Synthesis of Triacetic Acid Lactone by the Pigeon Liver Fatty Acid Synthetase Complex*

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SUMMARY

The synthesis of triacetic acid lactone (TAL) is effected by purified soluble pigeon liver fatty acid synthetase. This enzyme system synthesizes TAL from acetyl coenzyme A and malonyl coenzyme A in the absence of TPNH, and synthesizes palmitic acid in the presence of TPNH. The major product of the reaction in the absence of TPNH is TAL, and not free triacetic acid. Presumably triacetic acid, bound as a thioester to the 4'-phosphopantetheine prosthetic group of the fatty acid synthetase, is an intermediate in the formation of TAL. The latter compound is formed from 1 mole of acetyl-CoA and 2 moles of malonyl-CoA in the sequence, acetate-malonate-malonate (proceeding left to right from the methyl end of the molecule). The synthesis of TAL is inhibited by either TPN+ or TPNH. The very strong inhibition by both forms of the nucleotide implies that TAL is not synthesized in vivo by the avian soluble fatty acid synthetase.

Lynen (1) concluded in 1961, from a wide variety of data, that the specific arrangement of enzyme activities within the fatty acid synthetase complex determined whether fatty acids, aromatic compounds, or macrolide structures would be formed from malonyl coenzyme A and TPNH in the presence of a suitable "primer," such as acetyl-CoA. Lynen (1) also presented a hypothetical reaction scheme for 6-methylsalicylic acid synthesis by a multienzyme complex from Penicillium patulum. This scheme utilized malonyl-CoA, acetyl-CoA, and TPNH as reactants, and involved enzyme-bound triacetic acid as an intermediate.

Previous investigations in this laboratory (2, 3) established

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the synthesis of TAA1 or its lactone form, triacetic acid lactone, by a partially purified pigeon liver fatty acid synthetase. It was also shown that TAL synthesis involved enzyme-bound acetate, malonate, and acetoacetate as intermediates, and that the omission of TPNH was necessary for the synthesis of product. Later, Hsu, Wasson, and Porter (4) found that purified pigeon liver fatty acid synthetase formed only small amounts of TAA when the product was assayed by paper chromatography. It was concluded, therefore, that activity for the formation of TAA could be attributed either to the presence of a small amount of enzyme impurity which remained with the fatty acid synthetase throughout purification or to an integral part of the enzyme complex which had an activity much lower than that for fatty acid synthesis.

Brock and Bloch (5) also reported that a crude Escherichia coli fatty acid synthetase system reverted from fatty acid synthesis to TAL synthesis when the system was TPNH deficient. The ratio of fatty acids to TAL synthesized was controlled by the concentration of TPNH in the incubation system. Thus, when a TPNH-generating system was present long chain fatty acids were the major product, but when a TPNH-depleting system was present the reverse was found.

This paper presents further data on the synthesis of TAL by the pigeon liver fatty acid synthetase. It is shown that the activity for TAL synthesis remains with the pigeon liver fatty acid synthetase upon purification and that this activity is an integral part of the fatty acid synthetase complex. Information on the mechanism of synthesis of TAL and the effects of TPN+ and TPNH on the synthesis of this compound is also presented.

EXPERIMENTAL PROCEDURE

Materials

1-14C-Acetic anhydride and 2-14C-malonic acid were obtained from New England Nuclear, and coenzyme A, TPN+, and TPNH were purchased from P-L Biochemicals, Milwaukee. Dehydroacetic acid was provided by K and K Laboratories, New York.

Malonyl-CoA was prepared by the method of Trams and

1 The abbreviations used are: TAA, triacetic acid; TAL, triacetic acid lactone.
TAA was synthesized by the method of Harris and Harris (10). Each coenzyme A ester was purified as reported previously (8).

Preparation of Pigeon Liver Fatty Acid Synthetase

Pigeon liver fatty acid synthetase was purified by the procedure of Nau, Worek, and Porter (4) as modified by Butterworth et al. (11). Pigeon liver was homogenized in phosphate-bicarbonate buffer, pH 8.0, and then fractionated by differential centrifugation. The soluble protein (100,000 \times g supernatant solution) that precipitated between 25% and 40% of saturation with ammonium sulfate was treated with calcium phosphate gel (12). Protein not adsorbed when mixed with the gel in 0.005 M potassium phosphate buffer, pH 7.0, in a ratio of 2:1, protein to gel, was chromatographed on a DEAE-cellulose column. The protein was added to the column in 0.04 M potassium phosphate buffer, pH 7.0, and the desired fraction was eluted with 0.25 M potassium phosphate buffer. Protein in this fraction was precipitated between 26% and 32% of saturation with ammonium sulfate and then subjected to Sephadex G-100 gel filtration. The protein collected in the void volume from the Sephadex column was used routinely for assays for TAL synthesis.

When the first ammonium sulfate fraction and the calcium phosphate gel fraction were assayed for enzyme activity the phosphate concentration was immediately increased to 0.1 M, since the fatty acid synthetase is unstable at low ionic strength (4). Enzyme solutions were diluted before assay with 0.20 M potassium phosphate buffer, pH 7.0, and 1 mM EDTA and 1 mM dithiothreitol. Protein was determined by the biuret method of Gornall, Bardawill, and David (13).

Assays for TAL Synthesis

Spectrophotometric Assay—Most spectrophotometric assays were performed with a Beckman DU monochromator coupled to a Gilford model 200 optical density converter and a 10-mv Leeds-Northrup recorder equipped with an adjustable zero and a multiplexed chart drive. The recorder was run at chart speeds of 1 to 2 inches per min, and it was calibrated so that a full scale deflection of 10 inches represented an optical density change of 0.10 unit. Other spectrophotometric assays were performed with a Zeiss spectrophotometer coupled to a Gilford optical density converter and a Leeds-Northrup recorder. The recorder ran at a chart speed of 1 inch per min, and it was calibrated so that a full scale deflection of 0.1 inches represented an optical density change of 0.25 unit. Measurements were made in a 1-ml cuvette with a 1-cm light path at a wave length of 280 mp.

Incubation mixtures contained potassium phosphate buffer, pH 7.0, 100 \( \text{mM} \) EDTA, 3 \( \text{mM} \) acetyl-CoA, malonyl-CoA, and enzyme, at concentrations specified in the legends to the figures, in a final volume of 1 ml. Reaction mixtures were incubated at 30° or 38°.

Radiochemical Assay—Potassium phosphate buffer, pH 7.0, EDTA, pH 7.0, \( \text{C}^4\text{H}_8\text{H}_9\text{O}_2\text{C}^2\text{H}_5\text{OH} \), 40-60% acetyl-CoA, and nonradioactive malonyl-CoA were incubated with enzyme at the concentrations specified in the legends to the figures. The reactions (performed in ground glass-stoppered extraction tubes, 1.5 × 15 cm) were started by the addition of enzyme.

Extraction

The extraction procedure varied with the chemical form of the product assayed. Generally, the reaction was stopped by the addition of 0.25 ml of 1 N HCl. Carrier TAL (200 \( \mu \text{g} \)) was added and the incubation mixture was saturated with anhydrous sodium sulfate. TAL was then extracted with eight 2.5-ml aliquots of diethyl ether.

A modified procedure was used to determine the relative amounts of TAL, TAA, and esterified TAA formed in an incubation mixture. The initial extractions were effected at the pH of the incubation mixture (pH 7.0). The incubation was terminated by adding 2.5 ml of diethyl ether to the sample and the mixture was then agitated in a Vortex mixer. After carrier TAL and anhydrous sodium sulfate were added, eight extractions, each with 2.5 ml of diethyl ether, were made. The residual solution was then again extracted with diethyl ether, after acidification to pH 1.0 and addition of carrier TAA. Next, thiosteres of TAA were hydrolyzed. The residual solution was adjusted to 0.15 N in KOH and heated at 70° for 1 hour. The solution was again acidified, carrier TAA was added, and the liberated TAA was extracted with diethyl ether. Each ether extract was assayed by gas-liquid chromatography for TAL and TAA. The values obtained for each of these products in the first two extractions (extracts from neutral and acidified solutions) were added together to give total TAA and total TAL.

Assay of TAL by Gas Liquid Chromatography

The TAL-containing ether extract was evaporated to dryness under a stream of nitrogen and the residue was methylated with a minimum of ethereal diazomethane (14) (final volume, 0.4 to 0.5 ml). Approximately 50 \( \mu \text{l} \) of this solution were measured accurately in a 100-\( \mu \text{l} \) microsyringe. Then 20 \( \mu \)l were put directly into dioxane phosphor (15) for assay for radioactivity, and the solution remaining in the syringe was subjected to gas-liquid chromatography. The 20-\( \mu \)l aliquot was used to calculate the radioactivity subjected to chromatography and the total radioactivity in the methylated TAL solution.

Samples were chromatographed on a Barber-Colman model 10 gas chromatograph, containing a column of 28% butanediol succinate polyester (Wilkens Instruments and Research, Inc., Walnut Creek, California) on Chromosorb W (60 to 80 mesh), at a temperature of 200° and an argon gas flow rate of 100 ml per min. Retention times of the two enol methyl ethers of TAL (16) under these conditions were approximately 14 and 20 min. Radioactive material emerging from the column was trapped in S-shaped glass tubes, containing glass wool soaked in diethyl ether, in a Dry Ice-acetone bath. The material was eluted from the glass tubes into counting vials with 14 ml of dioxane phosphor and assayed for radioactivity. Total radioactivity in TAL was calculated by multiplying the total radioactivity in the methylated TAL solution by the percentage of the radioactivity put on the gas-liquid chromatograph that emerged from the column coincident with authentic carrier TAL.

The methylated ether extract was also chromatographed on a series 5000 Selecta-System Barber-Colman gas chromatograph, which measures both mass and radioactivity, to show that TAL is the major radioactive compound in the ether extract. The sample was chromatographed on the same column under the
same conditions as those used for manual trapping of radioactivity.

**Assay for TAA**

TAA was assayed as the methyl ester by the same gas-liquid chromatographic procedure used to assay for TAL. The retention time for methylated TAA was approximately 5 min under the conditions used in these assays.

**Assay for Palmitic Acid**

Palmitic acid and TAL were assayed in the same incubation mixture by the gas-liquid chromatographic method. Carrier palmitic acid was added with carrier TAL to the acidified reaction mixture and then the two compounds were extracted, methylated, and chromatographed. Radioactivity that emerged from the column coincident with carrier methyl palmitate was trapped by the same procedure used to trap TAL. The retention time of methyl palmitate was approximately 10 min.

**Degradation of TAL**

TAL was degraded to acetylacetone and carbon dioxide as described by Witter, Snyder, and Stotsz (18). 14C-TAL (5 or 6 mg) was dissolved in 14 ml of water and 1 ml of concentrated H2SO4 and then decarboxylated at 170° in a distillation apparatus connected to three carbon dioxide traps. Acetylacetone was collected in 10 to 12 ml of distillate, and carbon dioxide was trapped in 45 ml of 1 N NaOH. Nitrogen, introduced via a side arm in the reaction flask, was bubbled into the reaction mixture to carry the carbon dioxide to the sodium hydroxide solution.

The quantity of acetylacetone in the distillate was determined by the 0-phenylenediamine assay of Witter, Snyder, and Stotsz (18), and the radioactivity in acetylacetone was measured by the gas-liquid chromatographic method.

**Kuhn-Roth Oxidation of Methyl Ether of TAL**

The methyl ether of TAL (15 mg) was refluxed in 5 ml of oxidizing mixture (20) for 14 hours at 130° C. Acetic acid was distilled from the reflux mixture and then titrated with 0.01 N NaOH to determine the yield. An aliquot of the distillate was assayed for radioactivity in dioxane phosphor.

**Preparation of Iodoform from Acetylacetone Derived by Decarboxylation of TAL**

The solution of acetylacetone (in a conical centrifuge tube) was adjusted to an NaOH concentration of 2.5% by the addition of 10% NaOH. Iodine solution (75 g of KI and 17.5 g of I2 in 100 ml of water) was added dropwise until a brown color persisted. After 30 min the precipitated iodoform was collected by centrifugation and then washed twice with water. The iodoform was assayed for radioactivity in toluene phosphor (4 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 2,5-bis-(5-tert-butylbenzoxazolyl)-thiophene in 1 liter of toluene).

**RESULTS**

**Parallel Purification of Enzyme Activities for Fatty Acid and TAL Synthesis—Examination of the protein fractions at each step in the purification of soluble pigeon liver fatty acid synthetase for activity for the synthesis of TAL, as well as fatty acids, showed that those activities remained together during purification (Table I). Both activities exhibited a parallel decrease in specific activity at the calcium phosphate gel precipitation step and then parallel increases in activity through the remainder of the steps. The fatty acid synthetase was purified approximately 12-fold, as compared to 15-fold for the activity for TAL synthesis. However, the specific activity of the fatty acid synthetase for fatty acid synthesis was 4 times higher than the activity for TAL synthesis.**

**TABLE I**

<table>
<thead>
<tr>
<th>Purification step and activity assayed</th>
<th>Total fatty acid activity</th>
<th>Total TAL activity</th>
<th>Yield</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant solution (100,000 x g)</td>
<td>2.020</td>
<td>10.1</td>
<td>20,400</td>
<td>100</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>2.020</td>
<td>10.1</td>
<td>20,400</td>
<td>100</td>
</tr>
<tr>
<td>TAL</td>
<td>1.9</td>
<td>3,840</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate (25-40%)</td>
<td>17.6</td>
<td>11,600</td>
<td>57</td>
<td>1.7</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>660</td>
<td>2,310</td>
<td>60</td>
<td>1.8</td>
</tr>
<tr>
<td>TAL</td>
<td>3.5</td>
<td>2,310</td>
<td>60</td>
<td>1.8</td>
</tr>
<tr>
<td>Calcium phosphate gel</td>
<td>420</td>
<td>12.6</td>
<td>5,290</td>
<td>26</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>420</td>
<td>12.6</td>
<td>5,290</td>
<td>26</td>
</tr>
<tr>
<td>TAL</td>
<td>1.7</td>
<td>710</td>
<td>18</td>
<td>0.9</td>
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<tr>
<td>DEAE-cellulose</td>
<td>41</td>
<td>64.4</td>
<td>2,640</td>
<td>13</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>41</td>
<td>64.4</td>
<td>2,640</td>
<td>13</td>
</tr>
<tr>
<td>TAL</td>
<td>17.0</td>
<td>700</td>
<td>18</td>
<td>8.9</td>
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<tr>
<td>Sephadex G-100</td>
<td>25</td>
<td>120.2</td>
<td>3,000</td>
<td>15</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>25</td>
<td>120.2</td>
<td>3,000</td>
<td>15</td>
</tr>
<tr>
<td>TAL</td>
<td>20.4</td>
<td>705</td>
<td>19</td>
<td>15.4</td>
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</table>
 Dependence of TAL Synthesis on Protein Concentration and Time—The synthesis of TAL increased linearly with protein concentration (Fig. 1). However, there was a tendency toward an increased rate of TAL synthesis (upward curvature) as the protein concentration was increased, if the protein was not dialyzed. The formation of TAL was also linear with respect to protein concentration. The enzyme protein, in a final volume of 1.5 ml. The mixture was incubated at 38°C for 15 min. TAL was then extracted and the extract was methylated with diazomethane. Approximately 100 µg of authentic methylated TAL and 20,000 cpm of radioactivity were added to the chromatographic column in 50 µl of ether. The column and the conditions of chromatography are described in the text. The detector response during the first 2 min of chromatographic separation was damped to eliminate the off scale deflection caused by the solvent front. Mass Peak A is 6 methyl 4 methoxy-α-pyrene, and Mass Peak B is 6-methyl-2-methoxy-γ-pyrene.

Table II

<table>
<thead>
<tr>
<th>Product</th>
<th>1-14C-Acetyl-CoA incorporated %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacetone lactone</td>
<td>37.1</td>
</tr>
<tr>
<td>Triacetone acid</td>
<td>1.3</td>
</tr>
<tr>
<td>Esterified triacetone acid</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Identification of Ether-extractable Radioactivity—When the methylated, ether-extractable material obtained from an incubation mixture was chromatographed on the series 5000 Selecta-System Barber-Colman gas-liquid chromatograph, most of the radioactivity emerged coincident with authentic methylated carrier TAL (Fig. 2). This result shows that TAL is the major compound formed by the pigeon liver fatty acid synthetase from labeled acetyl- and malonyl-CoA in the absence of TPNH.

The authenticity of carrier TAL was confirmed by several methods. Chemically synthesized TAL (9), which had been recrystallized several times from water and from acetone, had a melting point of 187-188°C, the range reported in the literature (9, 16, 21). The ultraviolet light absorption data on TAL, λmax 283 μm in ethanol (log ε 3.84), were also in close agreement with published values (21, 22). Methylation of the chemically synthesized TAL with ethereal diazomethane (14) resulted in a product which yielded two mass peaks on gas-liquid chromatography (Fig. 2), which correspond to the two methoxy-aryl derivatives, 6-methyl-4-methoxy-α-pyrene and 6-methyl-2-methoxy-γ-pyrene (16), formed by this compound. The infrared spectrum of methylated TAL showed an absorption band at 5.8 to 5.85 μ (attributed to the lactone carbonyl group) and another band at 7.9 to 8.0 μ (for the methoxy group). The nuclear magnetic resonance spectrum of TAL in (CD)3SO yielded δ 4.05 (multiplet), 4.76 (J = 2 cpm), and 7.84 (d, J = 3 cpm) (22).

Assays for TAL, TAA, and Esterified TAA—Gas-liquid chromatographic assays of the methylated ether extract of a neutral incubation mixture showed the presence of both TAL and TAA. However, over 97% of the sum of the quantities of these compounds was TAL. Acidification and extraction of the residual reaction mixture with diethyl ether removed the remaining free TAL and TAA. Esterified TAA was then extracted after saponification and acidification of the residual solution. Esterified TAA represented less than 1% of the total product. The quantities of TAL and TAA were 96% and 3%, respectively, of the total products (Table II).

Thus, the lactone of TAA is the major product of the reaction. However, values for free TAA and esterified TAA may be low because TAA is not stable and may be partially destroyed during the isolation procedure. In our experiments TAA was not lactonized to TAL when extraction was performed at neutral...
pH or on acidification of the reaction mixture. These results are in agreement with reports that TAA is not readily converted to TAL (23, 24).

**Effect of TPNH on Synthesis of TAL and Palmitic Acid**—In a complete fatty acid-synthesizing system, the major product is palmitic acid. Only a small amount of TAL is formed. As the level of TPNH is reduced in the system, the synthesis of palmitic acid is decreased and then TAL synthesis is elevated (Fig. 3). However, TAL formation under optimum conditions of no TPNH is only one-fourth the rate of fatty acid synthesis under conditions that were similar except for the presence of TPNH (Table I).

**Effects of TPN+ and TPNH on Synthesis of TAL**—Fig. 4 shows that TPN+ is just as effective as TPNH as an inhibitor of TAL synthesis. This result and the fact that TAL synthesis is inhibited before palmitic acid synthesis is elevated (see Fig. 3) suggest that TPNH does not inhibit TAL synthesis solely by converting substrates to fatty acids at the expense of TAL synthesis.

Under the conditions of incubation reported for Fig. 4, the fatty acid synthetase synthesizes fatty acids from malonyl-CoA and acetyl-CoA in the presence of TPNH. In the presence of 100 mmoles of TPN+ and no TPNH, only a negligible amount of fatty acids was formed. This result shows that endogenous reduction of TPN+ does not occur and is therefore not responsible for the inhibition. Instead, TPN+ and TPNH must affect the synthesis of TAL in a different way, possibly by changing the conformation of the enzyme.

**Kinetic Assays**—Various kinetic parameters for TAL synthesis were determined from initial velocity studies. The initial velocity of TAL synthesis was calculated from the slope of tracings obtained by the continuous spectrophotometric assay described under "Methods." The effect of substrate concentration (acetyl-CoA and malonyl-CoA) and inhibitor concentration (TPN+ and TPNH) on initial velocity was evaluated from double reciprocal plots of initial velocity versus varying concentrations of one substrate at four fixed concentrations of the other substrate or the inhibitor. After inspection of the resulting patterns, the data were fitted to an appropriate rate equation, using the least squares method and assuming equal variance for the velocities (25). All fits were made by a digital computer with the use of Fortran programs which provide values of kinetic parameters and standard errors of their estimates (26).

Initial velocities were determined for a series of reactions in which the malonyl-CoA concentration was varied over a range from 6.4 to 32.0 μM at fixed acetyl-CoA concentrations of 8, 12, 20, and 40 μM. These data were then fitted to Rate Equation 1, which describes the initial velocities expected from a ping-pong mechanism (27) accompanied by competitive substrate inhibition by one of the substrates (acetyl-CoA). Michaelis constants for malonyl-CoA (Km) and acetyl-CoA (Ks) were 3.2 ± 1.6 μM and 4.9 ± 2.5 μM, respectively. The inhibitor constant (Ki) for acetyl-CoA was 4.2 ± 3.0 μM.

\[
\frac{v}{v_{max}} = \frac{YAB}{K_m B + K_s A + AB}
\]

The nature of the TPN+ and TPNH inhibition of TAL synthesis was evaluated kinetically. The effects of 0, 0.4, 0.8, and 1.25 μM concentrations of TPN+ or TPNH on initial velocities were determined for a series of reactions in which the malonyl-CoA concentration was varied from 8 to 40 μM and the acetyl-CoA concentration was held constant at 20 μM. The data were fitted to Rate Equation 2, which describes uncompetitive inhibition (TPN+ as inhibitor). Ks (intercept) for TPN+ was 0.37 ± 0.03 μM.
TPNH was a noncompetitive inhibitor of malonyl-CoA, and the data were fitted to Rate Equation 3. \( K_G \) (intercept) and \( K_a \) (slope) for TPNH were 0.50 \pm 0.13 \mu M and 0.72 \pm 0.26 \mu M, respectively.

\[
v = \frac{VA}{K + A(1 + I/K_a)}
\]

Although the standard errors of the estimates for these kinetic constants were relatively large, there was no question concerning the identity of the kinetic patterns.

Degradation of TAL—\( ^{14}C \)-Labeled TAL was synthesized enzymatically from \( ^{14}C \)-acetyl-CoA, \( ^{1}3^{14}C \)-malonyl-CoA, or \( ^2^{14}C \)-malonyl CoA and then chemically degraded (Fig. 5) to determine the sequence and molar amounts of acetate and malonate in this compound. The \( ^{14}C \)-TAL was synthesized in 3-ml incubation mixtures which contained 150 mmoles each of acetyl- and malonyl-CoA and 500 \mu g of protein. The resultant \( ^{14}C \)-TAL was extracted with diethyl ether, diluted with 50 mg of nonradioactive TAL, and then recrystallized twice from acetone/trimethyl. The specific radioactivity of TAL from each of the three labeled substrates is given in Table III.

The possible positions of the radioactive label from either \( ^{14}C \)-carboxyl-labeled malonate or acetate in TAL are carbon atoms 2, 4, and 6 of the pyrone ring (Fig. 5). Treatment of TAL with acid as described under "Methods" yields acetylacetone, which contains carbon atoms 4 and 6, and \( CO_2 \), which is derived from carbon atom 2 of the pyrone ring. When \( ^{14}C \)-TAL synthesized from \( ^1^{14}C \)-malonyl-CoA was degraded with acid, radioactivity was distributed equally between the acetylacetone and the \( CO_2 \) (Table III). The specific activity (disintegrations per min per \mu mole) of the acetylacetone was approximately half of the specific activity of the original TAL. In contrast, acid degradation of \( ^{14}C \)-TAL synthesized from \( ^1^{14}C \)-acetyl-CoA produced acetylacetone which contained nearly all of the added radioactivity. This product had a specific activity equal to that of the original TAL. These results show that TAL must provide carbon atoms 2 and 3 and either carbon atoms 4 and 5 or 6 and 7 of the TAL pyrone structure. Acetate must then provide either carbon atoms 4 and 5 or 6 and 7. Thus, it can be concluded from these data that the sequence of acetate and malonate in TAL is either acetate-malonate-malonate or malonate-acetate-malonate (from left to right from the methyl end of the molecule).

Kuhn-Roth oxidation was performed on the methyl ether of TAL to determine which substrate provided the 2-carbon unit at the methyl end of TAL. The Kuhn-Roth oxidation liberates carbon atoms 6 and 7 of the pyrone ring as acetic acid. Acetic acid derived from TAL that had been synthesized from \( ^1^{14}C \)-acetyl-CoA had a specific activity approximately equal to that of the degraded TAL (Table III). In contrast, acetic acid from TAL labeled with \( ^2^{14}C \)-malonyl-CoA contained a negligible amount of radioactivity. Thus, acetate must provide carbon atoms 6 and 7 of the TAL pyrone ring and, by deduction, malonate provides carbon atoms 4 and 5.

Formation of iodoform was used to verify that malonate provided the middle 2-carbon unit of TAL. When acetylacetone is treated with sodium hypoiodite, iodoform is formed from the methylene carbon atom (28). Thus, if iodoform is prepared

\[
\text{TABLE III}
\]

Degradation of triacetic acid lactone

<table>
<thead>
<tr>
<th>Labeled substrate</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trisacetic acid lactone</td>
<td>Acetylacetone</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td></td>
<td>dpm/\mu mole</td>
<td>\mu moles</td>
<td>dpm</td>
</tr>
<tr>
<td>( ^{14}C )-Acetyl-CoA</td>
<td>1,320</td>
<td>46.9</td>
<td>65,000</td>
</tr>
<tr>
<td>( ^{1}3^{14}C )-Malonyl-CoA</td>
<td>1,410</td>
<td>41.9</td>
<td>34,200</td>
</tr>
<tr>
<td>( ^{2}^{14}C )-Malonyl-CoA</td>
<td>1,660</td>
<td>58.2</td>
<td>69,600</td>
</tr>
</tbody>
</table>

a The recovery of acetylacetone was 88% of the theoretical.

b The presence of Ba(OH)\(_2\) in the BaCO\(_3\) precipitate prevented an accurate determination of the specific activity of the carbon dioxide.

c Iodoform was prepared from the acetylacetone in Experiment 1.

d Acetic acid was recovered from Kuhn-Roth oxidation performed on the methyl ether of TAL. The recovery of acetic acid was 75% of the theoretical.
from acetylacetone derived from the acid degradation of TAL, it would arise from carbon atom 5 of the pyrone ring. If carbon atoms 4 and 5 of the TAL pyrone ring are provided by malonate, then the isoform obtained from acetylacetone derived from TAL labeled with $^{14}$C-malonate-CoA should have a specific activity half that of the degraded TAL. This result was obtained on assay of isoform prepared from $^{14}$C-TAL synthesized from 2-$^{14}$C-malonate-CoA (Table III). Thus, the degradation experiments show that TAL is composed of acetate and malonate in the sequence, acetate-malonate-malonate (from left to right from the methyl end of the molecule).

**Discussion**

Long chain fatty acids are synthesized via enzyme-bound intermediates from acetyl- and malonyl-CoA and TPNH by the soluble pigeon liver fatty acid synthetase complex (3, 4). Injection of TPNH from the system results in the formation of TAL instead of long chain fatty acids (3, 5). The latter result suggests that acetoacetyl-enzyme is the branch point for the synthesis of TAL or fatty acids, depending on the presence or absence of TPNH. Further evidence that the activity for synthesis of TAL is an integral part of the fatty acid synthetase complex is the finding that this activity remains with that for fatty acid synthesis through several purification steps, to a protein which is homogeneous by several physical criteria (4, 11). In view of this finding and the fact that the specific activity of the purified synthetase complex for TAL synthesis is one-fourth the specific activity for long chain fatty acid synthesis, it appears unlikely that the TAL-synthesizing activity is a contaminant of this complex.

TAL, not TAA, is the major product synthesized by the pigeon liver fatty acid synthetase in the absence of TPNH. In this reaction TAA, bound as a thioester to 4'-phosphopantetheine intermediate in 6-methylsalicylic acid synthesis by P. patulum (22, 29), and a proposal that enzyme-bound TAA is an inducer of 6-methylsalicylic acid synthesis by P. patulum (22) have been suggested. Attempts to incorporate labeled TAL and TAA into 6-methylsalicylic acid with P. patulum and *Penicillium griseofulvum* have been made by Light, Harris, and Hendrickson (24, 25). Bentley and Zwitkowits (22) also studied the utilization of TAA and other polyketide lactones in tropolone formation by *Penicillium stipitatum*. Both of the latter groups concluded that TAA is the major product synthesized by the synthetase complex. Likewise, with the pigeon liver fatty acid synthetase system, TAL formation decreased and then palmitic acid formation increased as the concentration of TPNH was increased in the incubation mixture. However, TAL synthesis is inhibited by both TPNH and TPNH; and kinetic inhibition studies show that TPNH and TPNH inhibit differentially, thus eliminating the


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possibility that TPN⁺ inhibition was the result of endogenous reduction to TPNH.

Since the combined concentrations of these nucleotides in the cell should be higher than the concentrations required for effective inhibition, TAL synthesis probably does not occur in mammalian or avian systems under physiological conditions. A question is then raised as to the reason for the presence of this enzyme activity. A possible answer is that the enzyme for the inhibition, TAL synthesis probably does not occur in mammalian or avian systems under physiological conditions. A question is then raised as to the reason for the presence of this enzyme activity. A possible answer is that the enzyme for the inhibition, TAL synthesis probably does not occur in mammalian or avian systems under physiological conditions.

Since the fatty acid synthetase complex catalyzes the synthesis of both fatty acids and TAL, the same active sites are probably used in the synthesis of both products. Therefore, even though TAL synthesis may not be physiologically important, the study of the mechanism of TAL synthesis should help to elucidate the mechanism of fatty acid synthesis.

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Synthesis of Triacetic Acid Lactone by the Pigeon Liver Fatty Acid Synthetase Complex

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