Pyruvate Carboxylase

STUDIES OF ACTIVATOR-INDEPENDENT CATALYSIS AND OF THE SPECIFICITY OF ACTIVATION BY ACYL DERIVATIVES OF COENZYME A FOR THE ENZYME FROM RAT LIVER*

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SUMMARY

Oxalacetate synthesis catalyzed by pyruvate carboxylase from rat liver in the absence of acetyl-CoA exhibits a pH dependence and specificity for activation by univalent and divalent cations similar to that reported previously for acetyl-CoA-dependent oxalacetate synthesis by this enzyme (McClure, W. R., Lardy, H. A., and Kneifel, H. P. (1971) J. Biol. Chem. 246, 3569-3578). Fractionation studies have provided no indication that different species are responsible for catalysis in the presence or absence of this activator. However, linear Arrhenius and van't Hoff plots are observed for the temperature dependence of oxalacetate synthesis in the absence of acetyl-CoA over the range 10 to 50°C and E_a is obtained as 15.4 Cal per mole. In contrast in the presence of acetyl-CoA biphasic Arrhenius and van't Hoff plots are observed over this temperature range with the changes in slope occurring at approximately 25°C. The values obtained for E_a above and below 25°C are 9.1 and 31.5 Cal per mole, respectively. Hence the extent of activation of the maximal rate of oxalacetate synthesis by acetyl-CoA is a function of the temperature of observation.

The apparent K_A describing activation of rat liver pyruvate carboxylase by acetyl-CoA is a function of [pyruvate] but shows no significant dependence on [MgATP]^2- or [HCO_3^-]. The apparent K_A decreases from a value of 145 μM at [pyruvate]→ 0 to 50 to 55 μM as the pyruvate concentration is increased to saturation. These data, which may be relevant to in vivo regulation of the pyruvate→oxalacetate flux, indicate a specific interaction between the catalytic and activator sites of rat liver pyruvate carboxylase.

Examination of the specificity of activation of rat liver pyruvate carboxylase by acyl derivatives of coenzyme A and related compounds has shown that acetyl-CoA is the most potent activator of this enzyme. Effective activation is also observed in the presence of alkylacyl homologs in which the acyl chain contains 3 (propionyl-CoA) to 12 (n-dodecanoyl-CoA) carbon atoms while other derivatives, e.g., CoA-SH, adenosine 3':5':diphosphate, and phenylacetyl-CoA, are weak activators. Carboxylic derivatives of coenzyme A, e.g., succinyl-CoA, and derivatives in which the phospho-adenosine moiety is modified (e.g., acetyl-3':5'-diphospho-CoA, acetyldeamino-CoA, adenosine 2':5':diphosphate) act as inhibitors of rat liver pyruvate carboxylase. The Hill coefficient describing the relationship between initial rate and activator concentration approximates 2.0 for all activators except adenosine 3':5':diphosphate indicating that the presence of the 4'-phosphopantetheine portion of the CoA molecule is necessary for observation of cooperative interaction between activator sites. The presence of an alkylacyl moiety enhances the effectiveness of activation as indicated by an increased V_max and, in some instances, a more favorable apparent K_A. Since longer chain alkylacyl derivatives of coenzyme A act as inhibitors of chicken liver pyruvate carboxylase these data also indicate a marked difference in the response of the chicken and rat liver enzymes to the size of the acyl moiety.

Previous studies on pyruvate carboxylases purified from a wide spectrum of organisms have shown a remarkable constancy of catalytic properties for this enzyme which catalyzes Reaction 1 (cf. Ref. 1).

Pyruvate + MeATP^2- + HCO_3^- → oxalacetate + MeADP^2- + P_i

In contrast the effector properties of these enzymes vary widely (cf. 1) and striking differences in molecular structure, which may correlate with the variation in the effector properties, have also been reported (2, 3). All vertebrate liver pyruvate carboxylases studied thus far exhibit a requirement for activation by acetyl-CoA. In the case of the chicken liver enzyme no significant catalytic activity is observed in the absence of this activator (4). However recent studies have shown that pyruvate carboxylases purified from rat liver and sheep kidney cortex catalyze oxalacetate synthesis at a significant rate in the absence of acetyl-CoA (5, 6). Addition of all three substrates at high concentration is required for expression of maximal catalytic activity in the absence of the activator (5, 6) and it therefore appears unlikely that the property has any physiological significance. However, this observation has provided an approach to investigation of the relationship between the catalytic and activator sites on these pyruvate carboxylases. For example, direct evidence has been obtained for the allosteric nature of the activation by acetyl-CoA. Thus, incubation of
Pyruvate carboxylyases from rat liver, sheep kidney cortex, and Saccharomyces cerevisiae with amino group-specific reagents, e.g., trinitrobenzenesulfonate, has been shown to cause specific inactivation of the catalytic activity which is dependent on the presence of acetyl-CoA (7, 8). In the case of pyruvate carboxylyases from chicken liver and S. cerevisiae, inactivation of catalytic activity dependent on acetyl-CoA has no effect on the properties of regulatory inhibition by α-ketoglutarate and L-aspartate, respectively (7, 9). Additionally, quantitative differences are observed in the properties of the activation of pyruvate carboxylyase from mammalian and avian liver by acetyl-CoA. Activation of the avian liver enzymes is characterized by an apparent $K_a$ in the range of 2 to 4 mM, whereas for the mammalian liver and kidney cortex enzymes under comparable conditions approximating 3, whereas for the mammalian liver and kidney cortex enzymes under comparable conditions appropriate $K_a$ values and Hill coefficients in the range 15 to 25 μM and 2, respectively, are observed (10–12).

Such studies suggest that the properties of the activation site and of catalytic site–activator site interaction differ markedly in pyruvate carboxylylases obtained from these two phyla. Since the specificity of activation of pyruvate carboxylyase from chicken liver has been thoroughly characterized (13), it appeared of interest to perform a similar study for the enzyme from rat liver. The results of this study which are reported here indicate the existence of striking differences in activator specificity between the two enzymes. Data are also reported which provide further evidence for interaction between the catalytic and activator sites on rat liver pyruvate carboxylyase, and which define more completely the properties of catalysis by this enzyme in the absence of acetyl-CoA. A brief report of some of these data has been presented (14).

**MATERIALS AND METHODS**

Pyruvate carboxylylases were purified from chicken and rat liver mitochondria by procedures described previously (9, 15). The preparations employed in these studies had specific activities in the range 12 to 15 units per mg (chicken liver) or 7 to 12 units per mg (rat liver). When analyzed by polyacrylamide gel electrophoresis with the use of an enzymic activity stain the purity of these preparations was 60 to 70% (chicken liver) or 50 to 60% (rat liver) (cf. Fig. 2).

Enzymic activity was estimated from the initial rate of the decrease in absorbance at 340 nm in a system containing the components of the pyruvate carboxylylase reaction, excess malate dehydrogenase and pyruvate carboxylylase preparations by passage over columns (10 × 1 cm) of Sephadex G–25 equilibrated with 0.1 M Tris–Cl, pH 7.2, containing 0.2 mM KCl and 0.1 mM dithioerythritol. For the studies described in Table III these columns were equilibrated with 0.1 M (CH₃NH₂)₆HPO₄, pH 7.2, 0.2 mM (CH₃)₂NCl, and 0.1 mM dithioerythritol. Protein concentration was estimated from the relationship $\text{OD}_{280} = 0.5$ (chicken liver pyruvate carboxylylase) (17) or 0.8 (rat liver pyruvate carboxylylase) (11).

Polyacrylamide gel electrophoresis was performed as described by Gabriel (18). Gels (3.75%) were polymerized in 0.008 M Tris–Cl, pH 8.0. Pyruvate carboxylylase samples (0.02 to 0.01 unit; 2 to 10 μg of protein) equilibrated with 0.008 M Tris–phosphate, pH 7.2, containing 0.1 mM dithioerythritol were applied to the gels and subjected to electrophoresis in the Tris-phosphate buffer for 4 hours at a current of 3 mA per gel. The electrophoretic separation was performed in the cold room with precooled gels and buffers in order to minimize the increase in temperature. After electrophoresis, gels were stained for protein by incubation for 1 hour at 80° in 0.1% Coomassie blue dissolved in 50% methanol–7% acetic acid. Excess dye was removed by incubation at 80° in several changes of 50% methanol–7% acetic acid.

The assay media employed for detecting pyruvate carboxylylase activity on polyacrylamide gels contained either: Medium A, 100 mM K⁺–Hepes, pH 7.8, 5 mM sodium pyruvate, 2 mM ATP, 5 mM MgCl₂, 50 mM KHCO₃, and 0.1 mM acetyl-CoA (for detection of acetyl-CoA-dependent catalytic activity); or Medium B, 100 mM K⁺–Hepes, pH 7.8, 20 mM sodium pyruvate, 10 mM ATP, 15 mM MgCl₂, and 200 mM KHCO₃ (for detection of acetyl-CoA-independent catalytic activity). After incubation for 60 min at 25° in 2.5 ml of either Medium A or B the gels were then washed in distilled water. Oxalacetate production was detected by transfer of the gel to 2.5 ml of a solution containing fast violet B (1 mg per ml) and incubation for 15 to 20 min at 25° in the dark. Under these conditions the site of oxalacetate production appears as a red band. Nonspecific staining was minimized by washing the developed gel in distilled water to remove excess dye immediately following incubation with fast violet B. The stained gels were scanned at 530 nm (pyruvate carboxylylase activity) or 660 nm (protein) using a Gilford model 240 spectrophotometer equipped with a model 2410S linear transport.

Pyruvate carboxylylases were purified from chicken and rat liver mitochondria by procedures described previously (9, 15). The preparations employed in this study were obtained from P-L Biochemicals Inc., or in some cases (crotonyl-CoA, tiglyl-CoA, isovaleryl-CoA, n-valeryl-CoA, maleyl-CoA, glutaryl-CoA, diglycolyl-CoA) were the kind gift of Doctors C. H. Fung and M. F. Ting. The acetyl-CoA was purified by chromatography on DEAE-cellulose or DEAE-Sephadex A-25 (25°) and gel filtration on Sephadex G-10 as described by Fung (15). The concentration of the final solutions were estimated from the absorbance at 259 nm (19). (CH₃)₄N⁺N⁺OH₃ was prepared by adding excess Dicyclohexylcarbodiimide to a 1 M solution of (CH₃)₄N⁺NOH and incubating for 1 hour at 25°. The H⁺ concentration of the solution was estimated as described by Simon and Shemin (22).

The various acyl derivatives of coenzyme A and related compounds employed in this study were obtained from P-L Biochemicals Inc., or in some cases (crotonyl-CoA, tiglyl-CoA, isovaleryl-CoA, n-valeryl-CoA, maleyl-CoA, glutaryl-CoA, diglycolyl-CoA) were the kind gift of Doctors C. H. Fung and M. F. Ting. The acetyl-CoA was purified by chromatography on DEAE-cellulose or DEAE-Sephadex A-25 (25°) and gel filtration on Sephadex G-10 as described by Fung (15). The concentration of the final solutions were estimated from the absorbance at 259 nm (19) and as the acyl hydroxamate according to the procedure of Lipmann and Tuttle (21). An acetyl-CoA solution of known concentration was employed to construct the standard curve for the acyl hydroxamate assay. The concentrations as determined by these two procedures agreed to better than 5% for all the derivatives examined.

Acetyl-CoA, acetyldelphosphoacetyl-CoA, and acetyldaamino-CoA were prepared from coenzyme A, dephosphoacetyl-CoA, and deamino-CoA (Chromatopure grade, P-L Biochemicals Inc.) as described by Gabriel (23). Acetyl-CoA was assayed as the acetylhydroxamate (21). Adenosine 2'5'-diphosphate and adenosine 3'5'-diphosphate were obtained from P-L Biochemicals Inc. The purity of these derivatives was established by chromatography on thin layer cellulose plates (24) and by determination of the phosphate to adenine ratio (19, 25).

Pyruvic acid was purified by distillation in vacuo and was stored as a 1 M solution. After neutralization with the appropriate cation, the concentration was estimated enzymically as described by Butcher et al. (20). (CH₃)₄N⁺N⁺OH₃, (C₆H₅)₃NOH, and (C₅H₄)₂N⁺NOH were obtained from Eastman Kodak Co.; and triethanolamine from Mallinkrodt. All other chemicals and reagents were prepared or obtained as described previously (16).

**RESULTS**

Linearity of Initial Rate of Oxalacetate Synthesis with Enzyme Concentration in Presence and Absence of Acetyl-CoA—In their studies on pyruvate carboxylylase purified from sheep kidney cortex, Adunni et al. (6) observed that the specific activity for oxalacetate synthesis was greatest at an enzyme concentration of 2.5 mg per ml. At 4 mg per ml, the activity was reduced by about 50%.
acetate synthesis in the absence of acetyl-CoA (Reaction 1) was a nonlinear function of enzyme concentration, and suggested that this effect might be due to a difference in the enzyme forms which catalyze the effector-dependent and independent reactions. We have therefore examined the relationship between initial rate and enzyme concentration for rat liver pyruvate carboxylase. A linear relationship is observed over the entire range tested for catalysis in the absence of acetyl-CoA and in the presence of saturating (Fig. 1), or nonsaturating, concentrations of pyruvate, MgATP$^2$, and HCO$_3$\. In the light of the studies reported for the sheep kidney cortex enzyme (6), it is particularly significant that no deviation from linearity is observed even at the lowest enzyme concentration employed. For the acetyl-CoA-dependent reaction a linear relationship is observed under similar conditions up to an initial velocity of approximately 0.4 A per min (Fig. 1). The deviation at higher enzyme concentrations can be abolished by doubling the concentration of malate dehydrogenase in the assay and hence is probably due to saturation of the coupling system.

Identity of Species which Catalyze Oxalacetate Synthesis in Presence and Absence of Acetyl-CoA—Further evidence for the identity of the protein species which catalyze oxalacetate synthesis (Reaction 1) in the presence and absence of acetyl-CoA is provided by various fractionation studies. When purified rat liver pyruvate carboxylase is subjected to electrophoresis on polyacrylamide gels, and the gels are subsequently stained for protein and for enzymic activity in the presence and absence of acetyl-CoA as described under "Materials and Methods," the data shown in Fig. 2 were obtained. The single band of catalytic activity observed when the gel is incubated in an assay mix containing acetyl-CoA (A) is coincident with that obtained on incubation in a mix lacking acetyl-CoA (C) and also with the major band detected in the gel stained for protein (E). These data suggest that under the conditions employed the same single species catalyzes oxalacetate synthesis in the presence or absence of acetyl-CoA. The relative extent of oxalacetate synthesis in the two systems, as indicated by the areas under the peak in A and C, is approximately that expected from the relationship of

![Fig. 1. Relationship between the initial rate of oxalacetate synthesis measured in the presence or absence of acetyl-CoA and the concentration of rat liver pyruvate carboxylase. For assay of oxalacetate synthesis in the presence of acetyl-CoA the assay system contained 100 mM K$^+$-Hepes, pH 7.8, 5 mM sodium pyruvate, 2 mM ATP, 5 mM MgCl$_2$, 30 mM KHCO$_3$, 0.05 mM acetyl-CoA, 25 µg of malate dehydrogenase, and 0.2 mM NADH in a total volume of 1.0 ml. For assay of oxalacetate synthesis in the absence of acetyl-CoA the system contained 100 mM K$^+$-Hepes, pH 7.8, 25 mM sodium pyruvate, 10 mM ATP, 15 mM MgCl$_2$, 200 mM KHCO$_3$, malate dehydrogenase, and NADH as above in a total volume of 1.0 ml. After equilibration to 25$^\circ$C the reaction was initiated by addition of rat liver pyruvate carboxylase (specific activity = 15.0 units per mg) as indicated. In order to avoid possible complications due to salt effects, the enzyme solution employed in this experiment was equilibrated with 0.05 M Tris-Cl, pH 7.2, containing 0.1 M KCl.

![Fig. 2. Profiles observed for protein and pyruvate carboxylase activity in the presence or absence of acetyl-CoA after fractionation by polyacrylamide gel electrophoresis. Polyacrylamide gels (6 X 0.5 cm) were prepared as described under "Materials and Methods." Purified rat liver pyruvate carboxylase (specific activity = 10.8 units per mg) was placed on each gel in 0.5 M sucrose containing 0.008 M Tris-phosphate, pH 7.2, and 0.1 mM dithioerythritol. The protein loads were 2.4 µg (A, B); 7.2 µg (C, D); and 6.0 µg (E). Electrophoresis was conducted as described under "Materials and Methods." Gels were stained for pyruvate carboxylase activity in the presence or absence of acetyl-CoA and for protein as described under "Materials and Methods" and were scanned for protein at 660 nm and for oxalacetate production at 530 nm using a Gilford model 2410 Linear Transport mounted on a Gilford model 240 recording spectrophotometer. Unpublished studies indicate that an approximately linear relationship exists between the enzyme concentration and the intensity of the band over this range. The dashed lines extending vertically through all the gel scans indicate the coincidence of the major protein and the enzyme activity bands. The additional dashed lines on the protein scan (E) define the areas forming the basis for calculation of the purity of the enzyme preparation (see "Materials and Methods").]
activity or protein peaks were observed at the elution volumes predicted for the pyruvate carboxylase dimer and monomer on the basis of $V_e:V_0$ ratios observed for rabbit muscle pyruvate kinase (mol wt $= 237,000$) (29) and beef heart lactate dehydrogenase (mol wt $= 137,000$) (30). However only the monomer would have been clearly resolved from the tetramer in this experiment. Since the protein concentration of the solution applied to the G-200 column is an order of magnitude greater than that employed in the earlier studies (28), the apparent discrepancy may be due to this factor. The constancy of the ratio of catalytic activities observed in Fig. 3 provides no support for the suggestion that different enzyme species are responsible for catalysis of oxalacetate synthesis in the presence and absence of acetyl-CoA (6).

**pH Dependence of Oxalacetate Synthesis by Rat Liver Pyruvate Carboxylase in Absence of Acetyl-CoA**—Since addition of an effector often alters the pH dependence of the reaction catalyzed by an allosteric enzyme (cf. Ref. 31), it was of interest to determine the pH optimum for catalysis by rat liver pyruvate carboxylase in the absence of acetyl-CoA. When the relationship between pH and the apparent $V_{max}$ obtained from variation of initial velocity with the $HCO_3^-$ concentration was examined (Fig. 4), an optimum was observed at pH 7.8 which is similar to that reported for acetyl-CoA-dependent oxalacetate synthesis by McClure et al. (11). In addition, Fig. 4 describes the variation of the apparent $K_m$ for $HCO_3^-$ (plotted as $pK_m^A$ versus pH for oxalacetate synthesis in the absence of acetyl-CoA. The apparent $K_m$ becomes more favorable as the pH of the assay system is made more alkaline, and changes from 210 mM at pH 7.2 to a constant value approximating 9 mM at pH 8.4 and above. Analysis of these data (32) indicates that 1 or more residues of $pK_m = 8.3$ are involved in the interaction of $HCO_3^-$ with rat liver pyruvate carboxylase. It is of interest that a residue (or residues) of similar $pK_m$ have previously been implicated in the interaction of both rat and chicken liver pyruvate carboxylases with acetyl-CoA (4, 11).

The data presented in Fig. 4 provide an explanation for the discrepancy in the apparent $K_m$ values for $HCO_3^-$ for oxalacetate synthesis in the absence of acetyl-CoA as reported previously for rat liver pyruvate carboxylase (200 mM) (5) and for the enzyme from sheep kidney cortex (10 mM) (6). At the assay pH (8.4) employed by Ashman et al. (6) similar apparent $K_m$ values for $HCO_3^-$ are observed for the enzymes from these two species in the absence of the activator.

**Temperature Dependence of Oxalacetate Synthesis by Rat Liver Pyruvate Carboxylase in Presence and Absence of Acetyl-CoA**—Further insight into the mechanism of activation of oxalacetate synthesis by acetyl-CoA in the case of rat liver pyruvate carboxylase has been provided by comparison of the temperature dependence of catalysis in the presence or absence of the activator. In order to define the effects of variation of temperature more clearly, the variation of initial rate with $[HCO_3^-]$ was measured at a series of temperatures over the range 10-45$^\circ$. When apparent maximal rates of catalysis (apparent $V_{max}$) were plotted as a function of the reciprocal of the absolute temperature according to the Arrhenius relationship (Fig. 5A), a linear relationship is observed for oxalacetate synthesis in the absence of acetyl-CoA over the temperature range examined. The activation energy ($E_a$) is obtained from the slope of this relationship as 15.4 Cal per mole. In the presence of acetyl-CoA a biphasic Arrhenius plot is observed. At temperatures above 25$^\circ$ the variation of apparent $V_{max}$ with temperature corresponds to an activation energy of 9.1 Cal per mole in good agreement with the

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**Fig. 3.** Distribution of protein ($A_{280}$) and of oxalacetate synthesis measured in the presence or absence of acetyl-CoA observed on chromatography of partially purified rat liver pyruvate carboxylase on DEAE-Sephadex A-50 ($SO_4^{2-}$). Rat liver pyruvate carboxylase (350 mg; specific activity = 2.2 units per mg) was equilibrated with 0.02 M phosphate, pH 7.1, containing 0.05 M (NH$_4$)$_2$SO$_4$, 1 mM EDTA, 0.1 mM dithioerythritol, and 5 mM phenylmethylsulfonyl fluoride on a column ($50 \times 2.5$ cm) of Sephadex G-25. This enzyme was then applied to a column ($28 \times 2$ cm) of DEAE-Sephadex A-50 ($SO_4^{2-}$) equilibrated with the same buffer and the column was washed with this buffer until the absorbance of the effluent at 280 nm was less than 0.1. A concave gradient (27) (250 ml of buffer containing 0.05 → 0.25 M (NH$_4$)$_2$SO$_4$) was then applied to the column and fractions (6 ml) were collected for assay of their content of protein ($A_{280}$) and pyruvate carboxylase activity. Enzymic activity in the presence and absence of acetyl-CoA was assayed as described for Fig. 1. The maximal rates of catalysis in the presence and absence of acetyl-CoA (5). Fig. 2 provides a qualitative identification of the species responsible for catalysis of the effector-dependent and -independent reactions but a quantitative comparison of the distribution of these two activities is not obtained. Chromatographic studies were therefore performed under various conditions and some of the resultant data are summarized in Fig. 3. The ratio of catalytic activities observed in the absence and presence of acetyl-CoA is constant across the enzyme peak when partially purified rat liver pyruvate carboxylase is chromatographed on DEAE-Sephadex A-50 ($SO_4^{2-}$) using an (NH$_4$)$_2$SO$_4$ gradient (Fig. 3A) or when the purified rat liver enzyme is subjected to gel filtration on Sephadex G-200 in the presence of 0.03 M NH$_4$Cl or 1 M KCl. These latter conditions were employed since McClure (28) has reported that the rat liver enzyme dissociates into active dimers on treatment with high concentrations of alkali metal chlorides. In excess of 90% of the enzyme units applied to the G-200 column were however recovered in a peak with an elution volume of 45 ml ($V_e:V_0 = 1.07$) as predicted for a species having a molecular weight approximating 500,000 (11). No significant
The assay system contained 100 mM K+-Hepes at the pH indicated, 25 mM sodium pyruvate, 10 mM ATP, 15 mM MgCl₂, an appropriate concentration of KHCO₃ in the range 3 to 500 mM, 25 μg of malate dehydrogenase, and 0.15 mM NADH in a total volume of 1.0 ml. After equilibration to 25°C, the reaction was initiated by addition of 1 to 20 μg of rat liver pyruvate carboxylase (specific activity = 7.8 units per mg) and the initial rate obtained was measured as a decrease in absorbance at 340 nm. The apparent Vₕmax is expressed as ΔAₕ₄₅ per min per 4 μg. At each pH tested the variation of initial velocity with HCO₃⁻ concentration was examined over a range from 0.3 to 10 times the apparent Kₘ, and the apparent Kₘ and Vₕmax were determined from a plot of reciprocal velocity versus reciprocal [HCO₃⁻]. The pH of the complete assay system was checked before addition of pyruvate carboxylase and after completion of the reaction. No significant differences were observed between these determinations.

Fig. 4. pH dependence of oxalacetate synthesis by rat liver pyruvate carboxylase in the absence of acetyl-CoA. The assay system contained 100 mM K⁺-Hepes at the pH indicated, 25 mM sodium pyruvate, 10 mM ATP, 15 mM MgCl₂, an appropriate concentration of KHCO₃ in the range 3 to 500 mM, 25 μg of malate dehydrogenase, and 0.15 mM NADH in a total volume of 1.0 ml. After equilibration to 25°C, the reaction was initiated by addition of 1 to 20 μg of rat liver pyruvate carboxylase (specific activity = 7.8 units per mg) and the initial rate obtained was measured as a decrease in absorbance at 340 nm. The apparent Vₕmax is expressed as ΔAₕ₄₅ per min per 4 μg. At each pH tested the variation of initial velocity with HCO₃⁻ concentration was examined over a range from 0.3 to 10 times the apparent Kₘ, and the apparent Kₘ and Vₕmax were determined from a plot of reciprocal velocity versus reciprocal [HCO₃⁻]. The pH of the complete assay system was checked before addition of pyruvate carboxylase and after completion of the reaction. No significant differences were observed between these determinations.

value reported by McClure et al. (11) for variation of initial velocity with temperature (8.4 Cal per mole). At temperatures below 25°C a marked increase in the slope of the Arrhenius plot occurs. The activation energy calculated from the lower temperature range was 31.5 Cal per mole. A biphasic relationship similar to that shown in Fig. 5A has also been observed for pyruvate carboxylases from chicken and calf liver when initial velocities were measured as a function of [MgATP²⁻] over this temperature range studied. From the slope of this plot the enthalpy (ΔH°) and entropy (ΔS°) of activation are obtained as 6.2 Cal per mole and 18.3 e.u., respectively. In the presence of acetyl-CoA a biphasic relationship is observed (Fig. 5B) as is also the case for the Arrhenius plot (Fig. 5A). Furthermore the minimum in this van’t Hoff plot occurs at approximately the same temperature as the break in the Arrhenius plot (Fig. 5). The biphasic nature of the van’t Hoff plot obtained in the presence of acetyl-CoA (Fig. 5B) is clearly established by the minimal extent of variance between replicate determinations of the apparent Kₘ for HCO₃⁻ at several of the temperatures shown (cf. legend, Fig. 5D).

Specificity of Activation of Rat Liver Pyruvate Carboxylase by Univalent and Divalent Cations in Absence of Acetyl-CoA—In addition to acyl derivatives of coenzyme A, rat liver pyruvate carboxylase also exhibits requirements for activation by univalent and divalent cations. The specificities for activation by these cations in the presence of acetyl-CoA have been defined by McClure et al. (11). We have therefore determined the specificity for activation of oxalacetate synthesis by univalent and divalent cations in the absence of acetyl-CoA. The data obtained for activation by univalent cations is presented in Table I which also includes for purposes of comparison some observations on the activation of oxalacetate synthesis by univalent cations in the presence of acetyl-CoA. In all cases initial velocities were measured at several Me⁺ concentrations to ensure that the optimal extent of activation was observed. Univalent cations which are effective activators of acetyl-CoA-dependent catalysis (K⁺, NH₄⁺, Cs⁺, Rb⁺ (11), and Tl⁺) also activate oxalacetate synthesis in the absence of acetyl-CoA to approximately the same extent. Other alkali metal cations (Li⁺, Na⁺) appear ineffective or inhibitory in both systems. In this respect our data differ from the previous report of weak activation of rat liver pyruvate carboxylase by Li⁺ and Na⁺ in the presence of saturating levels of acetyl-CoA (11). We have been unable to obtain any evidence for activation by either Li⁺ or Na⁺ in the presence or absence of acetyl-CoA. Apparent Kₘ values for activation of oxalacetate synthesis by K⁺, Rb⁺, and NH₄⁺ in the absence of acetyl-CoA are shown in parentheses in Table I. These studies have also shown that, in the presence or absence of acetyl-CoA, rat liver pyruvate carboxylase catalyzes oxalacetate synthesis in a system containing (CH₃)₄N⁺ as the only added univalent cation at 10 to 15% of the rate observed in the presence of saturating concentrations of the most effective Me⁺ activator. The K⁺ (0.15 mM) and NH₄⁺ (0.32 mM) contents of the (CH₃)₄N⁺ assay system are inadequate to account for more than a small fraction of this rate of oxalacetate synthesis. Thus either (CH₃)₄N⁺ acts as a weak activator of rat liver pyruvate carboxylase or this enzyme exhibits significant catalytic potential in the absence of an activating univalent cation. In order to distinguish between these two possibilities we compared the rates of acetyl-CoA-dependent oxalacetate synthesis in systems which contained tetramethylammonium, tetraethylammonium, Tris⁺, triethanolamine, and tetrabutylammonium as the sole added univalent cation. Since the temperature stability of these pyruvate carboxylases differs strikingly (11, 33, 34) it seems improbable that the increase in activation energy observed at temperatures below 20–25°C is directly related to the inactivation which is observed in some instances on incubation at temperatures below 15°C.

These studies have also permitted determination of the temperature dependence of the apparent Kₘ for HCO₃⁻ in the presence and absence of acetyl-CoA (Fig. 5B). The absence of acetyl-CoA a linear van’t Hoff plot is obtained over the temperature range studied. From the slope of this plot the enthalpy (ΔH°) and entropy (ΔS°) of activation are obtained as 6.2 Cal per mole and 18.3 e.u., respectively. In the presence of acetyl-CoA a biphasic relationship is observed (Fig. 5B) as is also the case for the Arrhenius plot (Fig. 5A)
of acetyl-CoA is however observed when triethanolamine or tetrabutylammonium chloride are present. The activation is somewhat more marked for oxalacetate synthesis in the absence of acetyl-CoA. A further increase in rate is observed when K+ is added in addition to (C4H4)4N+ although the effect of these two cations does not appear to be strictly additive (Table I, Experiment B). Since both cations were added at concentrations which give maximal activation when added separately, the partial additivity observed suggests that activation by (C4H4)N+ may result from occupancy of a site other than that at which K+ interacts. These data are of particular interest since none of the alkylamine or alkylammonium cations tested here also activate pyruvate carboxylase from chicken liver despite the fact that this latter enzyme exhibits a similar "basal" rate of catalysis in a system containing (CH3)4N+ as the sole added univalent cation (20).

Less extensive studies have shown that Mg2+, Mn2+, and Co2+ are effective activators of oxalacetate synthesis in the absence of acetyl-CoA. A linear relationship is observed between reciprocal initial velocity and reciprocal [Mg2+]. The apparent $K_a$ for this metal ion is obtained as 1.75 mM in the presence of a nonsaturating concentration of MgATP2− (1 mM).

**Dependence of Apparent $K_a$ for Activation of Rat Liver Pyruvate Carboxylase by Acetyl-CoA on Concentration of MgATP2−, HCO3−, and Pyruvate**—The studies described in the previous sections document some further properties of catalysis by rat liver pyruvate carboxylase in the absence of acetyl-CoA. We have also examined the nature of the relationship between the catalytic and activator sites of this enzyme as expressed in the dependence of the apparent $K_a$ for acetyl-CoA on the concentration of the substrates of the pyruvate carboxylase reaction. In order to stimulate the in vivo environment more closely, these studies were performed at physiological pH (pH 7.2). Under such conditions the apparent $K_a$ for activation of the rat liver enzyme by acetyl-CoA shows no significant variation with [MgATP2−] over the range 0.05 to 2.0 mM, or with [HCO3−] over the range 0.6 to 100.0 mM in accord with data reported previously by McClure et al. (11) for experiments performed at the pH optimum (pH 7.8). However, variation of pyruvate concentration at physiological pH results in significant changes in the apparent $K_a$ for acetyl-CoA. As illustrated in Fig. 6 this parameter exhibits a hyperbolic dependence on [pyruvate] with the change in $K_a$ becoming especially marked at pyruvate concentrations below 0.5 mM. The Hill coefficient for acetyl-CoA is insensitive to variation in [pyruvate] over this range (Fig. 6). The apparent $K_a$ for acetyl-CoA at [pyruvate] → 0 is obtained as 145 μM by linear extrapolation from the lower range of pyruvate concentration (Fig. 6). The apparent $K_a$ in the presence of saturating concentrations of pyruvate may be estimated as 50 to 55 μM in reasonable agreement with the value determined previously at pH 7.2 by McClure et al. (11). The failure of these workers to observe pyruvate dependence of the apparent $K_a$ for acetyl-CoA may be due to a difference in the assay pH, and also possibly to the range of pyruvate concentrations employed in the two studies (11).

It is of interest that in the case of the chicken liver enzyme, which does not catalyze a significant rate of oxalacetate synthesis in the absence of acetyl-CoA (4), the apparent $K_a$ for acetyl-CoA is insensitive to variation of the concentrations of any of the three substrates over ranges similar to those employed here (13). Fig. 6 therefore indicates the existence of a catalytic site-activator site interaction in rat liver pyruvate carboxylase which does not appear to be present in the avian liver enzyme. These data are consistent with the activation of [14C]pyruvate/oxalacetate exchange by acetyl-CoA which has been described for rat liver pyruvate carboxylase (35, 36).

**Specificity of Activation of Rat Liver Pyruvate Carboxylase by Various Acyl Derivatives of Coenzyme A and Related Compounds**—
TABLE I

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative rate of catalysis</th>
<th>Relative rate of catalysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Acetyl-CoA</td>
<td>- Acetyl-CoA</td>
</tr>
<tr>
<td>None (added)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>A. + 25 mM Li⁺</td>
<td>0.88</td>
<td>1.0</td>
</tr>
<tr>
<td>+ 25 mM Na⁺</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>+ 25 mM NH₄⁺</td>
<td>3.8</td>
<td>3.8 (7.5)</td>
</tr>
<tr>
<td>+ 25 mM K⁺</td>
<td>4.5</td>
<td>4.3 (10.0)</td>
</tr>
<tr>
<td>+ 25 mM Rb⁺</td>
<td>4.4</td>
<td>5.5 (4.3)</td>
</tr>
<tr>
<td>+ 25 mM Cs⁺</td>
<td>3.6</td>
<td>3.8</td>
</tr>
<tr>
<td>+ 1 mM Tl⁺</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>B. + 25 mM Tris⁺</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>+ 50 mM (CH₃)₂N⁺</td>
<td>0.95</td>
<td>0.98</td>
</tr>
<tr>
<td>+ 50 mM (HOCH₂CH₂)N⁻</td>
<td>2.7</td>
<td>4.5</td>
</tr>
<tr>
<td>+ 50 mM (CH₃)₂N⁺</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>+ 50 mM (CH₃)₂N⁺ + 50 mM K⁺</td>
<td>4.8</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* Apparent $K_A$ values determined for several univalent cations from plots of $1/v - v_0$ versus $1/[Me⁺]$ are shown in parentheses.

Previous studies by Fung and Utter (cf. Ref. 13) have provided detailed insight into the specificity of activation of chicken liver pyruvate carboxylase by acyl derivatives of coenzyme A and related compounds. The studies described here, and in earlier publications (4, 5) indicate however that the nature of the interaction between the catalytic and activator sites differs in the mammalian and avian liver enzymes. Furthermore differences are also observed both in the properties of activation of chicken and rat liver pyruvate carboxylase by acetyl-CoA (10) and also in the effect of SO₄⁻ in these properties (11, 15). It was therefore of interest to define the activator specificity of rat liver pyruvate carboxylase. Such data, which are presented in Table II, reveal some interesting relationships which may shed further light on the activation process. Since adenosine 2':5'-diphosphate, 3'-AMP, and 5'-AMP do not activate the rat liver enzyme, an adenosine derivative bearing phosphate groups at the 3' and 5' positions possesses the minimal structural features necessary for activation of oxalacetate synthesis. Comparison of the properties of activation by adenosine 3':5'-diphosphate and CoA-SH indicates that the presence of the 4'-phosphopantetheine residue causes no increase in the relative $V_{max}$ but results in an

8-fold enhancement of the apparent $K_A$ and in induction of a sigmoid relationship between initial rate and activator concentration (Table IIID). Thus observation of cooperative interaction between the activator sites on rat liver pyruvate carboxylase appears dependent on occupancy of the 4-phosphopantetheine region of these activator sites. It is of interest that, in contrast to the chicken liver enzyme (10), the presence of an acyl thioester is not required for expression of the maximal Hill coefficient for activation of rat liver pyruvate carboxylase (Table II). However other parameters describing the activation of this enzyme are sensitive to the presence of the acyl group indicating the existence of a specific interaction involving the acyl thioester region. For example comparison of the properties of activation by CoA-SH and acetyl-CoA, which is the most effective activator, indicates that a 17-fold increase in the apparent $V_{max}$ and a 50-fold increase in the apparent $K_A$ result from the presence of the acyl group (Table II, A and D). As the chain length of the alkylacyl group is increased from 2 carbon atoms (acytly-CoA) to 5 carbon atoms (n-valeryl-CoA) a decrease in the efficiency of activation is observed as expressed in a trend to a decreased relative $V_{max}$ and a less favorable apparent $K_A$ (Table II, A to C). However, a further increase in the alkylacyl chain length

![Figure 9](http://www.jbc.org/) Dependent variable relationships for activation of rat liver pyruvate carboxylase by acetyl-CoA on the pyruvate concentration. The assay systems contained 100 mM K⁺-Hepes, pH 7.2, 2 mM ATP, 5 mM MgCl₂, 0.15 mM NADH, 25 μM of malate dehydrogenase, 0.15 mM NADH, the concentration of pyruvate as indicated, and a variable concentration of acetyl-CoA over a range from 0.2 to 10 times the apparent $K_A$. After equilibration to 25°C, the reaction was initiated by addition of 12 to 80 μg of rat liver pyruvate carboxylase (specific activity = 8.4 units per mg) and the initial velocity was estimated from the decrease in absorbance at 340 nm. At each pyruvate concentration the apparent $V_{max}$ was determined from a plot of reciprocal initial velocity versus the reciprocal of the square of the acetyl-CoA concentration. The apparent $K_A$ and the Hill coefficient ($n$) were then obtained by plotting the data according to the Hill equation.

For assay of oxalacetate synthesis in the presence of acetyl-CoA the system contained 100 mM (CH₃)₂N⁺-Hepes, pH 7.8, 5 mM (CH₃)₂N⁺-pyruvate, 2 mM (CH₃)₂N⁺-ATP, 5 mM MgCl₂, 2 mM (CH₃)₂N⁺CoA, 0.05 mM acetyl-CoA, 25 μg of malate dehydrogenase, and 0.15 mM NADH in a total volume of 1.0 ml. After equilibration to 25°C the reaction was initiated by addition of 4 to 30 μg of rat liver pyruvate carboxylase (specific activity = 7.9 units per mg) and the initial rate of catalysis determined from the decrease in absorbance at 340 nm. The initial rates observed in the absence of added univalent cation ($v_0$) (expressed as ΔA₄₅₀ nm per min per μg of enzyme) were 0.05 (+ acetyl-CoA) and 0.015 (- acetyl-CoA). The (CH₃)₂N⁺ concentration in this system is approximately 200 mM.

For assay of oxalacetate synthesis in the presence of acetyl-CoA the system contained 100 mM (CH₃)₂N⁺-Hepes, pH 8.4, 25 mM (CH₃)₂N⁺-pyruvate, 10 mM (CH₃)₂N⁺-ATP, 15 mM MgCl₂, 10 mM (CH₃)₂N⁺-HICO₃, 25 μg of malate dehydrogenase, and 0.15 mM NADH in a total volume of 1.0 ml. After equilibration to 25°C the reaction was initiated by addition of 4 to 30 μg of rat liver pyruvate carboxylase (specific activity = 7.9 units per mg) and the initial rate of catalysis determined from the decrease in absorbance at 340 nm. The initial rates observed in the absence of added univalent cation ($v_0$) (expressed as ΔA₄₅₀ nm per min per μg of enzyme) were 0.05 (+ acetyl-CoA) and 0.015 (- acetyl-CoA). The (CH₃)₂N⁺ concentration in this system is approximately 200 mM.
**Table II**

**Specificity of activation of rat liver pyruvate carboxylase by coenzyme A, various acyl derivatives of coenzyme A, and other analogs**

The assay mixture contained 100 mM K+ HEPES, pH 7.8; 10 mM sodium pyruvate, 2 mM ATP, 5 mM MgCl2, 50 mM KHC03, 25 μg of malate dehydrogenase, 0.15 mM NADH, and an appropriate range of concentration of the coenzyme A derivative in total volumes of 0.3 or 1.0 ml. After equilibration to 25° the reaction was initiated by addition of 8 to 40 μg of pyruvate carboxylase (specific activity 7 to 10 units per mg). The initial rate of reaction obtained from the decrease in absorbance at 340 nm was corrected for the minimal basal rate observed in the absence of any added CoA derivative. Except for adenosine 3'-5'-diphosphate the maximal rate of catalysis (Vmax) was estimated from a plot of reciprocal velocity versus reciprocal of the concentration which was linear in all instances for data obtained at concentrations in excess of the Kd. The apparent Kd and the Hill coefficient (n) were then obtained by plotting the data according to the empirical Hill equation (Equation 2).

\[
\log_{10} \left( \frac{V}{V_m} \right) = \log_{10} K - n \log_{10}[A]
\]

<table>
<thead>
<tr>
<th>Acyl coenzyme A (or coenzyme A)</th>
<th>Apparent Kd (μM)</th>
<th>Hill coefficient (n)</th>
<th>Relative Vmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Acetyl-CoA</td>
<td>22.7</td>
<td>2.1</td>
<td>100</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td>155</td>
<td>1.9</td>
<td>100</td>
</tr>
<tr>
<td>B. n-Butyryl-CoA</td>
<td>558</td>
<td>2.2</td>
<td>65</td>
</tr>
<tr>
<td>Crotonyl-CoA</td>
<td>206</td>
<td>2.1</td>
<td>67</td>
</tr>
<tr>
<td>Isovaleryl-CoA</td>
<td>228</td>
<td>2.0</td>
<td>29</td>
</tr>
<tr>
<td>Tiglyl-CoA</td>
<td>1440</td>
<td>2.0</td>
<td>24</td>
</tr>
<tr>
<td>C. n-Valeryl-CoA</td>
<td>755</td>
<td>2.2</td>
<td>29</td>
</tr>
<tr>
<td>n-Hexanoyl-CoA</td>
<td>840</td>
<td>2.0</td>
<td>30</td>
</tr>
<tr>
<td>n-Heptanoyl-CoA</td>
<td>822</td>
<td>2.0</td>
<td>26</td>
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<tr>
<td>n-Octanoyl-CoA</td>
<td>850</td>
<td>2.0</td>
<td>34</td>
</tr>
<tr>
<td>n-Nonanoyl-CoA</td>
<td>830</td>
<td>2.1</td>
<td>35</td>
</tr>
<tr>
<td>n-Decanoyl-CoA</td>
<td>822</td>
<td>2.0</td>
<td>28</td>
</tr>
<tr>
<td>n-Undecanoyl-CoA</td>
<td>730</td>
<td>2.0</td>
<td>29</td>
</tr>
<tr>
<td>n-Dodecanoyl-CoA</td>
<td>715</td>
<td>2.0</td>
<td>28</td>
</tr>
<tr>
<td>Phenylacetyl-CoA</td>
<td>450</td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td>D. CoA-SH</td>
<td>1070</td>
<td>1.9</td>
<td>6</td>
</tr>
<tr>
<td>Adenosine 3'-5'-diphosphate</td>
<td>8000</td>
<td>1.0</td>
<td>4</td>
</tr>
</tbody>
</table>

*a* No activity was detectable in the presence of tetradecanoyl- or palmitoyl-CoA over the range 50 to 200 μM (but see text).

*b* When CoA-SH was varied both the CoA-SH solution and the assay mix also contained 0.5 mM diethioerythritol. The apparent Vmax is expressed relative to that observed for acetyl-CoA in the same assay system. Inclusion of 0.5 mM diethioerythritol in the assay system causes no significant change in the apparent Kd for acetyl-CoA.

*c* No activation was observed in the presence of adenosine 2'-5'-diphosphate or 3'-AMP at concentrations in the range 0.5 to 10.0 mM.

When the acyl chain length exceeds 10 carbon atoms (n-dodecanoyl-CoA) causes no marked further change in either of these parameters which stabilize at 700 to 800 μM (apparent Kd) and approximately 30 (relative Vmax), respectively. Although the range of apparent Kd observed for these longer chain alkylacyl-CoAs does not differ markedly from that observed for activation by CoA-SH, the presence of the acyl thioester results in a 5-fold increment in the relative Vmax (Table II, C and D). Such an increment does not however appear to be observed when an aromatic acyl group is present as illustrated for phenylacetyl-CoA in Table II C. The analytical data presented under "Materials and Methods" and the failure of these longer chain homologs to serve as activators of chicken liver pyruvate carboxylase excludes the possibility that the activation observed for the rat liver enzyme (Table II) could be due to contamination of these preparations with CoA-SH or acetyl-CoA. Myristyl- and palmitoyl-CoA do not appear to activate rat liver pyruvate carboxylase when added at concentrations in the range 0.1 to 1.0 mM. However, this observation is equivocal since in the presence of undecanoyl- and dodecanoyl-CoA the relationship between Kd and time is linear over only a short time span especially when the acyl-CoA concentration exceeds 500 μM. Other studies indicate that irreversible inactivation of rat liver pyruvate carboxylase occurs on exposure to these acyl-CoAs. Hence the apparent failure to observe activation on addition of myristyl- or palmitoyl-CoA could be the result of very rapid inactivation of the enzyme rather than an inherent inability to activate catalysis. The inactivation probably results from the detergent properties of such longer chain acyl-CoAs (37) and hence is unlikely to be physiologically significant.

Certain comparative aspects of the data presented in Table II are of interest. For example the presence of a trans double bond in the acyl group (crotonyl-CoA) causes the apparent Kd for activation of rat liver pyruvate carboxylase to become more favorable as compared with that observed for the saturated analog (n butyryl CoA) (Table II B). The opposite trend in the apparent Kd is observed for the chicken liver enzyme (13), but for both enzymes the relative Vmax for activation by crotonyl-CoA is somewhat lower than that observed for n-butyryl-CoA (Table II B). When, in addition, a methyl group is present on the α-carbon of the unsaturated acyl-CoA, this analog (tiglyl-CoA) functions as a weak activator of rat liver pyruvate carboxylase (Table II B) but is a competitive inhibitor of chicken liver pyruvate carboxylase with respect to acetyl-CoA (13). However, the most striking differences between these two enzymes are observed in the effects of longer chain alkylacyl-CoAs on the activation process. Longer chain alkylacyl-CoAs which are effective activators of rat liver pyruvate carboxylase (Table II C), act as competitive inhibitors of the chicken liver enzyme with respect to acetyl-CoA in agreement with data presented previously for the effect of n-valeryl-CoA (13). The [I]50 and Hill coefficients obtained from analysis of the variation of initial rate with inhibitor concentration in a system containing a non-saturating concentration of acetyl-CoA (5 μM) are summarized in Table III. It is apparent that the properties of inhibition by these homologs are also essentially independent of acyl chain length. Thus as the chain length is increased from 5 (n-valeryl-CoA) to 10 (n-decanoyl-CoA) carbon atoms a minimal increase is observed in [I]50, while the Hill coefficient describing the inhibition remains in the range 1.5 to 1.8 except for n-decanoyl-CoA, and shows no consistent variation with the chain length of the acyl residue. The [I]50 and Hill coefficient shown in Table III for inhibition of the chicken liver pyruvate carboxylase by n-valeryl-CoA are in reasonable agreement with the values reported previously (apparent Kd = 40 μM, n = 2.1) (13). When the acyl chain length exceeds 10 carbon atoms, irreversible inactivation of the chicken liver pyruvate carboxylase by n-valeryl-CoA is sufficiently rapid to prevent observation of a linear initial rate of oxalacetate production. Hence it was not possible to examine the effects of longer chain homologs of acetyl-CoA on the catalytic activity of this enzyme.
Specificity of inhibition of chicken liver pyruvate carboxylase by various acyl derivatives of coenzyme A

The assay system contained 100 mM K+-Hepes pH 7.8, 10 mM sodium pyruvate, 2 mM ATP, 5 mM MgCl₂, 50 mM KHCO₃, 0.005 mM acetyl-CoA, 25 µg of malate dehydrogenase, and 0.15 mM NADH in a total volume of 0.3 ml. In order to remove SO₄⁻² from the malate dehydrogenase preparation this enzyme was equilibrated with 0.05 mM K⁺-Hepes, pH 7.2, containing 0.2 mM KCl by gel filtration on a column (20 x 1 cm) of Sephadex G-25. After equilibration to 25°C the reaction was initiated by addition of 2 to 3 µg of pyruvate carboxylase (chicken liver) (specific activity, 14.2 units per mg) and the initial velocity determined from the decrease in absorbance at 340 nm. The $I_{\text{B}0.5}$ and Hill coefficient ($n$) were determined by plotting the data according to the empirical Hill equation (Equation 3).

$$\log_{10} \frac{V_0 - V_i}{V_i - V_{\text{sat}}} = \log_{10} K - n \log_{10} [\text{acylcoenzyme-A}]$$  (3)

In Equation 3, $V_0$ is the initial rate observed in the absence of added inhibitory acyl coenzyme $A$; $V_i$, the rate observed in the presence of a given concentration of the inhibitory acyl coenzyme $A$; and $V_{\text{sat}}$, the rate observed in the presence of a saturating concentration of acyl coenzyme $A$. For all derivatives except decanoyl-CoA it was established experimentally that $V_{\text{sat}} \approx 0$ as predicted for competitive inhibition. $I_{\text{B}0.5}$ is defined as the concentration of inhibitor required to give $V_0 - V_i / V_i - V_{\text{sat}} = 1.0$ and is approximately equal to the apparent $K_i$ under these conditions.

<table>
<thead>
<tr>
<th>Acyl coenzyme $A^*$</th>
<th>$I_{\text{B}0.5}$</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Valeryl-CoA</td>
<td>28.0</td>
<td>1.6</td>
</tr>
<tr>
<td>n-Hexanoyl-CoA</td>
<td>29.8</td>
<td>1.5</td>
</tr>
<tr>
<td>n-Heptanoyl-CoA</td>
<td>32.7</td>
<td>1.6</td>
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<td>n-Octanoyl-CoA</td>
<td>44.0</td>
<td>1.7</td>
</tr>
<tr>
<td>n-Nonanoyl-CoA</td>
<td>42.0</td>
<td>1.7</td>
</tr>
<tr>
<td>n-Decanoyl-CoA</td>
<td>46.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>

a No significant activation is observed in the presence of any of these acyl coenzyme $A$s in the concentration range of $I_{\text{B}0.5}$.

Inhibition of Rat Liver Pyruvate Carboxylase by Analogos of Acetyl-CoA—Table IV summarizes $I_{\text{B}0.5}$ values and Hill coefficients observed for various acetyl-CoA analogs which act as inhibitors of rat liver pyruvate carboxylase. Analogos in which the 3':5'-diphosphoadenosine portion of the acetyl-CoA molecule is modified no longer activate rat liver pyruvate carboxylase and appear to act as competitive inhibitors with respect to acetyl-CoA. However for all analogs of this type which were examined the relationship between initial rate and inhibitor concentration is sigmoidal (Table IVB). As observed previously for the chicken liver enzyme (13), acetylpantetheine is a more effective inhibitor than acetyldephospho-CoA (Table IVB) thus providing further support for the postulate that the 3'-phosphate group has an important role in the interaction of both these enzymes with acetyl-CoA. In this context it is of interest that rat liver pyruvate carboxylases may be desensitized to activation by acetyl-CoA when incubated with reagents which react preferentially with lysyl residues (7).

Carboxyl-CoAs, e.g. glutaryl-CoA, succinyl-CoA, etc., also appear to act as competitive inhibitors with respect to acetyl-CoA but exhibit sigmoidal initial velocity [inhibitor] profiles with Hill coefficients approximating 2.0. Inhibition by these analogs is relatively weak with $I_{\text{B}0.5}$ values in the region of 1 mM being observed for all compounds tested (Table IVB). It is of interest that as observed for activation by the acyl homologs (Table IIIB), the presence of a double bond in the carboxyoacyl-CoA series results in some increase in the effectiveness of the analog as illustrated by comparison of $I_{\text{B}0.5}$ for succinyl-CoA and malonyl-CoA (Table IVB).

### Table III

<table>
<thead>
<tr>
<th>Compound</th>
<th>$I_{\text{B}0.5}$</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Acetylpantetheine</td>
<td>17.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Acetyldiphospho-CoA</td>
<td>1.11</td>
<td>1.4</td>
</tr>
<tr>
<td>Acetyldephospho-CoA</td>
<td>0.18</td>
<td>1.3</td>
</tr>
<tr>
<td>CoA-S-S-CoA</td>
<td>0.51</td>
<td>1.3</td>
</tr>
<tr>
<td>B. Methylmalonyl-CoA</td>
<td>0.77</td>
<td>2.1</td>
</tr>
<tr>
<td>Succinyl-CoA</td>
<td>1.58</td>
<td>1.8</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>0.80</td>
<td>2.1</td>
</tr>
<tr>
<td>Glutaryl-CoA</td>
<td>0.92</td>
<td>2.1</td>
</tr>
<tr>
<td>Diglycoyl-CoA</td>
<td>0.77</td>
<td>1.7</td>
</tr>
</tbody>
</table>

a Although adenosine 2':5'-diphosphate and 3'-AMP inhibit rat liver pyruvate carboxylase, the inhibition does not appear competitive with acetyl-CoA and may therefore result at least in part from interaction with the ATP region of the catalytic site.

Discussion

Previous studies by McClure et al. (11, 35, 38, 39) have shown that the catalytic properties of pyruvate carboxylase purified from rat liver are generally similar to those reported for the chicken liver enzyme (cf. 1), and few significant differences were detected in the regulatory properties. However, in this, and three earlier reports (5, 9, 20), a number of major differences have been described between these two enzymes which relate primarily to the properties of the interaction with various effectors and to the nature of the relationships between the catalytic and effector sites. Some of the more significant of these differences are summarized in Table V which also includes similar data for pyruvate carboxylase from S. cerevisiae. This latter enzyme can be rendered essentially insensitive to activation by acetyl-CoA in the presence of high concentrations of K⁺ and HCO₃⁻ (40). It is apparent from Table V that, in respect to effector properties, pyruvate carboxylase purified from rat liver differs markedly from the enzymes obtained from both chicken liver and S. cerevisiae. For example, a much wider spectrum of acyl derivatives of coenzyme A are effective activators of the pyruvate carboxylases from rat liver and S. cerevisiae than is the case for the enzyme from chicken liver. Derivatives in which the acyl chain length exceeds 4 carbon atoms, e.g. n-octanoyl-CoA, or in which a methyl group is present on the a-carbon, e.g. tiglyl-CoA, activate the rat liver and S. cerevisiae enzymes but
equally striking similarities are also apparent. All three enzymes are inhibited by carboxyacyl derivatives of CoA, e.g. succinyl-

in concert with the extent of effector-independent catalytic ac-
dephospho-Cob, acetyldeamino-CoA. The observed differences these three pyruvate carboxylases appear therefore to be focused in the specificities of activation by acyl derivatives of CoA in

The reaction components whose concentration has the greatest influence on the rate of oxalacetate synthesis in the absence of acetyl-CoA are indicated in parentheses.

act as inhibitors of the enzyme from chicken liver. However, the effect of an increase in acyl chain length on the properties of activation differs markedly for the pyruvate carboxylases from rat liver and S. cerevisiae. As illustrated in Table V by calculation of the relative $K_A$ (acetyl-CoA to octanoyl-CoA), longer chain acyl-CoAs are less effective activators of rat liver pyruvate carboxylase but more effective activators of the enzyme from S. cerevisiae. Furthermore, although CoA-SH is an activator of all three pyruvate carboxylases the effectiveness of activation by this metabolite relative to acetyl-CoA, as judged from calculation of the parameter relative $K_A$ (acetyl-CoA to CoA-SH), increases in concert with the extent of effector-independent catalytic activ-
tivity (Table V). Despite these marked differences, some equally striking similarities are also apparent. All three enzymes are inhibited by carboxyacyl derivatives of CoA, e.g. succinyl-CoA, and by analogs of acetyl-CoA in which the adenosine diphosphate portion of the molecule is modified, e.g. acetyl-
dehphospho-CoA, acetyldeamino-CoA. The observed differences in the specificities of activation by acyl derivatives of CoA in these three pyruvate carboxylases appear therefore to be focused in the properties of that portion of the activating site (or process) which accommodates, or responds to, the acyl moiety. From the present studies it is apparent that the requirements for activ-


tation of rat liver pyruvate carboxylase are much less stringent than those observed for the chicken liver enzyme in respect to the nature of the acyl moiety (Table II) (13). However, in contrast to pyruvate carboxylase from S. cerevisiae the apparent $K_A$ describing activation of the rat liver enzyme becomes less favorable, and subsequently invariant, as the length of the acyl chain is increased (Table II). Hence the region of the activator site on rat liver pyruvate carboxylase which accommodates the acyl group does not appear to have the same hydrophobic character as that which has been inferred for the enzyme from S. cere-
viesiae (cf. Ref. 40). Moreover, since the longer chain acyl-CoA (e.g. n-octanoyl-CoA) are competitive inhibitors of chicken liver pyruvate carboxylase with respect to acetyl-CoA (Table III), the qualitative difference in the effect of these derivatives on

act as inhibitors of the enzyme from chicken liver. However, the effect of an increase in acyl chain length on the properties of activation differs markedly for the pyruvate carboxylases from rat liver and S. cerevisiae. As illustrated in Table V by calculation of the relative $K_A$ (acetyl-CoA to octanoyl-CoA), longer chain acyl-CoAs are less effective activators of rat liver pyruvate carboxylase but more effective activators of the enzyme from S. cerevisiae. Furthermore, although CoA-SH is an activator of all three pyruvate carboxylases the effectiveness of activation by this metabolite relative to acetyl-CoA, as judged from calculation of the parameter relative $K_A$ (acetyl-CoA to CoA-SH), increases in concert with the extent of effector-independent catalytic activ-
tivity (Table V). Despite these marked differences, some equally striking similarities are also apparent. All three enzymes are inhibited by carboxyacyl derivatives of CoA, e.g. succinyl-CoA, and by analogs of acetyl-CoA in which the adenosine diphosphate portion of the molecule is modified, e.g. acetyl-
dehphospho-CoA, acetyldeamino-CoA. The observed differences in the specificities of activation by acyl derivatives of CoA in these three pyruvate carboxylases appear therefore to be focused in the properties of that portion of the activating site (or process) which accommodates, or responds to, the acyl moiety. From the present studies it is apparent that the requirements for activ-


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rating concentration of HCO₃⁻ for the studies of Table I. Our data have provided no indication of a change in the rank order for activation by univalent cations on addition of acetyl-CoA similar to that described by Ashman et al. (6) for pyruvate carboxylase from sheep kidney cortex. The properties of activation by divalent cations also appear similar in the presence or absence of acetyl-CoA. It is of particular interest that the relationship between initial rate and [Mg²⁺] is hyperbolic both in the absence of acetyl-CoA, and in the presence of non-saturating concentrations of this activator, in contrast to the sigmoidal relationship observed in studies on chicken liver pyruvate carboxylase (20). These data therefore indicate the absence of significant interaction between the activator sites for univalent cations, divalent cations, and acyl derivatives of CoA on rat liver pyruvate carboxylase in accord with conclusions drawn in previous studies on this enzyme (11). It is also apparent from Fig. 5A that the relationship between the maximal rates of oxalacetate synthesis in the presence and absence of acetyl CoA is a function of the temperature at which the studies are performed. Thus the maximal extent of activation by acetyl-CoA is observed at approximately 25°C (Fig. 5A).

Finally, the data presented here provide some further insight into the mechanisms which may be responsible for in vivo regulation of the pyruvate → oxalacetate flux in rat liver. Table V indicates that, in contrast to the enzymes from chicken liver and S. cerevisiae, a convincing negative effector has not yet been described for rat liver pyruvate carboxylase. However, in the case of this enzyme the apparent $K_a$ for acetyl-CoA is a function of the pyruvate concentration under conditions which may partially simulate the physiological environment (Fig. 6). The data suggest that, in the physiological range (43), an increase in pyruvate concentration in rat liver mitochondria could increase the rate of oxalacetate synthesis as a result of both increased substrate availability and also an enhanced efficiency of activation by acetyl-CoA. Such a system represents an unusual type of substrate activation. Furthermore, the apparent $K_a$ observed for acetyl-CoA (130 to 140 μM) when the pyruvate concentration approximates the physiological range (43) is in reasonable agreement with the range estimated by Williamson et al. (44) for the intramitochondrial acetyl-CoA concentration in perfused rat liver (163 ± 86 μM). Although such apparent agreement between the kinetic constant determined in vivo and the estimated in vivo concentration must be viewed with caution, the correlation appears consistent with data obtained in studies with rat kidney slices (45) and the perfused rat liver (46) which indicate that the rate of gluconeogenesis from 3-carbon precursors is activated by acetyl-CoA under in vivo conditions.

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