Biosynthesis of Branched-chain Fatty Acids in Bacillus subtilis

A DECARBOXYLASE IS ESSENTIAL FOR BRANCHED-CHAIN FATTY ACID SYNTHETASE*

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Branched long-chain fatty acids of the iso and anteiso series are synthesized in many bacteria from the branched-chain α-keto acids of valine, leucine, and isoleucine after their decarboxylation followed by chain elongation.

Two distinct branched-chain α-keto acid (BCKA) and pyruvate decarboxylases, which are considered to be responsible for primer synthesis, were detected in, and purified in homogenous form from Bacillus subtilis 168 strain by procedures including ammonium sulfate fractionation and chromatography on ion exchange, reversed-phase, and gel absorption columns.

The chemical and catalytic properties of the two decarboxylases were studied in detail.

The removal of BCKA decarboxylase, using chromatographic fractionation, from the fatty acid synthetase significantly reduced its activity. The synthetase activity was completely lost upon immunoprecipitation of the decarboxylase. The removal of pyruvate decarboxylase by the above two methods, however, did not affect any activity of the fatty acid synthetase. Thus, BCKA decarboxylase, but not pyruvate decarboxylase, is essential for the synthesis of branched-chain fatty acids. The very high affinity of BCKA decarboxylase toward branched-chain α-keto acids is responsible for its function in fatty acid synthesis.

Straight-chain fatty acids with 14 to 18 carbons are constituents of membrane lipids in most organisms.

The chemical mechanism of de novo synthesis of straight-chain fatty acids in these organisms is strikingly uniform: acetyl-CoA is the primer, malonyl-CoA is the chain extender of the primer, and palmitic acid is a major product (1-3). There is, however, an exception: certain bacteria possess branched-chain fatty acids of the iso and anteiso series as major acyl constituents of cellular lipids (50-98% of the total fatty acids) (4-7). In these bacteria the branched-chain fatty acids are synthesized using the branched-chain α-keto acids of valine, leucine, and isoleucine as primer sources and malonyl-CoA as the chain extender. The major products, in this case, are iso and anteiso fatty acids with 14-17 carbons. Little palmitic acid (5-10%) occurs in most of these bacteria. Extensive studies, mostly with Bacillus subtilis, have been done to elucidate the pathway of branched-chain α-keto acid incorporation into fatty acids, but the detailed mechanism is still obscure. Acyl-CoA esters with three to five carbons have been found in be good primers for the fatty acid synthetase of B. subtilis and other bacteria (8-10). A NAD-CoA-dependent dehydrogenase, which catalyses the formation of acyl-CoA esters from the α-keto acids of valine, leucine, and isoleucine has been detected in cell-free extracts of B. subtilis (11). These observations appear to support the hypothesis that CoA esters of isobutyrate, isovalerate, and 2-methylbutyrate would be primers for fatty acid synthesis from branched-chain α-keto acid substrates.

The overall reaction has been represented by the following equations:

\[
\alpha\text{-Ketoisovalerate} + \text{NAD}^{+} + \text{CoA} = \text{isobutyryl-CoA} + \text{NADH} + \text{CO}_{2} + \text{H}^{+} \quad (1)
\]

\[
\text{Isobutyryl-CoA} + 6 \space \text{malonyl-CoA} + 12 \space \text{NADPH} + 12 \text{H}^{+} = \text{isopalmitate} + 7 \space \text{CoA} + 6 \space \text{CO}_{2} + \text{5H}_{2}\text{O} + 12 \text{NADP}^{+} \quad (2)
\]

These reactions suggest that the synthetic mechanism of branched-chain fatty acids has been determined.

However, there are two specific observations which contradict the mechanism expressed by the above equations (12). First, the system of B. subtilis requires neither NAD nor CoA for fatty acid synthesis using branched-chain α-keto acids as a primer source. In fact, they were even inhibitory to the synthesis. Second, the system of B. subtilis does not lose any fatty acid synthesis activity after branched-chain α-keto acid dehydrogenase, a multi-enzyme complex, is removed from the system by ultracentrifugation. The decarboxylation of an α-keto acid substrate, however, is essential for fatty acid synthesis. Thus, the primer for fatty acid synthesis is likely to be produced from the α-keto acid substrate by decarboxylation rather than by the oxidative decarboxylation represented by Equation 1.

The amount of branched-chain fatty acid synthetase occurring in the bacteria is very small. For instance, the specific activity of the synthetase of B. subtilis extracts is only one-fifteenth of that of Escherichia coli extracts (9). This has been a major obstacle to a detailed study of the individually isolated enzymes of the fatty acid synthetase from B. subtilis. The recent major developments in high performance liquid chromatography have provided us with excellent tools to overcome this obstacle. The present paper reports the isolation of two distinct decarboxylases and then demonstrates which of the two decarboxylases is actually involved in branched-chain fatty acid synthesis in the organism.
two decarboxylases and the fatty acid synthetase are also described.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma, Aldrich, or Fisher Scientific. Radioactive substrates: [U-14C]l-isoleucine, [U-14C]l-phenylalanine, [U-14C]l-valine, [U-14C]l-lysine, [U-14C]l-valine, [U-14C]dihydroyacetone, and [U-14C]l-valine were purchased from Du Pont New England Nuclear. These radioactive amino acids were used to prepare the respective α-keto acids using L-α-amino oxidase from Sigma (13). Nonradioactive α-keto acids were prepared previously by the same method (11).

Enzyme Assays

Decarboxylation of α-keto acid substrates was routinely followed by the determination of 14CO2 from the radioactive carboxyl group of the substrate. The standard assay mixture contained, in mM, 66.7, triethanolamine-HCl (pH 7.5); 0.7, thiaminepyrophosphate; 8, MgCl2; 13.3, dithiothreitol; 0.05, [U-14C] or [U-14C]α-keto acid substrate (2--20 μCi/μmol) in a total volume of 0.3 ml. The reaction was carried out at 30°C for 90 min and stopped by the addition of 0.2 ml of 10% HClO4. The radioactive carbon dioxide produced was absorbed in 0.2 ml of 1 M hyamine (in methanol). The level of radioactivity was measured by a Beckman model LS-6800 liquid scintillation counter using a xylene-based scintillation fluid.

Alternatively, the decarboxylation was followed indirectly by the decolorization of 2,7-dichlorofluorescin (14). The assay mixture contained, in mM, 100, triethanolamine-HCl (pH 7.5); 1, MgCl2; 0.2, thiaminepyrophosphate; 0.1, dichlorophenolphthalein; and 0.2, substrate in a total volume of 1 ml. The decrease in absorbance at 600 nm was measured by a Gilford model 260 spectrophotometer at room temperature.

Fatty acid synthetase activity was measured by the amount of radioactive fatty acids produced from [U-14C]α-keto-β-methylvalerate. The radioactive fatty acids produced were isolated from the reaction mixture by a solvent extraction procedure. The standard assay mixture contained, in mM, 55, triethanolamine-HCl (pH 7.5); 0.6, thiaminepyrophosphate; 11, dithiothreitol; 0.6, NADPH-0.1, Malonyl-CoA; 0.0683, [U-14C]α-keto-β-methylvalerate; acyl carrier protein (12 μg), and enzyme in a total volume of 0.27 or 0.45 ml. In some cases, the substrate was replaced by [U-14C]α-ketoisovalerate or α-ketosuccinonitrile. The reaction mixture was incubated for 30 min at 30°C.

The reaction was stopped by the addition of 0.025 ml of 6 N HCl, and the 14CO2 produced was absorbed in 0.2 ml of 1 M hyamine in a closed system. After 60 min the radioactivity of the 14CO2 absorbed in Hamine was counted. Then 1 ml of methanol and 0.1 ml of oleic acid solution (5 mg/ml in methanol) were added to the acidified reaction mixture. If the amount of 14CO2 produced was not measured, the three reagents were added together at the end of incubation. The acidic mixture was centrifuged. The supernatant was searedated and mixed with 0.35 ml of H2O, 0.50 ml of methanol, and 3 ml of CCl4. The lower layer was washed twice with 2.5 ml of 50% aqueous methanol containing 0.05 N HCl. The washed CCl4 layer was placed under a nitrogen stream to evaporate the solvent and was counted by a Beckman model LS-6800 liquid scintillation counter.

Identification of Reaction Product

The reaction product from L-α-keto-β-methylvalerate by either decarboxylation in the presence of K3Fe(CN)6 was identified as a free acid.

Isolation of Product—The reaction mixtures contained, in mM, 66.7, triethanolamine-HCl (pH 7.5); 2, MgCl2; 0.6, thiaminepyrophosphate; 40, K3Fe(CN)6; 0.33, [U-14C]α-keto-β-methylvalerate, and either BCKA decarboxylase or pyruvate decarboxylase in a total volume of 0.3 ml. Either α-keto acid substrate or an enzyme was omitted from the complete reaction mixture for a blank run. The reaction was carried out at 30°C for 90 min and stopped by the addition of 50 μl of 1 N HCl. The acidified reaction mixture was extracted with 0.5 ml of ethyl ether twice and the volume of the combined ethyl ether extracts was reduced to 0.1 ml under a gentle nitrogen stream.

Identification of Product—Free fatty acid samples thus prepared were analyzed by gas-liquid chromatography and mass spectrometry.

The gas-liquid chromatograph used was a Hewlett-Packard model 5830 A equipped with a flame ionization detector. The column used was a fused boron capillary 20 μm capillary (15 m) (J & W, Scientific, Rancho Cordova, CA). The column temperature was initially 60°C for 5 min, raised to 100°C at a rate of 4°C/min and then raised to the final temperature of 150°C at a rate of 7.5°C/min.

The structural identification of free acid samples was done by a Finnigan Mat 4500 GC/MS with Super Iocos Data system (San Jose, CA). The column was used was a DBWAX capillary (80 m) (J & W, Scientific). The column temperature was initially kept at 35°C for 5 min and raised to 230°C at a rate of 5°C/min. The ionization of samples for mass spectrometry was done by electron impact at 70 eV for the determination of chemical structure and by chemical ionization using methane for the determination of molecular weight.

Protein Assay

The amount of protein was determined by the dye-binding method (15) using bovine serum albumin as a standard.

Column Chromatography

A Pharmacia LKB Biotechnology Inc fast protein liquid chromatography system was used for the estimation of molecular weights of two decarboxylases and for their purification. The columns used were Superose 12 prep-grade, Mono Q HR 10/10, and phenyl-Superose HR 5/5 from Pharmacia LKB Biotechnology Inc and Bio-Gel HPHT (hydroxylapatite) (100 × 7.5 mm) from Bio-Rad.

Estimation of Molecular Weights

The relative molecular weights of the two decarboxylases were measured by molecular sieve chromatography on a Superose column using 50 mM potassium phosphate buffer (pH 6.0) containing 0.15 M NaCl, 2 mM dithiothreitol, 2 mM EDTA, 0.1 mM thiaminepyrophosphate, and 10% glycerol. The Pharmacia LKB Biotechnology Inc protein standard contained ribonuclease (13,700), chymotrypsinogen (25,000), ovalbumin (43,000), albumin (bovine) (67,000), aldolase (158,000), catalase (230,000), and ferritir (440,000).

The purity and the relative molecular weights of the subunits of the two decarboxylases were determined by SDS-polyacrylamide gel electrophoresis (16).

Preparation of Crude Enzyme

Frozen cells of B. subtilis 168 supplied by Grain Processing Corp., Muscatine, IO were used as enzyme sources throughout the present work. A typical example is as follows.

Crude Extract—Fifty g of frozen cells were suspended in 2 volumes of 10 mM phosphate buffer (pH 7.0) containing 2 mM EDTA, 2 mM mercaptoethanol, and 10% glycerol. The suspension was ultrasonicated with ultrasound from a Biosonic III instrument (Brancwill Scientific, Rochester, NY) for three 1-min periods with 1-min cooling intervals. The irradiated resulting suspension was centrifuged at 48,000 × g for 20 min to yield about 100 ml of the supernatant.

Alternatively, in some cases B. subtilis suspensions were treated initially with lysozyme at a final concentration of 2.5 mg/ml at 37°C for 30 min and then with deoxyribonuclease and ribonuclease at a final concentration of 0.5 μg each/ml at 37°C for 30 min. The treated cell suspensions were centrifuged at 4°C to yield cell-free extracts (supernatant).

Ultrafiltration—The supernatant was again centrifuged at 151,000 × g for 2 h and then the upper two-thirds of its volume, about 60 ml, was removed.

Streptomycin—This supernatant was diluted to 10 mg of protein/ml with 10 mM phosphate buffer (pH 7.0) and mixed with 0.1 volume of 10% streptomycin sulfate. The precipitate was removed by centrifugation and discarded.

40–60% (NH4)2SO4—The supernatant obtained after streptomycin treatment had (NH4)2SO4 added to 40% saturation. The precipitate which formed was removed by centrifugation, and discarded. Then (NH4)2SO4 was added to the supernatant to bring it to 60% saturation. The precipitate was removed by centrifugation and dissolved in a minimum volume of 10 mM phosphate buffer (pH 7.0). The 40–60% (NH4)2SO4 fraction usually had 70 mg of protein/ml. It was stored at -70°C until needed.

Preparation of BCKA Decarboxylase

Mono Q—The 40–60% (NH4)2SO4 fraction containing 60–80 mg of protein was diluted 20 times with buffer and was applied to a Mono
Preparation of Crude Extracts

1. Mature male New Zealand rabbits were injected twice weekly for 3 weeks with a mixture of equal volumes of Freund's complete adjuvant and an approximately 170 μg of pure decarboxylase preparation of 8.1 M NaCl and 10 mM phosphate buffer (pH 7.0).

2. Injections were made twice weekly for 3 weeks to the animals and they were bled on the 33rd day.

Preparation of Antibodies

Antibodies against the two pure decarboxylases were raised in two male New Zealand rabbits using a mixture of equal volume of Freund's complete adjuvant and approximately 170 μg of a pure decarboxylase preparation of 8.1 M NaCl and 10 mM phosphate buffer (pH 7.0).

Injections were made twice weekly for 3 weeks to the animals and they were bled on the 33rd day.

Preparation of Crude Extracts

Mature male New Zealand rabbits were injected twice weekly for 3 weeks with a mixture of equal volumes of Freund's complete adjuvant and an approximately 170 μg of pure decarboxylase preparation of 8.1 M NaCl and 10 mM phosphate buffer (pH 7.0).

Injections were made twice weekly for 3 weeks to the animals and they were bled on the 33rd day.

**RESULTS**

**Purification of Two Decarboxylases**—Two decarboxylases were detected in the same fractions during the stage of (NH₄)₂SO₄ precipitation (Tables I and II). Then column chromatography on Mono Q separated BCKA decarboxylase from pyruvate decarboxylase. The former was eluted from the column at a concentration of 0.65 M NaCl whereas the latter eluted at a concentration of 0.57 M NaCl. BCKA decarboxylase required two more column chromatographic steps to purify it, but pyruvate decarboxylase was purified by the second Mono Q. Tables I and II summarize the purification of these decarboxylases. BCKA decarboxylase was purified 62.5-fold by the procedure with an overall recovery of 3.6% of the activity. Pyruvate decarboxylase was present in an amount approximately 10 times greater than BCKA decarboxylase judging from the peak heights of the two decarboxylase proteins on a Mono Q column. It was purified 47.8-fold by the procedure yielding 15% of the starting activity.

**B. subtilis** extracts used for the above experiments were prepared by treating cells with ultrasound. Essentially identi-
tical yields were obtained when the extracts were prepared using lysozyme.

**Molecular Weights of Two Decarboxylases and Their Subunits**—Pure preparations of the two decarboxylases were chromatographed on a molecular sieve (Superose 12) column to determine their relative molecular weights. $M_r$ values were estimated to be 175,000 for BCKA decarboxylase and 156,000 for pyruvate decarboxylase. Fig. 1 shows SDS-polyacrylamide gel electrophoresis of two decarboxylases. Both were electrophoretically homogeneous and were composed of two peptides in similar amounts. $M_r$ values were estimated to be 33,000 (A-subunit) and 39,800 (B-subunit) for the BCKA decarboxylase sample and 38,300 (A-subunit) and 41,500 (B-subunit) for the pyruvate decarboxylase sample (Table III). The expected $M_r$ values were calculated on the basis of the assumption that both decarboxylases are composed of a dimer of two subunits, $A_2B_2$. As shown on the last line of the table, the observed $M_r$ value and the calculated $M_r$ value do not match each other for BCKA decarboxylase. The figures differ by 30,000. This discrepancy is difficult to explain since the color intensity of A and B bands was essentially identical (Fig. 1). In the case of pyruvate decarboxylase the two values agree very well.

**Identification of Reaction Products**—The reaction products produced from L-α-keto-β-methylvalerate by BCKA decarboxylase and pyruvate decarboxylase were analyzed by gas-liquid chromatography and mass spectrometry. Caproic acid was routinely used as an internal standard giving a retention time of 18.3 min under the present experimental conditions.

The free acid sample extracted from the reaction mixture containing BCKA decarboxylase and L-α-keto-β-methylvalerate gave a major peak on the chromatogram. It had a retention time of 15.4 min, which was identical to that of an authentic sample of 2-methylbutyric acid. The chemical ionization mass spectrum of the peak component gave four major fragments (m/e) at the high end, 143 (51%), 131 (14.4%), 120 (71.8%), and 103 (100%). They correspond to [M + C$_2$H$_5$]+, [M + C$_2$H$_5$O]+, [M + H]+, and [M + H]+, respectively. Thus, the molecular weight of the peak component is 102. The electron impact mass spectrum of the peak component gave four major fragments (m/e): 87 (22.6%), 74 (100%), 57 (78.6%), and 41 (66.9%). These were identical to those of an authentic sample of 2-methylbutyrate. The same results were obtained when pyruvate decarboxylase was used. Thus, the reaction product produced from L-α-keto-β-methylvalerate was 2-methylbutyrate with both enzymes.

**Component Requirements**—The requirement for MgCl$_2$ shows a marked difference between two decarboxylases. BCKA decarboxylase did not require MgCl$_2$ for its activity, but MgCl$_2$ was essential for pyruvate dehydrogenase (Table IV). The addition of thiaminepyrophosphate stimulated both enzyme activities moderately. But dithiothreitol did not have any effect on either enzymes.

Because MgCl$_2$ was required by pyruvate decarboxylase, the effect of other metal ions on its activity was investigated. The replacement of MgCl$_2$ with FeCl$_2$, MnCl$_2$, CoCl$_2$, CaCl$_2$, FeCl$_3$, ZnCl$_2$, CuCl$_2$, and H$_2$O reduced activity to 97, 94, 72, 62, 41, 3, 2, and 3% of the control value, respectively. Both FeCl$_3$ and MnCl$_2$ are as good cofactors as MnCl$_2$. The increases by CoCl$_2$ and CaCl$_2$ were slightly less than these three metal ions but they were still good activators. The increase by FeCl$_3$ was less than half of that by FeCl$_2$. Apparently metals with divalent ions are more effective than the trivalent ion.

**pH Optimum**—Fig. 2 shows the effect of pH on the activities of the two decarboxylases. The optimum pH values for activities of the two enzymes differ slightly, pH 7.5 for BCKA decarboxylase and pH 7.8 for pyruvate decarboxylase.

**Substrate Specificity**—Activities of two decarboxylases with five α-keto acid substrates are listed in Table V. The order of activity of the substrates for BCKA decarboxylase was L-α-keto-β-methylvalerate, α-ketoisovalerate, α-ketoisocaproate, pyruvate, and α-ketoglutarate. The similar order for pyruvate decarboxylase was pyruvate, α-ketoisovalerate, L-α-keto-β-
Branched-chain Fatty Acid Synthetase

**TABLE III**

Molecular weights of two decarboxylases determined by gel filtration and gel electrophoresis

<table>
<thead>
<tr>
<th>Component</th>
<th>BCKA decarboxylase</th>
<th>Pyruvate decarboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel filtration</td>
<td>175,000 (n = 2)*</td>
<td>156,000 ± 11,200 (n = 3)</td>
</tr>
<tr>
<td>Gel electrophoresis</td>
<td>33,000 ± 410 (n = 4)</td>
<td>39,800 ± 600 (n = 4)</td>
</tr>
<tr>
<td></td>
<td>28,300 ± 500 (n = 4)</td>
<td>41,500 ± 300 (n = 4)</td>
</tr>
<tr>
<td>Calculated Mr</td>
<td>145,600</td>
<td>159,600</td>
</tr>
</tbody>
</table>

* n = number of determinations.

**TABLE IV**

Components required for the two decarboxylases

The reaction mixtures contained, in mM, 67, triethanolamine-HCl buffer (pH 7.5); 0.7, thiaminepyrophosphate; 8, MgCl₂; 13.3, diethiothreitol; 0.05 [U-¹⁴C]L-α-keto-β-methylvalerate or [¹⁴C]pyruvate; enzyme as indicated; but one component was omitted where indicated. The incubation was carried out for 30 min at 30 °C.

The reaction mixtures contained, in mM, 67, triethanolamine-HCl (A); the same components as described in Table IV, but triethanolamine-HCl buffer was replaced as follows: BCKA decarboxylase, phosphate buffer (B); pyruvate decarboxylase, phosphate buffer (O).

**TABLE V**

Substrate specificity of the two decarboxylases

The reaction mixtures contained the same components as described in Table IV, but the radioactive substrate was replaced as indicated. The incubation was carried out for 30 min at 30 °C.

**Effect of Chain Length of Substrates**—[¹⁴C]α-keto acids with four to six carbons were not available to us so we used an assay procedure based on the oxidation of dichlorophenolindophenol. BCKA decarboxylase and pyruvate decarboxylase showed the highest activity with α-ketobutyrate and pyruvate, respectively, among four straight-chain α-keto acids with three to six carbons. Fig. 3 shows activities of the two decarboxylases with five substrates normalized against their respective best substrates. In the case of BCKA decarboxylase, α-ketobutyrate was the best and two adjacent homologues, pyruvate and α-ketovalerate, were less active. In the case of pyruvate decarboxylase, pyruvate was the best, and the activity of α-keto acids decreased as their chain length increases. A noteworthy feature of BCKA decarboxylase is that the specific activity of the enzyme with L-α-keto-β-methylvalerate was approximately 3.5 times higher than that of α-ketobutyrate, the best straight-chain α-keto substrate. Thus, the enzyme is highly active for a branched-chain substrate. This feature will be looked at further under the “Discussion.” Pyruvate decarboxylase, however, did not show any preference toward the branched-chain substrate.

**Kinetic Constants**—Table VI lists Kₘ and Vₘₐₓ values of two decarboxylases with respect to four α-keto acid substrates. Kₘ values of BCKA decarboxylase with three branched-chain substrates were very small and were estimated to be approximately 0.2, 0.9, and 0.5 μM for L-α-keto-β-methylvalerate, α-ketoisovalerate, and α-ketoisocaproate, respectively. Accurate Kₘ values, however, could not be measured by the present assay procedure using radioactive substrates with specific activities of 10–20 μCi/μmol. A substrate with much higher specific activity is required. Such a substrate showed a high rate of spontaneous decarboxylation and as a result was not useful. An alternative method would be stopped-flow spectrophotometry, but this method required a large amount of enzyme. At the present we can only say that the Kₘ values of BCKA decarboxylase with three branched-chain α-keto acids are, in all cases, around 1 μM. The Kₘ value for pyruvate, on
Fig. 3. Effect of chain length of substrates on the activity of the two decarboxylases. The reaction mixtures contained, in mM, 100, triethanolamine-HCl, 0.2, thiaminepyrophosphate; 1, MgCl₂; 0.2, α-keto acid substrate; 0.1, dichlorophenolindophenol, and a decarboxylase. The reaction was followed by a decrease in absorbance at 600 nm at 22°C. The activity of BCKA decarboxylase (dark shade) was the highest with α-ketobutyrate, 30.2 nmol/min.mg, among α-keto acid substrates with straight chains tested, whereas that of pyruvate decarboxylase (light shade) was highest with pyruvate, 171 nmol/min.mg. KMV represents L-α-keto-β-methylvalerate.

### TABLE VI

**Kinetic constants for the two decarboxylases**

The reaction mixtures contained the same components as described in Table V, but the radioactive substrate was replaced as indicated at varying concentrations. The incubation was carried out for 30 min at 30°C. Kinetic constants for the two decarboxylases were determined by Lineweaver-Burk plots of the rates of ¹⁴CO₂ produced from ¹³C substrate at varying concentrations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>BCKA decarboxylase</th>
<th>Pyruvate decarboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵢ (μM)</td>
<td>Vₘₐₓ (nmol/min·mg)</td>
</tr>
<tr>
<td>L-α-Keto-β-</td>
<td>&lt;1</td>
<td>17.8</td>
</tr>
<tr>
<td>methylvalerate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Ketoisovalerate</td>
<td>&lt;1</td>
<td>13.3</td>
</tr>
<tr>
<td>α-Ketoisocaproate</td>
<td>&lt;1</td>
<td>5.6</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>51.1</td>
<td>15.2</td>
</tr>
</tbody>
</table>

the other hand, was approximately 50 times higher than that for branched-chain substrates. Thus BCKA decarboxylase was very high affinities for branched-chain substrates. The enzyme had a low affinity toward pyruvate but its Vₘₐₓ value was high being 85% of that of L-α-keto-β-methylvalerate. Pyruvate decarboxylase had high Kᵢ values with branched-chain substrates being three to nine times higher than that of pyruvate. The Vₘₐₓ value of α-ketoisovalerate was the highest among three branched-chain substrates and was slightly lower than that of pyruvate.

**Stereospecific Decarboxylation**—The specificity of the two decarboxylases toward the enantiomers of a substrate was studied by an isotopic dilution method. The amount of ¹⁴CO₂ produced from [U-¹⁴C]L-α-keto-β-methylvalerate (0.05 mM), by both enzymes, was measured in the presence or the absence of the same concentration of cold L- or D-α-keto-β-methylvalerate. The results are presented in Table VII. With both enzymes, the addition of cold L-substrate reduced the ¹⁴CO₂ production to half of the control value, but the addition of cold D substrate did not have any effect. This shows that BCKA decarboxylase and pyruvate decarboxylase both exhibit stereospecificity toward the L isomer of α-keto-β-methylvalerate.

**Effect of Ferricyanide on Two Decarboxylases**—Potassium ferricyanide is routinely used for the determination of dehydrogenase activities of α-keto acid dehydrogenase complexes, such as pyruvate, α-ketoglutarate, and α-ketoisovalerate (11). The ferricyanide added works as an oxidant to decarboxylate the α-keto acid substrate to the level which is comparable to the activity of the overall reaction measured in the presence of CoA and NAD. In many cases, the increase is 20 times or more of the control value.

The activities were increased by the addition of ferricyanide at a concentration of 40 mM to the maximum, 3.8 times of the control value for BCKA decarboxylase and 2.1 times for pyruvate decarboxylase. Thus, the effect of ferricyanide on the decarboxylases is not as large as that on the dehydrogenase complexes.

**Component Requirement for Fatty Acid Synthesis**—Table VIII shows the effect of components on the fatty acid synthesis by the fatty acid synthetase system. Malonyl-CoA, thiaminepyrophosphate, and dithiothreitol were essential for the synthetase. The effect of NADPH on fatty acid synthesis was somewhat less than that observed previously. The omission of NADPH from the system has reduced the synthesis to only

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**TABLE VII**

Stereospecific decarboxylation of [U-¹⁴C]L-α-keto-β-methylvalerate by the two decarboxylases

The reaction mixtures contained the same components as described in Table IV, but the radioactive substrate was replaced by 0.05 mM [U-¹⁴C]L-α-keto-β-methylvalerate and its cold D- or L-isomer was added as indicated. The incubation was carried out for 30 min at 30°C.

<table>
<thead>
<tr>
<th>Component</th>
<th>BCKA decarboxylase</th>
<th>Pyruvate decarboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>¹⁴CO₂ produced</td>
<td>Activity</td>
</tr>
<tr>
<td></td>
<td>nmol/min·mg</td>
<td>% nmol/min·mg</td>
</tr>
<tr>
<td>Complete</td>
<td>19.3</td>
<td>100</td>
</tr>
<tr>
<td>Plus cold D-α-Keto-β-methylvalerate</td>
<td>16.0</td>
<td>95</td>
</tr>
<tr>
<td>Plus cold L-α-Keto-β-methylvalerate</td>
<td>10.2</td>
<td>53</td>
</tr>
</tbody>
</table>

a The preparations of D- and L-isomers, prepared previously (11) had been racemized in part during storage at -20°C and had optical purity of 87 and 93%, respectively. Thus, the experimental values were adjusted accordingly.

**TABLE VIII**

Components required for fatty acid synthesis

The complete mixture contained, in mM, 55, triethanolamine-HCl buffer (pH 7.5); 0.6, thiaminepyrophosphate; 11, dithiothreitol; 0.6, NADPH; 0.1, malonyl-CoA; 0.0083, [U-¹³C]L-α-keto-β-methylvalerate, acyl carrier protein (12 μg), and fatty acid synthetase (4.1 mg) in a total volume of 0.45 ml. One component was omitted from or added to the complete mixture where indicated. The incubation was carried out for 30 min at 30°C.

<table>
<thead>
<tr>
<th>Component</th>
<th>Fatty acid synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min·mg %</td>
</tr>
<tr>
<td>Complete</td>
<td>5.23</td>
</tr>
<tr>
<td>Minus thiaminepyrophosphate</td>
<td>0.11</td>
</tr>
<tr>
<td>Minus dithiothreitol</td>
<td>0.45</td>
</tr>
<tr>
<td>Minus NADPH</td>
<td>1.22</td>
</tr>
<tr>
<td>Minus malonyl-CoA</td>
<td>0.17</td>
</tr>
<tr>
<td>Minus acyl carrier protein</td>
<td>5.22</td>
</tr>
<tr>
<td>Plus 2.2 mM MgCl₂</td>
<td>4.21</td>
</tr>
</tbody>
</table>
Branched-chain Fatty Acid Synthetase

5% of the control value (12). This probably is due to a different extraction procedure used in the present study. Thus, a portion of an intermediary product accumulated in the absence of NADPH may be extracted by the present procedure. This was verified by experiments using radioautography. The addition of acyl carrier protein and MgCl₂ did not affect any fatty acid synthesis by the B. subtilis synthetase system. This will be considered later in the discussion.

Identifying Primer Donating System by Chromatographic Fractionation—Branch-chain α-keto acids are incorporated into long-chain fatty acids after their decarboxylation (12). We have isolated two decarboxylases from B. subtilis. Initially the chromatographic approach had been used to identify which of the two decarboxylases is involved in the synthesis of branch-chain fatty acids from α-keto acids.

Fig. 4 shows the chromatogram of fatty acid synthetase fractionated on Mono Q. Two decarboxylase fractions, indicated as B (BCKA) and P (pyruvate), were well separated from each other and kept aside. The remaining fractions were combined, concentrated by ultrafiltration, and designated as basal enzyme.

Fig. 5 shows the separation by SDS-polyacrylamide gel electrophoresis of crude enzyme, basal enzyme, pyruvate decarboxylase, and BCKA decarboxylase preparations. Fatty acid synthetase (column 1), along with basal enzyme (column 2, synthetase minus the two decarboxylases), pyruvate decarboxylase (column 3), and BCKA decarboxylase (column 4) fractions from the Mono Q column were subjected to SDS-polyacrylamide gel electrophoresis on 12% gel and stained with Coomassie Brilliant Blue R-250. a and b indicated at the right side of the figure show the positions of two subunit peptides of pyruvate decarboxylase, whereas b and c correspond to those of BCKA decarboxylase.

**TABLE IX**

Decarboxylase activities of various enzyme fractions

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>CO₂ production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-α-keto-β-methylvalerate</td>
</tr>
<tr>
<td>Crude (0.41 mg)</td>
<td>0.3</td>
</tr>
<tr>
<td>Basal (0.31 mg)</td>
<td>0.1</td>
</tr>
<tr>
<td>BCKA decarboxylase (17 μg)</td>
<td>12.9</td>
</tr>
<tr>
<td>Pyruvate decarboxylase*</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*The amount of pyruvate decarboxylase fraction was 33 μg for L-α-keto-β-methylvalerate decarboxylation and 13 μg for pyruvate decarboxylation.

decarboxylase, respectively, are major ones. The BCKA decarboxylase fraction (lane 4) also gave 10 bands: bands b and c which correspond to peptides B and A of pure BCKA decarboxylase, respectively, are also major ones.

The decarboxylase activities of these four enzyme fractions were determined using L-α-keto-β-methylvalerate and pyruvate as substrates. Two decarboxylase activities, BCKA and

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M. Kojima and T. Kaneda, unpublished observations.
The basal reaction mixture contained, in mM, 55, triethanolamine-HCl buffer (pH 7.5); 0.6, thiaminepyrophosphate; 11, dithiothreitol; 0.6, NADPH; 0.1, malonyl-CoA; 0.0083, [U-14C]-a-keto-\beta\text{-}methylvalerate, acyl carrier protein (12 \mu g), and basal enzyme (3.1 mg) which was prepared from fatty acid synthetase by removing BCKA and pyruvate decarboxylase fractions using chromatographic fractionation on Mono Q. Two decarboxylase fractions thus separated were added back to the basal reaction mixture as indicated in the table. The reaction mixtures were a total volume of 0.45 ml and incubated for 30 min at 30 °C.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Fatty acid synthesis pmol/min.mg</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>3.83</td>
<td>100</td>
</tr>
<tr>
<td>Plus BCKA decarboxylase (47 \mu g)</td>
<td>11.13</td>
<td>291</td>
</tr>
<tr>
<td>Plus pyruvate decarboxylase</td>
<td>4.53</td>
<td>118</td>
</tr>
<tr>
<td>(96 \mu g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus BCKA decarboxylase, pyruvate decarboxylase</td>
<td>11.66</td>
<td>304</td>
</tr>
</tbody>
</table>

**Table X**

**Effect of decarboxylase fractions on the fatty acid synthesis by the basal enzyme system**

The reaction mixtures contained, in mM, 55, triethanolamine-HCl buffer (pH 7.5); 0.6, thiaminepyrophosphate; 11, dithiothreitol and either 0.0083, [U-14C]-a-keto-\beta\text{-}methylvalerate and pure BCKA decarboxylase (2.5 \mu g), or 0.050, [1-14C]pyruvate and pure pyruvate decarboxylase (2.5 \mu g) in a total volume of 0.27 ml. MgCl₂ was added for the pyruvate decarboxylase assay. The preincubation of BCKA decarboxylase with antibody was 15 min at 30 °C whereas the preincubation of pyruvate decarboxylase was 15 min at 0 °C because it was unstable at 30 °C. The reaction was initiated by the addition of radioactive substrate and was carried out for 30 min at 30 °C.

<table>
<thead>
<tr>
<th>Antibody added</th>
<th>BCKA decarboxylase pmol/min.mg</th>
<th>%</th>
<th>Pyruvate decarboxylase pmol/min.mg</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17.9</td>
<td>100</td>
<td>67.3</td>
<td>100</td>
</tr>
<tr>
<td>Preimmune serum (104 \mu g)</td>
<td>17.9</td>
<td>100</td>
<td>65.7</td>
<td>98</td>
</tr>
<tr>
<td>Anti-BCKA decarboxylase (89 \mu g)</td>
<td>0.1</td>
<td>0.6</td>
<td>66.9</td>
<td>99</td>
</tr>
<tr>
<td>Anti-pyruvate decarboxylase (84 \mu g)</td>
<td>18.6</td>
<td>104</td>
<td>10.6</td>
<td>16</td>
</tr>
</tbody>
</table>

**Table XI**

**Effect of the antibody preparations raised against the two decarboxylases on their respective enzymatic activities**

The reaction mixtures contained, in mM, 55, triethanolamine-HCl buffer (pH 7.5); 0.6, thiaminepyrophosphate; 11, dithiothreitol and either 0.0083, [U-14C]-a-keto-\beta\text{-}methylvalerate and pure BCKA decarboxylase (2.5 \mu g), or 0.050, [1-14C]pyruvate and pure pyruvate decarboxylase (2.5 \mu g) in a total volume of 0.27 ml. MgCl₂ was added for the pyruvate decarboxylase assay. The preincubation of BCKA decarboxylase with antibody was 15 min at 30 °C whereas the preincubation of pyruvate decarboxylase was 15 min at 0 °C because it was unstable at 30 °C. The reaction was initiated by the addition of radioactive substrate and was carried out for 30 min at 30 °C.

**Table XII**

**Effect of decarboxylase antibodies on fatty acid synthesis by fatty acid synthetase**

The reaction mixtures were the same as the complete reaction mixture indicated in Table VIII with the exception that one of [U-14C]-\alpha\text{-}keto-\beta\text{-}methylvalerate, \alpha\text{-}ketoisocaproate, or \alpha\text{-}ketoisovalerate was used as substrate. The amount of fatty acid synthetase was 3.3 mg/assay. Antibodies were added to the complete reaction mixture in the absence of U-14C substrate and were incubated for 15 min at 30 °C. The incubation mixtures were centrifuged to remove precipitates. The reaction was initiated by the addition of a U-14C substrate and was carried out for 30 min at 30 °C.

**Table XIII**

**Effect of NAD and CoA on fatty acid synthesis by the crude enzyme system**

The reaction mixtures were the same as the complete reaction mixture indicated in Table VIII but NAD and CoA were included as indicated. The amount of fatty acid synthetase was 2.5 mg/assay. The total volume was 0.27 ml and the incubation was the same as in Table VIII.

<table>
<thead>
<tr>
<th>Component</th>
<th>Fatty acid synthesis pmol/min.mg</th>
<th>%</th>
<th>CO₂ production pmol/min.mg</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>3.47</td>
<td>100</td>
<td>15.1</td>
<td>100</td>
</tr>
<tr>
<td>Plus CoA (0.5 mM)</td>
<td>2.07</td>
<td>60</td>
<td>16.1</td>
<td>106</td>
</tr>
<tr>
<td>Plus NAD (0.5 mM)</td>
<td>0.31</td>
<td>8.8</td>
<td>3.8</td>
<td>25</td>
</tr>
<tr>
<td>Plus CoA, NAD (0.5 mM each)</td>
<td>0.07</td>
<td>2.1</td>
<td>4.5</td>
<td>30</td>
</tr>
</tbody>
</table>

**FIG. 6. Immunoprecipitation of the two decarboxylases by their respective antibodies.** Two decarboxylases, pyruvate (P, 3 \mu g) and BCKA (B, 1.7 \mu g), were placed in the center wells. Wells 1 and 2 contained rabbit antibodies (84 \mu g) raised against pyruvate decarboxylase whereas wells 3 and 4 had rabbit antibodies (89 \mu g) raised against BCKA decarboxylase. Wells 5 and 6 contained serum from the rabbit before immunization with a decarboxylase (89 \mu g for pyruvate and 104 \mu g for BCKA).
was the inhibition of fatty acid synthesis. The antibody of BCKA decarboxylase, provides primers for fatty acid synthesis. The concentration of BCKA decarboxylase, but not pyruvate decarboxylase used at 0°C was somewhat less effective.

**FIG. 7. Effect of NAD and CoA on the decarboxylation of L-α-keto-β-methylvalerate by fatty acid synthetase.** The basic reaction mixtures contained, in mM, 133, potassium phosphate buffer (pH 7.0); 0.7, thiaminepyrophosphate; 8, MgCl₂; 0.6, dithiothreitol; 0.065 to 0.2, [U-¹⁴C]α-keto-β-methylvalerate and fatty acid synthetase (0.42 mg) in a total volume of 0.27 ml. The three curves represent activities of the basic reaction mixtures (○), the basic mixtures to which 0.5 mM NAD was added (O), and the basic mixtures to which 0.5 mM NAD and 0.5 mM CoA were added (△).

**TABLE XIV**

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>¹⁴CO₂ production pmol/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Pure BCKA decarboxylase</td>
<td>38.7</td>
</tr>
<tr>
<td>Fatty acid synthetase</td>
<td>37.3</td>
</tr>
<tr>
<td>Pure BCKA decarboxylase plus fatty acid synthetase</td>
<td>69.0</td>
</tr>
</tbody>
</table>

**Experiment 1**

| Pure pyruvate decarboxylase         | 90.3         | 81.3         |
| Fatty acid synthetase               | 71.7         | 25.3         |
| Pure pyruvate decarboxylase plus fatty acid synthetase | 152          | 45.3         |

The antibody of pyruvate decarboxylase has not been isolated in a homogeneous form (11). This was again looked at using the present enzyme of *B. subtilis* and confirmed. Table XIII shows that the addition of NAD to fatty acid synthetase reduced the fatty acid synthesis by 91% and CO₂ production by 75%. The addition of CoA suppressed the fatty acid synthesis by 40% but CO₂ production was unaffected.

**Inhibition of Decarboxylation by Various Nucleotides**—Since NAD inhibited the decarboxylation of L-α-keto-β-methylvalerate by fatty acid synthetase (Table XIII), a number of other nucleotides were examined for their inhibitory activity. NADH, NADPH, and NADP depressed activity of 2.90, and 90% of the control, respectively. CoA, in addition to AMP, ADP, and ATP, did not show any inhibition.

The inhibition of decarboxylation of L-α-keto-β-methylvalerate by NAD was further studied at varying concentrations up to 200 μM of the substrate (Fig. 7). The inhibitory activity of NAD was severe at low concentrations of substrate (75% inhibition at 10 μM) and was less severe at high concentrations (40% inhibition at 200 μM). This inhibition by NAD was reduced when CoA was present in the reaction mixture in a function of substrate concentrations. At 10 μM of substrate the CoA reduced NAD inhibition by 40%. At 25 μM of substrate the CoA suppressed NAD inhibition nearly completely. At substrate concentrations higher than 25 μM, the CoA decreased the CO₂ production beyond the control value. These results indicate that at low concentrations (5–20 μM) of L-α-keto-β-methylvalerate CO₂ production was mainly catalyzed by BCKA decarboxylase since its *Kₘ* value is very low. At high concentrations (25–200 μM) of the substrate, CO₂ production was largely catalyzed by BCKA dehydrogenase and pyruvate dehydrogenase, which require NAD and CoA for catalytic activity.

**Inhibitory Activity of NAD on Decarboxylases**—Pure decarboxylases were used to elucidate the inhibitory mechanism of NAD on the decarboxylation activity of fatty acid synthetase. In the absence of NAD in the reaction mixtures, the decarboxylation of L-α-keto-β-methylvalerate, determined in the presence of two enzymes (pure BCKA decarboxylase and fatty acid synthetase) was close to the sum of two enzyme activities measured individually (91% of the sum) (Table XIV). When NAD was added, CO₂ production by pure BCKA decarboxylase was unaffected, whereas CO₂ production by fatty acid synthetase was reduced to 24% of the control value. When the decarboxylase activity was measured in the presence of the two enzymes together, NAD inhibited the activity to 22% of the control value. This indicates that fatty acid synthetase contains a compound which becomes an active inhibitor against the decarboxylase only when NAD is present.

Experiment 2 was carried out with pyruvate as substrate. The same conclusion is drawn from the results of pyruvate decarboxylation.

**DISCUSSION**

As reported here, we have detected BCKA decarboxylase and pyruvate decarboxylase in cell-free extracts of *B. subtilis*. These enzymes are very similar in many respects. Both enzymes are composed of two subunits (A and B) in a tetrameric structure (A₂B₂). The molecular weights of the respective subunits and the native enzymes were similar.

A branched-chain α-keto acid dehydrogenase complex has been detected in *B. subtilis* extracts, but it occurs in a small amount and has not been isolated in a homogeneous form (11). The pyruvate dehydrogenase complex of *B. subtilis*, however, has been isolated in a homogeneous form, and its structure and catalytic properties have been studied. Pyruvate synthesis from the related α-keto acid by branched-chain α-keto dehydrogenase (11). This was again looked at using the present enzyme of *B. subtilis* and confirmed. Table XIII shows that the addition of NAD to fatty acid synthetase reduced the fatty acid synthesis by 91% and CO₂ production by 75%. The addition of CoA suppressed the fatty acid synthesis by 40% but CO₂ production was unaffected.

**Inhibition of Fatty Acid Synthesis by NAD and CoA**—NAD and CoA have been shown to inhibit fatty acid synthesis by fatty acid synthetase (12). They are required for acyl-CoA synthesis from the related α-keto acid by branched-chain α-keto dehydrogenase (11). This was again looked at using the present enzyme of *B. subtilis* and confirmed. Table XIII shows that the addition of NAD to fatty acid synthetase reduced the fatty acid synthesis by 91% and CO₂ production by 75%. The addition of CoA suppressed the fatty acid synthesis by 40% but CO₂ production was unaffected.

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dehydrogenase complex is composed of decarboxylase (or dehydrogenase) (E₁), lipote acetyltransferase (E₂), and dihydrolipoamide dehydrogenase (E₃). E₁ was determined indirectly by SDS-polyacrylamide gel electrophoresis of E₁, E₂, and E₃ complexes to be composed of E₁α (Mr, 42,500) and E₁β (Mr, 36,000) (14). These values are essentially identical to the molecular mass of the two polypeptide subunits composed of the pyruvate decarboxylase studied here: B (Mr, 42,000) and A (Mr, 38,000).

It is possible that the BCKA decarboxylase and pyruvate decarboxylase studied here might be artifacts shed from the respective dehydrogenases during the enzyme preparation. To examine this possibility, B. subtilis extracts were prepared by treating cells either with ultrasound which was used routinely or with lysozyme. We found no difference in the yields of both decarboxylases by the two procedures. Thus, this possibility is unlikely although these two decarboxylases and E₁ of the respective dehydrogenases may well be genetically related to one another.

Stereo-specificity is a unique feature of biological reactions catalyzed by enzymes. BCKA decarboxylase exhibited stereospecificity toward the L-isomer of α-keto-β-methylvalerate, which was the best substrate and related to L-isoleucine. BCKA dehydrogenase has been shown to have stereospecificity toward the same enantiomer (11). Pyruvate was the best substrate and related to L-isoleucine. BCKA decarboxylase has been shown to have stereospecificity toward branched-chain α-keto acid substrates. The concentration of α-keto acid substrates used in the present work was 8.3 μM giving approximately 80% of the maximum rate of fatty acid synthesis by B. subtilis (18). This concentration is sufficiently high for BCKA decarboxylase (Kₘ < 1 μM) to give its maximum activity, whereas it is too low for pyruvate decarboxylase (Kₘ = 58.2 μM) to give any significant activity (Table VI). Thus, the very high affinity of BCKA decarboxylase toward branched-chain α-keto acids makes it possible to function as the primer donating system for branched-chain fatty acid synthetase.

Fatty acid synthetases in nature are divided into two groups, one composed of separate individual enzymes (e.g. bacteria and higher plants) and the other composed of a few multifunctional proteins (e.g. yeast and animal) (19-21). B. subtilis fatty acid synthetase belongs to the first group (2), as does E. coli fatty acid synthetase.

The pathway shown in Fig. 8 is proposed for the biosynthesis of branched-chain fatty acids from α-keto acid substrates in B. subtilis. Step A is carried out by BCKA decarboxylase, and an aldehyde derivative rather than an acyl-CoA ester, is the primer for the synthesis. The exact chemical nature of the aldehyde primer remains to be identified, but it would be a thiaminepyrophosphate derivative or a complex with the protein.

In view of energy conservation, use of an aldehyde derivative as the primer in the biosynthesis of branched-chain fatty acid makes more sense than if an acyl-CoA ester were the primer. The former mechanism includes two fewer reactions than the latter mechanism in the fatty acid synthetase, one to yield an acyl-CoA ester from an aldehyde intermediate in the oxidative decarboxylation of an α-keto acid substrate, and the other to reduce the ketonic condensation product synthesized from the acyl primer and the chain extender (malonyl-CoA, see the next paragraph).

The primer, produced by step A, is condensed with the malonyl-CoA provided to the reaction mixture. E. coli fatty acid synthetase has been shown to use malonyl-CoA as the chain extender after it is converted to a malonyl-acyl carrier protein derivative (22-24). We could not detect any effect of acyl carrier protein on fatty acid synthesis by fatty acid synthetase in the present work. The effect, however, has been observed in the case of another strain of B. subtilis having a high synthetase activity if an extra procedure is included during the enzyme preparation to effectively remove more of the acyl carrier protein from the enzyme preparation. Unfortunately, the procedure considerably reduced the activity of the synthetase and was not applicable to the present work. Thus, we believe that step B requires acyl carrier protein and that the actual chain extender is malonyl-acyl carrier protein produced from malonyl-CoA substrate. The preparations of fatty acid synthetase, used in the present work would likely be contaminated with a sufficient amount of acyl carrier.
protein to support fatty acid synthesis. A detailed study concerning the elongation mechanism at step B is in progress.

Acknowledgments—We thank A. S. Green for his technical assistance, J. Bouvier for raising antibody in animals, and Dr. Ken Roy for critical reading of the manuscript.

REFERENCES