Fatty Acid Synthetase

A STEADY STATE KINETIC ANALYSIS OF THE REACTION CATALYZED BY THE ENZYME FROM PIGEON LIVER*

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The kinetic mechanism of pigeon liver fatty acid synthetase action has been studied using steady state kinetic analysis. Initial velocity studies are consistent with an earlier suggestion that the enzyme catalyzes this reaction by a seven-site ping-pong mechanism. Although the range of substrate concentrations that could be used was limited by several factors, the initial velocity patterns showing the relationship between the substrates acetyl coenzyme CoA, malonyl-CoA, and NADPH appear to be a series of parallel lines, regardless of which substrate is varied at fixed levels of a second substrate. However, two of the substrates, acetyl-CoA and malonyl-CoA, apparently exhibit a competitive substrate inhibition with respect to each other, but NADPH shows no inhibition of any kind. Product inhibition patterns suggest that free CoA is competitive versus acetyl-CoA and malonyl-CoA and is uncompetitive versus NADPH, and that NADP+ is competitive versus NADPH and uncompetitive versus acetyl-CoA or malonyl-CoA. These results are consistent with a seven-site ping-pong mechanism with intermediates covalently bound to 4'-phosphopantetheine (part of acyl carrier protein). Double competitive substrate inhibition by acetyl-CoA and malonyl-CoA is consistent with the rate equation derived for the overall mechanism. The kinetic mechanism developed from these results is capable of explaining the formation of fatty acids from malonyl-CoA and NADPH alone (Katiyar, S. S., Briedis, A. V., and Porter, J. W. (1974) Arch. Biochem. Biophys. 162, 412-420) and also the formation of triacetic acid lactone from either malonyl-CoA alone or acetyl-CoA plus malonyl-CoA.

The fatty acid synthetase from pigeon liver is a multienzyme complex which catalyzes the synthesis of long chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH. This reaction is initiated through the covalent binding of acetyl and malonyl groups to the enzyme (1). Subsequently these components are converted, in the presence of NADPH, to palmitic acid. The complete sequence of reactions involves the formation of a series of longer chain acyl intermediates which are bound covalently to sulphydryl binding sites on the enzyme (2). The stoichiometry for the over-all reaction in the formation of fatty acids is the following:

\[
\begin{align*}
\text{CH}_3\text{C-S-CoA} + 7 \text{ NADPH} + 14 \text{ H}^+ &\rightarrow \\
\text{CH}_3\text{(CH}_2\text{C-S})_n\text{COOH} + 8 \text{ CoASH} + 14 \text{ NADP}^+ + 7 \text{ CO}_2 + 6 \text{ H}_2\text{O} 
\end{align*}
\]

(1)

Integrated mechanisms for fatty acid synthesis by pigeon liver and yeast enzyme systems have been proposed by Porter and colleagues (3) and by Lynen (4), respectively. These mechanisms consist of a number of sequential reactions in which the intermediates are bound to the 4'-phosphopantetheine prosthetic group of the enzyme. Supporting evidence for these mechanisms has been obtained through tracer and enzymological studies. A further investigation of the reactions of fatty acid synthesis by kinetic methods of analysis, which might corroborate the proposed mechanism, has not been carried out on the fatty acid synthetase complexes isolated from pigeon or rat liver (5, 6) and yeast (7). Apparently this lack of investigation has been due to the complexity of the experimental and theoretical approaches. However, the kinetic mechanism of fatty acid synthetase action is of particular interest because this enzyme catalyzes a multisite reaction involving three substrates in the forward direction and four in the reverse direction.

The steady state kinetics of enzyme-catalyzed reactions involving three substrates has been developed and reviewed by Frieden (8), Cleland (9), and Keleti and Batke (10, 11). Recently Dalziel (12) carried out a systematic and comprehensive treatment of the analysis and interpretation of initial rate data for ter-reactant enzymes. However, each of these analyses is valid for enzymes that have only one active site operative at...
a time. The biotin-containing enzymes, like transcarboxylase (13) and pyruvate carboxylase (14, 15), have been shown to have two-site ping-pong mechanisms. The rate equations for such mechanisms have been derived by assuming that the reaction at each site between biotin or carboxybiotin and the other reactants is random sequential. Pyruvic dehydrogenase (16) has a three-site ping-pong mechanism in which lipoic acid is the bound carrier. Because two of the three sites have ping-pong rather than random sequential mechanisms, the derivation of rate equations needed a different approach as recently shown by Cleland (17).

Fatty acid synthetase contains a bound 4'-phosphopantetheine prosthetic group which transports the intermediates to various catalytic sites on the enzyme. This enzyme apparently also has seven sites at which the different reactions leading to the synthesis of fatty acids occur. In the present paper we report kinetic evidence that reactions at more than two of these sites are ping-pong. For such a complex reaction, standard ping-pong theory obviously has serious limitations. Hence, the recent method of Cleland (17) has been used to interpret what may be called a seven-site ping-pong mechanism. The present paper reports initial velocity and product inhibition studies which confirm this proposed kinetic mechanism.

**EXPERIMENTAL PROCEDURE**

**Materials**

Acetyl-CoA and malonyl-CoA were obtained from P-L Biochemicals, and NADPH, NADF-*, and coenzyme A were purchased from Sigma. Dithiothreitol was a product of Calbiochem. Other chemicals were obtained as follows: KHPO₄ from Mallinckrodt, EDTA from Fisher, and KH₂PO₄ from Matheson Coleman and Bell. All other inorganic reagents were of analytical grade. Deionized glass-distilled water was used for all of the experiments.

**Apparatus**

All spectrophotometric assays were carried out with a Gilford recording spectrophotometer, model 2400-S. This spectrophotometer was equipped with a thermostated 5-cm cuvette compartment and a recorder with an adjustable zero and a multispeed chart drive. The temperature of each cell compartment was maintained at 25° by circulating water from an external "Haake" bath. The light absorbances of the samples having concentrations of NADPH lower than 40 μM were measured in cuvettes of 5-cm path length. All absorption spectra were recorded with a Beckman DK2A ratio recording spectrophotometer. For other spectral measurements, a Gilford model 240 spectrophotometer was used. A radiometer pH meter, model 51, was used for pH measurements of buffers and other solutions; Fisher buffer was used for pH 7.0 was used as the standard.

**Methods**

**Enzyme Purification**—Pigeon liver fatty acid synthetase was purified according to the procedure of Hsu et al. (6) as modified by Butterworth et al. (2, 18). In addition, 1 mM dithiothreitol was used to replace 2-mercaptoethanol in all of the enzyme purification steps. This eliminated the possibility of oxidation of —SH groups of the enzyme. Purified enzyme was then stored frozen at —20° in 0.2 M potassium phosphate buffer, pH 7.0, 10 mM dithiothreitol, and 1 mM EDTA in an atmosphere of nitrogen. The protein content of all of the enzyme preparations was ascertained by paper chromatography of malonyl-CoA preparations was shown by assays by the recent method of Cleland (17) and pyruvate carboxylase (14, 15). Fatty acid synthetase contains a bound 4'-phosphopantetheine prosthetic group which transports the intermediates to various catalytic sites on the enzyme. This enzyme apparently also has seven sites at which the different reactions leading to the synthesis of fatty acids occur. In the present paper we report kinetic evidence that reactions at more than two of these sites are ping-pong. For such a complex reaction, standard ping-pong theory obviously has serious limitations. Hence, the recent method of Cleland (17) has been used to interpret what may be called a seven-site ping-pong mechanism. The present paper reports initial velocity and product inhibition studies which confirm this proposed kinetic mechanism.

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The lines in the figures are from fits of the data to the appropriate equation; the points are the actual experimental values.

RESULTS

Initial Velocity Studies—The initial velocity patterns were determined by holding the concentration of one substrate at a fixed level and varying the concentration of the second substrate at different fixed concentrations of the third substrate. Fig. 1 shows a set of patterns for one pair of reactants, acetyl-CoA and NADPH at 10 uM malonyl-CoA. Once any concentration of malonyl-CoA was chosen, it limited the acetyl-CoA concentrations that could be used because of the competitive inhibition between these substrates (see below) (24). The lines shown in Fig. 1 are parallel. Similarly, plots of reciprocal velocities against reciprocal concentrations of malonyl-CoA at various fixed concentrations of NADPH and at 15 uM acetyl-CoA are parallel (Fig. 2). These data and data from similar replicate experiments were fitted to Equation 3 and the Michaelis constants determined from fits of these primary data are listed in Table I. Double reciprocal plots for the third pair of substrates, acetyl-CoA and malonyl-CoA are also a set of parallel lines within limited ranges of these substrates (Figs. 3 and 4). Secondary plots of intercepts from the primary data with respect to the reciprocal concentrations of the fixed substrates were linear.

Substrate Inhibition—Significant inhibition of the reaction leading to the formation of fatty acids was shown by both acetyl-CoA and malonyl-CoA. When the rate of fatty acid synthesis was studied at increasing concentrations of malonyl-CoA, the results shown in Fig. 4 (lower set of data) were obtained. It can be seen that at higher concentrations of malonyl-CoA the parallel lines become sharply curved up-wards, indicating substrate inhibition by malonyl-CoA, which is competitive with acetyl-CoA binding. However, inhibition by malonyl-CoA is partial as a limiting rate is reached at higher concentrations of malonyl-CoA, which is identical with the rate of synthesis of fatty acids from NADPH and malonyl-CoA alone (25). Substrate inhibition was also observed with acetyl-CoA as the varied substrate (Figs. 3 and 5) and the degree of inhibition was stronger than with malonyl-CoA. Acetyl-CoA became inhibitory as soon as its concentration became more than one-half that of malonyl-CoA. At higher concentrations of acetyl-CoA inhibition was total. A double reciprocal plot showing the variation in the rate of the reaction was a function of acetyl-CoA concentration is shown in Fig. 5.

Product Inhibition—The products of the over-all reaction are palmitic acid, free coenzyme A, NADP+, and CO₂. Attempts to use palmitic acid as the inhibitor were unsuccessful due to the formation of micelles, even at low concentrations. CO₂ is not a suitable product for such studies due to the nonavailability of a reliable analytical method for its estimation. Consequently, coenzyme A and NADP+ were used as product inhibitors. The effects of including different fixed concentrations of free coenzyme A on the initial velocity at varying concentrations of one of the substrates; i.e. acetyl-CoA, malonyl-CoA, or NADPH, was investigated and the results are presented in Figs. 6, 7, and 8, respectively. Free coenzyme A was found to be a competitive inhibitor of acetyl-CoA (Fig. 6) and malonyl-CoA (Fig. 7). In contrast, coenzyme A was an

![Fig. 1 (left). Initial velocity patterns for the over-all reaction catalyzed by fatty acid synthetase complex (Reaction 1). Reciprocal velocities are plotted as functions of acetyl-CoA and NADPH as the varied substrates. Fixed concentrations of NADPH were 4 uM (A-A'), 5 uM (A-A'), 7 uM (O-O), and 10 uM (C-C). Malonyl-CoA was held constant at 10 uM and the enzyme concentration was 2 uM of protein per ml. Assay conditions were as described under "Experimental Procedure." The rate of the reaction was followed spectrophotometrically in a 5-cm cell. Units on the ordinate are (nanomoles of NADPH oxidized per min per mg of protein) -1 x 10⁴. The data are fitted to Equation 3.

![Fig. 2 (center). Initial velocity patterns for the substrate pair acetyl-CoA and malonyl-CoA and NADPH. Malonyl-CoA was varied at the following fixed concentrations of NADPH: 5 uM (A-A'), 7 uM (A-A'), 10 uM (O-O), and 15 uM (C-C). Acetyl-CoA was held constant at 15 uM. Other conditions were the same as those described in the legend to Fig. 1. The data are fitted to Equation 3.

![Fig. 3 (right). Initial velocity patterns for the substrate pair acetyl-CoA and malonyl-CoA. Acetyl-CoA was varied at the following fixed concentrations of malonyl-CoA: 10 uM (A-A'), 20 uM (O-O), 3 uM (A-A'), 4 uM (C-C), and 5 uM (C-C). NADPH was held constant at 30 uM. Other conditions were the same as those described in the legend to Fig. 1.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Michaelis constants from initial velocity patterns</th>
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<td>Data fitted to Equation 3.</td>
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<tr>
<td>Michaelis constant</td>
<td>NADPH and acetyl-CoA varied</td>
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<td>K_NADPH</td>
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<td>K_Acetyl-CoA</td>
<td>1.4 ± 0.2</td>
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<tr>
<td>K_Malonyl-CoA</td>
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*Malonyl-CoA = 10 uM.
*Acetyl-CoA = 15 uM.
FIG. 4. Double reciprocal plots of initial velocity of fatty acid synthesis with three substrates and two substrates. Malonyl-CoA is the varied substrate. The lower pattern was obtained at 30 μM NADPH, 2 μg per ml of enzyme protein, and the following fixed concentrations of acetyl-CoA: 1.0 μM (Δ—Δ), 1.25 μM (○—○), 1.5 μM (■—■), 2.0 μM (□—□), 3.0 μM (●—●), and 4.0 μM (▲—▲). The upper pattern was obtained in the absence of acetyl-CoA and the following fixed concentrations of NADPH: 5.0 μM (▲—▲), 7.0 μM (Δ—Δ), 10.0 μM (●—●), 15 μM (○—○), and 30.0 μM (□—□). Enzyme protein was 5 μg/ml.

FIG. 5. Reciprocal plot of enzyme activity under conditions in which acetyl-CoA was the variable substrate. Malonyl-CoA was 10 μM; NADPH, 30 μM; and enzyme protein, 2 μg/ml. Other conditions were the same as described in the legend to Fig. 1.

uncompetitive inhibitor against NADPH (Fig. 8). Inhibition by NADP⁺ of the rate of fatty acid synthesis produced a competitive pattern with NADPH (Fig. 9) as the varied substrate, and an uncompetitive pattern with acetyl-CoA (Fig. 10) and malonyl-CoA (Fig. 11) as the varied substrates. Secondary plots of slopes or intercepts with respect to concentrations of products were linear. The inhibition constants for coenzyme A and NADP⁺ obtained by fitting the data to Equations 4 or 5 are summarized in Table II.

DISCUSSION

The initial velocity patterns showing the relationships between the three different pairs of substrates are a series of parallel lines. These results indicate that the reaction catalyzed by pigeon liver fatty acid synthetase is ping-pong in nature; i.e., that the first product is released from the enzyme surface before the second substrate combines. For the acetyl-CoA and malonyl-CoA pairs at higher concentrations of acetyl-CoA, a pronounced substrate inhibition by acetyl-CoA was observed. Similarly, when malonyl-CoA was the varied substrate, higher concentrations of malonyl-CoA produced inhibition. However, the inhibition was less than that shown by acetyl-CoA. Thus, acetyl-CoA and malonyl-CoA show mutually competitive substrate inhibition (Figs. 3 and 4); this feature is common with ping-pong mechanisms. In such a mechanism, substrate inhibition arises as a result of binding of the inhibitory substrate to the wrong form of the stable enzyme. When substrates bind to a common site, the binding of the inhibitory substrate to the enzyme competes with the binding of the other substrate, resulting in competitive inhibition. The inhibitory effects observed in our study are in disagreement with the earlier data of Plate et al. (26) who reported that malonyl-CoA inhibition is noncompetitive with respect to acetyl-CoA and competitive with respect to NADPH. We did not find NADPH substrate inhibition of any kind.

Despite the complications of marked double competitive substrate inhibition, the results obtained with two pairs of substrates; i.e., NADPH and acetyl-CoA, NADPH and malonyl-CoA, obeyed the equation for a standard Ping-Pong Bi Bi reaction (9). This suggests that the kinetic behavior of the fatty acid synthetase involves a covalently modified form of the enzyme as the intermediate and proceeds through the formation, conversion, and breakdown of an ordered series of binary complexes, but without the formation of a kinetically significant amount of ternary complex. Earlier chemical studies in Porter's laboratory (3, 27, 28) on the mechanism of fatty acid synthesis have demonstrated the formation of enzyme-4'-phosphopantetheine-acetyl and enzyme-4'-phosphopantetheine-malonyl intermediates. These studies are thus consistent with the ping-pong mechanism indicated by the initial velocity pattern.

The problem of assigning a kinetic mechanism for fatty acid synthetase action therefore transforms to that of determining the kind of ping-pong mechanism that is operative. This mechanism must also incorporate the formation of intermediates like acetyl-4'-phosphopantetheine-enzyme, malonyl-4'-phosphopantetheine-enzyme, and other intermediates outlined later in the various reactions occurring at different catalytic sites. The mechanism should be able to explain the double competitive substrate inhibition by acetyl-CoA, malonyl-CoA, and the observed product inhibition patterns. Recently Cleland (17) proposed a new approach for the derivation of the rate equations for multisite ping-pong mechanisms. He has derived equations for a three-site ping-pong mechanism in which one or two of the individual sites have a ping-pong.
FIG. 6 (left). Inhibition of fatty acid synthesis by free coenzyme A when acetyl-CoA was the varied substrate. The concentrations of added free coenzyme A were 0 μM (O—O), 5.0 μM (●—●), 7.5 μM (Δ—Δ), and 10.0 μM (◆—◆). Malonyl-CoA and NADPH were kept constant at 10 μM and 30 μM, respectively. Enzyme protein was 2.0 μg/ml. Other conditions were the same as described in the legend to Fig. 1. Inset, a secondary plot of the slopes of the lines versus the concentration (μM) of free coenzyme A. The data are fitted to Equation 4.

FIG. 7 (center). Inhibition of fatty acid synthesis by free coenzyme A when malonyl-CoA was the varied substrate. The concentrations of added free coenzyme A were 0 μM (O—O), 2.5 μM (●—●), 5.0 μM (Δ—Δ), and 10 μM (◆—◆). Acetyl-CoA and NADPH were held constant at 10 μM and 30 μM, respectively. Enzyme protein was 2.0 μg/ml. Other conditions were the same as described in the legend to Fig. 1. Inset, a secondary plot of the slopes of the lines versus the concentration (μM) of free coenzyme A. The data are fitted to Equation 5.

FIG. 8 (right). Inhibition of fatty acid synthesis by free coenzyme A when NADPH was the varied substrate. The concentrations of the added free coenzyme A were 0 μM (O—O), 10 μM (●—●), 20 μM (Δ—Δ), and 30 μM (◆—◆). Acetyl-CoA and malonyl-CoA were held constant at 10 μM and 30 μM, respectively. Enzyme protein was 2.0 μg/ml. Other conditions were the same as in the legend to Fig. 1. Inset, a secondary plot of the intercepts of the lines versus the concentration (μM) of free coenzyme A. The data are fitted to Equation 4.

FIG. 9 (left). Inhibition of fatty acid synthesis by NADP⁺ when NADPH was the varied substrate. The concentrations of the added NADP⁺ were 0 μM (O—O), 2.5 μM (●—●), 5.0 μM (Δ—Δ), and 10.0 μM (◆—◆). Acetyl-CoA and malonyl-CoA were held constant at 10 μM and 30 μM, respectively. Enzyme protein was 2.0 μg/ml. Other conditions were the same as described in the legend to Fig. 1. Inset, a secondary plot of the slopes of the lines versus the concentration (μM) of NADP⁺. The data are fitted to Equation 4.

FIG. 10 (center). Inhibition of fatty acid synthesis by NADP⁺ when acetyl-CoA was the varied substrate. The concentrations of the added NADP⁺ were 0 μM (O—O), 10 μM (●—●), 20 μM (Δ—Δ), and 40 μM (◆—◆). Malonyl-CoA and NADPH were held constant at 10 μM and 30 μM, respectively. Enzyme protein was 2.0 μg/ml. Other conditions were the same as described in the legend to Fig. 1. Inset, a secondary plot of the intercepts of the lines versus the concentration (μM) of NADP⁺. The data are fitted to Equation 5.

FIG. 11 (right). Inhibition of fatty acid synthesis by NADP⁺ when malonyl-CoA was the varied substrate. The concentrations of the added NADP⁺ were 0 μM (O—O), 10 μM (●—●), 20 μM (Δ—Δ), and 40 μM (◆—◆). Acetyl-CoA and NADPH were held constant at 3 μM and 30 μM, respectively. Enzyme protein was 2.0 μg/ml. Other conditions were the same as described in Fig. 1. Inset, a secondary plot of the intercepts of the lines versus the concentrations (μM) of NADP⁺. The data are fitted to Equation 5.

rather than random, sequential mechanism. This equation predicts a parallel initial velocity pattern regardless of which substrate is varied at fixed levels of a second substrate. This type of plot has been found in the present study with the pigeon liver fatty acid synthetase. The reaction of fatty acid synthesis is known to involve at least seven sites and our evidence suggests that at least three of the sites catalyze ping-pong reactions.
ably an acyl ester of serine) is formed (3). Covalently to the enzyme as demonstrated by earlier CoA-acyl-CoA exchange studies (3, 27, 29, 30). Then enzyme-bound 4'-phosphopantetheine binds to the transacylase site, transfer the transport of various intermediates of the reaction to the catalytic sites. The site reactions have been attributed to the 4'-phosphopantetheine group on the enzyme. It has now been established that 4'-phosphopantetheine is a part of acyl carrier protein (ACP) and thus all the reactions in which the fatty acyl chain is elongated, reduced, and dehydrated appear to occur while the chain is in acyl linkage to ACP.

**Site Reactions**—The complete reaction leading to formation of fatty acids consists of seven reactions occurring at different sites. The site reactions are given below:

**Site 1**

**Acetyl transacylase** → **Acyl transfer**

Acetyl-CoA + E + 4'-PP → Acetyl-4'-PP-E + CoA

**Site 2**

**Malonyl transacylase**

Malonyl-CoA + E + 4'-PP → Malonyl-4'-PP-E + CoA

**Site 3**

Acetyl-4'PP-E + ES → Acetyl-ES + 4'-PP-E

**Site 3'**

β-Ketoacyl synthase

Acetyl- (or acyl-) E + ES → Acetoacetyl- (or β-ketoacyl-) malonyl-4'-PP-E

**Site 4**

β-Ketoacyl reductase

β-Ketoacyl-4'-PP-E + NADPH + H⁺ → Reduction 1

**Site 5**

β-Hydroxacyl dehydrase

β-Hydroxacyl-4'-PP-E → Dehydration

**Site 6**

Enoyl reductase

Enoyl-4'-PP-E + NADPH + H⁺ → Reduction 2

**Site 7**

Palmitoyl transacylase

Palmitoyl-4'-PP-E → Palmitoyl transfer in the vicinity of the transacylase site. This over-all process is a ping-pong event as shown below:

The reaction of malonyl-CoA and 4'-phosphopantetheine at the other transacylase site (Site 2) is also a ping-pong event as shown below, inasmuch as CoA-malonyl-CoA exchange occurs (1, 27, 29, 30):

**Site 2**

E + E-Mal' + 4'-PP-Malonyl-4'-PP

**Site 3**

Acetyl-4'-phosphopantetheine formed in the Site 1 reaction adds onto a catalytic site adjacent to cysteine and the acetyl moiety is transferred to the cysteine (3, 4). 4'-Phosphopantetheine then dissociates from this enzymatic site (Site 3) and goes on to Site 2, where it binds a malonyl moiety in the process described above. Malonyl-4'-phosphopantetheine thus formed then combines at the decarboxylation-condensation site (Site 3'), which is in the vicinity of the acetyl-cysteine. The products of this reaction dissociate from their respective enzyme sites as shown below:

This composite reaction at Sites 3 and 3' must be ping-pong because only one 4'-phosphopantetheine is present per molecule of fatty acid synthetase (18), and this molecule had to obtain a malonyl moiety after transferring an acyl group to the cysteine site. This kind of interlocking mechanism is critical for the over-all reaction because it allows the existence of only one 4'-phosphopantetheine per molecule and it prevents the initiation of new chains until the elongation process in the synthesis of a molecule of fatty acid is complete. The two transacylase reactions are thus separated from each other by an interlocking ping-pong reaction.

The proposal detailed above describing the kinetic events leading to the transfer of the acetyl moiety to cysteine via an acetyl 4'-phosphopantetheine intermediate, and then the transfer of the malonyl moiety to 4'-phosphopantetheine is in agreement with the results of previous chemical studies (3, 29) which demonstrated the binding of the acetyl moiety to hydroxyl, 4'-phosphopantetheine, and cysteine and the binding of the malonyl moiety to hydroxyl and 4'-phosphopantetheine but not to cysteine. The assignment of ping-pong mechanisms to the reactions forming acetyl- and malonyl-4'-phosphopantetheine (Sites 1 and 2) is also consistent with the demonstration (27, 28) that acetyl-CoA or malonyl-CoA yield acetyl- or malonyl-enzyme when added to pigeon liver fatty acid synthetase having the cysteine and 4'-phosphopantetheine sites blocked by iodoacetamide. This result clearly demonstrates that free CoA is released from the site of the transacylation reaction.

β-Ketoacyl-4'-phosphopantetheine, formed at Site 3', and NADPH combine at the reductase 1 site (Site 4) with the subsequent formation of β-hydroxyacyl-4'-phosphopantetheine as the product of reduction. In earlier chemical studies (31, 32)
it was established that the binding of NADPH to the enzyme is independent of the covalent binding of either acetyl or malonyl groups. The reaction at Site 4 may thus be random sequential or ordered with NADPH adding first. A ping-pong reaction at this site is ruled out on the basis of earlier data (31, 32) on the NADPH binding to fatty acid synthetase. Although there is stereospecific transfer of hydrogen from the B side of NADPH to substrate (33), no direct hydride transfer from NADPH to enzyme was found and fluorescence of NADPH increased with increased NADPH binding (31, 32). β-Hydroxyacyl-4'-phosphopantetheine is then transported to catalytic Site 5, where it is dehydrated with the formation of α,β-unsaturated acyl-4'-phosphopantetheine. The product of dehydration at Site 5 then dissociates from this site and combines along with NADPH at Site 6. At this site α,β-unsaturated acyl 4'-phosphopantetheine is reduced to acyl-4'-phosphopantetheine in a reaction that may be random sequential or ordered with NADPH adding first. (There is direct transfer of hydrogen from the A side of NADPH to substrate (33).) The nature of the reactions at these two reductase sites, whether random sequential or ordered, does not change the general form of the rate expression for a multisite ping-pong reaction (17).

The reactions occurring at Sites 2 and 6 repeat six more times to yield palmityl 4' phosphopantetheine, which is then deacetylated at Site 7 to give palmitic acid as the final product of the reaction. In shorthand notation the complete sequence of reactions for the formation of fatty acids may be written as l(323'456)7.

**Product Inhibition**—The product inhibition patterns in multisite ping-pong mechanisms (17) call for each product to be competitive versus the substrates combining at that site, irrespective of whether the reaction at that site is random sequential or ping-pong. The observed product inhibition patterns for free CoA with varied acetyl-CoA, for NADP+ with varied NADPH, and for free CoA with varied malonyl-CoA are competitive and are thus in agreement with the predicted patterns. The general rate equation (17) also predicts that each product will be uncompetitive against other substrates combining at other sites, except that it will be noncompetitive against the substrate that combines at the next site in the over-all sequence if the reaction at that site is random sequential. With fatty acid synthetase, NADP+ is uncompetitive versus both acetyl-CoA and malonyl-CoA, and free CoA is uncompetitive versus NADPH. These inhibition patterns are consistent with a multisite ping-pong mechanism. It may be noted that free CoA is a common product at two transacylase sites, and according to the predictions of multisite ping-pong mechanisms, one might expect that CoA should be noncompetitive versus malonyl-CoA because CoA should combine at both transacylase sites, and neither CoA ester could by itself eliminate all inhibition by CoA. However, if neither transacylase is normally rate-limiting, the combination of CoA at the one not involving the variable substrate will have little kinetic effect, whereas combination at the same site as the variable substrate will of course produce competitive inhibition. It is known that the acetyl and malonyl transacylase reactions are much faster at 0° than the over-all rate of fatty acid synthesis at 30° (34).

A pictorial model showing the sites of all enzyme activities with kinetic events leading to the formation of the various intermediates and products described above is shown in Fig. 12. The 4'-phosphopantetheine in this diagram is shown as an arm-like structure. The substrate sites for acetyl-CoA, malonyl-CoA, and free CoA are in the vicinity of the transacylase sites, whereas the NADPH and NADP+ binding sites are presumably in the vicinity of reductase 1 and reductase 2 sites. This model is also capable of explaining the dissociation of the multienzyme complex (35) into two nonidentical subunits and the respective capabilities of each of the subunits to carry out the partial reactions.

**Kinetic Analysis of Substrate Inhibition**—Double competitive substrate inhibition by acetyl-CoA and malonyl-CoA has been observed in the reactions leading to the synthesis of fatty acids (24). This enzyme also catalyzes the formation of palmitic acid from malonyl-CoA and NADPH in the absence of acetyl-CoA (25). However, with two substrates no inhibition of the rate of fatty acid synthesis by malonyl-CoA or NADPH is observed. The two-substrate reaction is consistent with the conclusion arrived at earlier (25) that the malonyl group bound to the 4'-phosphopantetheine of the enzyme, is slowly decarboxylated, presumably at Site 3', to give acetyl-4'-phosphopantetheine. This acetyl group is then transferred to the cysteine—SH, thus leaving 4'-phosphopantetheine free to receive a malonyl moiety and to continue the synthesis of palmitic acid according to the mechanism detailed above. The double reciprocal plots of initial velocity with three substrates and two substrates (in the absence of acetyl-CoA) are shown in Fig. 4. The lower set of data show the initial velocity pattern in the presence of all three substrates, and the upper set of data show the same pattern with two substrates; i.e. in the absence of acetyl-CoA. It can be seen in Fig. 4 that the lines curve upward as the malonyl-CoA concentration increases. On extrapolation these lines meet at a point on the ordinate which corresponds to the maximum velocity of the two-substrate reaction. Further, the initial velocity pattern with the two substrates is also a parallel one (Fig. 4).

An abbreviated steady state model for the fatty acid synthetase reaction is shown in Fig. 13. This model shows only
Fig. 13. Steady state model for the reactions catalyzed by the fatty acid synthetase complex. Steps involving products are shown as irreversible processes, because the forward reaction resulting in the formation of palmitate is shown in the absence of products. A and B represent substrates acetyl-CoA and malonyl-CoA, respectively. Fig. 13a shows all the steps involving acetyl-CoA and malonyl-CoA required in the formation of fatty acids from three substrates and two substrates (in the absence of acetyl-CoA). Further abbreviation of this model, as shown by broken lines, results in the model (b). The primary sequence of reactions and it considers in detail only those steps in which the substrates acetyl-CoA and malonyl-CoA are involved. This model (Fig. 13b) will now be utilized for deriving a steady state equation which will predict and explain the effects of acetyl-CoA and malonyl-CoA on the fatty acid synthetase reaction. This model shows six enzyme species: $E$, $(1)$, $(3)$, $(3)A$, $(4)$, and $(6)$. $E$ is free enzyme, $(1)$ and $(6)$ represent $E$-acetyl-CoA and $E$-malonyl-CoA complexes, respectively, $(3)$ and $(3)A$ represent $E$-$S_{cyx}$-acetyl intermediate and $E$-$S_{cyx}$-acetyl complex, respectively, and $(4)$ represents $E$-$S_{cyx}$-acetyl complex. $A$ and $B$ in this figure represent substrates acetyl-CoA and malonyl-CoA, respectively. The rate equation (see "Appendix") obtained by King's method (36) for initial velocities in the absence of products but at fixed acetyl-CoA and malonyl-CoA concentrations is of the following form:

$$\frac{1}{v} = \frac{a + b (1/B) + c (1/B^2)}{d + e (1/B)}$$  \hspace{1cm} (18)$$

where $a$, $b$, $c$, $d$, and $e$ are constants in terms of a group of kinetic constants (see "Appendix"). Equation 18 yields the expressions for the asymptote intercept, true intercept, initial slope, and asymptote slope given under "Appendix." The expression for asymptote intercept (Equation 25, Appendix) predicts that as the concentration of acetyl-CoA approaches zero, the asymptote intercept will be positive only if the numerator of Equation 25 is positive, which means that

$$k_{a2} k_{b1} (k_{a2} + k_{b1}) (k_{a3} + k_{b1}) > 0$$  \hspace{1cm} (19)$$

The true and asymptote intercepts are thus positive quantities as predicted by the steady state model, and they have been found experimentally to be positive as seen in Fig. 4.

The expression for initial slope (Equation 27, Appendix), reduces to the following as the concentration of acetyl-CoA approaches zero:

$$\frac{k_{a2} + k_{b1} + k_{a3} + k_{b1}}{k_{a2} k_{b1} (k_{a2} + k_{b1}) (k_{a3} + k_{b1})}$$  \hspace{1cm} (20)$$

This gives a positive slope indicating a decrease in the initial rate with decreasing malonyl-CoA concentration for the two-substrate reaction. The experimental data in Fig. 4 are consistent with this prediction. However, at higher concentrations of acetyl-CoA Equation 27 reduces to

$$k_{a2} k_{b1} k_{a3} k_{b1} (k_{a2} + k_{b1}) (k_{a3} + k_{b1}) (k_{a2} - k_{b1})$$  \hspace{1cm} (21)$$

and predicts a negative initial slope for the three-substrate reaction when $k_a$ is greater than $k_{11}$. A reference to Fig. 13a shows that the prediction of a larger magnitude for $k_a$ as compared to $k_{11}$ is reasonable; $k_a$ represents the reaction from enzyme forms (1) to (3):

$$E \rightleftharpoons E-4'\text{-PP}-\text{acyetyl} \rightleftharpoons E-4'\text{-PP}-\text{acyetyl}$$  \hspace{1cm} (22)$$

whereas $k_{11}$ refers to the reaction from enzyme forms (6) to (3):

$$E-4'\text{-PP}-\text{acyetyl} \rightleftharpoons E-4'\text{-PP}-\text{malonyl} \rightleftharpoons E-4'\text{-PP}-\text{acyetyl}$$  \hspace{1cm} (23)$$

In the absence of acetyl-CoA, the malonyl group is bound to 4'-phosphopantetheine and at Site 3' the attack on water slowly decarboxylates it to acetyl-4'-phosphopantetheine. The decarboxylation of malonyl-4'-phosphopantetheine at Site 3' is presumably a slower reaction than the other reactions shown in Equation 23.

Formation of Triacetic Acid Lactone—The mechanism developed above is capable of explaining another reaction catalyzed by the fatty acid synthetase complex; namely, the formation of triacetic acid lactone. Triacetic acid lactone is formed from 2 molecules of malonyl-CoA and 1 molecule of acetyl-CoA (37). Presumably the formation of acetoacetyl-4'-phosphopantetheine enzyme is on this pathway. In the absence of NADPH, the acetoacetyl group would then be transferred to the cysteine from 4'-phosphopantetheine. This would presumably be a slow process. Then a condensation with malonyl-4'-phosphopantetheine would yield 3,5-diketohexanoyl-4'-phosphopantetheine. The latter compound would then cyclize either spontaneously, or more likely catalytically, to yield triacetic acid lactone. Where this cyclization reaction occurs is not clear at present. The complete sequence of reactions for the formation of triacetic acid lactone can be written from the kinetic mechanisms developed above as follows:

$$1(323') (323') \text{(cyclize, release of lactone)}.$$
APPENDIX

Derivation of rate equation predicting effect of substrates

King's method (36), when applied to the steady state portion of the model shown in Fig. 13b, yields the following rate equation:

$$\frac{1}{a} - \frac{1}{b} = \frac{1}{c} \left( \frac{1}{k_{11}} \right)$$

where

$$a = k_4 k_9 (k_4 + k_9) (k_4 + k_9)$$

$$b = \left( k_4 k_9 + k_9 k_4 + k_4 k_4 + k_9 k_9 \right)$$

$$c = k_4 k_9 (k_4 + k_9) (k_4 + k_9)$$

For the experimental condition of Fig. 4 that is a different fixed concentration of acetyl-CoA and varying concentrations of malonyl-CoA, Equation 18 yields the following expressions for asymptote intercept, true intercept, initial slope, and asymptote slope.

Asymptote intercept = \[ \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9)}}} \]

Asymptote slope = \[ \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9)}}} \]

Initial slope = \[ \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9) (k_4 + k_9)}{1 + \frac{k_4 k_9 (k_4 + k_9) (k_4 + k_9) (k_4 + k_9)}}} \]

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