acetoacetyl-ACP (Goldman *et al*, 1963b; Goldman, 1964). Acetoacetyl-ACP has an ultraviolet-absorption peak at 303 mμ characterstic of β-ketothiolesters, and this spectral characteristic has been used to measure the condensation reaction. Thus the requirements for the over-all condensation reaction are shown in Table II.

TABLE II
REQUIREMENTS FOR THE FORMATION OF ACETOACETYL-ACP[a]

System	Acetoacetyl-ACP Formed (mμmoles)
Complete	3.66
-acetyl-CoA	0
-malonyl-CoA	0
-ACP	0
Complete (boiled acetyl transacylase)	0.30
Complete (boiled malonyl transacylase)	0.10

[a] The complete system contained, in 0.5 ml, 0.05 μmole of acetyl-CoA, 0.05 μmole of malonyl-CoA, 50 μmoles of imidazole-HCl buffer, *p*H 6.1, 2 units of acetyl transacylase, 15 units of malonyl transacylase containing condensing activity, and 8 mμmoles of ACP. After 15 minutes at 30° the mixture was assayed for acetoacetyl-ACP as described under Methods.

In the complete system, which included acetyl-CoA, malonyl-CoA, ACP, and the two purified transacylase fractions, 3.66 mμmoles of acetoacetyl-ACP were formed. There is total dependence of the reaction on the three substrates, acetyl-CoA, malonyl-CoA, and ACP. Boiling either the acetyl or malonyl transacylase essentially inactivated the condensation reaction. However, it should be pointed out that malonyl

TABLE III
CONVERSION OF ACETYL-ACP TO ACETOACETYL-ACP[a]

Time (min)	[^{3}H]Acetate Bound (mμmoles)	Aceto-acetyl-ACP (mμmoles)
0	3.20	0
2	2.78	0.191
10	3.26	0.729
20	3.15	1.19
120	3.18	3.05

[a] Each reaction mixture (0.4 ml) contained 3.2 mμmoles [^{3}H]acetyl-ACP, 0.04 μmole (0.5 μc/μmole) of [2-^{14}C]-malonyl-CoA, 40 μmoles of imidazole-HCl buffer, *p*H 6.1, and 12 units of malonyl transacylase containing condensing activity. At the times indicated a 0.1-ml aliquot was transferred to 0.5 ml of 5% perchloric acid and assayed for radioactivity as described under Methods. The remainder of the reaction mixture was treated as described under Methods for the identification of acetoacetyl-ACP.

transacylase activity is stable to boiling since this boiled preparation still catalyzed the binding of malonate to ACP. It is only the condensing activity (reaction 3) of the malonyl transacylase fraction which is inactivated. It is not yet established whether these two activities are catalyzed by a single protein. Acetyl transacylase activity is heat labile.

Substitution of Acetyl-ACP for Acetyl-CoA and Acetyl Transacylase.—The requirement for acetyl-CoA and acetyl transacylase in the condensation reaction can be replaced by acetyl-ACP. This compound was prepared by incubating 150 mμmoles of ACP (which had been reduced with 2-mercaptoethanol), 30 units (0.27 mg) of acetyl transacylase, 1 μmole of [^{3}H]acetyl-CoA (10 μc/μmole), and 500 μmoles of imidazole-HCl buffer, *p*H 6.1, in a total volume of 5.0 ml. The reaction mixture was incubated at 30° for 20 minutes and the reaction was terminated by boiling. Acetyl-ACP was precipitated by acidification with HCl to *p*H 1. The resulting precipitate was collected by centrifugation and washed with 0.1 N HCl until the wash showed negligible radioactivity. The precipitate was then dissolved in 1.5 ml of 0.1 M imidazole HCl, *p*H 6.1. All the radioactivity in the solution at this time was protein bound. The yield was 77 mμmoles of [^{3}H]acetyl-ACP. As indicated in Table III, incubation of [^{3}H]acetyl-ACP with malonyl-CoA and the protein fraction containing malonyl transacylase and condensing activity resulted in the formation of acetoacetyl-ACP. During the 2-hour incubation period there was essentially no change in the amount of tritium ([^{3}H]acetyl) bound to ACP, suggesting that a direct conversion of acetyl-ACP to acetoacetyl-ACP occurred without the intermediate release of the acetyl moiety from ACP. [^{3}H]Acetyl-ACP, 3.2 mμmoles, was stoichiometrically converted to 3.05 mμmoles of acetoacetyl-ACP during this experiment.

Chemically prepared acetyl-ACP can react similarly in the condensation reaction. Thus acetoacetyl-ACP was formed when chemically prepared acetyl-ACP was incubated with malonyl-CoA and the same enzyme preparation. However, this reaction required the addition of free ACP, since the acylation of ACP by acetic anydride causes the quantitative conversion of ACP to acetyl-ACP. There was, therefore, no free ACP available for the formation of malonyl-ACP, and additional ACP was required for the condensation reaction. The addition of ACP was not required when enzymatically synthesized acetyl-ACP was utilized since enzymatic acetylation of ACP never goes to completion (Majerus, *et al.*, 1964), and therefore some free ACP is always available.

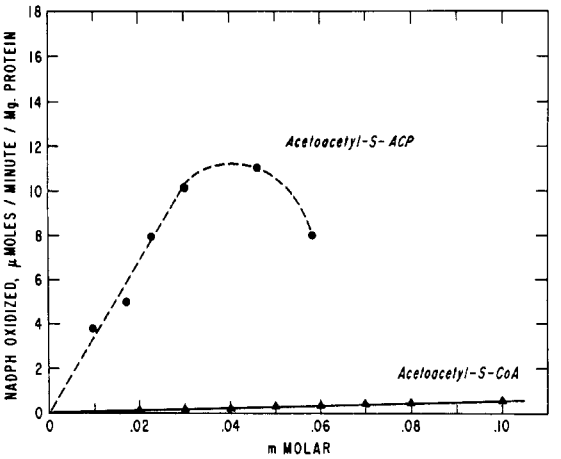

FIG. 4.—Comparison of β-ketoacyl-ACP reductase activity with acetoacetyl-ACP and acetoacetyl-CoA. Reactions were carried out as detailed under Methods except that the concentrations of acetoacetyl thiolesters were varied as indicated.

TABLE IV
CONDENSATION OF MALONYL-ACP AND ACETYL-ACP[a]

Time (min)	Aceto-acetyl-ACP (mμmoles)
0	0
5	1.30
10	1.69
30	2.78

[a] Each reaction mixture (0.4 ml) contained 5 mμmoles of acetyl-ACP, 5 mμmoles of malonyl-ACP, 40 mμmoles of imidazole-HCl buffer, *p*H 6.1, and 12 units of malonyl transacylase containing condensing activity. At the times indicated the reaction mixture was assayed for acetoacetyl-ACP as described under Methods.

Substitution of Malonyl-ACP for Malonyl-CoA.—An experiment which indicates that malonyl-ACP, the product of the malonyl transacylation reaction (reaction 2), condensed with acetyl-ACP to form acetoacetyl-ACP (reaction 3) is indicated in Table IV. Malonyl-ACP was prepared enzymatically by incubation of 1.0 μmole of [2-^{14}C]malonyl-CoA (0.5 μc/μmole) with 150 mμmoles of ACP and 30 units (0.05 mg) malonyl transacylase under conditions similar to those used in the synthesis of acetyl-ACP. The yield in this case was 74 mμmoles of [2-^{14}C]malonyl-ACP. When [2-^{14}C]malonyl-ACP was incubated with enzymatically prepared acetyl-ACP and the condensing enzyme fraction, acetoacetyl-ACP was formed (Table IV). Chemically synthesized malonyl-ACP reacted equally well in the condensation reaction. The condensation of acetyl-ACP with malonyl-ACP is catalyzed by the protein fraction which contains both condensing and malonyl transacylase activities.

β-Ketoacyl-ACP Reductase.—Preliminary experiments with the crude *E. coli* fraction A, which contains all the proteins necessary for fatty acid synthesis except ACP, indicated the presence of a β-ketoacyl thiolester reductase (reaction 4). This enzyme was purified 157-fold (Table V) with an overall recovery of about 30%. The reductase is very stable when stored at −20°.

Specificity studies with this enzyme indicated that acetoacetyl-ACP, the product of the first condensation reaction in the fatty acid biosynthetic sequence (reaction 3), is the most active substrate of the acetoacetyl

TABLE V
β-KETOACYL-ACP REDUCTASE PURIFICATION

Fraction	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Recovery of Activity (%)	Purification (-fold)
Crude extract	72.7	23,950	0.003		
Ammonium sulfate, 0.45–0.75%	58.2	4,820	0.0122	80	4.03
Calcium phosphate gel eluate	50.6	1,539	0.033	69.6	11.0
Pooled DEAE-cellulose column fractions	21.5	45.5	0.472	29.6	157

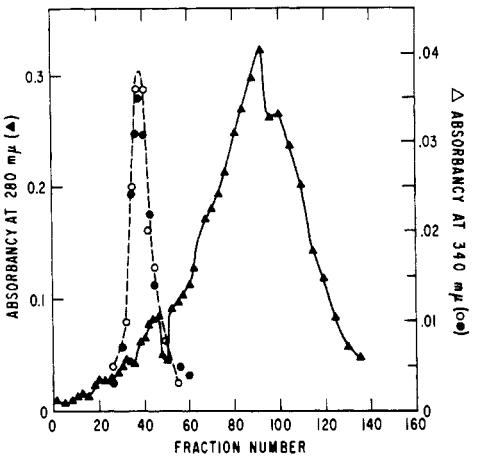

FIG. 5.—DEAE-cellulose-column chromatography of β-ketoacyl-ACP reductase. The enzyme was chromatographed on DEAE-cellulose as in the last step of the purification described under Methods. Enzyme activity with acetoacetyl pantetheine (solid circles) was assayed as described under Methods using 0.1-ml aliquots of the enzyme fractions. Assays in which acetoacetyl-ACP was used as substrate (open circles) contained 0.001-ml aliquots of the enzyme fractions. Decrease per minute in absorbancy at 340 mμ was recorded.

thiolesters that were tested. The relative rates of NADPH oxidation using acetoacetyl-ACP and acetoacetyl-CoA as substrates are shown in Figure 4. There is a linear increase in reaction rate with increasing concentrations of acetoacetyl-ACP up to about 0.03 mM. Concentrations of acetoacetyl-ACP above 0.05 mM are inhibitory to this enzyme. When these data are plotted according to the method of Lineweaver and Burk (1934), the Michaelis-Menten constant for acetoacetyl-ACP is approximately 1.2×10^{-4} M, whereas that for acetoacetyl-CoA is 6.6×10^{-4} M. The calculated maximal velocities for acetoacetyl-ACP and acetoacetyl-CoA are 5.0×10^{-5} and 4.0×10^{-6} moles/min per mg protein, respectively. Thus it is seen that the higher reactivity of acetoacetyl-ACP in this reaction is due to both a higher affinity for the enzyme and a higher maximal velocity. Acetoacetyl pantetheine, the compound routinely utilized in the assay of this enzyme, has approximately the same affinity and maximal velocity as acetoacetyl-CoA. The lack of specificity of the reductase toward the β-ketoacyl thiolesters allows both CoA and pantetheine derivatives to act as model compounds in this reaction in which acetoacetyl-ACP is the preferred substrate. However, the enzyme reacts specifically with NADPH; there is no reaction when NADH is substituted for NADPH.

The possibility that separate β-ketoacyl thiolester reductases catalyze the reduction of acetoacetyl-ACP and acetoacetyl-CoA was tested by assaying with each substrate all the fractions from the DEAE-cellulose-column-chromatography step of the purification procedure. As noted in Figure 5, a single peak of enzyme activity was eluted and the reductase activities, using either acetoacetyl-ACP or acetoacetyl-CoA as substrates, were superimposed. Inasmuch as this step yielded a 14-fold purification, this experiment suggests that both substrates are reduced by the same enzyme.

This reductase does not behave as a sulfhydryl enzyme since it is stable when stored in the absence of added sulfhydryl compounds and since it is not stimulated at any stage of purification by the addition of mercaptans. Moreover, incubation of the enzyme for 15 minutes with either 10^{-3} M *p*-mercuriphenylsulfonate, 10^{-2} M iodoacetamide, or 2×10^{-3} M *N*-ethylmaleimide caused no inhibition when the enzyme was then tested in the routine assay. This reductase, therefore, does not have a functionally significant sulfhydryl group.

Product of β-Ketoacyl-ACP Reductase.—Experiments were carried out to identify the reaction product. Incubation mixtures contained 50 μmoles of potassium phosphate (*p*H 7), 10 μmoles of NADPH, 1.3 μmoles of acetoacetyl-ACP (13 mg protein), and 0.09 mg reductase in a volume of 2.0 ml. After 15 minutes of incubation at 37°, the reaction mixture was diluted 20-fold with water and poured onto a DEAE-cellulose column (1.0 × 2.0 cm) which had been equilibrated with 0.01 M potassium phosphate buffer, *p*H 6.3. After the column had been washed with 20 ml of the same buffer, the acyl-ACP was eluted with 0.5 M LiCl. The acyl-ACP was concentrated by acid precipitation (*p*H 1) and either converted to the hydroxamic acid derivative or hydrolyzed in 0.5 N NaOH. A single hydroxamic acid product was identified as β-hydroxybutyryl hydroxamate by paper chromatographic comparison to authentic β-hydroxybutyryl hydroxamate. β-Hydroxybutyric acid, which was released from ACP by the alkaline hydrolysis, was extracted into ether and identified by paper chromatographic comparison to an authentic standard. Similar chromatographic results were obtained, using the model compounds, acetoacetyl-CoA or acetoacetyl pantetheine. Therefore it is clear that the acetoacetyl-ACP is reduced to β-hydroxybutyryl-ACP by this enzyme.

Stoichiometry of Reductase Reaction.—Stoichiometry experiments with the reductase were of particular interest because the chemically prepared acetoacetyl-ACP thus could be examined quantitatively in a relatively specific enzymatic reaction. As it has been established that ACP contains a single sulfhydryl group, chemical synthesis of acetoacetyl-ACP should yield the thiolester in amounts approximating the sulfhydryl content of the starting material. In separate experiments, 0.14 and 1.4 μmoles of ACP (measured as sulfhydryl groups) gave rise to 0.096 and 1.6 μmoles of acetoacetyl-ACP, respectively. Thus approximately stoichiometric yields of thiolester were obtained. When acetoacetyl-ACP was reacted with NADPH and the reductase, and

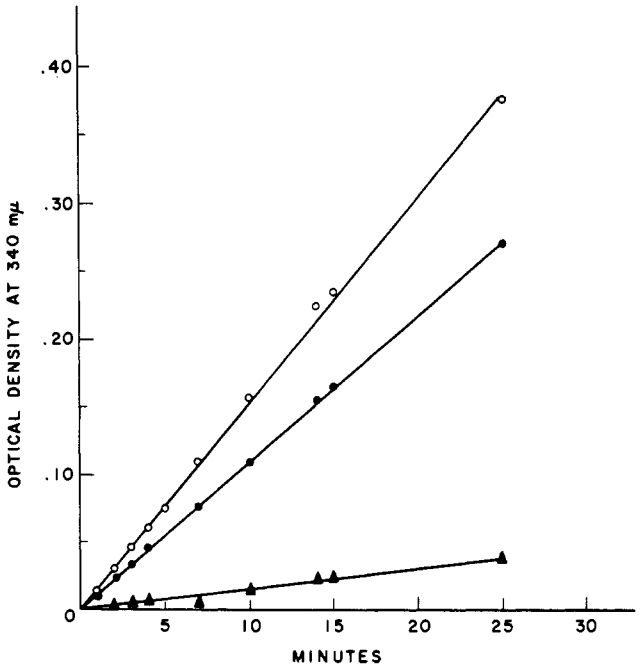

FIG. 6.—Stereoisomer specificity of β-ketoacyl-ACP reductase. Reaction mixtures contained 200 μmoles of Tris-HCl buffer, *p*H 9.0, 1 μmole of $NADP^+$, 0.015 mg of reductase, and 1.0 μmole of either D(−)- (open circles), racemic (closed circles), or L(+)-β-hydroxybutyryl pantetheine (triangles) in a volume of 1.0 ml. Optical density at 340 mμ was recorded after addition of enzyme.

TABLE VI
REDUCTION OF ACETOACETYL-ACP BY *E. coli* AND ANIMAL ENZYME PREPARATIONS[a]

Enzyme System	AcAc-ACP Added (mμmoles)	Reduced Pyridine Nucleotide Oxidized (mμmoles)	Reduced Pyridine Nucleotide Oxidized/ AcAc-ACP Added
β-Ketoacyl-ACP Reductase	5.0	5.8	1.16
	7.5	7.6	1.01
	10.0	11.0	1.10
	20.0	19.9	0.99
	30	29.8	0.99
Rat fatty acid synthetase	1.1	1.95	1.77
	2.2	4.5	2.04
	3.3	6.65	2.02
β-Hydroxyacyl-CoA Dehydrogenase	1.1	1.3	1.18
	2.2	2.2	1.00
	4.4	4.3	0.98

[a] Assays were carried out as described in Figures 4, 7 and 8 for the β-ketoacyl-ACP reductase, rat fatty acid synthetase, or β-hydroxyacyl-CoA dehydrogenase, respectively. However, reactions were allowed to continue until reduced pyridine nucleotide oxidation ceased. Total reduced pyridine nucleotide that was oxidized and the ratio of reduced pyridine nucleotide oxidized/acetoacetyl-ACP in the reaction mixture are indicated.

the reactions were allowed to go to completion, a stoichiometric amount of NADPH was oxidized (Table VI). Similar results were obtained when acetoacetyl-CoA was used as substrate. Thus all the sulfhydryl groups of the ACP were equivalent when they were chemically esterified and the resulting acetoacetyl-ACP was reduced with the purified reductase.

Reversibility of Reductase Reaction.—Incubation of the β-ketoacyl-ACP reductase at high *p*H with chemically prepared racemic β-hydroxybutyryl pantetheine and

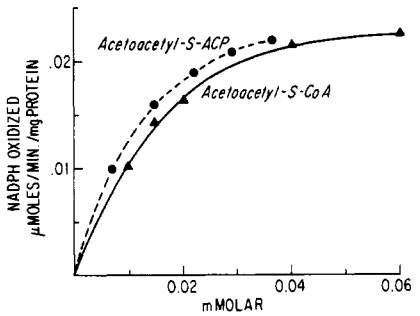

FIG. 7.—Rates of acetoacetyl-ACP and acetoacetyl-CoA reduction by rat fatty acid synthetase. Reaction mixtures contained 10 μmoles of potassium phosphate, *p*H 7.0, 20 mμmoles of NADPH, 0.063 mg of enzyme, and the indicated concentrations of either acetoacetyl-ACP or acetoacetyl-CoA in a volume of 0.15 ml. Decrease in optical density at 340 mμ was recorded every 30 seconds and the rates of oxidation of NADPH were calculated from the initial velocities.

$NADP^+$ led to the rapid oxidation of the substrate to form acetoacetyl pantetheine coincident with the formation of one equivalent of NADPH. The reaction, therefore, is readily reversible and it could be utilized to determine the configuration of the β-hydroxybutyryl thiolester preferred by the enzyme. As noted in Figure 6, which compares NADPH formation when racemic, D(−)-, or L(+)-β-hydroxybutyryl pantetheine was used in the reverse reaction, the enzyme is relatively specific for D(−)-β-hydroxybutyryl pantetheine. Oxidation of the L(+)-β-hydroxybutyryl pantetheine occurs at a much slower rate.

Mammalian Fatty Acid Synthetase.—The mechanism of long-chain fatty acid synthesis in animal enzyme systems is thought to be similar to that of bacterial systems. Thus the β-ketoacyl-CoA reductase, which has been shown to copurify with the fatty acid synthetase isolated from rat adipose tissue (Robinson *et al.*, 1963a; Martin and Vagelos, 1964), might be expected to catalyze the reduction of acetoacetyl-ACP. The *E. coli* ACP derivative was tested with the fatty acid synthetase purified from rat adipose tissue (Martin *et al.*, 1961). The relative rates of NADPH oxidation, using acetoacetyl-ACP or acetoacetyl-CoA as substrate, are shown in Figure 7. Although the rate of oxidation of NADPH at each of the substrate concentrations with either substrate is essentially the same, it was interesting to note that when the reactions had gone to completion 2 moles of NADPH were oxidized per mole of acetoacetyl-ACP added (Table VI). On the other hand, when acetoacetyl-CoA was the substrate, only 1 mole of NADPH was oxidized per mole of substrate added. Therefore it is clear that the mammalian fatty acid synthetase does catalyze the reduction of *E. coli* acetoacetyl-ACP, but the reduction probably proceeds all the way to butyryl-ACP (reactions 4, 5, and 6). The product of this series of reactions has not yet been identified. Of particular significance is the fact that the model compound, acetoacetyl-CoA, undergoes only a single reduction step using this enzyme preparation. Thus, although there is undoubtedly a handicap of species specificity when the *E. coli* ACP derivative is metabolized by the animal enzyme system, the acetoacetyl-ACP appears to undergo further metabolism than the CoA derivative which undergoes only a single reduction.

Reduction of Acetoacetyl-ACP by Crystalline β-Hydroxyacyl-CoA Dehydrogenase.—The possibility that ACP is involved in the β-oxidation of fatty acids was examined by testing one of the β-oxidation enzymes,

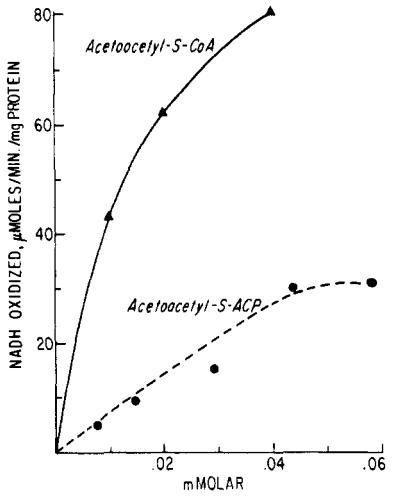

FIG. 8.—Rates of acetoacetyl-ACP and acetoacetyl-CoA reduction by crystalline β-hydroxyacyl-CoA dehydrogenase. Assays were done as described in Fig. 7, except that NADH and 0.04 μg of β-hydroxyacyl-CoA dehydrogenase were substituted for NADPH and fatty acid synthetase.

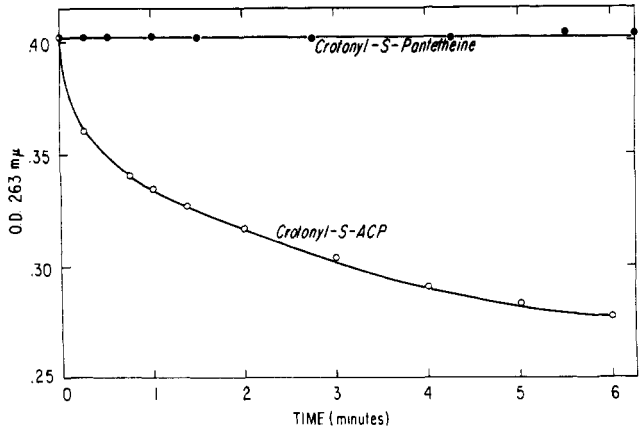

FIG. 9.—Disappearance of crotonyl-ACP catalyzed by enoyl-ACP hydrase. Reaction mixtures contained 1.2 μmoles potassium phosphate, pH 7.6, 3 mμmoles crotonyl ACP, and 0.5 μg enzyme fraction in final volume of 0.12 ml. Reaction mixtures using crotonyl pantetheine contained 3–30 mμmoles of this substrate and were otherwise identical with the above.

pig heart crystalline β-hydroxyacyl-CoA dehydrogenase, with acetoacetyl-ACP. This enzyme is relatively specific for NADH and forms specifically L(+)-β-hydroxybutyryl thiolesters (Lehninger and Greville, 1953). The relative rates of NADH oxidation when this enzyme was incubated with acetoacetyl-CoA or acetoacetyl-ACP are shown in Figure 8. It is obvious that the CoA derivative is more active than the *E. coli* ACP derivative.

Enoyl-ACP Hydrase.—The ultraviolet-absorption peak at 263 mμ, characteristic of α,β-unsaturated acyl thiolesters (Lynen, 1953), provided a convenient assay for enoyl-ACP hydrase activity (reaction 5). As shown in Figure 9, there was rapid decrease in absorbancy at 263 mμ when the hydrase preparation was added to the reaction mixture. The product of this reaction has been tentatively identified as β-hydroxybutyryl-ACP. Enoyl-ACP hydrase reacts specifically with the ACP derivative (Fig. 9); there was no activity detected when crotonyl pantetheine was substituted for crotonyl-ACP.

DISCUSSION

The involvement of ACP as the acyl carrier in fatty acid biosynthesis was established earlier with the demonstration of the formation of acetoacetyl-ACP and the conversion of this compound to long-chain fatty acids (Goldman *et al.*, 1963b). The intermediate steps in the condensation reaction include the transfer of the acetyl and malonyl moieties of acetyl-CoA and malonyl-CoA to ACP to form acetyl-ACP and malonyl-ACP, respectively. Acetyl-ACP and malonyl-ACP are the condensing units in the biosynthesis of fatty acids. Malonyl-CoA cannot substitute for malonyl-ACP in the condensation reaction. Thus, when malonyl-CoA was incubated with the malonyl transacylase–condensing enzyme fraction and chemically prepared acetyl-ACP (where the sulfhydryl groups were totally acylated), condensation did not occur until free ACP was added. With the addition of ACP, the transfer of the malonyl group from CoA to ACP occurred rapidly, forming malonyl-ACP which then condensed with acetyl-ACP to form acetoacetyl-ACP.

The involvement of acyl-ACP rather than acyl-CoA derivatives in fatty acid biosynthesis is emphasized by comparison of the relative reactivity of the various substrates with the enzymes of this pathway. Thus acetoacetyl-ACP is reduced approximately sixty times faster than acetoacetyl-CoA or acetoacetyl pantetheine by β-ketoacyl-ACP reductase. Crotonyl-ACP is rapidly metabolized by enoyl-ACP hydrase, whereas crotonyl pantetheine is not metabolized under the same conditions.

The studies with the rat adipose fatty acid synthetase and *E. coli* acetoacetyl-ACP indicate that the *E. coli* protein can serve as a model compound for the mammalian enzyme system. In this system, acetoacetyl-CoA undergoes a single reduction to form β-hydroxybutyryl-CoA whereas acetoacetyl-ACP is presumably converted to butyryl-ACP. If *E. coli* acetoacetyl-ACP is metabolized through three enzymatic reactions (reactions 4–6), this would suggest that the *E. coli* protein is a better model than CoA for the mammalian fatty acid synthetase. The finding that acetoacetyl-ACP is metabolized by crystalline β-hydroxyacyl-CoA dehydrogenase suggests that protein-bound intermediates may play a role in fatty acid oxidation as well. Attempts to isolate an ACP-like protein from mammalian tissue are in progress.

These studies indicate the general significance of ACP. It is the acyl carrier for all the intermediates of *E. coli* fatty acid synthesis, and the studies with the mammalian enzymes suggest that a similar protein functions in mammalian fatty acid synthesis. Studies with avocado mesocarp by Overath and Stumpf (1964) indicate that plant fatty acid synthesis is stimulated by *E. coli* ACP, and a protein similar to the bacterial ACP has been demonstrated in the avocado system. Further, Pugh, Sauer, and Wakil[3] have reported that *E. coli* protein-bound malonate is metabolized by a crude avian liver fatty acid synthetase.

Acetyl transacylase and malonyl transacylase have been shown previously to be sulfhydryl enzymes (Alberts *et al.*, 1963; Vagelos, 1964). As reported earlier, it is the acetyl (or fatty acyl) transacylase which is most sensitive to alkylating agents and which can be protected against inactivation by alkylating agents by acyl thiolesters. It is possible, therefore, that the acyl group of acetyl-CoA is transferred to the acetyl transacylase

[3] Presented at the Federation of American Societies for Experimental Biology, Chicago, April, 1964; E. L. Pugh, F. Sauer, and S. J. Wakil.

sulfhydryl group prior to its transfer to ACP. It is of interest that a different fatty acyl transacylase which catalyzes the transfer of longer-chain-length acyl groups from CoA to ACP now has been identified.[4] The significance of this enzyme has not yet been determined, but it is possible that it is involved in the metabolism of long-chain fatty acids, i.e., in elongation, desaturation, oxidation, or esterification reactions.

The availability of substrate quantities of acyl-ACP derivatives has permitted the study of β-ketoacyl-ACP reductase and enoyl-ACP hydrase activities in the *E. coli* system. Similar reactions have been studied previously in fatty acid synthetase preparations from yeast (Lynen, 1961, 1962), rat brain (Robinson *et al.*, 1963b), rat adipose tissue (Robinson *et al.*, 1963a; Martin and Vagelos, 1964), and avian liver (Wakil and Bressler, 1962). In those cases acyl thiolesters of CoA, pantetheine, and *N*-acetyl cysteamine were used as substrates which apparently acted as model compounds for the protein-bound intermediates.

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O-Phosphorylethanolamine: A Component of Lipopolysaccharide in Certain Gram-negative Bacteria*

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O-Phosphorylethanolamine has been isolated from acid hydrolysates of polysaccharide prepared from the cell-wall lipopolysaccharide of a UDP-D-galactose-4-epimeraseless mutant of *Salmonella typhimurium*. The material was obtained in crystalline form and identified by melting point, elemental analysis, and infrared spectrum, and was detected by paper chromatography in hydrolysates of the lipopolysaccharide of certain other Gram-negative bacteria. Together with L-glycero-D-mannoheptose, 3-deoxyoctulosonate, and phosphate, *O*-phosphorylethanolamine appears to be an integral component of the phosphorylated polysaccharide to which glucose and other sugars composing the antigenic side chains are attached. It contains 50% of the total phosphate in the polysaccharide and appears to be linked to the polymer through phosphodiester bridges.

Glucose, glucosamine, galactose, mannose, rhamnose, and abequose are well-known components of the lipopolysaccharide *O*-antigen of *Salmonella typhimurium* (Kauffmann *et al.*, 1960). Recently, 3-deoxyoctulosonate (Heath and Ghalambor, 1963; Osborn, 1963) and L-glycero-D-mannoheptose (Kauffman *et al.*, 1960; M. J. Osborn and B. L. Horecker, unpublished observations) were identified as carbohydrate components of the *O*-antigen, and a mutant strain of *S. typhimurium* was isolated whose lipopolysaccharide contained only these two sugars in addition to glucose (Nikaido, 1962; Osborn *et al.*, 1962). It has been suggested that heptose,

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