

# Pyruvate Carboxylase

## IX. SOME PROPERTIES OF THE ACTIVATION BY CERTAIN ACYL DERIVATIVES OF COENZYME A\*

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### SUMMARY

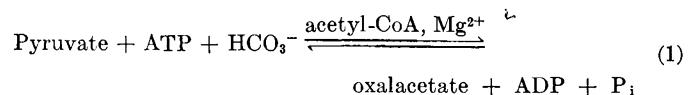
Pyruvate carboxylase purified from chicken liver shows an essentially absolute requirement for activation by an acyl coenzyme A in all preparations and under all conditions tested thus far. The enzyme is activated by formyl-, acetyl-, propionyl-, crotonyl-, and iso-butyryl-CoA, but carboxylated analogues of the activating acyl derivatives of CoA and certain other acetyl thioesters are potent competitive inhibitors with respect to acetyl-CoA. Examination of the initial reaction rate as a function of activator concentration reveals a sigmoid relationship, suggesting that more than 1 molecule of activator per active site is required for the activation process or that cooperative interactions between the bound activator molecules occur.

Inactivation analyses show an interaction of acetyl-CoA with pyruvate carboxylase, with resultant marked alterations in the tertiary and quaternary structures of the protein as compared with the free enzyme. For example, the rate of inactivation of this biotin enzyme by avidin is markedly increased in the presence of acetyl-CoA concentrations similar to those required for activation of the over-all reaction. Acetyl-CoA also affords almost complete protection of this enzyme against denaturation at 2° or by low concentrations of urea and sodium dodecyl sulfate. These findings are consistent with the proposal that acetyl-CoA and the other activator acyl-CoA derivatives act as allosteric effectors for pyruvate carboxylase. However, acetyl-CoA in the presence or absence of other reaction components has no significant effect either on the sedimentation properties of pyruvate carboxylase in the range from 0.5 to 5.0 mg of protein per ml or on the absorption spectrum of this enzyme.

Pyruvate carboxylase catalyzes a very slow hydrolysis of acyl thioesters. An apparent relationship between thioester hydrolysis and the activation of the over-all reaction is suggested by studies of the specificity of hydrolysis and of the effects of other reaction components on the rate of hydrolysis. The evidence appears to exclude the possibility that the activated species of pyruvate carboxylase is an acyl-enzyme, and the observed thioester hydrolysis may therefore be related indirectly to the activation process.

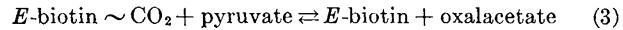
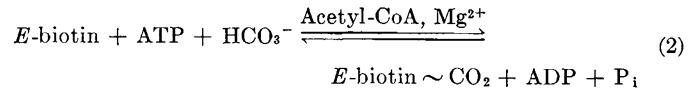
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The most striking feature of the reaction catalyzed by pyruvate carboxylase (pyruvate:CO<sub>2</sub> ligase (ADP), EC 6.4.1.1) purified from chicken liver (Reaction 1) is the essentially absolute requirement of this enzyme for an acyl coenzyme A, e.g. acetyl-CoA, as an added cofactor (1).



All pyruvate carboxylases purified thus far from mammalian and avian species have been found to be essentially inactive in the absence of an acyl-CoA (2).<sup>1</sup> In contrast, the pyruvate carboxylases purified from *Pseudomonas citronellolis* (3) and *Aspergillus niger* (4) show maximal activity in the absence of an acyl-CoA. The enzyme purified from bakers' yeast (5, 6) is stimulated by the addition of either acetyl-CoA or CoA-SH but shows significant enzymic activity in the absence of these cofactors. A wide divergence in the requirement for the addition of this activator therefore exists for pyruvate carboxylases purified from different sources.

Studies of the exchange reactions catalyzed by pyruvate carboxylase purified from chicken liver and isolation of the enzyme-bound intermediate (7) have permitted the formulation of a minimal mechanism for this enzyme (Reactions 2 and 3).



The requirement for activation by acetyl-CoA is localized in the formation of *E*-biotin  $\sim$  CO<sub>2</sub> from ATP and HCO<sub>3</sub><sup>-</sup> (Reaction 2). Further dissection of Reaction 2 has shown that an enzyme-ATP complex may be formed in the absence of acetyl-CoA (8). Added acetyl-CoA also has no effect on the pyruvate-oxalacetate exchange reaction (Reaction 3) catalyzed by the pyruvate carboxylase purified from bakers' yeast (5).

The essentially absolute requirement of pyruvate carboxylase purified from chicken liver for activation by an acyl-CoA requires the consideration of reaction mechanisms in which the cofactor participates as an intermediate in the reaction sequence. Evidence has previously been presented that the acetyl carbon of acetyl-CoA is not incorporated into oxalacetate and that acetyl-

<sup>1</sup> M. C. Scrutton, unpublished observations.

CoA is not broken down by pyruvate carboxylase at a significant rate compared with that of oxalacetate formation (1). Several reaction sequences for the pyruvate carboxylase reaction which are consistent with these findings have been proposed, and some of the evidence suggesting that acyl-CoA activation cannot be described by these sequences has been considered previously (9). Further evidence is presented here to show that the acyl-CoA is probably not involved as an intermediate of the pyruvate carboxylase reaction.

Activation of pyruvate carboxylase by an acyl-CoA could also result from an alteration in the tertiary or quaternary structure of the protein in the presence of the activator. Many enzymes are now known with catalytic activity that is altered in the presence of an effector molecule, and such systems have been designated as "allosteric" enzymes by Monod, Changeux, and Jacob (10). All allosteric enzymes described in detail thus far possess subunit structure, and similar studies on pyruvate carboxylase from chicken liver have shown that the active enzyme ( $s_{20,w}^0 = 14.8$  S;  $M_w = 660,000$ ) (11) is dissociated by exposure at 2° to give subunits ( $s_{20,w}^0 = 6.75$  S) with an estimated molecular weight of 165,000 (11). Both the biotin (11) and manganese (12) contents of this enzyme, which approximate 4 moles per mole of enzyme, are consistent with the proposal that the pyruvate carboxylase molecule contains four approximately equivalent subunits. Purified pyruvate carboxylase has been examined in the electron microscope by negative staining techniques (13). The active enzyme molecules are seen as tetrameric structures with four similar subunits arranged at the corners of squares. The electron micrographs are consistent with a molecule possessing either one tetrad or three dyad axes of symmetry. Although no significant effects of acetyl-CoA can be demonstrated on the sedimentation properties or ultraviolet absorption spectrum of pyruvate carboxylase, or on the structure of the enzyme as observed in the electron microscope, evidence is presented here suggesting that a noncovalent enzyme-acetyl-CoA complex is formed in which marked changes occur in both the environment of the biotin residues and the interaction between the 6.75 S subunits as compared with the free enzyme. These findings are consistent with the proposal that acetyl-CoA functions as an allosteric effector for pyruvate carboxylase.

A preliminary report of some of these data has appeared (9, 14).

#### METHODS

Formyl-CoA was synthesized as described by Sly and Stadtman (15). Malonyl-CoA and methylmalonyl-CoA were prepared by the procedure of Beck and Ochoa (16). All other thioesters were prepared from the respective anhydrides and thiols by the procedure of Simon and Shemin (17). After exhaustive extraction with ether at pH 2, the thioester solutions were neutralized to pH 5.0 and concentrated by lyophilization. They were further purified by chromatography on Whatman No. 3MM paper at 4° with ethanol-potassium acetate, pH 4.5 (1:1), as the developing solvent (18). This further purification did not affect their properties when examined for activation or inhibition of the pyruvate carboxylase reaction, and in some cases was omitted. Pantetheine was prepared by reduction of pantethine with excess NaBH<sub>4</sub> and was used immediately for preparation of acetyl pantetheine as above. Acetyl-CoA tritiated in the acetyl group was prepared from CoA and tritiated acetic anhydride (250 μC per μmole) as described above.

Thioesters except for formyl-CoA and acetyl-CoA were assayed as their hydroxamates (19). Formyl-CoA, which gives a low and variable color yield in the hydroxamate procedure, was assayed by measurement of the decrease in absorbance at 232 mμ on addition of 10 μmoles of hydroxylamine (15). Acetyl-CoA was assayed with the use of citrate synthetase as described by Ochoa (20).

Pyruvate carboxylase was prepared and assayed spectrophotometrically as described previously (1, 11). For the initial rate studies, the points represent the mean of at least two determinations which showed less than 3% deviation. Specific activities are expressed as micromoles of product per min per mg of protein at 25° except where indicated. Protein was estimated spectrophotometrically (21).

Acetyl-CoA hydrolysis was measured either by the decrease in absorbance at 232 or 240 mμ, with the extinction coefficients given for the thioester bond by Stadtman (18) (Assay I), or by the liberation of nonprotein sulfhydryl groups (Assay II). For Assay I, the incubation mixture contained, in 1 ml, 100 μmoles of Tris-sulfate (pH 7.8), 0.1 to 0.2 μmole of acetyl-CoA, and 100 to 200 μg of pyruvate carboxylase. Control cuvettes contained identical amounts of Tris-sulfate and either acetyl-CoA or pyruvate carboxylase. The change in absorbance at 232 or 240 mμ was estimated with the digital readout of a Gilford model 2000 spectrophotometer. For Assay II, the incubation mixture contained, in 0.5 ml, 50 μmoles of Tris-sulfate (pH 7.8), 0.05 μmole of acetyl-CoA, 100 to 200 μg of pyruvate carboxylase, and additions as described for each experiment. After incubation for 10 min, the reaction was stopped by addition of 0.5 ml of 10% metaphosphoric acid, and the precipitated protein was removed by centrifugation. The precipitate was washed with 0.2 ml of 0.5 M Tris-sulfate, pH 7.8, and the washings were combined with the supernatant fraction. After addition of 0.8 ml of 1 N KOH, the pH was adjusted with 0.1 N KOH to 8.0 ± 0.05, and 20 μl of DTNB<sup>2</sup> (4 mg per ml) were added. The absorbance at 412 mμ was determined rapidly after neutralization and the addition of DTNB. Accurate pH adjustment in Assay II is essential since the absorbance of the p-nitrothiophenol anion released is markedly pH-dependent. For both Assays I and II, appropriate controls were included to estimate the nonenzymic hydrolysis of acetyl-CoA under the conditions used, and all values given are corrected accordingly. The rate of acetyl-CoA hydrolysis by pyruvate carboxylase measured by either Assay I or Assay II is approximately 0.015% of the rate of CO<sub>2</sub> fixation. Disappearance of acetyl-CoA at a rate equivalent to the appearance of nonprotein sulfhydryl (Assay II) was demonstrated by specific assay of residual acetyl-CoA with citrate synthetase (20). A close correlation was observed, indicating that acetyl-CoA is the substrate for the reaction under these conditions.

Tritiated acetic anhydride (250 mC per mmole) was obtained from Volk; CoA, from either P-L Biochemicals or Farmochimica Cutolo Calosi; acetic, propionic, n-butyric, isobutyric, and n-valeric anhydrides, from Eastman; crotonic anhydride, from L. Light and Company, Ltd; and DTNB, from Aldrich. Citrate synthetase was prepared by the procedure of Srere and Kosicki (22). All other compounds were obtained as described previously (1, 7, 8, 11).

<sup>2</sup> The abbreviation used is: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

## RESULTS

## Activation Studies

**Activation of CO<sub>2</sub> Fixation by Acyl-CoA Derivatives**—Pyruvate carboxylase purified from chicken liver is one of a small group of enzymes possessing an essentially absolute requirement for activation by a non-metal ion cofactor. The rate of CO<sub>2</sub> fixation by this enzyme in the absence of an acyl-CoA has a maximal value of 0.04% of the rate observed in the presence of saturating concentrations of this cofactor. The rate obtained in the absence of an acyl-CoA is unaffected by the addition of very high concentrations of the other reaction components, e.g. 1 M KHCO<sub>3</sub>. The requirement for an acyl-CoA is characteristic of all preparations (at least 200) of this enzyme obtained thus far, and at all stages of the purification procedure.

The requirement for acyl-CoA activation is found over wide ranges of pH and ionic strength or on treatment of the enzyme with low concentrations of denaturing agents, e.g. urea or sodium dodecyl sulfate. Although the possibility cannot be excluded that pyruvate carboxylase purified from chicken liver might show significant activity in the absence of an acyl-CoA under certain conditions, there is no evidence to support this possibility.

The reversibility of the activation of pyruvate carboxylase has previously been shown by experiments in which citrate synthetase was used to remove acetyl-CoA (9). More detailed studies of the time courses of activation of pyruvate carboxylase by acetyl-CoA and of the reversal of this activation have been performed with dilution techniques as described by Chen, Brown, and Plaut (23). The time course of activation was examined by addition of 5  $\mu$ l of pyruvate carboxylase (12.6 mg per ml; specific activity, 18.4) to 1 ml of the spectrophotometric assay mixture (1). Similar results were observed by addition of acetyl-CoA to a system containing pyruvate carboxylase and the assay mixture minus acetyl-CoA. For examination of the time course of deactivation, 20  $\mu$ l of a solution containing pyruvate carboxylase (3.2 mg per ml; specific activity, 18.4) and acetyl-CoA (2.5 mM) were added to 1 ml of the spectrophotometric assay mixture lacking acetyl-CoA. Rapid mixing was achieved by means of a modification of the apparatus described by Strittmatter (24). The change in absorbance at 340 m $\mu$  was recorded in a Gilford model 2000 automatic recording spectrophotometer with a chart speed of 6 inches per min. Under these conditions, the earliest time of accurate measurement was 1 sec. The rate of change of absorbance with time showed no deviation from linearity after the earliest time of observation, indicating that complete activation and deactivation occurs within 1 sec. Therefore, at pH 7.8 and low protein concentration (5 to 10  $\mu$ g per ml), the activated (*E*-acetyl-CoA) complex is freely dissociable with a lifetime of less than 1 sec.

The observed results further indicate that, if a change in protein structure occurs in the presence of acetyl-CoA, this transition occurs in less than 1 sec after the exposure to or removal of acetyl-CoA. This finding is in contrast with the results obtained for some other allosteric enzymes (23). In further accord with these findings, gel filtration of an incubation mixture containing pyruvate carboxylase and acetyl-CoA tritiated in the acetyl group, in the presence or absence of other reaction components, showed the absence of significant enzyme-bound radioactivity under conditions of high protein concentration at 4° and pH 7.8.

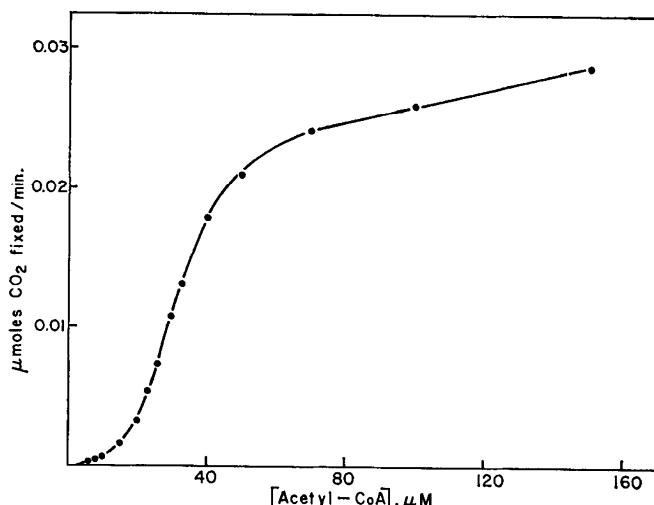


FIG. 1. The initial rate of CO<sub>2</sub> fixation measured as a function of the acetyl-CoA concentration at pH 7.8 and 25°. CO<sub>2</sub> fixation was measured spectrophotometrically as described previously (1). All other reaction components were present at concentrations at least 10-fold in excess of their Michaelis constants.

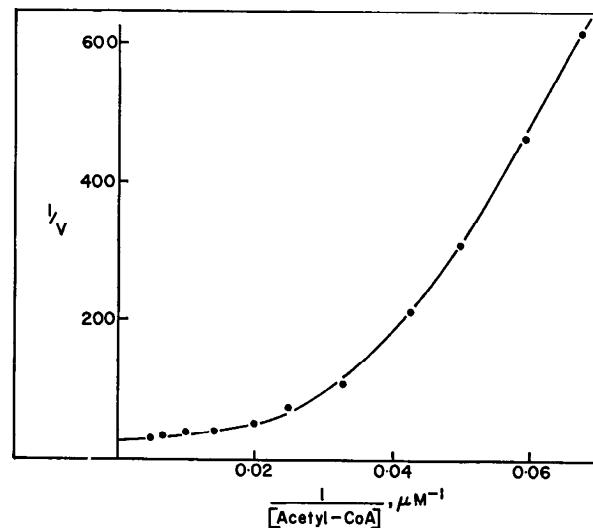


FIG. 2. The reciprocal initial rate of CO<sub>2</sub> fixation measured as a function of the reciprocal acetyl-CoA concentration at 25° and pH 7.8. Other conditions were as described for Fig. 1.

Other experiments have shown that acetyl-CoA cannot be covalently bound to pyruvate carboxylase by reduction with NaBH<sub>4</sub> at pH 7.8 and that pyruvate carboxylase is not activated by incubation with acetylating agents such as acetic anhydride or *N*-acetylimidazole.

**Initial Rate Studies of Activation Process**—An analysis of the initial rate of pyruvate carboxylation as a function of the acetyl-CoA concentration shows that this relationship is markedly sigmoid in character (Fig. 1). This finding is in accord with similar results obtained for the pyruvate carboxylase from rat liver (25) and sheep kidney cortex (26), but is at variance with results reported earlier for the enzyme purified from chicken liver (27). The relationship of reciprocal initial rate at pH 7.8 to reciprocal acetyl-CoA concentration shows marked change in slope only at acetyl-CoA concentrations lower than 20 to 30  $\mu$ M (Fig. 2). The concentration range used in the earlier experiments

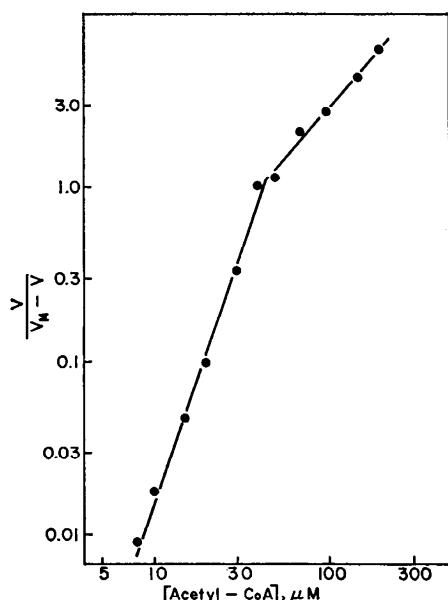


FIG. 3. Initial rate data for acetyl-CoA plotted as  $\log_{10} [v / (V_{\max} - v)]$  with respect to  $\log_{10}$  acetyl-CoA. The maximal rate of activation was taken as the observed rate at activator concentrations 20-fold greater than  $K_A$ . The conditions were as described for Fig. 1.

did not extend below 20  $\mu\text{M}$ , and therefore the marked deviations illustrated in Fig. 2 were not observed.

Monod *et al.* (10) have proposed the use of Hill's empirical equation (Equation 4) to describe the sigmoid relationships between initial rate and reactant concentration which are observed for many allosteric enzymes.

$$\log_{10} \frac{v}{V_{\max} - v} = n \log_{10} S - \log_{10} K \quad (4)$$

In Equation 4,  $n$  is an experimental parameter related to intersite interaction.

The initial rate data for variation of acetyl-CoA concentration in the pyruvate carboxylase system at pH 7.86, plotted according to Equation 4, are shown in Fig. 3. Two distinct linear regions are observed. At acetyl-CoA concentrations below 40  $\mu\text{M}$ , the value of  $n$  is calculated as 2.9, while above 40  $\mu\text{M}$  this value decreases to 1.09. The latter concentration range is the region of linearity observed in Fig. 2. The extent of this linear region is pH-dependent, since it is absent at pH 6.95 but extends to 9  $\mu\text{M}$  acetyl-CoA at pH 9.0. Similar analysis of the initial rate data obtained for activation of the sheep kidney cortex enzyme by acetyl-CoA gives a value for  $n$  of 2, and no change in slope occurs at levels of acetyl-CoA which approach saturation (26).

Analysis of the effect of pH on the activator constant and value of  $n$  at low concentrations of acetyl-CoA has also been undertaken (Fig. 4A). The value of  $n$  decreases from 3.0 at pH 6.95 to 2.03 at pH 9.0, and this decrease is associated with a decrease in the activator constant from 170  $\mu\text{M}$  at pH 6.95 to 9.5  $\mu\text{M}$  at pH 9.0. In all cases the initial rates were measured at activator concentrations from at least 6-fold below to 20-fold above the activator constant,  $K_A$ . This is illustrated for the determinations at the extremes of pH in Fig. 4B. Over a similar range of pH, the Michaelis constants for ATP and  $\text{HCO}_3^-$  varied not more than 2-fold,<sup>3</sup> and the  $K_m$  for pyruvate decreased with lowered pH (26).

Saturating concentrations of these components were therefore present at all times. The variation of  $K_A$  with pH may be analyzed as described by Dixon and Webb (28). When fitted with tangents having slopes of 0 and 1, the relationship of  $\text{p}K_A$  and pH (inset, Fig. 4A) shows a clear inflection point at pH 8.6, indicating the involvement of an ionizing group having this  $\text{p}K$  value. The data also suggest the involvement of a group with a  $\text{p}K$  value of approximately 7 but are inadequate to establish this point. The close correlation between the variation of  $n$  and  $K_A$  with pH above 8.0 suggests that the ionization of the group having a  $\text{p}K$  of 8.6 may be associated with the cooperative interactions observed for acetyl-CoA (Fig. 1).

The value for the activator constant obtained from Fig. 1 is 33  $\mu\text{M}$  (Table I). Similar values have been obtained with other preparations of acetyl-CoA, but one preparation of this activator gave a markedly lower value (11  $\mu\text{M}$ ). This variation, which appears to be related to the purity of the CoA preparations used, is under further investigation.

**Specificity of Activation Process**—Previous studies have shown that propionyl-CoA, crotonyl-CoA, and *n*-butyryl-CoA can replace acetyl-CoA as activators of pyruvate carboxylase (27), and, more recently, activation of this enzyme by formyl-CoA has also been demonstrated. All the activators show marked deviation from Michaelis-Menten behavior, and their activator constants and  $n$  values, together with the maximal reaction velocities relative to acetyl-CoA as 100, are summarized in Table I. At acyl-CoA concentrations above  $K_A$ , the  $n$  value for formyl-CoA decreases to 0.95, a finding similar to that shown for acetyl-CoA (Fig. 3). The other activators do not show similar decreases in the  $n$  value at high concentration.

Propionyl-CoA and crotonyl-CoA closely resemble acetyl-CoA in the extent of maximal activation and the value of  $n$  at pH 7.8, but show increased activator constants. However, formyl-, *n*-butyryl-, and *n*-valeryl-CoA are much weaker activators of pyruvate carboxylase, with  $V_{\max}$  values less than 12% of that found for acetyl-CoA. These weaker activators also have lowered values of  $n$  (1.0 to 1.7) as compared with the value of approximately 3 observed for acetyl-CoA. Isobutyryl-CoA, with a  $V_{\max}$  59% of that found for acetyl-CoA and an  $n$  value of 2.1, represents an intermediate case. Although no functional significance can be ascribed to the value of  $n$  at this time, a relationship appears to exist between its value and the relative  $V_{\max}$  obtained for a given activator (Table I). Maximal activation occurs only when the acyl-CoA added possesses a terminal methyl group which carries significant residual positive charge as a result of electron withdrawal by the thioester group, and the extent of activation appears to be related in part to the extent of electron withdrawal from the terminal methyl group. This is indicated most clearly by comparison of the results obtained for crotonyl-CoA, isobutyryl-CoA, and *n*-butyryl-CoA. Additionally comparison of the  $K_A$  values obtained for *n*-valeryl-CoA and CoA-SH suggests that the acyl group may be involved in binding of the activators.

Although no other acyl thioesters tested thus far activate pyruvate carboxylase, carboxylated analogues of the activators, e.g. malonyl-CoA, and acetylated analogues of CoA, e.g. acetyl-pantetheine, are potent inhibitors of this enzyme. The linear relationship between reciprocal initial rate at pH 7.8 and the reciprocal acetyl-CoA concentration at acetyl-CoA concentrations exceeding 30  $\mu\text{M}$  (Fig. 2) has permitted investigation of the characteristics of the inhibition by these analogues. As shown

<sup>3</sup> M. C. Scrutton and M. F. Utter, unpublished observations.

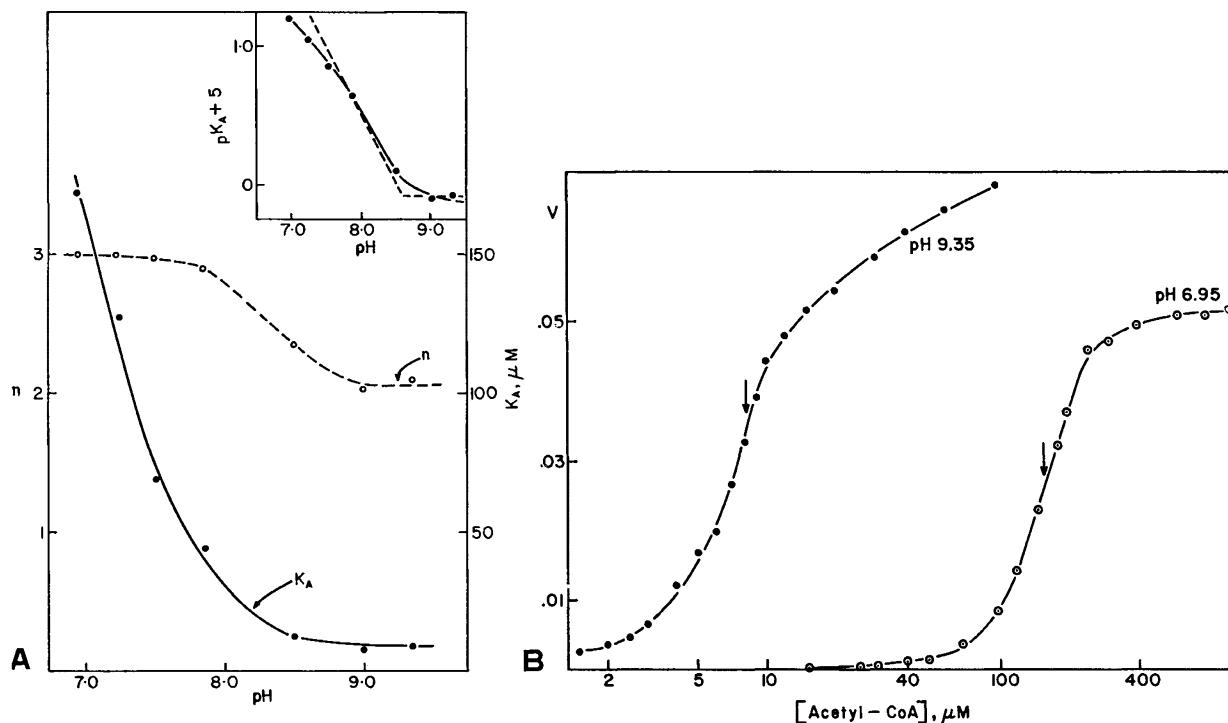


FIG. 4. A, variation of the activator constant and  $n$  for acetyl-CoA as a function of pH. The activator constant was taken as the acetyl-CoA concentration required to give 50% maximal activation, based on the  $V_{max}$  obtained as described in Fig. 3. The values for  $n$  were obtained from the slopes of plots similar to Fig. 3. Other conditions of measurement were as described for Fig. 1. The pH was maintained constant by use of Tris-Cl buffers throughout the range shown. The pH value shown is that of the assay mixture before addition of acetyl-CoA and enzyme. Acetyl-CoA was added to the assay mixture immediately prior to the addition of enzyme. Linear rates were observed at all pH values and all

acetyl-CoA concentrations, suggesting that hydrolysis of acetyl-CoA at the more alkaline pH values is not sufficiently rapid to affect the results. Measurement of pH on the assay mixtures after completion of the reaction showed that the pH never changed by more than 0.1 pH unit as compared with the initial value. B, the initial rate of  $\text{CO}_2$  fixation as a function of the logarithm of the acetyl-CoA concentration at pH 6.95 and pH 9.35.  $\text{CO}_2$  fixation was measured spectrophotometrically (1) at 25°. Other conditions were as described for Fig. 1. The arrows indicate the acetyl-CoA concentration required for 50% maximal activation ( $K_A$ ).

for malonyl-CoA (Fig. 5) and acetylpanthenone (Fig. 6), the inhibition shows competitive characteristics with respect to acetyl-CoA, and inhibitor constants obtained from such data are summarized in Table II. The significance of the observation that carboxylated analogues of the activator acyl-CoA derivatives are potent competitive inhibitors of pyruvate carboxylase is discussed in a later section. The effect of these inhibitors has also been examined as a function of acetyl-CoA concentration in the sigmoidal region of the saturation curve. The results obtained do not, however, appear to be susceptible to interpretation at this time.

The data for the acetylated analogues of acetyl-CoA suggest that both the acyl thioester and the nucleotide portions of the acetyl-CoA molecule are required for activation of pyruvate carboxylase from chicken liver. An analogue lacking the nucleotide portion of the molecule, e.g. acetylpanthenone, however, is able to bind at the activation site (or sites), since competitive inhibition by this compound is observed with respect to acetyl-CoA (Fig. 6). The acyl thioester group itself is not sufficient to permit binding at this site, since no significant inhibition by acetylethanethiol or acetylthioglycolate is observed (Table II), and a further section of the acetyl-CoA molecule must also be required for binding at this site. In this regard, the results ob-

TABLE I  
Specificity of activation of pyruvate carboxylase at pH 7.8

Activator <sup>a</sup>	Activator constant, $K_A^b$	$n^c$	Relative $V_{max}$
<i>M</i>			
Formyl-CoA.....	$1.9 \times 10^{-4}$	1.7	11
Acetyl-CoA.....	$3.3 \times 10^{-5}$	2.9	100
Propionyl-CoA.....	$1.1 \times 10^{-4}$	3.05	94
Crotonyl-CoA.....	$1.35 \times 10^{-4}$	2.9	82
Isobutyryl-CoA.....	$4.1 \times 10^{-4}$	2.1	59
<i>n</i> -Butyryl-CoA.....	$2.8 \times 10^{-4}$	1.43	4
<i>n</i> -Valeryl-CoA.....	$2.7 \times 10^{-4}$	1.0	1.3
CoA.....	$10^{-2}$		0.5

<sup>a</sup> The following compounds are ineffective as activators of pyruvate carboxylase: acetyl carnitine, acetyl acyl carrier protein, panthenone, mercaptosuccinate, thioglycolate, and benzoyl-CoA.

<sup>b</sup>  $K_A$  is the concentration of activator required to produce 50% maximal activation as obtained from plots similar to Fig. 1. The maximal rate of activation was determined as described for Fig. 3.

<sup>c</sup> The  $n$  values are obtained from plots of  $\log_{10} [v/(V_{max} - v)]$  with respect to  $\log_{10}$  activator as shown in Fig. 3.

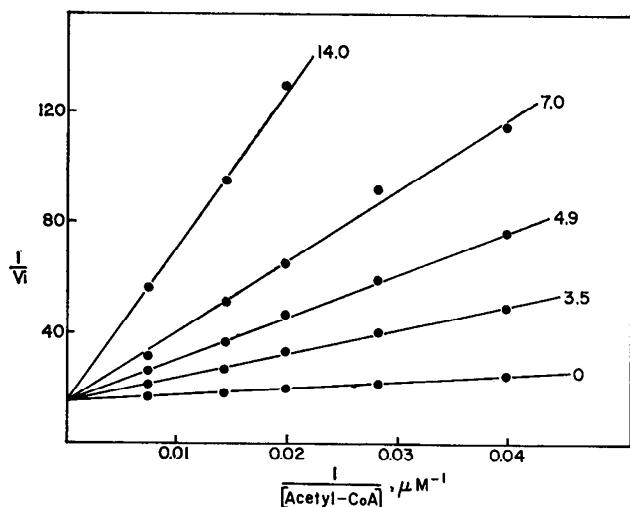


FIG. 5. Inhibition of pyruvate carboxylase by malonyl-CoA. The initial rate of  $\text{CO}_2$  fixation was measured spectrophotometrically (1) as a function of the acetyl-CoA concentration in the presence at pH 7.8 and 25° and in the micromolar concentrations of malonyl-CoA indicated by the numbers by the lines. Other conditions were as described for Fig. 1.

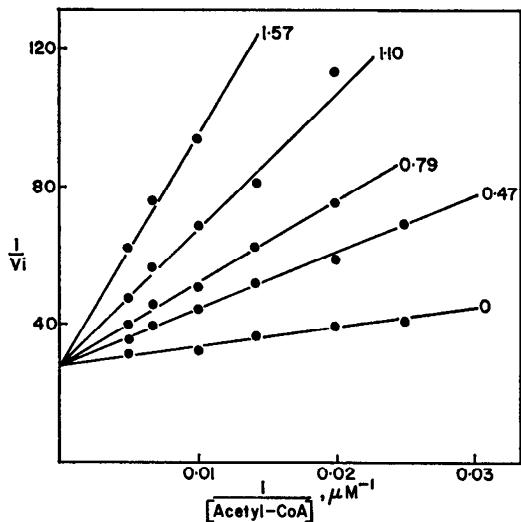


FIG. 6. Inhibition of pyruvate carboxylase by acetylpanthenone. The initial rate of  $\text{CO}_2$  fixation was measured spectrophotometrically (1) as a function of the acetyl-CoA concentration at pH 7.8 and 25° and in the presence of the millimolar concentrations of acetylpanthenone indicated by the numbers by the lines. Other conditions were as described for Fig. 1.

tained with acetylmercaptosuccinate are confusing, since this thioester is not a strict analogue of this section of the acetyl-CoA molecule and further experiments are required to clarify this aspect. The absence of inhibition by acetyl acyl carrier protein is perhaps surprising, since the active site of this cofactor has been reported to contain 4'-phosphopantetheine (29). However, sufficient concentrations were not available to establish the absence of inhibition at higher levels of acetyl acyl carrier protein. For the chicken liver enzyme, the specificity for an acyl thioester is nearly absolute, since CoA is a very weak activator of the over-all reaction with respect to both  $K_A$  and relative  $V_{\max}$  (Table I). In accord with this finding, CoA is also a weak competitive inhibitor with respect to acetyl-CoA, and its  $K_i$  (Table II) agrees well with the observed  $K_A$ . It

should be noted that for pyruvate carboxylase purified from bakers' yeast the specificity for an acyl thioester is less stringent and the reaction is markedly activated by either CoA or acetyl-CoA (6).

#### Demonstration and Properties of Interaction of Acetyl-CoA with Pyruvate Carboxylase

The properties of the activation of pyruvate carboxylase by acetyl-CoA suggest the formation of a noncovalent enzyme-acetyl-CoA complex in which the structure of the protein is altered. More direct evidence for the formation of this complex in the absence of other reaction components is presented below.

*Effect of Acetyl-CoA on Rate of Inactivation of Pyruvate Carboxylase by Avidin*—The rate of inactivation of pyruvate carboxylase by avidin is markedly decreased in the presence of ATP (8) and is increased in the presence of pyruvate or oxalacetate (30). The effect of acetyl-CoA on this rate of inactivation has also been examined, and a biphasic relationship is observed (Fig. 7). Increased inactivation rates are observed in the presence of 20 to 200  $\mu\text{M}$  acetyl-CoA, the latter concentration giving a maximal increase of 5.1-fold in the pseudo-first order rate constant. Further increase in acetyl-CoA concentration causes a decrease in this pseudo-first order rate constant until, at concentrations of 3 mM, the inactivation rate approximates that found for the free enzyme. These effects are qualitatively similar to the effect of NADPH on the inactivation of glutamate dehydrogenase from bovine liver (31) and of *N*-acetylglutamate on the thermal inactivation of carbamyl phosphate synthetase (32). The data, however, are not described either by the equation derived previously for the effect of ATP and other nucleoside triphosphates on the rate of inactivation by avidin (8) or by the equation derived by Frieden (31) to account for the effect of NADPH on the inactivation of glutamate dehydrogenase. Both these equations require that the reaction orders for enzyme sites and for inactivator be unaffected in the presence of the effector. Preliminary evidence suggests that, although the re-

TABLE II  
Inhibitor constants for acyl thioesters, carboxylated analogues of activators, and CoA

The initial rate of  $\text{CO}_2$  fixation was measured as a function of the acetyl-CoA concentration as described for Figs. 5 and 6 in the presence of varying concentrations of these inhibitors.  $K_i$  was obtained from a plot of the reciprocal initial rate with respect to inhibitor concentration. In all cases the inhibition showed competitive characteristics.

Inhibitor <sup>a</sup>	$K_i$
Malonyl-CoA.....	$8.3 \times 10^{-6}$
Methylmalonyl-CoA.....	$1.0 \times 10^{-4}$
Acetylpanthenone.....	$2.8 \times 10^{-4}$
Acetylmercaptosuccinate.....	$7.4 \times 10^{-6}$
CoA.....	$5.3 \times 10^{-3}$

<sup>a</sup> Acetylthioglycolate, acetylethanethiol, acetylmethanethiol, acetylcarnitine, and benzoyl-CoA show no significant inhibition at 5  $\mu\text{M}$ . Acetyl acyl carrier protein shows no significant inhibition at 10  $\mu\text{M}$ . All determinations were made over a range of acetyl-CoA similar to that shown for Figs. 5 and 6.

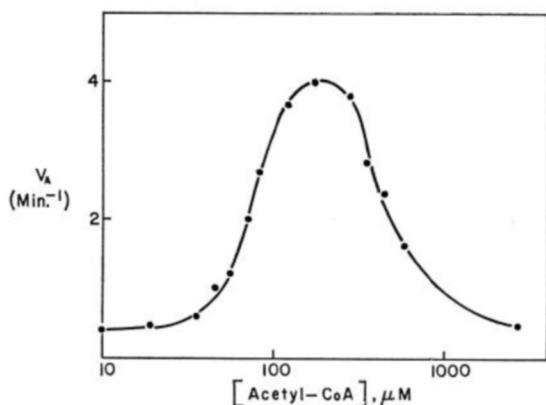


FIG. 7. The rate of inactivation of pyruvate carboxylase by avidin as a function of the concentration of acetyl-CoA. The system contained, in 0.2 ml, 7.5  $\mu$ moles of Tris-sulfate (pH 7.8), 10  $\mu$ moles of  $(\text{NH}_4)_2\text{SO}_4$ , 63  $\mu$ g of pyruvate carboxylase (specific activity, 14.1), 70  $\mu$ g of avidin (3.3 units per mg), and the acetyl-CoA concentrations indicated. Residual enzyme activity was measured after 2 and 4 min of incubation at 23° by dilution of a 5- $\mu$ l aliquot into 1.0 ml of the spectrophotometric assay mixture for  $\text{CO}_2$  fixation (1). These data were analyzed to give the pseudo-first order rate constants for inactivation by avidin as described previously (8).

action order for enzyme sites is unaffected in the presence of acetyl-CoA, the reaction order for avidin is altered. In this case the data would not be susceptible to simple analysis. Despite the failure to obtain a dissociation constant for the enzyme-acetyl-CoA complex from these data, the known specificity of avidin for the biotin moiety in the active site of pyruvate carboxylase strongly suggests that the observed effect of acetyl-CoA is related to a change in the environment of the biotin residues in the active site of this enzyme. This conclusion is supported by the observation that the concentration range in which acetyl-CoA increases the rate of inactivation by avidin is similar to the range of the activator constant obtained as the concentration required for 50% maximal activation of the over-all reaction (Fig. 1).

The decrease in the rate of inactivation by avidin observed at higher concentrations of acetyl-CoA (0.3 to 3.0 mM) may be due to nonproductive binding of this activator (33) in a manner analogous to substrate inhibition. This explanation is suggested by the finding that binding sites exist for both the acyl thioester and nucleotide moieties of acetyl-CoA, as discussed above. Since the over-all reaction is not significantly inhibited by high acetyl-CoA concentrations, this nonproductive binding may not occur in the presence of the other reaction components.

**Effect of Acetyl-CoA on Denaturation of Pyruvate Carboxylase by Sodium Dodecyl Sulfate**—In the presence of 0.02 M sodium dodecyl sulfate, pyruvate carboxylase has been shown to dissociate into subunits ( $s_{20,w}^0 = 2.7 \text{ S}$ ) (11). At concentrations of this detergent 25 to 50 times lower than those used to induce dissociation of the protein, a time-dependent inactivation of the enzyme is observed which is second order in enzyme sites (Fig. 8). The presence of acetyl-CoA (30 to 200  $\mu$ M) protects the enzyme against this inactivation by low concentrations of sodium dodecyl sulfate. Sedimentation analysis of pyruvate carboxylase in the presence of 0.6 mM sodium dodecyl sulfate shows that under these conditions the 14.8 S component is completely lost (Fig. 9B2). No 2.7 S material is formed under these conditions, and there is no increase in the 6.75 S fraction

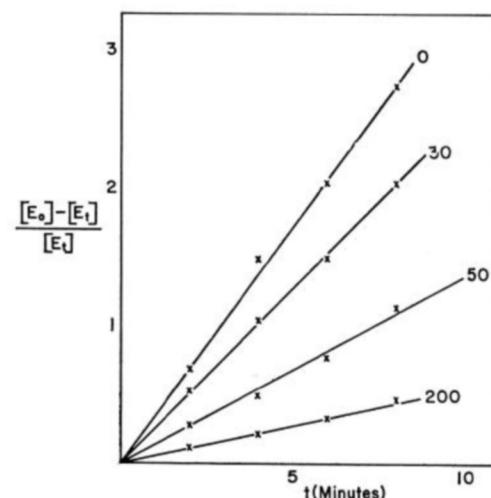


FIG. 8. The effect of acetyl-CoA on the inactivation of pyruvate carboxylase by sodium dodecyl sulfate. The system contained, in 0.2 ml, 10  $\mu$ moles of Tris-sulfate (pH 7.2), 8  $\mu$ moles of  $(\text{NH}_4)_2\text{SO}_4$ , 84  $\mu$ g of pyruvate carboxylase (specific activity, 10.6), 36  $\mu$ moles of sodium dodecyl sulfate, and the micromolar acetyl-CoA concentrations indicated by the numbers by the lines. Residual enzymic activity ( $E_1$ ) was determined at the times ( $t$ ) indicated as described for Fig. 7. The initial enzymic activity ( $E_0$ ) was obtained in a system lacking sodium dodecyl sulfate.

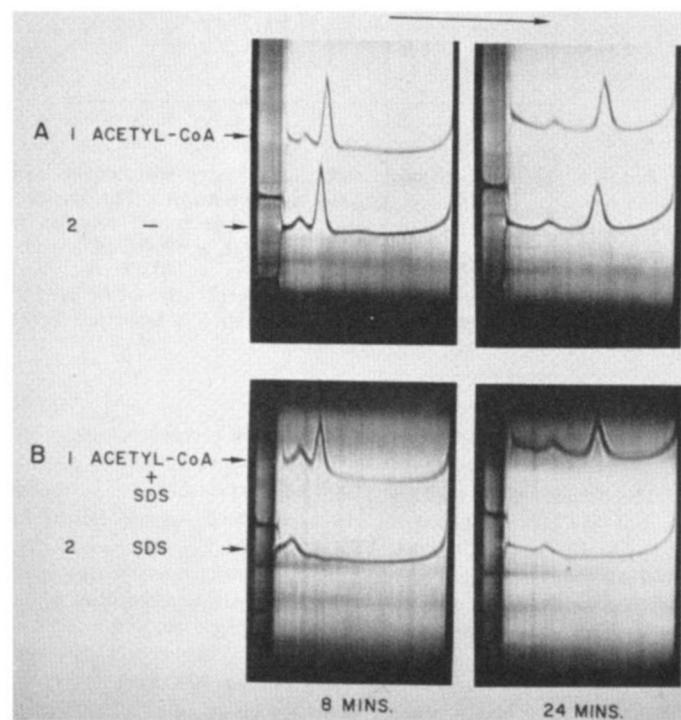


FIG. 9. The effect of acetyl-CoA on the loss of the 14.8 S component induced by low concentrations of sodium dodecyl sulfate (SDS). A, pyruvate carboxylase (4.0 mg per ml; specific activity, 24.2) in 0.05 M Tris-Cl, pH 7.2, containing 0.06 M  $(\text{NH}_4)_2\text{SO}_4$ ; B, pyruvate carboxylase in 0.05 M Tris-Cl, pH 7.2, containing 0.06 M  $(\text{NH}_4)_2\text{SO}_4$  and 0.5 mM sodium dodecyl sulfate. The upper schlieren pattern in both A and B, which corresponds to the wedge cell, is given by the systems described above with the addition of 0.3 mM acetyl-CoA. Sedimentation was in the direction of the arrow at 60,000 rpm and 22°. The schlieren photographs were taken at 8 and 24 min, and at phase plate angles of 55° (8 min) and 50° (24 min).

which is observed in all the experiments. In the presence of 0.3 mM acetyl-CoA, the loss of the 14.8 S component is partially prevented (Fig. 9B1), and the sedimentation pattern qualitatively resembles that observed for pyruvate carboxylase sedimented in the presence of the same concentration of acetyl-CoA (Fig. 9A1), but shows a decrease in the percentage of the 14.8 S component present. Measurement of the catalytic activity in the same samples show that at the times indicated approximately 70% of the original activity remained in the sample corresponding to Fig. 9B1, but that less than 5% remained in the sample corresponding to Fig. 9B2. No significant loss of activity occurred in the samples corresponding to Fig. 9, A1 and A2. Similar experiments, in which 0.02 M sodium dodecyl sulfate was used, show that acetyl-CoA has no effect on the dissociation

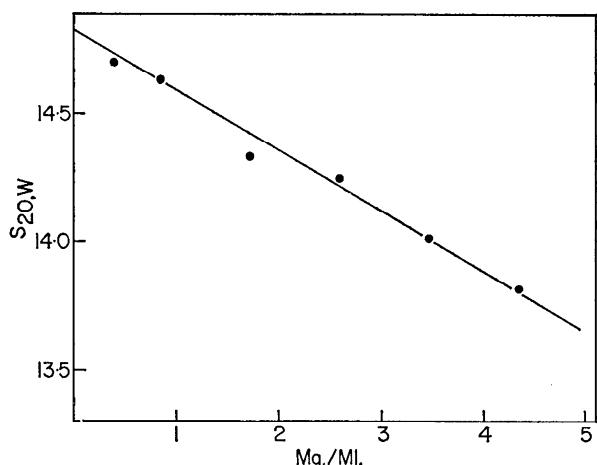


FIG. 10. The sedimentation coefficient of pyruvate carboxylase measured as a function of protein concentration. The various concentrations of pyruvate carboxylase (specific activity, 16.0) in 0.1 M Tris-sulfate, pH 7.2, containing 0.06 M  $(\text{NH}_4)_2\text{SO}_4$ , were examined by analytical ultracentrifugation at 60,000 rpm and 20–23° in 12-mm cells with aluminum centerpieces and an Spino AN-D rotor. The plates were analyzed with a Gaertner comparator.

TABLE III  
Effect of acetyl-CoA and other reaction components on sedimentation properties of pyruvate carboxylase

The system contained, in 1 ml, 0.1 M Tris-sulfate, pyruvate carboxylase (specific activity, 16 to 17), and, where indicated, 0.22 mM acetyl-CoA, 4.0 mM ATP, 10 mM  $\text{MgCl}_2$ , 10 mM  $\text{KHCO}_3$ , or 5 mM potassium pyruvate. The ionic strength was maintained at a value of 0.48 by addition of the appropriate amount of 1.0 M  $(\text{NH}_4)_2\text{SO}_4$ . All sedimentation data were obtained and analyzed as described for Fig. 10. The  $s_{20,w}^0$  values obtained were plotted against protein concentration as shown in Fig. 10 to give  $s_{20,w}^0$  from the ordinate intercept and  $-k$  from the slope.

System	$s_{20,w}^0$	$-k$
	$s$	
Pyruvate carboxylase.....	14.8	0.23
+ Acetyl-CoA.....	14.75	0.21
+ ATP.....	14.85	0.22
+ ATP, $\text{Mg}^{2+}$ , $\text{HCO}_3^-$ .....	14.96	0.21
+ ATP, $\text{Mg}^{2+}$ , $\text{HCO}_3^-$ , acetyl-CoA, pyruvate.....	14.8	0.2

of pyruvate carboxylase to small (2.7 S) subunits, which is induced by this concentration of the detergent.

Other studies<sup>4</sup> (11) indicate that acetyl-CoA prevents both the loss of catalytic activity and the dissociation of pyruvate carboxylase to 6.75 S subunits induced by exposure to 2° or in the presence of 0.4 M urea. The effect of acetyl-CoA on these three methods of denaturation of pyruvate carboxylase suggests that this cofactor is involved in the maintenance of subunit interactions, as will be discussed in a later section.

*Sedimentation Properties and Absorption Spectrum of Enzyme-Acetyl-CoA Complex*—Studies on allosteric enzymes have shown that in many cases the effector alters the sedimentation properties of the protein as well as its catalytic activity, as illustrated, for example, by the effect of citrate on acetyl-CoA carboxylase purified from rat adipose tissue (34) and chicken liver (35), the effect of ADP on NAD<sup>+</sup>-specific isocitrate dehydrogenase purified from bovine heart (23), and that of 5'-AMP on L-threonine dehydratase purified from *Escherichia coli* (36). In similar studies on pyruvate carboxylase, the sedimentation coefficient was measured as a function of protein concentration over the range from 0.5 to 5.0 mg per ml and extrapolated to infinite dilution to give  $s_{20,w}^0$  (intercept) and  $-k$  (slope). The relationship of  $s_{20,w}$  with protein concentration is linear over this range (Fig. 10), indicating that no significant dissociation of this enzyme occurs on dilution to 500  $\mu\text{g}$  per ml at pH 7.2 and 23°. The effects of acetyl-CoA and other reaction components on the sedimentation properties of pyruvate carboxylase are summarized in Table III as  $s_{20,w}^0$  and slope values taken from data plotted as shown in Fig. 10. No significant change is observed in either parameter under any of the conditions used, and other studies have shown that ATP,  $\text{Mg}^{2+}$ , and pyruvate added separately do not affect the sedimentation properties of pyruvate carboxylase. Furthermore, no detectable redistribution of protein between the 14.8 S and 6.75 S species found in all pyruvate carboxylase preparations (11) occurs on addition of the reaction components. These data exclude the possibility that major changes in the molecular weight of pyruvate carboxylase result from its activation by acetyl-CoA in the presence or absence of other reaction components. However, these studies do not exclude minor changes in sedimentation properties which might result from alterations in the relative orientation or affinity of the subunits of this enzyme in the presence of acetyl-CoA. Such changes would be similar to those proposed for the aspartate transcarbamylase system (37).

Alterations in the tertiary or quaternary structure of a protein in the presence of an allosteric ligand may also affect the environment of the aromatic residues and hence give rise to a ultraviolet difference spectrum on addition of the ligand. Such effects have been observed for carbamyl phosphate synthetase in the presence of *N*-acetylglutamate (32). Examination for similar alterations in the spectrum of pyruvate carboxylase at pH 7.8 in the presence of 10 to 100  $\mu\text{M}$  acetyl-CoA, by means of the "tandem" cell technique described by Herskovits and Laskowski (38), has failed to show any significant effect. Additionally, no marked ultraviolet difference spectrum resulting from the interaction of the adenine moiety of acetyl-CoA with pyruvate carboxylase is observed at acetyl-CoA concentrations in the region of the activator constant (20 to 50  $\mu\text{M}$ ). Therefore,

<sup>4</sup> J. J. Irias and M. F. Utter, unpublished observations.

neither the aromatic residues of the protein nor the adenine ring system of acetyl-CoA are markedly perturbed by the binding of this allosteric ligand to pyruvate carboxylase from chicken liver, suggesting that the environment of the aromatic residues is not significantly affected either by binding of acetyl-CoA or by changes in enzyme structure induced by this binding.

#### Hydrolysis of Acyl Thioesters Catalyzed by Pyruvate Carboxylase

**Demonstration and Properties of Reaction**—Highly purified pyruvate carboxylase preparations catalyze a very slow hydrolysis of acetyl-CoA and other acyl thioesters. This reaction is measured either by the decrease in absorbance at 232 m $\mu$  (Assay I) or by appearance of nonprotein sulphydryl groups (Assay II) as described in "Methods." Assay I is linear with time for 2 hours in the presence of 3 units of pyruvate carboxylase (Fig. 11A) and with enzyme concentration to 10 units over a 30-min incubation period. In the presence of other reaction components, Assay II is linear with time for 20 min with 2.5 units of pyruvate carboxylase (Fig. 11B), and with enzyme concentration to 20 units when a 10-min incubation period is used. The decreased region of linear response with time shown by Assay II as compared with Assay I may be due to the presence of the other reaction components.

The rate of hydrolysis of acetyl-CoA by pyruvate carboxylase in the absence of other reaction components is approximately 0.015% of the rate of CO<sub>2</sub> fixation but is stimulated 2- to 2.5-fold by their addition (Table IV). Maximal stimulation requires the presence of pyruvate, ATP, and Mg<sup>2+</sup>, but omission of pyruvate has a less marked effect on the rate than omission of ATP or Mg<sup>2+</sup>. Omission of HCO<sub>3</sub><sup>-</sup> does not affect the rate of hydrolysis, but no precautions were taken to remove or exclude endogenous

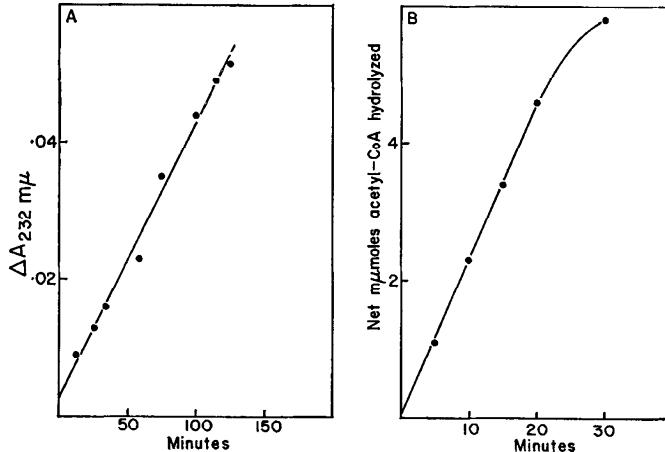


FIG. 11. Measurement of the time course of acetyl-CoA hydrolysis by pyruvate carboxylase. In A, the system contained, in 1.0 ml, 100  $\mu$ moles of Tris-sulfate (pH 7.8), 0.1  $\mu$ mole of acetyl-CoA, and 162  $\mu$ g of pyruvate carboxylase (specific activity, 11.8). Control cuvettes lacked either acetyl-CoA, pyruvate carboxylase, or both. The decrease in absorbance at 232 m $\mu$  was measured, and the results were corrected for nonenzymic hydrolysis of acetyl-CoA. In B, the system contained, in 0.5 ml, 100  $\mu$ moles of Tris-sulfate (pH 7.8), 0.1  $\mu$ mole of acetyl-CoA, 1.25  $\mu$ moles of ATP, 2.5  $\mu$ moles of MgCl<sub>2</sub>, 7.5  $\mu$ moles of KHCO<sub>3</sub>, 5  $\mu$ moles of potassium pyruvate, and 51  $\mu$ g of pyruvate carboxylase (specific activity, 15.1). After the times of incubation shown, nonprotein —SH was estimated as described in "Methods."

TABLE IV

#### Effect of reaction components on hydrolysis of acetyl-CoA by pyruvate carboxylase

The system contained, in 0.5 ml, 50  $\mu$ moles of Tris-sulfate (pH 7.8), 0.1  $\mu$ mole of acetyl-CoA, and, where indicated, 1.25  $\mu$ moles of ATP, 2.5  $\mu$ moles of MgSO<sub>4</sub>, 7.5  $\mu$ moles of KHCO<sub>3</sub>, 5  $\mu$ moles of potassium pyruvate, and 405  $\mu$ g of pyruvate carboxylase (specific activity, 15.1). Incubation was conducted for 10 min at 23°, after which the protein was precipitated and nonprotein —SH was estimated in the supernatant fraction as described in "Methods" (Assay II).

System	Net hydrolysis of acetyl-CoA
	$\mu$ moles/10 min
Enzyme + acetyl-CoA	7.2
Enzyme + reaction mixture	16.7
— ATP	9.3
— Mg <sup>2+</sup>	8.7
— Pyruvate	11.2
— HCO <sub>3</sub> <sup>-</sup>	16.8

TABLE V

#### Specificity of hydrolysis of acyl thioesters by pyruvate carboxylase

The system contained, in 0.5 ml, 50  $\mu$ moles of Tris-sulfate (pH 7.8) and either 276  $\mu$ g of pyruvate carboxylase (specific activity, 18.2) (Experiment A) or 432  $\mu$ g of pyruvate carboxylase (specific activity, 14.8) (Experiment B). Thioesters were added at the concentrations indicated, and ATP, Mg<sup>2+</sup>, HCO<sub>3</sub><sup>-</sup>, and potassium pyruvate were present at the concentrations indicated in Table IV. After incubation for 10 min at 23°, nonprotein —SH was assayed as described in "Methods" (Assay II).

Thioester	Net hydrolysis of acyl thioester	
	Enzyme + thioester	Enzyme + thioester + ATP, Mg <sup>2+</sup> , HCO <sub>3</sub> <sup>-</sup> , and pyruvate
	$\mu$ moles/10 min	
Experiment A		
Acetyl-CoA, 0.1 mM	3.7	9.5
Propionyl-CoA, 0.36 mM	4.3	12.1
Crotonyl-CoA, 0.3 mM	3.1	7.1
Experiment B		
Acetylmercaptosuccinate, 0.23 mM	7.0	7.0
Acetylpanthenotheine, 1.57 mM	4.2	2.5
Methylmalonyl-CoA, 0.35 mM	3.1	3.1
Acetyl-CoA, 0.1 mM	7.5	17.9

CO<sub>2</sub> from the system. Under these conditions the rate of CO<sub>2</sub> fixation is 30 to 50% of the rate observed in the presence of saturating concentrations of HCO<sub>3</sub><sup>-</sup>.

Examination of the specificity of the reaction showed that other acyl thioesters which are activators of the pyruvate carboxylase reaction, e.g. propionyl-CoA or crotonyl-CoA, are also hydrolyzed, and their rate of hydrolysis is stimulated by addition of the other reaction components (Table V, Experiment A). In contrast, inhibitory acyl thioesters, e.g. acetylmercaptosuccinate or methylmalonyl-CoA, do not show stimulation by the

TABLE VI

*Effect of inhibitors of pyruvate carboxylase on hydrolysis of acetyl-CoA*

The complete system contained, in 1.0 ml, 100  $\mu$ moles of Tris-sulfate (pH 7.8), 0.1  $\mu$ mole of acetyl-CoA, 2.5  $\mu$ moles of ATP, 5  $\mu$ moles of MgSO<sub>4</sub>, 15  $\mu$ moles of KHCO<sub>3</sub>, 10  $\mu$ moles of potassium pyruvate, and approximately 500  $\mu$ g of pyruvate carboxylase (specific activity, 15). After incubation for 10 min at 23°, the protein was precipitated and nonprotein —SH was measured as described in "Methods" (Assay II).

Inhibitor	Relative rate of hydrolysis
None	100
Oxalate, 0.5 mM	102
Avidin, 1 unit (preincubated 30 min at 23°)	110
DTNB, 0.1 mM	60-70

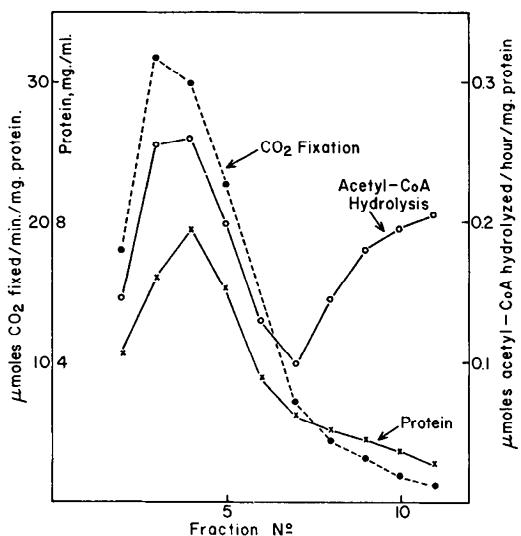


FIG. 12. Correlation of CO<sub>2</sub> fixation and acetyl-CoA hydrolysis by gel filtration on Sephadex G-200. CO<sub>2</sub> fixation and protein were measured spectrophotometrically, and acetyl-CoA hydrolysis was determined by the decrease in absorbance at 240  $\mu$ m as described in "Methods."

reaction components although they are hydrolyzed (Table V, Experiment B). Specific inhibitors of pyruvate carboxylase, e.g. avidin (27) or oxalate (30), do not affect the rate of hydrolysis, with the exception of DTNB, which causes partial inhibition (Table VI). The observation that the other reaction components stimulate the hydrolysis only of those acyl-CoA analogues that activate the over-all reaction suggests a relationship between thioester hydrolysis and pyruvate carboxylase. However, the studies with the other thioesters and with specific inhibitors of pyruvate carboxylase do not provide any further support for this proposal.

*Correlation of Acetyl-CoA Hydrolysis with Pyruvate Carboxylase*—More definitive evidence for a relationship between the observed thioester hydrolysis and pyruvate carboxylase was sought by protein fractionation studies similar to those used previously to correlate exchange reactions with this enzyme (7, 8). CO<sub>2</sub> fixation and acetyl-CoA hydrolysis (Assay I) were measured in a series of fractions obtained by gel filtration of Stage 5 pyruvate carboxylase (11) on a Sephadex G-200 column. The fractions

showed a 26-fold range of specific activity, with a maximal value of 31.7 when assayed for CO<sub>2</sub> fixation. A clear correlation of the thioester hydrolysis with CO<sub>2</sub> fixation is observed in the early fractions (Fractions 2 through 5) (Fig. 12), but hydrolytic activity is also present in the later fractions (Fractions 8 through 11), which have little pyruvate carboxylase activity. Previous experiments (11) have shown that Stage 5 preparations of pyruvate carboxylase contain two components with sedimentation coefficients that, when extrapolated to infinite dilution, are 14.8 S and 6.75 S, respectively, and that pyruvate carboxylase activity is confined to the 14.8 S component. The 6.75 S component, however, is believed to be related, at least in part, to a subunit of pyruvate carboxylase on the basis of biotin content (11), manganese content (12), and ATP-ADP exchange studies (8). Electron micrographic and sedimentation studies on the dissociation of this enzyme at 2° provide definitive evidence for the presence of subunits of this size in the pyruvate carboxylase protein (13). Fig. 12 suggests, therefore, that thioester hydrolysis in the absence of other reaction components is a property of both the subunit and the active enzyme.

Further studies on a series of pyruvate carboxylase fractions obtained from another Sephadex G-200 column show that a similar relative distribution of CO<sub>2</sub> fixation and acetyl-CoA hydrolysis is observed in the earlier fractions (Fractions 1 through 7) when hydrolysis is measured by the release of nonprotein sulphydryl (Assay II) in the presence or absence of the other reaction components (Fig. 13). The stimulation of acetyl-CoA hydrolysis by the other reaction components, which approximates 2.4-fold in Fractions 1 through 6, is also correlated with pyruvate carboxylase. The data also indicate that the stimulation of the hydrolysis reaction by other reaction components is a property of the 6.75 S subunit, since an increased specific activity for both the stimulated and unstimulated reactions is observed in Fractions 10 through 15. This finding is in accord with previous findings which indicate that ATP, Mg<sup>2+</sup>, and pyruvate can bind to this subunit (8, 12).

The data shown in Figs. 12 and 13 strongly suggest that the observed hydrolysis of acetyl-CoA and other thioesters is a property of pyruvate carboxylase but do not clarify the relationship of this reaction to the activation of pyruvate carboxylation by an acyl-CoA. The specific stimulation of activator acyl-CoA hydrolysis by the other reaction components (Table V, Experiment A) suggests such a relationship when compared with inhibitory acyl thioesters, e.g. methylmalonyl-CoA or acetyl-mercaptosuccinate, the rates of hydrolysis of which are not stimulated under these conditions (Table V, Experiment B).

A possible mechanism for the hydrolysis reaction is shown in Scheme 1. The over-all and hydrolysis reactions might most simply be related by the proposal that the acyl-enzyme (Complex III) is the activated species, but several arguments exclude this possibility. First, assuming four sites for thioester hydrolysis, the lifetime of the acyl-enzyme at pH 7.8 and in the presence of the other reaction components may be calculated from the observed rate of hydrolysis as approximately 30 sec. This is clearly inconsistent with the lifetime of the activated complex, which does not exceed 1 sec when measured by dilution techniques as described above. Second, certain acetylated analogues of acetyl-CoA, e.g. acetylpanthenone, which are hydrolyzed by pyruvate carboxylase (Table V, Experiment B) and therefore give rise to an acetyl-enzyme (Complex III), are potent competitive inhibitors of the over-all reaction when the initial rate

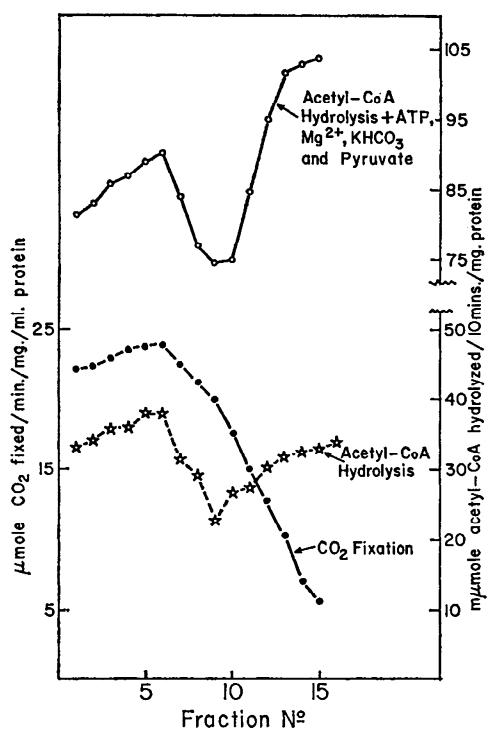


FIG. 13. Correlation of  $\text{CO}_2$  fixation and acetyl-CoA hydrolysis in the presence and absence of other reaction components by gel filtration on Sephadex G-200.  $\text{CO}_2$  fixation and protein were measured spectrophotometrically. Acetyl-CoA hydrolysis was measured by release of nonprotein —SH (Assay II) as described in "Methods," but with 5% trichloroacetic acid to precipitate the protein. For the assay of acetyl-CoA hydrolysis in the presence of reaction components, the system contained, in 0.5 ml, 50  $\mu\text{moles}$  of Tris-Cl (pH 7.8), 0.1  $\mu\text{mole}$  of acetyl-CoA, 1.25  $\mu\text{moles}$  of ATP, 2.5  $\mu\text{moles}$  of  $\text{MgCl}_2$ , 7.5  $\mu\text{moles}$  of  $\text{KHCO}_3$ , and 10  $\mu\text{moles}$  of Tris-pyruvate.

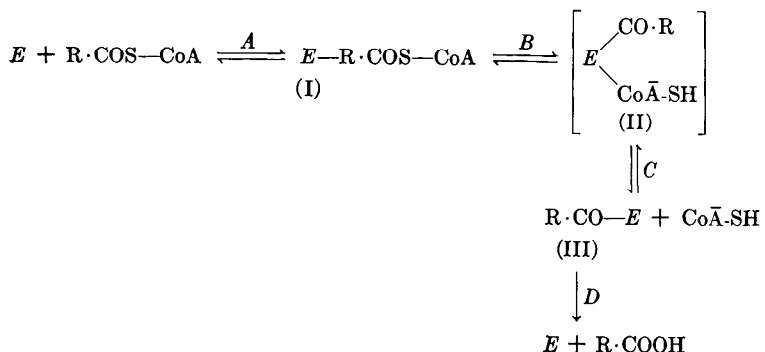
specific stimulation of activator acyl-CoA hydrolysis by other reaction components (Table V, Experiment A) indicates that either Complex I or II may represent the activated species. The process of activation may then cause the thioester bond to show an increased sensitivity to nucleophilic attack by hydroxyl ion, resulting in the observed slow hydrolysis.

The data obtained for the hydrolysis reaction also point to a requirement for both the acyl thioester and nucleotide portions of the molecule, as discussed earlier for the over-all reaction. Stimulation of the rate of hydrolysis by other reaction components (Table V) is observed only when both these portions of the molecule are unmodified.

#### DISCUSSION

The results described here provide further insight into the mechanism by which acyl-CoA derivatives activate pyruvate carboxylase purified from chicken liver, but do not permit a complete understanding of this phenomenon. The purified enzyme shows an essentially absolute requirement for an acyl-CoA under all conditions tested thus far. The studies of Krebs *et al.* (39) further indicate that pyruvate carboxylase in rat kidney cortex slices is markedly stimulated by certain of the acyl-CoA analogues which activate the enzyme purified from chicken liver (Table I). The latter findings are consistent with the conclusion that the requirement of pyruvate carboxylase for activation by an acyl-CoA is not an artifact of the procedures used in the purification of this enzyme.

Whereas an essentially absolute requirement for activation by an acyl-CoA is a feature of many mammalian and avian pyruvate carboxylases, similar enzymes purified from microbial sources show either a partial requirement for acetyl-CoA, *e.g.* bakers' yeast (5, 6), or a complete absence of this requirement, *e.g.* *P. citronellolis* (8). Studies of the exchange reactions catalyzed by the pyruvate carboxylases purified from chicken



SCHEME 1. A possible reaction sequence for hydrolysis of acetyl-CoA analogues and other thioesters by pyruvate carboxylase

is measured as a function of the acetyl-CoA concentration (Fig. 6). Finally, pyruvate carboxylase is not activated by incubation with acetylating agents such as acetic anhydride and *N*-acetylimidazole. This conclusion has been confirmed in preliminary experiments, which show that the acetyl-enzyme isolated at pH 7 is inactive in the absence of acetyl-CoA.<sup>5</sup> The pathway shown in Scheme 1 may therefore be related only indirectly to the activation of the over-all reaction by an acyl-CoA. However, the relationship between thioester hydrolysis and the activation of the over-all reaction suggested by the

liver (7), bakers' yeast (5), and *P. citronellolis* (3) suggest, however, that a very similar minimal mechanism is involved in all cases (Reactions 2 and 3). For the enzymes purified from chicken liver and bakers' yeast, the activation by acetyl-CoA is localized in Reaction 2, the formation of *E*-biotin  $\sim$   $\text{CO}_2$  from ATP and  $\text{HCO}_3^-$  (5, 7). When an essentially absolute requirement for acyl-CoA activation is found, mechanisms must be considered in which the acyl-CoA is utilized and regenerated during the reaction sequence. Two possible classes of mechanisms of this type may be proposed: (a) sequences in which pyruvate reacts with the acyl-CoA to form an activated species,

<sup>5</sup> C. H. Fung and M. F. Utter, unpublished observations.

*e.g.* pyruvyl-CoA, which then accepts CO<sub>2</sub> from the *E*-biotin ~ CO<sub>2</sub> complex, and (*b*) sequences in which the acyl-CoA acts as a CO<sub>2</sub> carrier during the reaction via the formation of its carboxylated analogue, *e.g.* malonyl-CoA.

The first type of mechanism is inconsistent with the finding that acetyl-CoA activation is localized in Reaction 2, the formation of *E*-biotin ~ CO<sub>2</sub> from ATP and HCO<sub>3</sub><sup>-</sup>, and that the exchange of pyruvate with oxalacetate occurs in the absence of any added reaction components. The latter finding requires that pyruvate itself accept CO<sub>2</sub> from the *E*-biotin ~ CO<sub>2</sub> complex. Evidence against the participation of the second type of mechanism is provided by the failure to observe release of tritium from acetyl-CoA when oxalacetate formation is catalyzed in the presence of acetyl-CoA labeled in the acetyl group with tritium (9). Additionally, both malonyl-CoA and methylmalonyl-CoA are potent inhibitors of the pyruvate carboxylase reaction (Table II), and show competitive characteristics when the initial reaction rate is measured as a function of the acetyl-CoA concentration (Fig. 5). This observation is not consistent with the proposal that the carboxylated analogues of the activators are intermediates of the pyruvate carboxylase reaction.

The elimination of the more probable mechanisms providing a direct role for the activator acyl-CoA in the reaction sequence has led to the proposal that the activation of pyruvate carboxylase occurs as a result of a change in the structure of the protein in the presence of the activator acyl-CoA (9). This proposal is more consistent with the wide divergence in the requirement of pyruvate carboxylases purified from various sources for activation by an acyl-CoA, as contrasted with their apparent similarity in minimal mechanism noted above. Although direct evidence for an allosteric mode of interaction is lacking for the acyl-CoA, in that spatial separation of the catalytic and activator sites has not yet been demonstrated for the pyruvate carboxylase system, a considerable body of indirect evidence supports this proposal. First, examination of the initial rate as a function of the acyl-CoA concentration reveals a sigmoid relationship (Fig. 1 and Table I), suggesting that this enzyme possesses multiple interacting sites for the activator. Preliminary evidence indicates that ATP, HCO<sub>3</sub><sup>-</sup>, and pyruvate do not show this behavior over a wide range of acetyl-CoA concentration.<sup>6</sup> The present evidence may be interpreted to indicate that if two forms of the enzyme exist in equilibrium with each other, as suggested in the general theory of allosteric transitions proposed by Monod, Wyman and Changeux (40), only the acyl-CoA possesses markedly different affinities for these two forms of the enzyme. The results obtained for pyruvate carboxylase may be contrasted with the initial rate data for isocitrate dehydrogenase purified from yeast (41) where sigmoid relationships exist for both the substrates and the activators. In terms of the model proposed by Atkinson, Hathaway, and Smith (41), it may be suggested that interaction occurs only between the activator sites and not between the catalytic sites of pyruvate carboxylase.

A second line of evidence derives from the finding that marked effects of acetyl-CoA are observed on the rates of inactivation of pyruvate carboxylase either by denaturation, *e.g.* by cold, urea, and sodium dodecyl sulfate, or by specific binding of the biotin residues by avidin. The acceleration of the rate of in-

activation by avidin in the presence of acetyl-CoA concentrations (Fig. 7) which are similar to those required for 50% maximal activation of CO<sub>2</sub> fixation suggests that marked alterations in the environment of the biotin residues are induced by binding of acetyl-CoA. Previous studies (7) are consistent with the identification of the enzyme-bound intermediate of pyruvate carboxylase as 1'-*N*-carboxybiotin and therefore implicate the biotin residues of this enzyme as one part of the active site. The enhancement of the bound manganese of pyruvate carboxylase, however, is not affected in the presence of acetyl-CoA at concentrations approximating K<sub>A</sub> (30). Evidence has previously been presented to indicate that the bound manganese forms another part of the active site of this enzyme (30).

In contrast to the acceleration of inactivation by avidin, acetyl-CoA protects pyruvate carboxylase against denaturation at 2° and in the presence of low concentrations of urea and sodium dodecyl sulfate. Denaturation under these conditions involves alterations in the interaction between the subunits of pyruvate carboxylase which may be expressed as dissociation (2°, low urea) or loss of the 14.8 S component (low sodium dodecyl sulfate). A further effect of acetyl-CoA on pyruvate carboxylase may therefore involve the maintenance of subunit interactions which are required for stabilization of the catalytically active structure. The similarity in the concentration ranges over which acetyl-CoA activates the over-all reaction, accelerates the rate of inactivation by avidin, and protects this enzyme against denaturation suggests a relationship among these effects.

The relatively specific requirement for activation by acetyl-CoA shown by pyruvate carboxylases purified from avian and mammalian sources may be of considerable importance in connection with the proposed key role of this enzyme in the regulation of gluconeogenesis in liver and kidney (9, 39). Pyruvate carboxylase and phosphoenolpyruvate carboxykinase provide a pathway by which pyruvate may be converted to phosphoenolpyruvate without invoking reversal of the pyruvate kinase reaction (42), and studies of both these enzymes in a variety of dietary and hormonal states have shown that, while the level of phosphoenolpyruvate carboxykinase may be changed dramatically (43), the variations in the level of pyruvate carboxylase either are much less marked (44-46) or may not occur. Recently Williamson (47) has examined the rapid changes in rat liver metabolite levels resulting from administration of glucagon. The observed changes are consistent with the identification of a control point in hepatic gluconeogenesis at the pyruvate carboxylase level. Physiological control of the pyruvate carboxylase reaction may therefore be exerted more effectively by fluctuations in the levels of the substrates and cofactors of this enzyme, and alterations of the level of total enzyme activity may be less important than is the case for phosphoenolpyruvate carboxykinase. This proposal is consistent with the apparent absence of an allosteric effector for phosphoenolpyruvate carboxykinase. The studies of Krebs *et al.* (39) indicate that the rate of glucose formation from lactate in rat kidney cortex slices may be effectively controlled by alterations in the acetyl-CoA level, and the specificity of the observed effects suggests a key role for pyruvate carboxylase. Williamson *et al.* (48) have further shown that the total acetyl-CoA concentration in rat liver rises 2- to 3-fold when the rate of gluconeogenesis is increased by fasting or administration of glucagon and that the level in untreated control animals approxi-

<sup>6</sup> M. C. Scrutton, C. H. Fung, and M. F. Utter, unpublished observations.

mates the activator constant observed for pyruvate carboxylase. Other workers have also presented evidence suggesting that the total acetyl-CoA concentration in liver and kidney cortex lies in a range from 17 to 50  $\mu\text{moles}$  per g, wet weight (39, 49, 50).

Although all of these considerations are consistent with the proposed role for acetyl-CoA in the regulation of pyruvate carboxylase and, hence, of gluconeogenesis, the interpretation based on the observed tissue levels of acetyl-CoA must be accepted with reservations since the major mechanisms for acetyl-CoA production *in vivo* are largely intramitochondrial, whereas many of the pathways which utilize this metabolite, *e.g.* fatty acid synthesis and cholesterol synthesis, are extramitochondrial. The intramitochondrial acetyl-CoA levels might therefore be expected to be higher than values obtained for the whole cell.

Some evidence has been presented suggesting that the ATP:ADP ratio might also play a role in the regulation of pyruvate carboxylase (9), and the potent inhibition of pyruvate carboxylase by malonyl-CoA reported here suggests that control by the ratio of acetyl-CoA to malonyl-CoA should be considered. Fifty per cent inhibition of pyruvate carboxylation would be achieved in the presence of an acetyl-CoA to malonyl-CoA ratio of 2:1, in contrast to the requirement for an ATP:ADP ratio of 1:10 to give a similar degree of inhibition. Although control by the ratio of acetyl-CoA to malonyl-CoA would represent a very sensitive mechanism, its physiological significance may be questionable since the major pathways involved in the production and utilization of malonyl-CoA are believed to be extra-mitochondrial.

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