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PYRUVATE CARBOXYLASE: AN EVALUATION OF THE RELATIONSHIPS BETWEEN STRUCTURE AND MECHANISM AND BETWEEN STRUCTURE AND CATALYTIC ACTIVITY

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I. Introduction

Pyruvate carboxylase, which catalyzes reaction 1, was first detected in chicken liver during a study of the initial reactions of gluconeogenesis from pyruvate (1–3).

$$\text{Pyruvate} + \text{MgATP}^{2-} + \text{HCO}_3^- \xrightleftharpoons{(\text{Acyl-CoA}),\ \text{Me}^{2+},\ \text{Me}^{+}} \text{Oxalacetate} + \text{MgADP}^- + \text{P}_i^{2-} \quad (1)$$

Since this initial finding, pyruvate carboxylase has been found at high levels of activity in all species of liver and kidney that have been examined (4,5). Less extensive evidence suggests that the enzyme is present at reasonably high levels in adipose tissue (6–8) and at lower but significant levels in brain (3,6,9) and mammary tissue (6,10). The enzyme has also been found in insects (11); yeast [*Saccharomyces* (4,12, 13)]; mold [*Penicillium* (14); *Rhizopus* (15)]; fungi [*Aspergillus*, (16–18); *Agaricus* (19); *Neocosmospora* (20); *Neurospora* (Taylor, B. L. and Utter, M. F., unpublished observation); *Verticillium* (21); *Helminthosporum* (22)]; bacteria [*Arthrobacter* (23); *Bacillus* (24–26); *Rhodopseudomonas* (27,28); *Azotobacter* (29); *Micrococcus* (30); *Clostridium* (31); *Pseudomonas* (32–34); *Chromatium* (35); *Streptococcus* (36); *Peptostreptococcus* (37)] and in other simple organisms [*Plasmodium* (38); *Crithidia* (39)]. In addition to its gluconeogenic role in liver, kidney, and some microorganisms, pyruvate carboxylase appears to carry out an anaplerotic function (40) in other species of microorganisms and perhaps in brain and certain other animal tissues. It has also been postulated that pyruvate carboxylase can serve as part of a cyclic process that furnishes NADPH for fatty acid synthesis in adipose tissue (41).

In view of the widespread distribution of pyruvate carboxylase, its apparent absence from certain sources is worth noting. The enzyme has not been demonstrated in higher plants nor in a number of bacterial genera which include *Escherichia, Aerobacter,* and *Salmonella.* These bacteria as well as higher plants often contain phosphoenolpyruvate carboxylase which provides an alternative to pyruvate carboxylase for the anaplerotic synthesis of oxalacetate (42).

As written above, reaction 1 indicates that all species of pyruvate carboxylase tested thus far require the presence of a divalent and a monovalent cation for activity. There is a wide difference, however, in the requirement for an acyl-CoA. Such compounds have a strong activating effect on all species of the enzyme of animal origin. In some species, such as the enzyme from chicken liver, the requirement for an acyl-CoA activator is essentially absolute (4,43). In species obtained from microorganisms a wide variety of responses to the presence of an acyl-CoA has been observed. In some species [*Arthrobacter* (Jones, K. M. and Utter, M. F., unpublished observation); *Bacillus* (25)] there is a very strong activation by the acyl-CoA or perhaps even a complete dependence on its presence; in others [*Saccharomyces* (44)] addition of an acyl-CoA causes a modest stimulation of activity, and in still other species [*Pseudomonas* (45,46); *Aspergillus* (16,17); *Azotobacter* (29)] there is no response to the addition of the acyl-CoA. The various species of pyruvate carboxylase also respond quite differently to various inhibitors. A number of types of the enzyme of microbial origin [*Saccharomyces* (47); *Arthrobacter* (Jones, K. M. and Utter, M. F., unpublished observation); *Bacillus* (25)] are strongly inhibited by aspartate, but other microbial enzymes [*Pseudomonas* (33,46); *Azotobacter* (29)] appear to be unaffected by aspartate or other potential metabolic effectors. None of the varieties of the enzyme derived from animal sources is significantly affected by aspartate, although a number of acyl-CoA compounds can act as inhibitors (4,48). However, the specificity and the degree of the inhibition appear to be species-dependent. It is clear that the varieties of pyruvate carboxylase exhibit a wide spectrum of regulatory properties.

The overall reaction catalyzed by pyruvate carboxylase is relatively complex since it involves three substrates—MgATP, HCO_3^-, and pyruvate—and three products—MgADP, phosphate, and oxalacetate (reaction 1). In addition to the freely dissociable substrates, products, and activators, two enzyme bound cofactors have been implicated in the reaction (4,48). The enzyme contains covalently bound biotin in a stoichiometric ratio that approaches 4 moles of biotin per mole of protein in tetrameric varieties of the enzyme isolated from liver (49–51) and yeast (52) and 2 moles of biotin per mole of enzyme for the dimeric enzyme from *Pseudomonas citronellolis* (33). When pyruvate carboxylase from chicken liver is incubated with ATP, Mg^{2+}, [^{14}C] HCO_3^-, and acetyl-CoA, the formation of the enzyme-biotin-$^{14}COO^-$ intermediate

can be detected (53), and the transfer of the labeled carboxyl group from the isolated intermediate to pyruvate can also be demonstrated. These observations show the biotin plays an obligatory role in the CO_2 fixation reaction catalyzed by pyruvate carboxylase. Thus, the carboxyl-carrier function of biotin in this reaction appears to be the same as that in other biotin-enzymes [cf. recent reviews on biotin-enzymes by Knappe (54) and Moss and Lane (55)].

All pyruvate carboxylases examined thus far except the enzyme from *P. citronellolis* have been shown to contain a tightly bound transition metal (4). The situation with the bacterial enzyme is still not clear, but the enzymes from animal liver contain Mn or Mg or both (50,51,56), and the enzyme from yeast contains Zn (52). Extensive investigations of the role of Mn in pyruvate carboxylase from chicken liver (57–60) suggested that the metal ion may be involved in catalysis at the pyruvate site, although more recent findings (61,62) have served to somewhat modify the earlier hypothesis.

The present chapter will be limited mainly to a discussion of the relationship of the structural features of this group of enzymes to their mechanism of action and to the control of their catalytic activities. Therefore, individual sections on the catalytic properties of the enzymes, the mechanistic implications of their structure, and the interrelationships of structure and catalytic activity follow in that order. The discussion will not deal extensively with the physiological roles of these enzymes, nor with the physiological regulation of the various types of enzymes. Earlier reviews have treated these topics and have also discussed certain aspects of the earlier structural and mechanistic studies in more detail than will be possible here (4,5,48,63).

II. Catalytic Properties of Pyruvate Carboxylase

A. REACTION MECHANISM

1. Minimal Mechanism

A minimal reaction for pyruvate carboxylase can be proposed on the basis of isotope exchange studies. With all species of the enzyme examined thus far, demonstration of isotopic exchange between $^{32}P_i$ and ATP requires the presence of ADP, HCO_3^-, Me^+, and Me^{2+} (53, 64–66). On the other hand, isotopic exchange between [^{14}C]pyruvate and oxalacetate occurs in the absence of other reaction components (53,

65,66). These observations, together with the isolation of a enzyme-biotin-CO_2 complex (53), indicate that the overall reaction catalyzed by pyruvate carboxylase is the sum of two partial reactions (reactions 2 and 3):

$$E\text{-biotin} + MgATP^{2-} + HCO_3^- \xrightleftharpoons{Me^+ \cdot Me^{2+}} E\text{-biotin-}CO_2 + MgADP + P_i^{3-} \quad (2)$$

$$E\text{-biotin-}CO_2^- + \text{pyruvate} \rightleftharpoons E\text{-biotin} + \text{oxalacetate} \quad (3)$$

According to the terminology of Cleland (67), the minimal mechanism represented by reactions 2 and 3 is described as Ping-Pong Bi-Bi Uni-Uni.

A complete description of the reaction mechanism for pyruvate carboxylase should include the following information: (*1*) the order in which substrates add to the enzyme and products dissociate from the enzyme, and (*2*) the composition of enzyme-reactant complexes and the significant interconversions among these complexes. Information of this type can be gained from steady-state kinetic studies if experimental procedures such as those outlined by Cleland (68) are followed. A thorough investigation of the steady-state kinetics of pyruvate carboxylase from chicken liver has been reported (69) and much of the following discussion pertaining to the reaction mechanism of pyruvate carboxylase is based on these studies. The enzymes from rat liver and sheep kidney have also been investigated, although in these cases the results of both steady-state kinetic studies and isotope exchange kinetic studies were required in order to obtain a clear description of the reaction mechanism (65,70,71). Feir and Suzuki have also studied the enzyme from *Aspergillus niger* (16). Thus far no major differences in reaction mechanism have been detected among the species of pyruvate carboxylase that have been examined by kinetic methods, with the possible exception of certain aspects of the mechanism of the enzyme from *A. niger*. This matter will be discussed later.

2. *Initial Velocity Studies of CO_2 Fixation and Oxalacetate Decarboxylation*

For the experiments with pyruvate carboxylase from chicken liver (69) or rat liver (65, 70) which furnish the major basis for this discussion of the reaction mechanism, a saturating concentration of acetyl-CoA was used in all initial velocity and product inhibition experiments. Thus, the catalytic properties observed under these conditions are those of the

enzyme-modifier complex in which all kinetically significant modifier sites are occupied. As will be noted in Section II. B, kinetic experiments with less than saturating concentrations of acetyl-CoA have been performed with pyruvate carboxylase from chicken liver (72) or in the absence of activator in species from sheep kidney (42), yeast (44), and *A. niger* (16). These experiments, in which the properties of the free enzyme (as contrasted with the enzyme-activator complex) can be observed, suggest that the fundamental mechanism of action is not modified by the presence of the activator. On the other hand, presence of the activator may alter the maximal velocity of the reaction and the ability of the enzyme to bind certain substrates, or both, depending on the species of enzyme involved. These relationships are discussed in Section II.B below.

According to the minimal mechanism cited above (reactions 2 and 3), the overall reaction catalyzed by pyruvate carboxylase is the sum of two partial reactions, one with a sequential Bi-Bi kinetic mechanism and the other with a Uni-Uni mechanism. When the initial velocity pattern for each possible pair of substrates in both the direction of CO_2 fixation and the direction of oxalacetate decarboxylation was determined with the enzyme from chicken liver, the results in every case were consistent with the patterns which were predicted for a Ping-Pong Bi-Bi Uni-Uni mechanism (69–71).

Although the results of the initial velocity studies were consistent with the mechanism originally proposed on the basis of isotope exchange experiments, it was not possible to decide which of two fundamentally different types of Ping-Pong Bi-Bi Uni-Uni mechanisms was applicable to pyruvate carboxylase. The distinction between the two types of mechanisms depends on the number of separate subsites that are assumed to be operative within each active site. Classical mechanisms assume that the active site functions as a single catalytic entity, for example, the glutamate-oxalacetate transaminase mechanism (73). An active site of this sort as applied to the pyruvate carboxylase reaction is represented schematically in Figure 1*a*. On the other hand, nonclassical mechanisms assume that two functionally independent catalytic subsites are present within each active site; or more specifically, that a separate subsite exists for the reactants of each partial reaction. A schematic representation of an active site of this type is shown for pyruvate carboxylase in Figure 1*b*. It is assumed that the biotinyl residue, which is located on a flexible chain of approximately 14 Å length and which

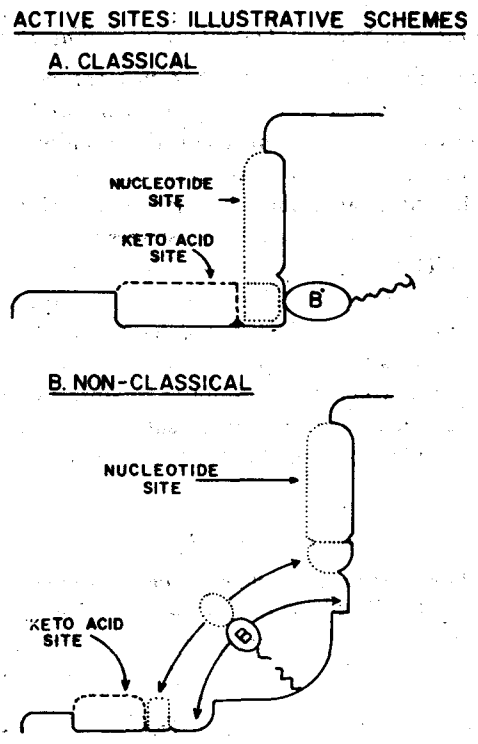

Fig. 1. Illustration of classical and nonclassical active sites as applied to the pyruvate carboxylase reaction.

functions as a carboxyl carrier (55), can link the two separate catalytic subsites and thus provide the element that unifies the active site.

The classical concept of the active site (i.e., Fig. 1*a*) has been utilized successfully in describing the kinetic behavior of a large number of enzymes. On the other hand, except for multienzyme complexes, the nonclassical concept of the active site (i.e., Fig. 1*b*) had been incorporated into the reaction mechanism of only one enzyme, methylmalonyl-CoA transcarboxylase (74,75), prior to initiation of kinetic studies with pyruvate carboxylase. Methylmalonyl-CoA transcarboxylase, which is also a biotin-enzyme, exhibits several catalytic and structural parameters that are analogous to those of pyruvate carboxylase.

3. Product Inhibition Studies: The "Two-site" Ping-Pong Mechanism

The classical and nonclassical mechanisms are most readily distinguished by examination of certain key pairs of product inhibitor and varied substrate which should show competitive inhibition patterns. The predicted patterns for either the classical or nonclassical mechanisms will be influenced by the assumptions concerning the Bi-Bi portion of the reaction. The weight of the evidence suggests that the addition of $MgATP^{-2}$ and HCO_3^-, and the release of $MgADP^-$, and P_i^{2-}, can best be described as a rapid equilibrium random mechanism (69), and the predictions of patterns in Table I are based on this type of Bi-Bi mechanism. In the classical mechanism (Fig. 1*a*), it can be predicted that $MgADP^-$ will be a competitive inhibitor against pyruvate since they would combine at the same site with the same form of the enzyme. The same situation would hold for $MgATP^{2-}$ as a product inhibitor and

TABLE I[a]

Comparison of Selected Product Inhibition Patterns Predicted by Classical and Nonclassical Ping-Pong Mechanisms with Observed Patterns

Relationship between varied substrate and product inhibitor	Varied substrate	Product inhibitor	Predicted patterns: Classical rapid equilibrium	Predicted patterns: Nonclassical rapid equilibrium	Observed patterns
Partial reaction					
Same	MgATP	MgADP	UC[b]	C	C
Same	MgATP	P_i	UC	C	C
Same	MgADP	MgATP	UC	C	C
Same	P_i	MgATP	UC	C	C
Same	Pyruvate	Oxalacetate	NC	C	C
Same	Oxalacetate	Pyruvate	NC	C	C
Partial reaction					
Different	Pyruvate	MgADP	C	UC	UC
Different	MgADP	Pyruvate	C	NC	NC
Different	Oxalacetate	MgATP	C	UC	UC
Different	MgATP	Oxalacetate	C	NC	NC

[a] Adapted from Table II of ref. 69.

[b] NC designates noncompetitive; UC, uncompetitive; C, competitive.

oxalacetate as the varied substrate. As shown by these and other examples in Table I, competitive inhibition will occur only when the varied substrate and the product inhibitor are involved in different partial reactions. In contrast, the nonclassical mechanism (Fig. 1*b*) predicts that $MgADP^-$ should be an uncompetitive inhibitor against pyruvate since they would combine at different sites on the same form of the enzyme. The nonclassical mechanism predicts competitive interactions only between substrates and products that are in the same partial reaction; for example, between pyruvate and oxalacetate (Table I). The kinetic behavior predicted for the nonclassical mechanism is a direct consequences of the assumption that a separate catalytic subsite exists for the reactants of each partial reaction.

The results of a comprehensive set of product inhibition experiments for pyruvate carboxylase from chicken liver have been reported (69). Selected examples from this more complete study are shown in Table I. As shown in the first part of this table, when the product inhibitor and the varied substrate belong to the same partial reaction the observed patterns are all competitive. This is consistent with the nonclassical rapid equilibrium mechanism and is inconsistent with the predictions of the classical rapid equilibrium mechanism. As shown in the second half of the table, when the varied substrate and product inhibitor are from different partial reactions, the nonclassical mechanism predicts that these patterns should be either noncompetitive or uncompetitive. In each case, the observed pattern fits the prediction of the nonclassical rapid equilibrium mechanism. These results strongly suggest that the reaction catalyzed by pyruvate carboxylase is best described as a nonclassical or "two-site" Ping-Pong Bi-Bi Uni-Uni mechanism. As discussed below, some of the product inhibition studies involving HCO_3^- give patterns that are inconsistent with either the classical or nonclassical rapid equilibrium mechanisms of Table I. Minor modifications of the nonclassical mechanism have been required to reconcile these results. Product inhibition experiments were not used extensively in probing the mechanism of pyruvate carboxylase from rat liver and sheep kidney, but the results reported (70,71) are consistent with those obtained with the enzyme from chicken liver.

The competitive patterns observed when $MgATP^{2-}$ interacts kinetically with $MgADP^-$ or phosphate (Table I) (69,70) argue that the binding of reactants at the Bi-Bi site can be described by a rapid equilibrium random type of mechanism. The observation of equal exchange

rates for the [^{14}C]ADP, ATP pair, and the [^{32}P]phosphate, ATP pair with the rat liver enzyme also suggested a rapid equilibrium random type of mechanism (65). However, a simple rapid equilibrium random Bi-Bi mechanism is not completely adequate, at least for the enzyme from chicken liver. Such a mechanism requires that HCO_3^- and $MgATP^{2-}$ show the same kinetic behavior, but HCO_3^- shows noncompetitive interactions with $MgADP^-$ and phosphate , not the competitive interactions exhibited by $MgATP^{2-}$ (69). The kinetic behavior of HCO_3^- can be explained if it is assumed that abortive reactant complexes form with the enzyme in which HCO_3^- and a product of the Bi-Bi partial reaction are simultaneously bound to the enzyme. If the formation of two abortive complexes—namely, E-HCO_3^--P_i and E-HCO_3^--$MgADP^-$—is combined with a rapid equilibrium random Bi-Bi mechanism, a rate equation can be derived for the overall reaction (Section II.A.5) which predicts the product inhibition patterns observed with pyruvate carboxylase (69), except for a single case (discussed below). The two proposed abortive complexes might be expected to form at a site that binds $MgATP^{2-}$ and HCO_3^- in the productive sequence. Analogous abortive complexes are commonly encountered in kinetic studies of other enzymes that have random mechanisms (68).

An uncompetitive pattern is predicted when HCO_3^- is a product inhibitor of oxalacetate decarboxylation and oxalacetate is the varied substrate, but a noncompetitive pattern is observed (69). This apparent inconsistency can be explained if the rate of the reaction in the direction of oxalacetate decarboxylation is not limited solely by the interconversion of the central complex but also in part by release of $MgATP^{2-}$ from the enzyme. Under such circumstances, HCO_3^- could react with E-$MgATP^{2-}$ and biotin to regenerate the carboxy-biotin forms of the enzyme. This will result in an inhibition of the reaction that cannot be overcome by increasing the concentration of oxalacetate, hence yielding a noncompetitive pattern. Evidence of the existence of an E-$MgATP^{2-}$ complex from which the release of $MgATP^{2-}$ is relatively slow was obtained in isotope exchange studies of the enzyme from rat liver (65).

4. Other Evidence Supporting a "Two-Site" Ping-Pong Mechanism

The proposed existence of two separate subsites per active site for pyruvate carboxylase is also supported by other studies on the properties of the interaction of substrates, activators, and inhibitors with the enzymes from chicken or rat liver. First, the properties of the interaction

of the bound Mn with the substrates of the Uni-Uni partial reaction (reaction 3) are unaffected by the presence of the substrates and cofactors of the Bi-Bi partial reaction (reaction 2) (57,60). Second, oxalate acts as a specific inhibitor of the exchange of [^{14}C]pyruvate with oxalacetate (reaction 3) but has no significant effect on the rate of exchange of [^{32}P]phosphate with $MgATP^{2-}$ (reaction 2) (57). Third, $MgADP^-$, phosphate, and Mg^{2+}, which are all reactants of the Bi-Bi partial reaction (reaction 2), have no effect on the exchange of [^{14}C]pyruvate with oxalacetate (53,65). Fourth, the exchange of [^{14}C]ADP with ATP is accelerated by α-ketobutyrate, a dead-end inhibitor that binds at the site for the Uni-Uni partial reaction (65). This effect of a dead-end inhibitor is most readily rationalized if one assumes the existence of a two-site mechanism, as discussed by Northrop and Wood (75). Fifth, with the chicken liver enzyme, the activator, acetyl-CoA, is required for isotopic exchange between reactants of the Bi-Bi partial reaction (reaction 2) (53); but, the exchange of [^{14}C]pyruvate with oxalacetate is unaffected by acetyl-CoA.

5. *The Rate Equation*

In summary, the major features of the reaction mechanism proposed for pyruvate carboxylase are the following: (*a*) the overall reaction is the sum of two partial reactions and can be described as Ping-Pong Bi-Bi Uni-Uni, (*b*) separate catalytic subsites exist for the reactants of each partial reaction, (*c*) the two subsites are connected by a biotinyl residue that carries the carboxyl group from one subsite to the other, (*d*) the mechanism of the Bi-Bi partial reaction is rapid equilibrium random, (*e*) the additional existence of two kinetically significant abortive complexes (E-HCO_3^--P_i and E-HCO_3^--MgADP) is postulated, at least for the chicken liver enzyme.

The derivation of rate equations for such complex reaction mechanisms has been greatly facilitated by the development of a procedure that combines features of the steady-state and rapid equilibrium methods (76). Application of this method in the case of pyruvate carboxylase had to take into account the general features of the mechanism as described above. The additional assumption required was that the substrates and products bind equally well to their respective sites, whether or not the biotinyl residue is carboxylated. The complete rate equation obtained for pyruvate carboxylase from chicken liver using this procedure may be found in reference 69. This general procedure had

previously been used by Northrop to derive a rate equation for methylmalonyl-CoA transcarboxylase (74), a somewhat similar, but less complex, reaction.

B. KINETIC BEHAVIOR OF ACTIVATORS AND THEIR INTERACTIONS WITH EACH OTHER AND WITH SUBSTRATES

Although the available evidence suggests that the basic reaction mechanism of the various species of pyruvate carboxylase is very similar or identical, the mechanism of activation by acyl-CoA compounds appears to vary considerably among the species of enzyme. These differences are manifest in the degree of dependence on acyl-CoA for activity, on the degree of interaction between the acyl-CoA activator and the substrates, and on the degree of interaction between the acyl-CoA activator and the monovalent cation activator. This complex topic has not yet been completely explored, but striking differences have already been found among the different types of pyruvate carboxylase that have been examined.

Table II presents, in summary form, a comparison of the properties of the activation process in several different classes of pyruvate carboxylase. The chicken liver enzyme represents the extreme example of dependence on an acyl-CoA activator. Ashman et al. (43) have reported that this variety of the enzyme does have measureable activity in the absence of an activator, but the ratio of this rate to the rate of the activated enzyme is only about $\frac{1}{1000}$. This situation becomes more evident when compared with other types of pyruvate carboxylases, such as those of mammalian origin. This type of enzyme is represented in Table II by the enzymes from rat liver and sheep kidney. Under appropriate conditions, that is, very high concentrations of substrates and K^+, the acyl-CoA-independent activity of these enzymes may approach 25% of the activity in the presence of acetyl-CoA (43,77). Even under more usual assay conditions (43,65), the activity of these enzymes in the absence of activator may approach 2–4% of that of the activated enzyme. The enzyme from yeast shows a further decrease in dependence on an acyl-CoA activator. At moderate concentrations of substrate and K^+, the stimulation achieved by the addition of an acyl-CoA is only two to three-fold (44,78,79) and with other conditions the degree of activation may decline still further. The enzyme from *P. citronellolis* represents still another class of pyruvate carboxylases that has no dependence on an acyl-CoA activator under any conditions (33,45,46).

TABLE II

Variations of the Nature of the Acyl-CoA Activation Process Among Different Species of Pyruvate Carboxylase[a]

Species	Maximal activity in absence of acetyl-CoA (%)[b]	Interaction of acyl-CoA substrates	Interaction of acyl-CoA with K^+	Interaction of K^+ with substrates
Chicken liver	0.07	Little or none	Little or none	Yes (HCO_3^-)
Rat liver	2–25	Strong (HCO_3^-)	Strong	Yes (HCO_3^-)
Sheep kidney	4–25	Strong (HCO_3^-, pyruvate)	Strong	Yes (HCO_3^-)
Yeast	33–100	Very strong (HCO_3^-)	Very strong	Yes (HCO_3^-)
P. citronellolis	100	None	None	Yes

[a] These data represent a summation of published reports (43–47, 70–72,77) and unpublished data kindly made available by M. C. Scrutton; L. C. Ashman and D. B. Keech; B. Tolbert and M. R. Young; and C.-H. Fung.

[b] Percent of V_{max} in presence of saturating concentrations of acetyl-CoA.

Let us examine in more detail the possible bases of the widely varying dependence on acyl-CoA activation exhibited by the various species. The enzyme from chicken liver probably represents a rather limited class that includes other avian enzymes, such as the one from turkey liver (Wallace, J. C. and Utter, M. F., unpublished results) and may include certain varieties of microbial origin, for example, the enzymes from *Arthrobacter globiformis* (23), and *Bacillus stearothermophilis* (80). With this type of pyruvate carboxylase, the relationship between the initial rate of CO_2 fixation and the acyl-CoA concentration is highly cooperative, since plots of initial velocity versus acyl-CoA concentration show marked sigmoidicity. Analysis of the data according to the empirical equation of Hill (81,82) yields values for the Hill coefficient (n_H) approaching 3.0 [Jones, K. M. and Utter, M. F., unpublished observation (83)]. With the enzyme from chicken liver at least, the n_H is