# PYRUVATE CARBOXYLASE FROM RAT LIVER: CATALYTIC PROPERTIES IN THE ABSENCE, AND AT LOW CONCENTRATIONS, OF ACETYL-CoA*

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Summary: Initial velocity studies of the reaction catalyzed by pyruvate carboxylase from rat liver have defined the properties of oxalacetate synthesis in the absence of acetyl-CoA. The maximal rate of oxalacetate synthesis under these conditions is 21% of that observed in the presence of saturating concentrations of this activator. The studies have also indicated that marked changes are observed in the apparent $K_M$ ($HCO_3^-$), or velocity/concentration profile ($MgATP^{2-}$), for the substrates of the carboxylation partial reaction as a function of [acetyl-CoA] over the range 0 - 10μM with little change in the apparent $V_{MAX}$.

Previous studies by McClure et al (1) demonstrated that, in contrast to the avian liver enzyme (2), pyruvate carboxylase purified from rat liver catalyzes a slow rate of OAA[1] synthesis in the absence of added acetyl-CoA (Reaction 1):

$$\text{Pyruvate} + \text{MeATP}^{2-} + HCO_3^- \rightleftharpoons^{Me^{2+},\,Me^{+}} \text{OAA} + \text{MeADP}^- + P_i \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Isotopic exchange studies (3,4) further indicated that (i) both the carboxylation (Reaction 2) and transcarboxylation (Reaction 3) partial reactions proceed at significant rates in the absence of acetyl-CoA; and (ii) acetyl-CoA stimulates both partial reactions although the extent of stimulation is greater for the carboxylation partial reaction:

$$\text{E-biotin} + \text{MeATP}^{2-} + HCO_3^- \rightleftharpoons^{Me^{2+},\,Me^{+}} \text{E-biotin}\sim CO_2 + \text{MeADP}^- + P_i \quad \ldots\ldots\ldots (2)$$

$$\text{E-biotin}\sim CO_2 + \text{Pyruvate} \rightleftharpoons \text{E-biotin} + \text{OAA} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

These observations contrast with earlier studies on pyruvate carboxylase from avian liver which indicated that Reaction 2 was the sole site of activation by acetyl-CoA (5).

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[1]The abbreviations used are: OAA, oxalacetate; HEPES, N-2-hydroxyethylpiperazine- N-2-ethanesulfonate; DTNB, 5,5-dithiobis-(2-nitrobenzoate).

In this communication we define the conditions necessary for expression of maximal catalytic activity by rat liver pyruvate carboxylase in the absence of acetyl-CoA and examine the effect of acetyl-CoA on the kinetic parameters of this enzyme with special reference to effects observed in the presence of low concentrations (<10μM) of this cofactor.

Methods: Pyruvate carboxylase was purified from rat liver mitochondria as described by Scrutton and Fung (6). These preparations exhibited specific activities in the range 7-15 units (μmoles $HCO_3^-$ fixed/min. at 25°)/mg and hence appear approximately 50% pure as compared with the best preparations obtained by McClure et al (1). Contaminating activities, e.g. lactate dehydrogenase, acetyl-CoA deacylase, which might have interfered with the studies described, were absent from the preparations.

Pyruvate carboxylase was assayed spectrophotometrically in the presence of malate dehydrogenase and NADH as described previously except for use of $K^+$HEPES[1] in place of Tris-Cl (7). Substrates and cofactors were obtained, purified and assayed as described previously (6,7).

Results: Although McClure et al (1) reported that rat liver pyruvate carboxylase catalyzed a slow rate of OAA synthesis in the absence of acetyl-CoA, these workers made no attempt to characterize this activity or to define its relationship to acetyl-CoA-dependent OAA synthesis by this enzyme. Several observations are summarised here which define the relationship of the acetyl-CoA-independent activity to pyruvate carboxylase. First, OAA synthesis in the absence of acetyl-CoA exhibits an absolute requirement for pyruvate, ATP and $Mg^{2+}$ and is inactivated by prior incubation of the enzyme preparation with avidin. However, prior incubation of the preparation with citrate synthetase and OAA has no effect on OAA synthesis indicating that the rate observed in the absence of acetyl-CoA is not attributable to the presence of bound acetyl-CoA. Second, good correlation is observed between the acetyl-CoA-independent and acetyl-CoA-dependent rates of OAA synthesis in fractions obtained when

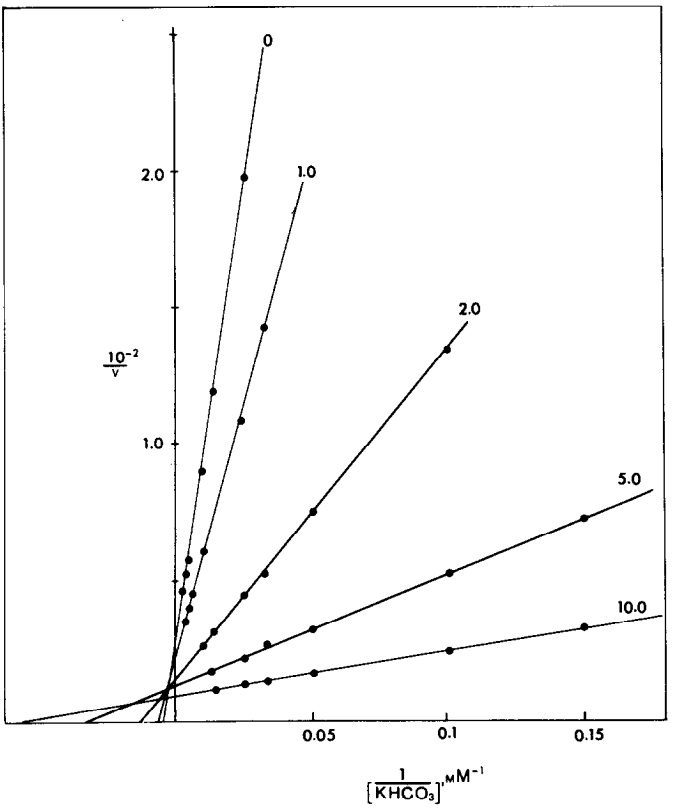


Fig. 1 Initial velocity study of OAA synthesis with $HCO_3^-$ as the variable substrate at several changing fixed concentrations (μM) of acetyl-CoA. The assay system contained in 1.0ml, 100μmoles $K^+$ HEPES pH 7.8, 5μmoles $Na^+$ pyruvate, 2μmoles $Na^+$ pyruvate, 2μmoles $MgATP^{2-}$, 3μmoles $Mg^{2+}$, 25μg. malate dhydrogenase, 0.03-0.15 μmoles NADH, $KHO_3$ and acetyl-CoA as indicated and pyruvate carboxylase (4.6 - 18.4μg.; sp. act.; 9.6). Velocity is expressed as Δabsorbance/minute at 340nm.

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partially purified pyruvate carboxylase from rat liver is chromatographed on DEAE-Sephadex A-50 ($SO_4^{2-}$). Third, the acetyl-CoA-independent and acetyl-CoA-dependent activities exhibit similar patterns of inactivation when the purified rat liver enzyme is incubated with (i) a 20-fold molar excess of DTNB[1]; (ii) 1M KCl at 2°; or (iii) guanidine-HCl over the range 0.1-1.0M.

Initial rate studies indicate that higher substrate concentrations are required for a given rate of OAA synthesis in the absence of acetyl-CoA than when this activator is present at saturating levels. The apparent $K_M$ for

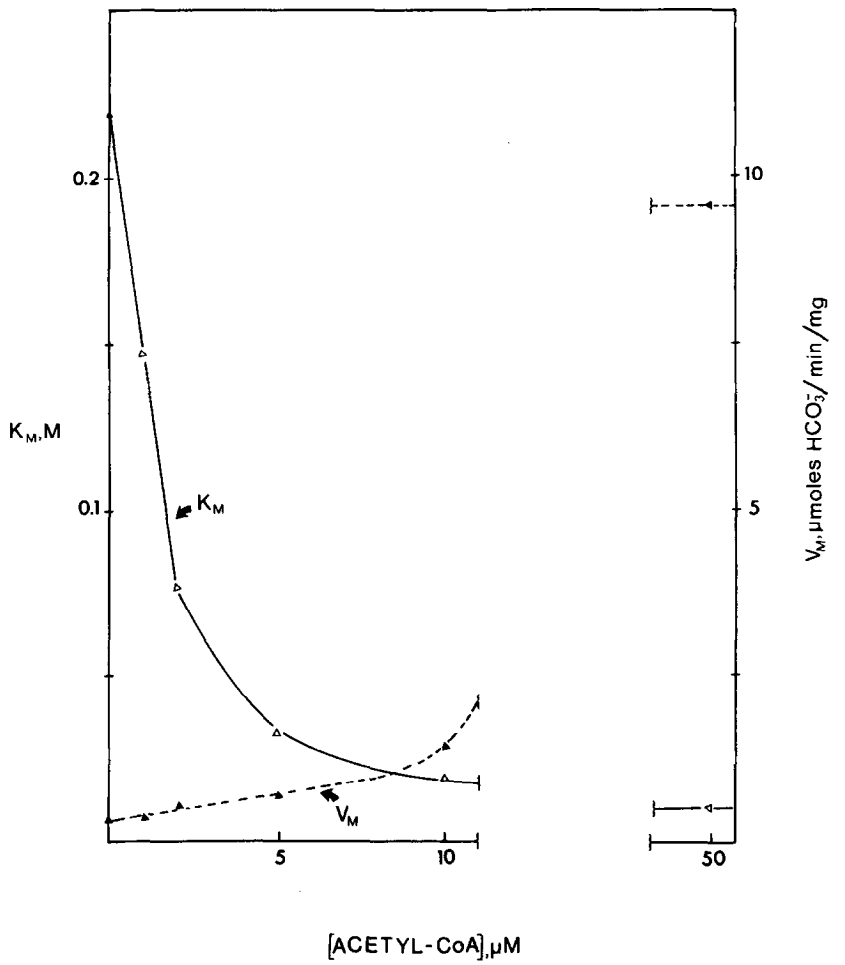


Fig. 2 Apparent $V_{MAX}$ and apparent $K_M$ for $HCO_3^-$ from Fig. 1 (with additional data) as a function of [Acetyl-CoA].

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$HCO_3^-$ is obtained as 200mM in the absence of acetyl-CoA, as compared with 3mM in the presence of a saturating concentration (50µM) of this cofactor (Fig. 1 and 2). Furthermore, the apparent $K_M$ for $HCO_3^-$ changes dramatically without a significant change in apparent $V_{MAX}$ as the acetyl-CoA concentration is increased from 0 to 10µM (Fig. 2). Subsequently as [acetyl-CoA] is increased to and above the region of the apparent $K_A$ (20-25µM) a marked increase is observed in the apparent $V_{MAX}$ while the apparent $K_M$ for $HCO_3^-$ changes less markedly over this range (Fig. 2).

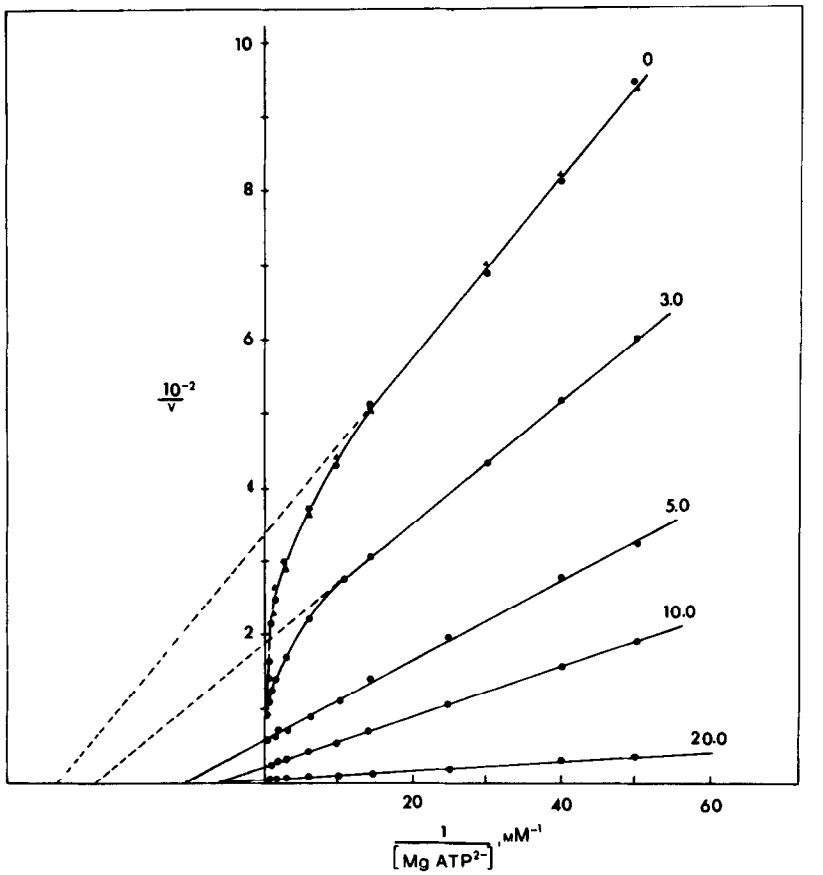


Fig. 3 Initial velocity study of OAA synthesis with $MgATP^{2-}$ as the variable substrate at several changing fixed concentrations (μM) of acetyl-CoA. The assay system was as described for Fig. 1 with the addition of $KHCO_3$ at 100mM and with pyruvate carboxylase (8.2-10.2μg: sp. act.; 10.4). The concentrations of $MgATP^{2-}$ and acetyl-CoA were varied as indicated.

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A somewhat similar pattern of behavior is observed when the relationship between reciprocal initial velocity and reciprocal $[MgATP^{2-}]$ is examined as a function of [acetyl-CoA] (Fig. 3). In the absence of acetyl-CoA a non-classical "convex-down" relationship is observed which cannot be subjected to simple analysis. However, extrapolated "apparent $K_M$'s" of 0.04mM and 2.5mM may be estimated from the linear lower and upper concentration regions respectively. The extent of deviation from classical behavior decreases as [acetyl-CoA] is increased and a linear relationship between reciprocal vel-

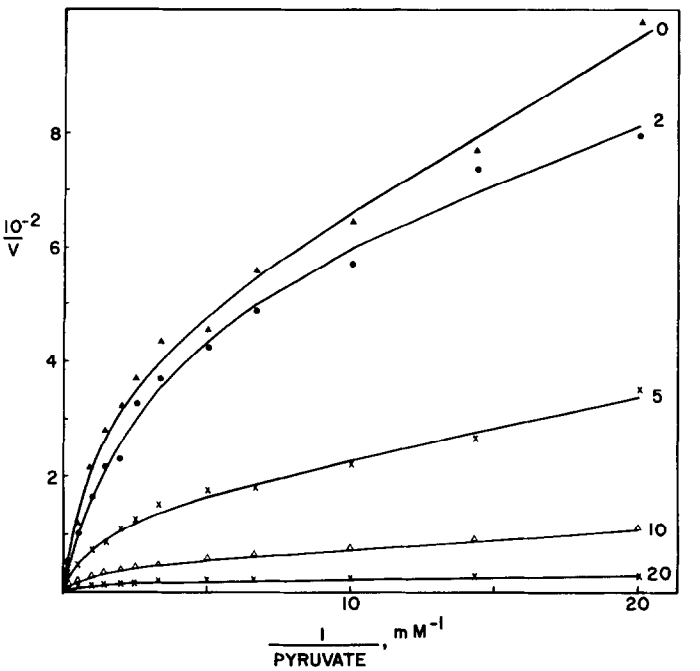


Fig. 4 Initial velocity study of OAA synthesis with $Na^+$ pyruvate as the variable substrate at several changing fixed concentrations (μM) of acetyl-CoA. The assay system was as described for Fig. 1 with the addition of $KHCO_3$ at 100mM and with pyruvate carboxylase (9.4μg: sp. act., 9.6). The concentrations of pyruvate and acetyl-CoA were varied as indicated. The lines shown were drawn from a computer fit of the experimental data to the 2/1 equation (8).

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ocity and reciprocal [Mg. $ATP^{2-}$] is observed at concentrations of this cofactor above 5μM. However, little change in the apparent $V_{MAX}$ occurs over the range 0-5μM acetyl-CoA (Fig. 3). Furthermore, when the concentration of the activator exceeds 10μM the apparent $K_M$ for $MgATP^{2-}$ becomes independent of [acetyl-CoA].

In contrast to the marked effects observed for the substrates ($HCO_3^-$, $MgATP^{2-}$) of the carboxylation partial reaction (Reaction 2), variation of [acetyl-CoA] over the range 0-10μM has no significant effect on the extrapolated "apparent $K_M$'s" which describe the non-linear relationship between reciprocal initial velocity and reciprocal pyruvate concentration (Fig. 4). However, at higher concentrations of acetyl-CoA in the region of, and above, the

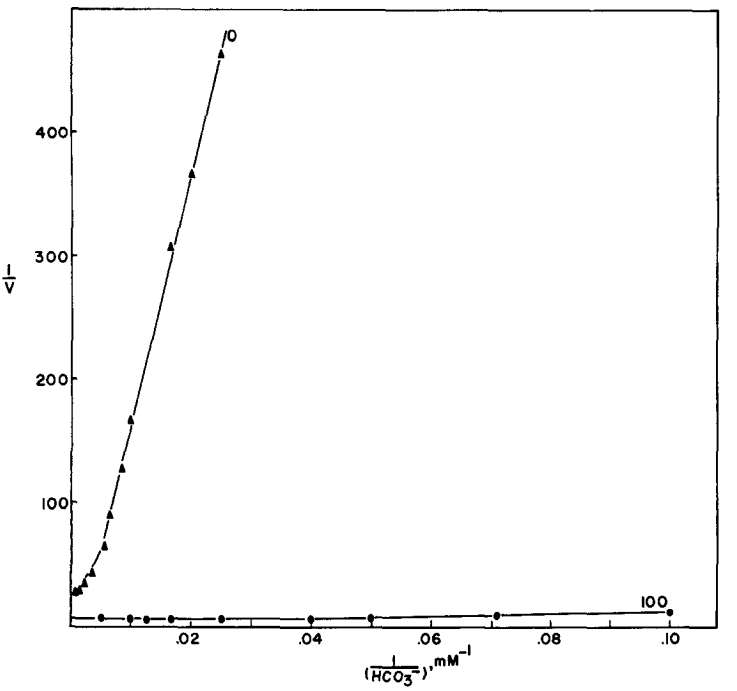


Fig. 5 Initial velocity study of OAA synthesis as a function of the concentrations of $HCO_3^-$, $MgATP^{2-}$ and pyruvate varied in a constant ratio of 100:10: 1 in the absence and in the presence of 100μM acetyl-CoA. The other components were present as indicated for Fig. 1 with 10.5μg. pyruvate carboxylase (sp. act., 7.4). The data are plotted as a function of $[HCO_3^-]$.

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apparent $K_A$, the relationship between reciprocal initial velocity and reciprocal pyruvate concentration exhibits an increasing degree of linearity and, in the presence of 100mM $HCO_3^-$, becomes linear at acetyl-CoA concentrations exceeding 50μM.

The maximal rate of OAA synthesis in the absence of acetyl-CoA was estimated by measuring the initial velocity as a function of the concentration of all three substrates ($HCO_3^-$, $MgATP^{2-}$, pyruvate) varied at constant ratio approximating the relative $K_M$'s observed for the acetyl-CoA independent reaction (Figs. 1-4). The $V_{MAX}$ for OAA synthesis in the absence of acetyl-CoA obtained from a double reciprocal plot of these data is 21% of that observed when a similar experiment is performed at a saturating concentration (100μM) of this

cofactor (Fig. 5).

Discussion: Two conclusions can be drawn from the data presented here. First, pyruvate carboxylase from rat liver catalyzes a substantial rate of OAA synthesis in the absence of acetyl-CoA. In this respect, as well as in the marked effect of acetyl-CoA on the apparent $K_M$ for $HCO_3^-$ (Figs. 1 and 2), rat liver pyruvate carboxylase resembles the enzyme purified from Saccharomyces cerivisiae more closely than that from avian liver (cf. 9). Although due to the conditions required for its expression, OAA synthesis in the absence of acetyl-CoA appears unlikely to posses physiological significance, the detection of a significant level of this activity is of importance since it will permit investigation of the relationship between the catalytic and activator sites of rat liver pyruvate carboxylase by the classical techniques of protein modification (cf. 10). Also, detailed kinetic analysis may be possible since the non-linear double reciprocal plots obtained with pyruvate as the variable substrate (Fig. 4) can be fitted to the 2/1 equation[2]. Second, examination of the concentration dependence of the effects of acetyl-CoA on OAA synthesis by rat liver pyruvate carboxylase has revealed a specific action of very low concentrations (0-10μM) of this cofactor. Over this region of concentration the enzyme behaves essentially as a K system (11) with respect to activation by acetyl-CoA and the effect of this activator is apparently localized in the carboxylation partial reaction (Reaction 2) (Figs. 1-3). At higher acetyl-CoA concentrations (10-50μM) a marked increase in apparent $V_{MAX}$ is observed (Fig. 2) which appears to be associated with modification of the kinetic behavior of pyruvate, the substrate of the transcarboxylation partial reaction (Reaction 3), although some change is also observed in the apparent $K_M$ for $HCO_3^-$ over this range (Fig. 2). These observations appear in accord with the prior demonstration that acetyl-CoA activates both partial reactions in the case of the rat liver enzyme (3,4), but indicate that the nature of the

[2]V. L. Schramm and M. C. Scrutton. Unpublished observations.

changes in the conformation of the active site of pyruvate carboxylase which are induced appear to be dependent on the degree of saturation by acetyl-CoA. Alternatively the reported effects might be observed if the enzyme carries kinetically non-identical sites for this activator.

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