(Arfin and Koziell, 1971) and that *N*-ethylmaleimide protects the threonine dehydratase from *C. tetanomorphum* (Phillips, 1968) from serine inhibitions, along with the results reported here, would suggest that alkylation of an enzyme-generated intermediate could be responsible for the L-chloroalanine and L-serine inhibition of many of the threonine dehydratases.

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Studies on the Intramolecular and Intermolecular Kinetic Isotope Effects in Pyruvate Carboxylase Catalysis†

Yak-Fa Cheung‡ and Christopher Walsh*,§

ABSTRACT: A deuterium kinetic isotope effect of 2.1 was observed when [$^2H_3$]pyruvate was used as the substrate for pyruvate carboxylase. The effect is on V_{max}/K_m alone and disappears at infinite substrate concentration. This is interpreted to mean that the slowest step in the overall catalysis is in the half-reaction involving the carboxylation of enzyme–biotin by ATP and HCO_3^-. A tritium *intra*molecular isotope effect of 4.8 and an *inter*molecular effect of 1.2 were also observed. The former was interpreted as the isotope effect on the "effective k_{cat}", while the latter the one on V_{max}/K_m. With these data, the rate constant for binding of pyruvate was estimated to be 4.5×10^6 M^{-1} min^{-1}, and the deuterium kinetic isotope effect on the catalytic step to be 3.1. Relative values for various rate constants were also obtained. Fluoropyruvate was also shown to be a substrate, reacting six times slower. A deuterium kinetic isotope effect of 1.5 was observed, which remained even at infinite substrate concentration. This is interpreted to mean that the slowest step in the overall catalysis is now the carboxylation of fluoropyruvate.

Pyruvate carboxylase (EC 6.4.1.1) is a biotin-containing enzyme purified from liver mitochondria (Utter and Keech, 1963). It catalyzes the ATP[1]-dependent carboxylation of pyruvate by HCO_3^-, forming oxalacetate, ADP, and inorganic phosphate. The methyl group of pyruvate is converted to the C3 prochiral methylene group of oxalacetate. Our interests in this enzyme stem from two unique features of this class of enzymatic reactions: (i) pyruvate carboxylase is conveniently used in establishing the absolute configurations of chirally labeled [^{1}H,^{2}H,^{3}H]pyruvate samples (Cheung and Walsh, 1976a; Creighton and Rose, 1976). We have been interested in determining the kinetic isotope effect with respect to the *intra*molecular discrimination against deuterium, and thus be able to predict accurately (for chiral pyruvate samples) the theoretical ratio of (3*R*)- to (3*S*)-[3-^{3}H]oxalacetate formed, and after enzymatic reduction 3*R*- and 3*S*-[3-^{3}H]-L-malate in such experiments (Cornforth et al., 1969; Luthy et al., 1969). (ii) This enzymatic reaction, involving reaction at a torsiosymmetric methyl group, offers a unique situation where one can separately determine the kinetic isotope effects on both the catalytic step (an *intra*molecular effect) and the overall reaction (an *inter*molecular effect), thus probing certain kinetic aspects of this enzyme which is not possible experimentally in many other cases.

We report here results on these two issues and relate this information to some of the previous kinetic information on the enzyme (Scrutton and Young, 1972; Utter et al., 1975).

Experimental Section

Materials. Sodium pyruvate, sodium fluoropyruvate, lactic dehydrogenase, and malate dehydrogenase were purchased

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[1] Abbreviations used are: ATP, ADP, adenosine tri- and diphosphate; NMR, nuclear magnetic resonance; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NADH, reduced nicotinamide adenine dinucleotide; lhs and rhs, left and right hand side.

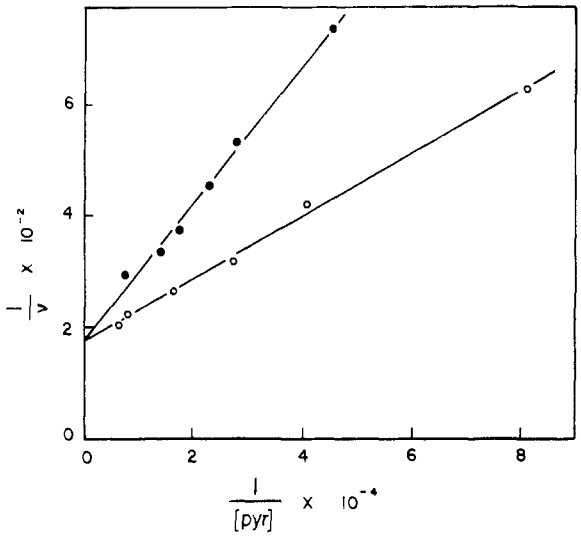


FIGURE 1: Double-reciprocal plots of initial velocities of pyruvate carboxylase, expressed in μmol/min vs. concentrations of [$^1H_3$]- (○) and [$^2H_3$]pyruvate (●). The incubations contained (in μmol) Tris buffer (6.5, pH 7.8), $KHCO_3$ (15), $MgCl_2$ (5), ATP (2.5), acetyl-CoA (0.1), NADH (2.5), 30 units of malate dehydrogenase, 7.7 μg of pyruvate carboxylase in a total volume of 0.8 ml. The reaction was started by the addition of various amount of [$^1H_3$]- or [$^2H_3$]pyruvate at 30 °C. The rate was monitored by the decrease of A_{340} on a Gilford spectrophotometer.

from Sigma Chemical Co. All other chemicals and solvents were of commercial reagent grade.

Pyruvate carboxylase (specific activity, 12 μmol min^{-1} mg^{-1}) from chicken liver was a generous gift from Professor M. F. Utter and Dr. D. Myer.

[^{3}H]Pyruvate (~10^6 cpm/μmol) was prepared by the procedure of Rose (1960). [2-^{14}C]Pyruvate (6.7 Ci/mol) was purchased from New England Nuclear and purified as reported (Cheung et al., 1975). [$^2H_3$]Pyruvate was prepared as reported (Cheung et al., 1975).

[$^2H_2$]Fluoropyruvic acid was prepared by the following procedure. Sodium fluoropyruvate (160 mg) was dissolved in 5 ml of $2H_2O$; excess ^2HCl was added. The solution was heated in a sealed tube placed in refluxing toluene for 8 h. Solvent was removed on a rotovaporator. The solid residue was sublimed (60–75 °C, 0.2 mm Hg). White-crystalline solid (65 mg) was collected, mp 75–77 °C (lit. 86 °C, Nair and Busch, 1958). Nuclear magnetic resonance (NMR) analysis showed at least 95% deuteration. The compound so obtained assayed quantitatively with lactic dehydrogenase.

Methods. [^{3}H]Pyruvate, [2-^{14}C]pyruvate, and carrier pyruvate were mixed such that the final specific radioactivities were 2.9 × 10^5 cpm/μmol in ^{3}H, and 2 × 10^5 cpm/μmol in ^{14}C. The solution was passed through a Dowex 1 (Cl^-) column (0.5 × 2 cm), which was extensively washed with water and then developed with 25 mM HCl. The most concentrated fraction was 8.2 mM in pyruvate by lactic dehydrogenase assay. In order to accurately determine the $^3H/^{14}C$ ratio of the sample, some [2-^{14}C, 3-^{3}H]pyruvate was first converted to [2-^{14}C, 3-^{3}H]lactate by the action of lactic dehydrogenase before chromatographic purification. The incubation contained (in μmol) Tris buffer (90, pH 7.8), NADH (0.35), and [2-^{14}C, 3-^{3}H]pyruvate (0.41). Reaction was started by the addition of trace amount of lactic dehydrogenase. Seven 100-μl aliquots were withdrawn along the time course, and each pipetted into 0.5 ml of boiling water to stop the reaction. The seven sample solutions, each with various amounts of [2-^{14}C, 3-^{3}H]lactate, were loaded separately on Dowex 1 (Cl^-) columns (3 × 0.5 cm). After extensive washing with water, the [2-^{14}C, 3-^{3}H]lactate samples were eluted with 5 mM HCl. Unreacted pyruvate remained on the columns. The amount of ^{3}H and ^{14}C in each sample was determined by scintillation counting. A graph was plotted with ^{3}H against ^{14}C radioactivity (similar to Figure 2), yielding a straight line, the slope of which is the $^3H/^{14}C$ ratio of the sample.

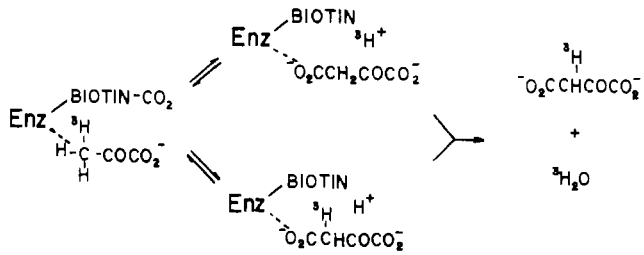
Scintillation counting was performed in 10 ml of Beckman Cocktail D, consisting of 100 g of naphthalene and 5 g of diphenyloxazole/l. of dioxane solution. Each sample was adjusted to contain 1.0 ml of 5 mM HCl, in order to correct for quenching effect. Net ^{3}H radioactivities were obtained by correcting for the ^{14}C crossover, which was separately standardized.

Results

In contrast to the related biotin-dependent enzyme-catalyzed carboxylations of propionyl-CoA by both propionyl-CoA carboxylase and transcarboxylase (Prescott and Rabinowitz, 1968; Cheung et al., 1975) which show no kinetic isotope effects, the carboxylations of pyruvate catalyzed by transcarboxylase (Cheung et al., 1975) and pyruvate carboxylase (see Figure 1) do exhibit detectable kinetic isotope effects in the overall reaction. However, in the case of transcarboxylase, the deuterium isotope effect of 2.1 is on both V_{max} and V_{max}/K_m, since the K_m for [$^1H_3$]- and [$^2H_3$]pyruvate remain the same (Cheung et al., 1975). On the other hand, the deuterium isotope effect in pyruvate carboxylase action, present at subsaturating substrate concentrations, disappears at infinite substrate level, indicating that the effect is now solely on V_{max}/K_m instead (Cleland, 1975). As shown in Figure 1, the Lineweaver–Burke lines for [$^1H_3$]- and [$^2H_3$]pyruvate intersect at the y axis (common V_{max}), but cross the x axis at different points (different K_m's). The ratio of the two slopes yields the deuterium V_{max}/K_m effect of 2.1.

The determination of the two tritium isotope effects in pyruvate carboxylase catalysis (i.e., on the catalytic step, and on the overall reaction) is more complicated. We will first look at the effect on the catalytic step.

Scheme I



Scheme I shows the stoichiometry of the carboxylation reaction. In the process, one hydrogen species from the tritiated pyruvate is released into the medium, and two retained in the product oxalacetate. Since all three hydrogens are equivalent in a freely rotating methyl group (in contrast to a prochiral methylene group where enzymes are stereospecific for one of the hydrogen pair), exactly one third of the tritium level would be released as ^3H_2O if there were *no* kinetic isotope effect in this catalytic step. However, Figure 1 shows there is an isotope effect; consequently, one expects: (i) tritium will be preferentially retained in the product oxalacetate, and (ii) the specific radioactivity of the total products, namely ^3H_2O and [3-^{3}H]oxalacetate will be *lower* than the substrate [^{3}H]pyruvate. Suppose the carboxy-biotin-enzyme binds a pyruvate molecule with one tritium atom in the methyl group (the probability of

having two or more tritiums on the same pyruvate molecule is effectively zero). The enzyme can then replace any of the three hydrogen species (two protons and one triton) with a carboxyl group. Yet, given a tritium isotope effect of x on the catalytic step, reaction will occur x times faster in the removal of a proton than a triton. And, since there are two protons and only one triton, the end result is there will be $2x$ times as much tritium in oxalacetate as in ^3H_2O. Thus, half of the ratio of the specific radioactivity in oxalacetate to that of the proton released into water gives the value for the tritium isotope effect. As shown in Figure 2, the ratio of the two slopes is 9.7 (up to 13% substrate conversion), indicating the tritium effect in the catalytic step to be 4.8. In another experiment (data not shown) where 30% of the [^{3}H]pyruvate was carboxylated, the same number of 4.8 was again obtained. In general, as will be discussed later, one cannot calculate from the tritium effect the deuterium isotope effect on this catalytic step by the relationship of Swain et al. (1958) (eq 9), since the effective rate constant may be a complex one rather than a simple rate step.

On the other hand, the tritium effect on the overall reaction, essentially on V_{max}/K_m (Simon and Palm, 1966), can be calculated by taking the ratio of the specific radioactivity of the starting material (i.e., [^{3}H]pyruvate) to that of the product, in this case, the sum of the tritium released into water and tritium retained in oxalacetate. In all these experiments, we have not used specific radioactivities directly, but have utilized double labels of ^{14}C and ^3H, and compared only $^3H/^{14}C$ ratios. Furthermore, the ratios were obtained by plotting graphs comprised of multiple individual time points, thus eliminating errors caused by any possible radioactive impurities in the [^{14}C, ^{3}H]pyruvate (which should remain constant in equal aliquots withdrawn). The $^3H/^{14}C$ ratio of the starting material [^{14}C, ^{3}H]pyruvate was determined by enzymatic reduction to lactate by lactic dehydrogenase. The [^{14}C, ^{3}H]lactate samples were isolated and the $^3H/^{14}C$ ratio determined as described under the Experimental section. A graph (not shown) similar to Figure 2 was plotted, yielding a $^3H/^{14}C$ ratio of 1.38. As given by the slopes of the respective graphs in Figure 2, the $^3H/^{14}C$ ratio of oxalacetate is 1.05, and the ratio of ^3H_2O to [^{14}C]-oxalacetate is 0.11. Thus, the tritium kinetic isotope effect on the overall reaction is given by $1.38/(1.05 + 0.11) = 1.19 \simeq 1.2$. The meaning of this number will be analyzed under the Discussion. It is not necessary to correct for the possible increase in specific radioactivity in the substrate, since the reaction shows only such a small tritium isotope effect and completion only proceeded to about 13%.

We also tested fluoropyruvate as a substrate for pyruvate carboxylase. It reacts about six times slower than pyruvate, with V_{max} of 2 μmol min^{-1} mg^{-1}. We next proceeded to determine the deuterium kinetic isotope effect for this substrate. A Lineweaver-Burke plot (not shown) was made for both [$^1H_2$]- and [$^2H_2$]fluoropyruvate under identical experimental conditions (as described in the legend of Figure 1). In contrast to the pyruvate case, the deuterium kinetic isotope effect of 1.5 remains at V_{max}, while the K_m's for both protio and deutero-species are virtually the same, at 1.5×10^{-4} M.

Discussion

In analogy to other biotin-dependent carboxylases (Alberts and Vagelos, 1972; Wood, 1972) the reaction catalyzed by pyruvate carboxylase can be broken down into two discrete half-reactions (Scrutton and Young, 1972; Utter et al., 1975), namely the formation of the carboxy-biotin enzyme intermediate utilizing ATP as the energy donor, and the subsequent

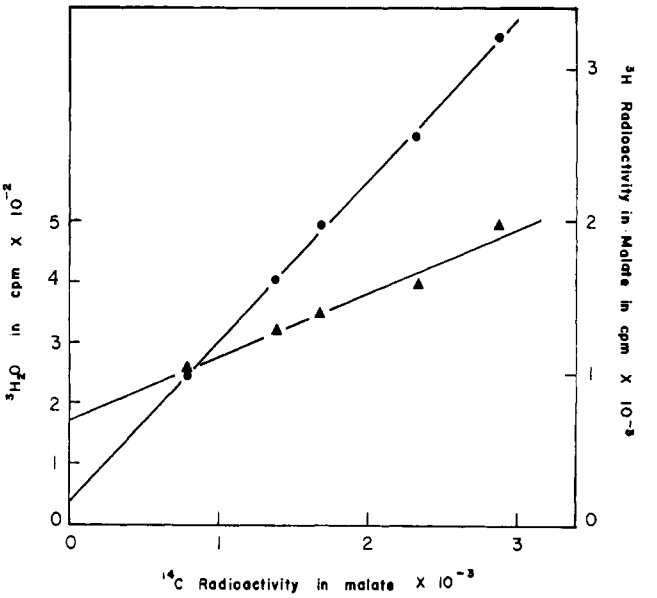
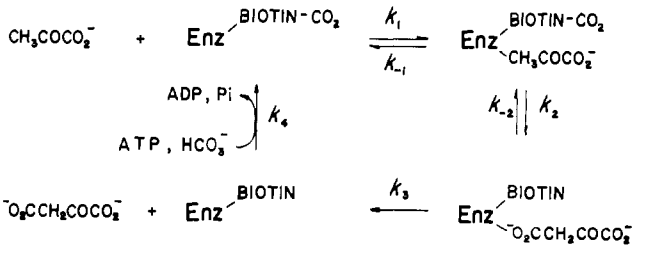


FIGURE 2: Intra- and intermolecular tritium kinetic isotope effects in pyruvate carboxylase reaction, represented by ^3H_2O released vs. [^{14}C]-malate formed (▲), and tritium retained in malate vs. [^{14}C]malate formed (●). The incubation contained (in μmol) Tris buffer (45, pH 7.8), $KHCO_3$ (7.5), $MgCl_2$ (2.5), ATP (2), NADH (3), acetyl-CoA (0.1), 30 units of malate dehydrogenase, [2-^{14}C, 3-^{3}H]pyruvate (1.8, containing 2.1×10^5 cpm of ^{3}H, and 1.46×10^5 cpm of ^{14}C) in a total volume of 0.67 ml. The reaction was started by the addition of 15 μg of pyruvate carboxylase at 30 °C. Five 100-μl aliquots were withdrawn in the time course. The reaction was stopped by pipetting the aliquot sample into 75 μl of a solution containing 50 μmol of Tris buffer (pH 7.8), 50 units of lactic dehydrogenase, and excess NADH. ^3H_2O released during the pyruvate carboxylase reaction was collected by lyophilization of each sample. The residue was dissolved in 1 ml of water. The container was rinsed with 2 × 1 ml of water. All three aqueous aliquots were loaded onto a Dowex 1 (Cl^-) column (0.5 × 6 cm). The unreacted material, now as [2-^{14}C, 3-^{3}H]lactate, was eluted completely in 8 ml of 5 mM HCl. The [2-^{14}C, 3-^{3}H]malate was eluted by 3 ml of 40 mM HCl (we could not detect any radioactivity present as pyruvate, which nevertheless would elute only later). The solution was collected directly into scintillation vials, and lyophilized to remove excess HCl. The residue was taken up in 1.0 ml of 5 mM HCl. The ^{3}H and ^{14}C radioactivities in each sample were determined by scintillation counting.

transfer of that carboxyl group to pyruvate. Under initial velocity conditions and saturating levels of ATP and HCO_3^-, essentially all enzyme molecules are converted to the carboxy-biotin intermediate (driven by release of ADP and inorganic phosphate). Thus, our studies on kinetic isotope effects with isotopically labeled pyruvate deal essentially only with the second half-reaction, as represented in Scheme II.

Scheme II



Under initial conditions, the steps represented by k_3 and k_4 are essentially irreversible and k_{-3} and k_{-4}, the respective rate constants for back reaction, are omitted.

As shown under the Results, this enzymatic reaction exhibits a deuterium kinetic isotope effect on V_{max}/K_m, the bimolecular rate constant at low substrate concentrations, but none on V_{max}

itself (Cleland, 1975). This is the second example of this phenomenon, the first being the reduction of acetaldehyde by (4*R*)-[^{2}H]NADH catalyzed by horse liver alcohol dehydrogenase (Bush et al., 1973). As pointed out by Cleland (1975), this situation is possible only if some step after the first irreversible step is rate limiting. In Scheme II, under initial velocity conditions, the step of k_3, involving release of a product, is irreversible, and the subsequent steps described by k_4 are then held to be rate determining. We have designated k_4 to include several processes: the possible isomerization of enzyme molecules (e.g., movement of the biotinyl "swinging arm" between subunits), the binding of ATP and HCO_3^-, the carboxylation of the biotin, and the release of ADP and phosphate (Scrutton and Young, 1972). It is unclear which of these step(s) is(are) the slow one(s) and our studies reported here provide no information on these points.

The linearity in the double reciprocal plots in Figure 1 warrants comments at this point. Whereas a biphasic double reciprocal plot was observed by Scrutton et al. (1965) in studying the exchange of [2-^{14}C]pyruvate into oxalacetate (the break occurred at ca. 0.2 mM pyruvate), an essentially linear one was reported by the same authors (Barden et al., 1972) in studying the product inhibition pattern by oxalacetate. In the absence of oxalacetate, the double reciprocal plot was linear up to ca. 2 mM pyruvate (Barden et al., 1972). This fact is completely consistent with our data presented in Figure 1.

The analysis of the isotope effects on V_{max}/K_m is more complicated. We have adapted the approach of Cleland (1975), and thus V_{max}/K_m is given by the following expression (relating to Scheme II):

$$V_{max}/K_m = k_1 \frac{k_2'}{k_{-1} + k_2'} \quad (1)$$

where k_2' is the effective or net rate constant for the actual catalytic step:

$$k_2' = k_2 \frac{k_3}{k_{-2} + k_3} \quad (2)$$

The catalytic step represented by k_2 involves the removal of one proton from carbon 3 of pyruvate and transfer of the carboxy group from the biotin coenzyme to that carbon of pyruvate. In view of recent evidence (Rose et al., 1976) indicating the likelihood that this process may be a concerted one, k_2 can be assumed to involve only one elementary step. When pyruvate is labeled with hydrogen isotopes (i.e., ^{2}H or ^{3}H), one would expect it to react with a kinetic isotope effect on k_2. However, what one can actually measure is the isotope effect on k_2' instead, unless $k_3 \gg k_{-2}$. In the other extreme, if $k_{-2} \gg k_3$, one would be dealing with a preequilibrium case, and the most one would see would be an equilibrium isotope effect of value very close to 1.0. When [^{3}H]pyruvate is the substrate, if there is a kinetic isotope effect on k_2', one would expect to find more than two-thirds (i.e., the statistical factor) of tritium label retained in the product oxalacetate, and less than one third released as ^3H_2O. Obviously, the validity of our experimental results here depends on the quantitative recovery of ^3H_2O during lyophilization. We have checked our methodology by lyophilizing known amounts of ^3H_2O under identical conditions. Recoveries have consistently been greater than 99%. We have also successfully used the same lyophilization technique in a few stereochemical studies (Cheung and Walsh, 1976a,b; Cheung et al., 1975).

Since tritium is present only as a tracer, i.e., only an extremely small number of pyruvate molecules actually contain a tritium label (and none of those contain more than one ^{3}H per methyl group), we have to make a distinction here between two kinds of kinetic isotope effects that will be experienced on k_2', namely the *intra-* and *inter*molecular ones. The *intra*molecular isotope effect is the discrimination between removal of a triton or one of two protons from the same methyl group, while the *inter*molecular effect is the rate difference between molecules containing one triton, two protons and molecules containing three protons in the methyl group. The *intra*molecular effect is directly observed in the distribution of tritium in the products, i.e., the ratio of amount of tritium label retained in oxalacetate to that released as ^3H_2O. After correcting for the statistical factor (one ^{3}H, two ^{1}H per methyl group) of 2, we calculated this *intra*molecular effect on k_2' to be 4.8. On the other hand, the *inter*molecular kinetic isotope effect will be much smaller, because a pyruvate molecule containing three protons will react only $(3 \times 4.8)/(2 \times 4.8 + 1) = 1.36$ times faster than a molecule containing one tritium. Or in another word, $k_2'(^1H)/k_2'(^3H) = 1.36$. This *inter*molecular effect (on k_2') is *indirectly* reflected in the lowering of the specific radioactivity of the products with respect to the starting material. In turn, *that* ratio of specific activities is of course the tritium kinetic isotope effect on V_{max}/K_m (Cleland, 1975; Simon and Palm, 1966). We have experimentally determined this V_{max}/K_m effect to be 1.2.

In the case of [$^2H_3$]pyruvate, where essentially all substrate molecules contain three deuterons, one should see only an *inter*molecular kinetic isotope effect on k_2', indirectly expressed in the effect on V_{max}/K_m which we have found experimentally to be 2.1.

Having determined all these values, one can proceed to estimate the relative magnitudes of k_{-1} and k_2' using eq 1. This knowledge in turn allows the calculation of k_1, the association constant for pyruvate and carboxybiotinyl enzyme. From eq 1, the expression for V_{max}/K_m for [$^1H_3$]pyruvate is given by:

$$V_{max}/K_m(^1H) = k_1 \frac{k_2'(^1H)}{k_{-1} + k_2'(^1H)} \quad (3)$$

and that for [^{3}H]pyruvate is:

$$V_{max}/K_m(^3H) = k_1 \frac{k_2'(^3H)}{k_{-1} + k_2'(^3H)} \quad (4)$$

Dividing eq 3 into 4, one gets:

$$\frac{V_{max}/K_m(^1H)}{V_{max}/K_m(^3H)} = \frac{k_2'(^1H)[k_{-1} + k_2'(^3H)]}{k_2'(^3H)[k_{-1} + k_2'(^1H)]} = \frac{\frac{k_2'(^1H)}{k_2'(^3H)} k_{-1} + k_2'(^1H)}{k_{-1} + k_2'(^1H)} \quad (5)$$

The left hand side of eq 5 is nothing but the ratio of the specific radioactivity of the substrate to that of the product (Simon and Palm, 1966), which we determined to be 1.19. And as described above, $k_2'(^1H)/k_2'(^3H)$ is estimated to be 1.36. Thus, eq 5 becomes:

$$1.19 = \frac{1.36k_{-1} + k_2'(^1H)}{k_{-1} + k_2'(^1H)}$$

$$\text{i.e., } k_{-1} = 1.12k_2' \simeq 1.1k_2'(^1H) \quad (6)$$

This value means that after the pyruvate molecule binds, it is only about 50% committed to catalysis (i.e., it will come back off the enzyme without reacting one out of two times it binds).

Substituting eq 6 into eq 3 yields the following expression:

$$k_1(^1H) = 2.1 V_{max}/K_m(^1H) \quad (7)$$

Under our experimental conditions at 30 °C, V_{max} was 12 μmol min^{-1} mg^{-1} [2] and K_m 7.1 × 10^{-1} M. Assuming the molecular weight of 125 000 (Barden et al., 1975) per catalytic subunit, k_1 can be estimated to be 4.5 × 10^6 M^{-1} min^{-1} for pyruvate binding. (We do not yet know the K_D for pyruvate. This knowledge would allow evaluation of both k_{-1}, the off rate, and also k_2', the net forward rate constant.)

The deuterium kinetic isotope effect on k_2' was not determined experimentally. However, one can estimate its value by knowing the deuterium V_{max}/K_m effect. Similar to the above derivation, one obtains the following expression for [$^2H_3$]-pyruvate:

$$\frac{V_{max}/K_m(^1H)}{V_{max}/K_m(^2H)} = \frac{\frac{k_2'(^1H)}{k_2'(^2H)} k_{-1} + k_2'(^1H)}{k_{-1} + k_2'(^1H)} \quad (8)$$

We have experimentally determined the deuterium V_{max}/K_m effect to be 2.1, i.e., the left hand side of eq 8 = 2.1. Substituting this value and eq 6 into 8, the value of $k_2'(^1H)/k_2'(^2H)$ can be calculated to be 3.1.

This value of 3.1 for the deuterium kinetic isotope effect on the net forward catalytic rate constant is a useful number to have. In particular, this should be the number to use if one wants to estimate the partition of tritium label into the two prochiral positions at C3 of malate when using pyruvate carboxylase to assay samples of chiral [^{1}H, ^{2}H, ^{3}H]pyruvate in stereochemical determinations (Cheung, and Walsh, 1976a; Creighton, and Rose, 1976). It would predict a split of 76% of the tritium in one prochiral C3 position and 24% in the other from pyruvate samples of complete chiral purity.

At this point, one must be cautioned that the above estimations can be quite approximate. Even though the values for deuterium V_{max}/K_m effect and tritium k_2' effect can be determined to satisfactory accuracy (as evidenced by the data presented), the tritium V_{max}/K_m effect involved a considerable degree of uncertainty in that it is obtained by taking the ratio of two numbers of approximate magnitudes (see Results). We have also ignored any possible secondary kinetic isotope effects in our calculations.

If one compares the *intra*molecular tritium effect of 4.8 and the *estimated* deuterium effect of 3.1 on k_2' one sees that they indeed fit the Swain relationship (1957) (eq 9) to within experimental uncertainty. Predicted from eq 9 and the tritium effect of 4.8, the deuterium effect would be 3.0.

$$\ln\left(\frac{k_{1H}}{k_{3H}}\right) = 1.44 \ln\left(\frac{k_{1H}}{k_{2H}}\right) \quad (9)$$

This equivalence suggests that the rate constant k_2' is very close to the elementary step's rate constant k_2, i.e., $k_3 \gg k_{-2}$. In other words, the release of oxalacetate is much faster than the reverse catalytic step. On the other hand, deviation from the Swain relationship (eq 7) would have suggested k_{-2} and k_3 are of approximately equal magnitude.[3]

Recently, Northrop (1975) discovered the very interesting relationship between the true isotope effects on the catalytic step (with the assumption that it is irreversible, i.e., $k_2' \sim k_2$ in our case) and the *observed* ones on V_{max} and V_{max}/K_m. One of the expressions he has come up with is:

$$\frac{\frac{V_{max}/K_m(^1H)}{V_{max}/K_m(^2H)} - 1}{\frac{V_{max}/K_m(^1H)}{V_{max}/K_m(^3H)} - 1} = \frac{\frac{k_2(^1H)}{k_2(^2H)} - 1}{\frac{k_2(^1H)}{k_2(^3H)} - 1} \quad (10)$$

If our values for the deuterium and tritium isotope effects on the catalytic step (right hand side of eq 8) are correct, the equality should hold. Before we can compare to see if they conform to this relationship, we *have* to create a hypothetical situation where all [^{3}H]pyruvate molecules contained tritium quantitatively. If one could use such a tri-tritio substrate in subsaturating concentration, one would observe a V_{max}/K_m effect, which can be predicted by the k_2' effect of 4.8 and eq 1, to be 2.8. Now if one substitutes the numbers we have into eq 10

$$\text{lhs} = \frac{2.1 - 1}{2.8 - 1} = 0.61$$

$$\text{rhs} = \frac{3.1 - 1}{4.8 - 1} = 0.55$$

With all the experimental uncertainties, and various approximations made in all the calculations, the equality apparently holds here. This apparent agreement is consistent with our conclusion above that $k_2' \simeq k_2$, and any kinetic isotope effect on k_2 is fully expressed on k_2' (but not on the overall reaction rate).

Now let us turn to the results with fluoropyruvate as a substrate. Fluoropyruvate was attributed to be an efficient inhibitor by Mildvan et al. (1966). We have now shown that it is in fact a substrate. The fact that it reacts six times slower at V_{max} can only mean that now instead of k_4, k_2' is the rate-determining step, since k_4 has to remain constant regardless of whether pyruvate or fluoropyruvate is the substrate. In another word, with fluoropyruvate, the rate of the catalytic step (k_2') has been reduced so much that k_2' is now the slowest. There are at least two reasons why fluoropyruvate should react slowly: (i) it only has two hydrogens for reaction; (ii) fluorine, being much more electronegative than hydrogen, must have perturbed the electron distribution in the molecule such that carboxylation is now less favorable.

As one would have predicted, the deuterium kinetic isotope effect is now on V_{max} (and of course also on V_{max}/K_m), since V_{max} is determined largely by k_2', and the K_m's remain essentially the same for both [$^1H_2$]- and [$^2H_2$]fluoropyruvate.

Finally, we would contrast the mechanisms of pyruvate carboxylase and transcarboxylase. Whereas the carboxylation of pyruvate by pyruvate carboxylase is not rate limiting in the overall reaction, that by transcarboxylase has to be at least partially rate determining, since a deuterium kinetic isotope effect of 2.1 was observed at V_{max} (Cheung et al., 1975). The rate of the half reaction involving methylmalonyl-CoA (Northrop, 1969) must be fast relatively. In other words, in the transcarboxylase reaction, kinetic isotope effect was observed *only* in the carboxylation of pyruvate, but not in that of propionyl-CoA (Cheung, et al., 1975). A full investigation on transcarboxylase similar to the one reported here is being conducted.

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[2] This value is the highest specific activity we have obtained. Upon storage at 0 °C for a month, the activity dropped to 7 μmol min^{-1} mg^{-1}.

[3] An alternate possibility is that k_2 is actually comprised of two or more elementary steps, with the actual proton removal only partially rate limiting (with respect to k_2).

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Reinvestigation of the Phenacyl Bromide Modification of α-Chymotrypsin†

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ABSTRACT: The modification of α-chymotrypsin with phenacyl bromide has been reinvestigated over a wide pH range. Evidence is presented that indicates that the nature of the phenacyl-modified enzymes prepared by this reaction is dependent upon the pH of the reaction medium. The phenacyl α-chymotrypsin produced at low pH is most probably the Met-192 phenacylsulfonium salt, as proposed earlier, since it readily undergoes dealkylation using 2-mercaptoethanol. However, the phenacyl-enzyme prepared at neutral pH possesses a much reduced enzymatic activity and does not react with 2-mercaptoethanol to regenerate native α-chymotrypsin.

In addition, incubation of the Met-192 phenacyl sulfonium enzyme at neutral pH causes a smooth irreversible change to the new phenacyl-enzyme as monitored by changes in enzymatic activity, susceptibility to dealkylation using 2-mercaptoethanol, and ultraviolet difference absorption spectral properties. The stoichiometries of both the low and neutral pH modification reactions have been determined, using [*carbonyl*-^{14}C]phenacyl bromide, to be 1 phenacyl group/enzyme molecule. In efforts to obtain information about the nature and mechanism of formation of the phenacyl α-chymotrypsin produced at neutral pH, alkylation reactions of modified α-chymotrypsins produced by His-57 functionalization with tosylphenylalanine chloromethyl ketone and by Met-192 oxidation to the sulfoxide have been investigated. The combined results of these studies have been initially interpreted in terms of a neutral pH, phenacyl bromide modification resulting in formation of a new modified enzyme via the Met-192 sulfonium salt.

Specific chemical modification of the methionine-192 amino acid residue of α-chymotrypsin using a variety of alkylating agents has been the subject of numerous investigations (Schramm and Lawson, 1963; Lawson and Schramm, 1965; Kezdy et al., 1967; Sigman et al., 1967, 1969; Gibian et al., 1969; Stevenson and Smillie, 1970; Jones and Hysert, 1972; Naider and Bohak, 1972; Alazard et al., 1973; Hille and Koshland, 1967). Schramm and Lawson (1963) were the first to provide evidence for the nature of the modification of α-chymotrypsin using hydrophobic alkyl halides at pH 5.1. The site of alkylation was deduced from amino acid analysis data that showed the absence of one of the two methionine residues of the native enzyme. Further evidence to support the postulate that modification of α-chymotrypsin with phenacyl bromide

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