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Equilibrium Perturbation by Isotope Substitution†

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ABSTRACT: When malic enzyme is added to a mixture of malate-*2-d,* TPN, CO_2, pyruvate, and TPNH at concentrations calculated to be at equilibrium, the TPNH level first drops and then increases slowly to its original level. This equilibrium perturbation is caused by slower cleavage of C–D than C–H bonds during hydride transfer as malate-*2-d* and TPNH are partly converted into TPND and malate-*2-h* in the process of establishing isotopic equilibrium. With malate-*2-d,* isotope effects for malic enzyme at pH 7.1 and malate dehydrogenase at pH 9.3 of 1.45 and 1.70–2.16 (depending on oxaloacetate level) were determined with this method, while the corresponding isotope effects on V/K_{malate} and V for the chemical reactions were 1.5–1.8 and 1.0, and 1.9 and 1.5 for the two enzymes. The advantage of this method is its extreme sensitivity, and the lack of interference from various artifacts. The sensitivity is sufficient to permit determination of ^{13}C and ^{15}N isotope effects in favorable cases, and values of 1.031 for malic enzyme with $^{13}CO_2$, and 1.047 for glutamate dehydrogenase with $^{15}NH_4^+$ have been determined. In the course of this work it was discovered that the equilibrium constants for oxidation by DPN, and oxidative decarboxylation by TPN are lower for malate-*2-d* than for malate-*2-h* by a factor of 0.76–0.82. Changes in K_{eq} upon deuterium substitution, which are predicted by the calculations of Hartshorn and Shiner (1972), should be observed for many other reactions as well.

It is well established that cleavage of C–D bonds is slower than cleavage of C–H bonds, and thus that dehydrogenases and other enzymes where C–H bond cleavage is involved show deuterium isotope effects (v_H/v_D) varying from 1 to as high as 15 (Bright and Gibson, 1967). The study of isotope effects has been given considerable impetus by the recent discovery of Northrop (1975) that comparison of the apparent deuterium and tritium isotope effects on V/K will permit calculation of the true isotope effects on the bond-breaking step, regardless of how many other steps precede or follow the bond breaking one, and thus a determination of how rate limiting the bond-breaking step really is. The usual method of determining deuterium isotope effects is to vary both deuterated and nondeuterated substrates, and compare the slopes (V/K effect) and intercepts (V effect) of the resulting reciprocal plots. This method requires concentrations to be accurately known (for comparison of V/K values), and that neither substrate contains any inhibitors (for comparison of V values), and is only capable of determining effects greater than about 1.1. Tritium isotope effects are determined by comparing the specific activity of the substrate with that of the first portion of tritiated product formed (which measures the V/K effect only), and realistically only values above 1.05 are meaningful.

While some enzymes show very nice isotope effects when the above methods are applied, others do not. Thus at pH 7 with malic enzyme the deuterium effects on V/K and V are 1.5–1.8 and 1.0, although the V effect does approach 3 at high and low pH; the V/K effect is nearly pH independent (Schimerlik and Cleland, 1975). With glutamic dehydrogenase we have seen in unpublished experiments no effect on either V or V/K with glutamate-*2-d* as substrate, although Fisher et al. (1970) have seen an effect of 1.5–1.8 on the pre-steady-state burst of TPNH production on the enzyme (norvaline-*2-d,* on the other hand, gives effects of 6 and 3.2 on V and V/K). These low values for enzymes where there certainly should be effects on the bond-breaking steps are caused by the rate-limiting step at high (V) or low (V/K) substrate concentrations being some other step than the bond-breaking one. It would thus be of considerable value to have a method for determining isotope effects lower than 1.1, or for measuring them under conditions where they were more fully expressed than in the usual experiments where initial velocities of the chemical reaction are determined.

When reaction mixtures are made up at concentrations calculated to be near equilibrium, and enzyme is then

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added, the reaction proceeds to equilibrium by a first-order process, and there is no net change if exact equilibrium concentrations are used. When one substrate contains deuterium in a position that undergoes cleavage during reaction, but the corresponding product contains hydrogen, the reaction will not stay at equilibrium when enzyme is added, but will be perturbed in the direction of the deuterated substrate, and then return to equilibrium. This perturbation is caused by the more rapid reaction of hydrogen than deuterium molecules as isotopic equilibrium is attained, and the driving force is the decreased entropy of the initial localization of deuterium in one reactant and hydrogen in the other relative to the final state of isotopic mixing. Similar perturbations are also seen in favorable cases with ^{13}C and ^{15}N as the heavy isotope. In this paper we will derive the equations which describe these perturbations, and present experimental data for several enzymes. We will also present evidence that deuterium substitution alters the equilibrium constant for reactions with pyridine nucleotides, an effect predicted by the calculations of Hartshorn and Shiner (1972), and one which must be taken into account when determining deuterium isotope effects.

Theory

Consider the following general scheme where k_7 and k_8 are for the bond-breaking steps affected by isotopic substitution, and B and R are the reactants that contain the isotopic atoms involved (B contains the heavier isotope):

$$E_1 \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_5 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_6 \underset{k_{12}P}{\overset{k_{11}}{\rightleftharpoons}} E_7 \underset{k_{14}Q}{\overset{k_{13}}{\rightleftharpoons}} E_8 \underset{k_{16}R}{\overset{k_{15}}{\rightleftharpoons}} E_1 \quad (1)$$

For the moment we will assume that k_7 and k_8 are equally affected by the change in isotope (that is, there is no change in K_{eq} for the reaction. This assumption is actually not true, and we will discuss below the resulting effects). All other rate constants are unaffected by isotopic substitution. For malic enzyme, for example, A, B, P, Q, and R would be TPN, malate-*2-d*, CO_2, pyruvate, and TPNH. When enzyme is added to such a system at a point near equilibrium, the rate of movement of deuterium from B to R is given by eq 2, where B_2 and R_2 represent concentrations of the deuterated molecules:

$$\frac{dR_2}{dt} = \left(\frac{k_3k_5k_{7H}[B_2][E_2]}{k_4k_6} - \frac{k_2k_{8H}k_{10}k_{12}k_{14}k_{16}[P][Q][R_2][E_2]}{k_1k_9k_{11}k_{13}k_{15}[A]}\right) \Big/ \alpha\Delta \quad (2)$$

where $\Delta = 1 + b$, and

$$\alpha = \frac{k_{7H}/k_{7D} + b}{1 + b}$$

$$b = \frac{k_{7H}}{k_6}\left(1 + \frac{k_5}{k_4}\right) + \frac{k_{8H}}{k_9}\left(1 + \frac{k_{10}}{k_{11}}\left[1 + \frac{k_{12}[P]}{k_{13}}\left(1 + \frac{k_{14}[Q]}{k_{15}}\right)\right]\right) \quad (3)$$

Note that α, the apparent isotope effect, will be less than k_{7H}/k_{7D}, the actual isotope effect on the bond breaking step, whenever b is finite, as it usually is. The constant b is made up of ratios of rate constants that control partitioning of the enzyme forms during reaction.

Since $[B_2] = [B_0] - [R_2]$, where $[B_0]$ is the initial concentration of deuterated B:

$$\frac{d[R_2]}{dt} = \frac{k_3k_5k_{7H}[E_2]([B_0] - [R_2] - [R_2]/K)}{k_4k_6\alpha\Delta} \quad (4)$$

where

$$K = \frac{k_1k_3k_5k_{7H}k_9k_{11}k_{13}k_{15}[A]}{k_2k_4k_6k_{8H}k_{10}k_{12}k_{14}k_{16}[P][Q]} = \frac{[R_{eq}]}{[B_{eq}]} \quad (5)$$

and $[R_{eq}]$ and $[B_{eq}]$ are concentrations of total R and B at equilibrium. If the concentrations of A, P, and Q are all much more than the smaller of $[B_0]$ or $[R_0]$, then K can be considered constant during the perturbation (we will consider later the case where this is not true). Then by defining:

$$k = \frac{k_3k_5k_{7H}[E_2](1 + K)}{k_4k_6\Delta K} \quad (6)$$

and integrating, we obtain:

$$[R_2] = \frac{[B_0]K(1 - e^{-kt/\alpha})}{(1 + K)} \quad (7)$$

Similarly the equation for movement of hydrogen from TPNH to malate (with $[R_1]$ and $[B_1]$ being the concentrations of hydrogen containing species) is:

$$\frac{-d[R_1]}{dt} = \left(\frac{k_2k_{8H}k_{10}k_{12}k_{14}k_{16}[P][Q][R_1][E_2]}{k_1k_9k_{11}k_{13}k_{15}[A]} - \frac{k_3k_5k_{7H}[B_1][E_2]}{k_4k_6}\right) \Big/ \Delta \quad (8)$$

or, since $[B_1] = [R_0] - [R_1]$, and K has the definition given above:

$$\frac{-d[R_1]}{dt} = \frac{k_3k_5k_{7H}[E_2]([R_1] + [R_1]/K - [R_0])}{k_4k_6\Delta} \quad (9)$$

which after substituting the value of k integrates to:

$$[R_1] = \frac{[R_0](K + e^{-kt})}{(1 + K)} \quad (10)$$

The concentration of total R is now given by the sum of eq 7 and 10:

$$[R] = \frac{K([B_0] + [R_0]) + [R_0]e^{-kt} - [B_0]Ke^{-kt/\alpha}}{1 + K} \quad (11)$$

If the reaction mixture is at the exact calculated equilibrium point when enzyme is added, $[R_0] = [B_0]K$, and eq 11 becomes:

$$[R] = [R_0] + [R'_0](e^{-kt} - e^{-kt/\alpha}) \quad (12)$$

where

$$[R'_0] = 1/(1/[B_0] + 1/[R_0]) \quad (13)$$

This equation predicts that [R] will first decrease, and then return to its original level.

It might appear that the simplest method of solving for α would be to fit the observed [R] values as a function of time to eq 11 or 12 by the least-squares method, since α is the ratio of the rate constants for the two exponential terms. Such fits are very ill conditioned, however, and the contours of equal residual least squares form a steep-walled, but flat-bottomed valley which permits a wide range of α values to give essentially the same residual least squares. What does vary with α is the size of the perturbation. At the maximum point of the curve described by eq 12:

$$kt_{max} = \frac{\alpha}{(\alpha - 1)} \ln \alpha \quad (14)$$

and thus the value of [R] at the maximum point is given by:

$$[R_{max}] = [R_0] + [R'_0](\alpha^{-\alpha/(\alpha-1)} - \alpha^{-1/(\alpha-1)}) \quad (15)$$

which, if ($[R_{max}] - [R_0]$) is the absolute value of the perturbation, gives:

$$\frac{[R_{max}] - [R_0]}{[R'_0]} = \alpha^{-1/(\alpha-1)} - \alpha^{-\alpha/(\alpha-1)} \quad (16)$$

A plot of eq 16 is given in Figure 1. At values of α below 1.1, this equation reduces to:

$$\frac{[R_{max}] - [R_0]}{[R'_0]} = \frac{\alpha - 1}{e}$$

or

$$\alpha = 1 + 2.72 \frac{[R_{max}] - [R_0]}{[R'_0]} \quad (17)$$

When the reaction mixture is not exactly at equilibrium when enzyme is added, these equations all become more complex, but the correct value of α is given very closely by calculating the perturbation from the average value of $[R_0]$ and $[R_\infty]$, rather than from $[R_0]$. Thus $[R_{max} - ([R_0] + [R_\infty])/2]$ replaces ($[R_{max}] - [R_0]$) in eq 16 and 17.

The above derivation assumes 100% heavy isotope in B at the start. If the actual percent label is less than this, the maximum perturbation is reduced accordingly, and the apparent value of $1 - \alpha$ must be divided by the fraction of heavy isotope originally in B to give the true value. Thus for 90% label, and an apparent α of 1.09, 0.09 is divided by 0.90 to give the true α of 1.10.

In setting up experiments to look for perturbations, it is useful to remember that the maximum point will occur at a kt value (as given by eq 14) only slightly higher than one, and at low α values, eq 14 reduces to:

$$kt_{max} = (\alpha + 1)/2 \quad (18)$$

If α is set equal to 1 in eq 11, it is clear that a kt value of 1 corresponds to 63% of the way to equilibrium for a mixture not quite at equilibrium to start with, and one can thus use reaction mixtures with hydrogen-containing substrates that are not quite at equilibrium to adjust the enzyme level to put kt at a convenient place on the chart. With this enzyme level, and reaction mixtures containing labeled reactant, one then changes the concentrations of one reactant so that the equilibrium position is approached as closely as possible (this is best done by making a premix containing all reactants at calculated equilibrium levels, and adding various amounts of a dilute solution of one reactant until exact equilibrium is obtained). The time course of the reaction must be followed until 10 kt_{max} or longer, in order to be sure of the final equilibrium value of [R].

The above derivation assumes no effect of isotopic substitution on K_{eq}. However, when equilibrium perturbation is carried out with a deuterated reactant, one must consider the change in equilibrium constant that accompanies the deuterium substitution, which can be as high as 20–30% (see below). By making the following definitions in terms of mechanism 1:

$$\beta = \frac{K_{eqD}}{K_{eqH}} = \frac{(k_{8H}/k_{8D})}{(k_{7H}/k_{7D})} \quad (19)$$

$$K = ([R_1]/[B_1])_{eq} \qquad K\beta = ([R_2]/[B_2])_{eq} \quad (20)$$

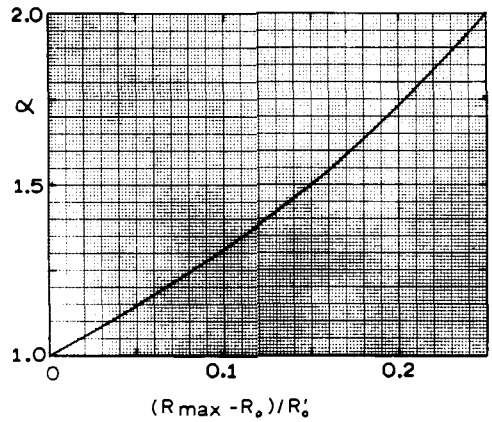

FIGURE 1: Calculation of isotope effect (α) from perturbation size according to eq 16.

$$\alpha = \frac{k_{7H}/k_{7D} + x + y/\beta}{1 + x + y} \quad (21)$$

$$\alpha\beta = \frac{k_{8H}/k_{8D} + \beta x + y}{1 + x + y} \quad (22)$$

where x and y are the portions of b (given by eq 3) that include k_{7H} and k_{8H}, respectively, we can calculate that for the system to come to the starting point after the perturbation:

$$[R_0]/[B_0] = \frac{K\beta(1 + K)}{(1 + K\beta)} \quad (23)$$

Thus one picks an initial ratio of $[R_0]/[B_0]$ which lies between K and $K\beta$, depending on the value of K. In eq 12, 16, and 17, $[R'_0]$ is then replaced by $[R_0]/(1 + K)$ and α by appα, where:

$$\text{app}\alpha = \frac{\alpha(\beta + K\beta)}{(1 + K\beta)} = \alpha\beta\frac{(1 + K)}{(1 + K\beta)} \quad (24)$$

Although β can be determined independently, K must normally be calculated with eq 23 from β and the experimental value of $[R_0]/[B_0]$ that produces a perturbation that returns to the starting point. In practice this means that for use in eq 12, 16, 17, and 34 (see below), $[R'_0]$ is calculated by eq 25, rather than 13, and α, the apparent isotope effect in the forward direction, is calculated by eq 26 from the appα value from eq 16 or 17:

$$[R'_0] = 2/\left(\frac{1}{[R_0]} + \frac{1}{[B_0]} + \left[\left(\frac{1}{[R_0]} + \frac{1}{[B_0]}\right)^2 + 4[(1/\beta) - 1](1/[B_0])(1/[R_0])\right]^{1/2}\right) \quad (25)$$

$\alpha = (\text{app}\alpha)z$, where

$$z = \frac{1 - [B_0]/[R_0] + [(1 - [B_0]/[R_0])^2 + 4[B_0]/(\beta[R_0])]^{1/2}}{2} = \frac{2/\beta}{1 - [R_0]/[B_0] + [(1 - [R_0]/[B_0])^2 + 4[R_0]/(\beta[B_0])]^{1/2}} \quad (26)$$

(Use the first expression for z when $[B_0]/[R_0] < 1$, and the second when $[B_0]/[R_0] > 1$; if $[B_0] = [R_0]$, $z = 1/\beta^{1/2}$.) The apparent isotope effect in the reverse direction is then $\alpha\beta$. When determining ^{13}C or other heavy isotope effects, β

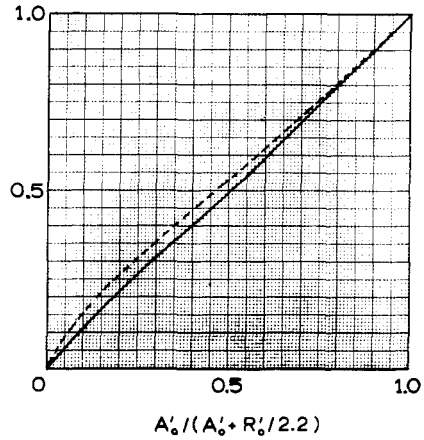

FIGURE 2: Variation of perturbation size and time of maximum perturbation when reactants other than those involved in the perturbation are not at high concentration. (—) Ratio of observed value of ($[R_{max}] - [R_0]$) to that calculated from eq 17; (- - -) ratio of observed value of t_{max} to that calculated from eq 18.

will be near unity (in the range of 0.99–1.01 for ^{13}C, based on the calculations of Hartshorn and Shiner (1972)), and eq 25 reduces to eq 13, and α = appα.

When the concentrations of A, P,[1] and Q are not much greater than $[R'_0]$, as has been assumed until now, their concentrations will change appreciably during the perturbation in such a way that the size of the perturbation is reduced. This situation will always occur when measuring heavy isotope effects, and will usually be a slight problem with deuterium effects. If x is the size of the perturbation at any time:

$$x = [R_1] + [R_2] - [R_0] \tag{27}$$

then:

$$\begin{aligned} [A] &= [A_0] + x = [A_0](1 + x/[A_0]) \\ [P] &= [P_0] - x = [P_0](1 - x/[P_0]) \\ [Q] &= [Q_0] - x = [Q_0](1 - x/[Q_0]) \end{aligned} \tag{28}$$

and by assuming K in eq 5 to include $[A_0]$, $[P_0]$, and $[Q_0]$, and thus to remain constant, we can write eq 4 as:

$$\frac{d[R_2]}{dt} = \frac{k_3k_5k_{7H}[E_2]}{k_4k_6\alpha\Delta} \times \left([B_0] - [R_2] - \frac{[R_2](1 - x/[P_0])(1 - x/[Q_0])}{K(1 + x/[A_0])}\right) \tag{29}$$

Since at low values of x where terms in x^2 or x^3 are negligible:

$$\frac{(1 - x/[P_0])(1 - x/[Q_0])}{(1 + x/[A_0])} \frac{(1 - x/[A_0])}{(1 - x/[A_0])} = 1 - x/[A'_0] \tag{30}$$

where:

$$[A'_0] = 1/(1/[A_0] + 1/[P_]] + 1/[Q_0]) \tag{31}$$

eq 29 can be written:

$$\frac{d[R_2]}{dt} = \frac{k_3k_5k_{7H}[E_2]}{k_4k_6\alpha\Delta} \times \left([B_0] - [R_2] - \frac{[R_2]}{K}\left(1 - \frac{[R_1] + [R_2] - [R_0]}{[A'_0]}\right)\right) \tag{32}$$

Application of similar logic permits eq 9 to be written as:

$$\frac{d[R_1]}{dt} = \frac{k_3k_5k_{7H}[E_2]}{k_4k_6\Delta} \times \left([R_0] - [R_1] - \frac{[R_1]}{K}\left(1 - \frac{[R_1] + [R_2] - [R_0]}{[A'_0]}\right)\right) \tag{33}$$

These two differential equations must now be solved simultaneously to give $[R_1]$ and $[R_2]$ as a function of time. We have been unable to do this analytically, but have obtained numerical solutions with the aid of a program available at our local computer center. The empirical results of these solutions are shown in Figure 2. Thus the observed value of $[R_{max}] - [R_0]$ should be divided by the value obtained by entering the graph with the calculated value of $[A'_0]/([A'_0] + [R'_0]/2.2)$ in order to obtain the correct value to use in eq 16 or 17. Alternatively, $[R'_0]$ in eq 16 and 17 can be replaced by $[R''_0]$, whose value is given by:

$$[R''_0] = 1/(1/[R'_0] + 1/y[A'_0]) \tag{34}$$

where the value of y is 2.2 when $[A'_0]/[R'_0]$ is over 0.3, but is changed to 2.3 below 0.3, to 2.4 below 0.16, and to 2.5 below 0.08 if the empirical data in Figure 2 are to be closely approximated.

It should also be noted from Figure 2 that as $[A'_0]/([A'_0] + [R'_0]/2.2)$ drops, the time of maximum perturbation also decreases, although the relationship is not linear.

One further problem can lower the size of the observed perturbation as the result of lowering the value of α. Equation 3 shows that b is a function of the concentrations of P and Q in mechanism 1, and this will be true for all reactants in an ordered mechanism that add between the points of combination of the perturbing molecules, and in random mechanisms for all reactants that slow down the release of the perturbing molecules from the enzyme. The equation for y (that portion of b that involves k_{8H}; see eq 21 and accompanying text) can be rewritten:

$$y = \frac{k_8}{k_9} + \frac{k_8k_{10}}{k_9k_{11}} + \frac{k_8k_{10}[P]}{k_9k_{13}K_{ip}} + \frac{k_8k_{10}[P][Q]}{k_9k_{15}K_{ip}K_{iq}} \tag{35}$$

where $K_{ip} = k_{11}/k_{12}$ and $K_{iq} = k_{13}/k_{14}$. At very low [P] and [Q] the value of α given by eq 21 is identical with $(V/K)_H/(V/K)_D$ when B is the variable substrate, but at high levels of [P] and [Q], α is lowered because of the increased value of y in eq 21, and the observable perturbation is correspondingly decreased. Thus the high levels of [P] and [Q] one might be tempted to use to keep $[A'_0]$ large would only lower α and decrease, rather than increase the size of the perturbation.

Note that the size of the last two terms in eq 35 depends not only on the ratio of [P] and [Q] to their dissociation constants, but also on the relative values of k_{13}, k_{15}, and k_{11}. These terms are likely to be important only when either k_{15} or k_{13} are considerably less than k_{11}, and when the ratios k_8/k_9 and k_{10}/k_{11} are not far below unity. Thus the levels of [P] and [Q] should generally be kept at or below their

[1] Since CO_2 and bicarbonate equilibrate more rapidly than the rate of the perturbation (apparent k about 2.4 min^{-1} at 25° (Gibbons and Edsall, 1963), which is at least ten times faster than any of the perturbations reported here), their combined concentrations must be taken as P for purposes of this calculation. Where the perturbation occurs in less time or at lower temperature, or one wishes the greatest possible accuracy, carbonic anhydrase could be added. The same situation applies to eq 13 when the perturbation involves $^{13}CO_2$ as a perturbing molecule and CO_2 and bicarbonate must be considered as a common pool of ^{13}C and ^{12}C. Fortunately the fractionation factor for ^{13}C between CO_2 and bicarbonate (1.007 in favor of bicarbonate (Thode et al., 1965)) is small enough not to affect the results appreciably.

dissociation constants to minimize these effects, but the exact levels that give the optimal size of perturbation must be determined empirically.

If the value of α is measured at several appropriate levels of [P] and [Q], α will be a hyperbolic function of [P] or [Q], and a plot of $1/[(V/K)_H/(V/K)_D - \alpha]$ vs. $1/[P]$ or $1/[Q]$ should give a linear plot with a vertical intercept of $1/[(V/K)_H/(V/K)_D - 1/\beta]$. Such a plot provides a means of checking the internal consistency of the experimentally determined values of β, α, and $(V/K)_H/(V/K)_D$.

In summary, the analysis of perturbations is carried out as follows. *A. Heavy Isotopes* (^{13}C, ^{15}N, *etc.*). (1) $[A'_0]$ calculated from eq 31 and $[R'_0]$ from eq 13 are combined to give $[R''_0]$ according to eq 34, with the correct value of y for use in eq 34 determined from the value of $[A'_0]/[R'_0]$. (y is 2.2 for values of $[A'_0]/[R'_0]$ over 0.3, 2.3 between 0.16 and 0.3, 2.4 between 0.08 and 0.16, and 2.5 below 0.08.) (2) The observed perturbation and $[R''_0]$ are used to determine α from eq 17.[2] (3) If the label used was less than 100%, $\alpha - 1$ is divided by the fraction of heavy isotope used to get the true value of α.

B. Deuterium. (1) The effect of deuterium substitution on $K_{eq}(\beta)$ must be determined first. (2) $[A'_0]$ calculated from eq 31 and $[R'_0]$ from eq 25 are combined to give $[R''_0]$ according to eq 34, with y determined as usual. (3) appα is determined from the observed perturbation and $[R''_0]$ by eq 17 when appα is less than 1.1, or from Figure 1 if it is greater than 1.1.[2] (4) The isotope effect in the forward direction (α) is then determined from eq 26, and that in the reverse direction is $\alpha\beta$.

Materials and Methods

Pigeon liver malic enzyme was isolated by the method of Hsu and Lardy (1967). D_2O (99.8%), pig heart malate dehydrogenase, glutamate-oxaloacetate transaminase, glutamic dehydrogenase, and other biologicals were from Sigma. $^{13}CO_2$ (90% ^{13}C) was a gift from Dr. M. H. O'Leary, and $(^{15}NH_4)_2SO_4$ (98.5% ^{15}N) was a gift from Dr. R H. Burris.

Aspartate was deuterated at C-2 by allowing up to 5 g of potassium L-aspartate, 2–5 mg of glutamate-oxaloacetate transaminase, and a trace amount of α-ketoglutarate to equilibrate in 10 ml of D_2O at room temperature for 48–72 hr at a pD of 7.5–8.0. The pH was adjusted to 1 with HCl and after addition of 0.5 ml of CCl_4 the solution was vortexed vigorously to precipitate the protein. After centrifugation the supernatant was added to a 1.5 × 20 cm column of Dowex-50-X8-H^+, 200–400 mesh, and α-ketoglutarate was eluted with 100 ml of water. Aspartate-*2-d* was then eluted with 0.2 *M* aniline at the point where aniline first emerged from the column. Aniline was extracted with ether, and the solution flashed to dryness to give a crystalline product, which was recrystallized twice from water–ethanol. Total deuterium substitution at C-2 was confirmed by nuclear magnetic resonance (NMR) spectroscopy.

Aspartate-*2-d* was deaminated by the procedure of Horton and Philips (1973) to yield malate-*2-d*. Cations were removed by passing the reaction mixture through a 2 × 30 cm column of Dowex-50-X8-H^+, 200–400 mesh, and washing with water until all the malate was eluted, and the eluate was then flashed to dryness. The residue was taken up in a small amount of water, adjusted to pH 7–8, and adsorbed on a 1 × 10 cm column of Dowex-1-X8-Cl^-, 200–400 mesh. Strongly colored contaminants remained at the top of the column while malate-*2-d* was eluted with 0.1 *M* HCl. The eluate was flashed to a syrup and crystallized in vacuo over P_2O_5. The melting point of malate-*2-d* was 123–126°, compared to 103–105° for L-malic acid, and 121–124° for DL-malic-d_6 acid purchased from Aldrich. Malate synthesized in this manner contained an unknown contaminant that inhibited malate dehydrogenase strongly at pH 8 but only weakly at pH 9.3, and did not affect malic enzyme. This inhibitor was removed by eluting the L-malate-*2-d* with a linear gradient of 0–2 *M* formic acid from a 1 × 20 cm column of Dowex-1-X8-formate, 200–400 mesh. The malate-*2-d* was shown to be fully deuterated at C-2 by NMR spectroscopy. L-Malate-*2-d* concentrations were determined by enzymatic analysis using malic enzyme with an excess of TPN. A comparison of enzymatically determined L-malate and titratable equivalents indicated that the ratio of L-malate to D-malate in the deuterated preparation was 80:20.

Data Fitting. The data from Figure 5 for the kinetic isotope effects on malate dehydrogenase were fitted to eq 36 by the method of least squares, assuming equal variances for the velocities, and using a Fortran program and a digital computer:

$$v = V_H[A]/[K_H(1 + f[(V/K)_H/(V/K)_D - 1]) + [A](1 + f(V_H/V_D - 1))] \quad (36)$$

where f is the fraction of deuterium in the substrate (zero for normal malate; 1.0 for malate-*2-d*) and [A] is the concentration of whichever form of malate was used.

Determination of β Values. Equilibrium constants with malate-*2-d* and malate-*2-h* were determined for malic enzyme at pH 7.1 in 3-ml ground glass stoppered cuvettes by allowing a mixture of malate, pyruvate, CO_2 (added as $KHCO_3$), TPN, and the same buffer described in Figure 3 to come to equilibrium, and measuring the TPNH or TPND formed spectrophotometrically at 340 nm. CO_2 concentrations were calculated with a pK for bicarbonate of 6.4, and K_{eq} evaluated as [pyruvate][CO_2][TPNH]/[L-malate][TPN]. In one set of experiments $K_{eqH} = 0.031 \pm 0.001$ *M* and $K_{eqD} = 0.026 \pm 0.001$ *M* (average for three experiments), and thus $\beta = K_{eqD}/K_{eqH} = 0.84 \pm 0.04$. A second set of experiments gave $K_{eqH} = 0.0295 \pm 0.0015$ *M* and $K_{eqD} = 0.0235 \pm 0.0005$ *M* (average of two experiments), with $\beta = 0.80 \pm 0.04$. The average value of β from these two sets of experiments was 0.82 ± 0.04.

The same procedure was used for malate dehydrogenase at pH 9.3. Here K_{eq} = [oxaloacetate][DPNH]/[L-malate][DPN], and the observed values of $K_{eqH} = 1.17 \pm 0.04 \times 10^{-3}$ and $K_{eqD} = 8.90 \pm 0.23 \times 10^{-4}$ (average of four experiments) give $\beta = 0.76 \pm 0.04$.

Results

Malic Enzyme. When malic enzyme was added to reaction mixtures calculated to be at equilibrium and containing TPNH and malate-*2-d*, a burst of TPNH disappearance was followed by a slow return to equilibrium (Figure 3). Using eq 31, 25, 34 and Figure 1, α values of 1.43, 1.43, and 1.48 and $\alpha\beta$ values of 1.17, 1.17, and 1.22 were obtained for three experiments. The average values are $\alpha = 1.45 \pm 0.03$ and $\alpha\beta = 1.19 \pm 0.03$.

[2] For highest precision, a table of values for eq 16 should be used. This table, and a short Fortran program which makes all of the calculations described here, is available on request from the senior author (W.W.C.).

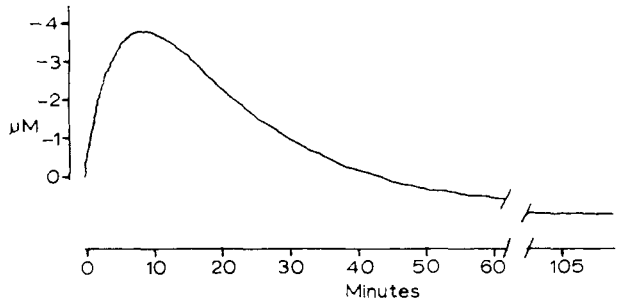

FIGURE 3: Equilibrium perturbation caused by malate-*2-d* with malic enzyme. The reaction mixture contained in 3.0-ml volume in a ground glass stoppered cuvette 20 m*M* $KHCO_3$ (3.8 m*M* CO_2), 20 m*M* $MgSO_4$, 79 μM TPNH, 102 μM TPN, 3.83 m*M* pyruvate, 0.419 m*M* L-malate-*2-d*, 67 μg/ml of bovine serum albumin, and a buffer consisting of 25 m*M* each of glycine, acetate, hydroxyethylpiperazineethanesulfonate, and cacodylate (K^+, pH 7.1). (This buffer was designed for pH studies of malic enzyme which will be reported at a later date.) Full scale, 0.1 OD; chart speed, 0.1 in./min. Reaction at 25° started with 0.4 unit of malic enzyme and followed at 340 nm. Calculated α = 1.48, $\alpha\beta$ = 1.22.

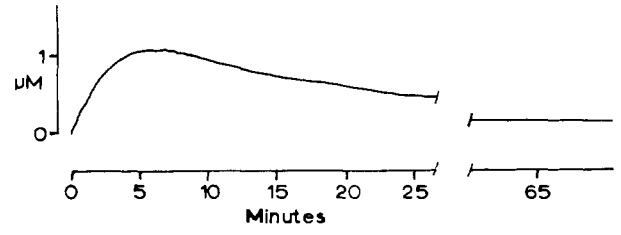

FIGURE 4: ^{13}C isotope effect with malic enzyme. The reaction mixture in 2.5-ml volume in a ground glass stoppered cuvette contained 102 μM TPN, 102 μM TPNH, 24.6 m*M* $MgSO_4$, 10 m*M* pyruvate, 1.25 m*M* malate, 82 μg/ml of bovine serum albumin, the same buffer used in Figure 3 (pH 7.8), and 2.36 m*M* $^{13}CO_2$ (total CO_2 plus bicarbonate, 61.4 m*M*; 90% ^{13}C). Full scale, 0.05 OD; chart speed, 0.3 in./min. Reaction at 25° started with 0.3 unit of malic enzyme. Calculated α = 1.027.

When similar experiments were carried out with normal malate, but with $^{13}CO_2$, a burst of TPNH formation was observed, followed by a slow return to equilibrium (Figure 4). The α values calculated from eq 31, 13,[3] 34, and 17, and corrected for the 90% ^{13}C label were: 1.021, 1.027, 1.032, 1.034, and 1.040, with an average value from these five experiments at pH 7.8–8.0 of 1.031. At pH 7.1 the slow breakdown of TPNH makes observations more difficult, but two experiments gave values of 1.030 and 1.063.

Malate Dehydrogenase. L-Malate-*2-h* and L-malate-*2-d* are compared as substrates for malate dehydrogenase at pH 9.25 in Figure 5. A fit of these data to eq 36 gives V_H/V_D = 1.5 ± 0.1 and $(V/K)_H/(V/K)_D$ = 1.9 ± 0.1. At pH 8, a similar experiment gave 1.10 ± 0.03 and 1.30 ± 0.06 as the effects on V and V/K. The low effect on V at pH 8 is consistent with previous studies that have suggested that DPNH release is the rate-limiting step at this pH (Raval and Wolfe, 1962). To test the dependence of α on the concentration of a reactant released before one of the perturbing molecules, mixtures near equilibrium were prepared with either 100 μM oxaloacetate (plus 1.97 m*M* DPN, 530–640 μM L-malate-*2-d*, and 15–40 μM DPNH), or 10.8 μM oxaloacetate (plus 400 μM DPN, 1.14 m*M* L-malate-*2-d*, and 90–140 μM DPNH). Four experiments at 100 μM oxaloacetate gave α values of 1.72, 1.85, 1.63, and 1.60 with an average of 1.70 ± 0.11, and $\alpha\beta$ values of 1.31, 1.41, 1.24, and 1.22 with an average of 1.30 ± 0.09 (see Figure 6). For three experiments at 10.8 μM oxaloacetate, α values were 2.11, 2.17, and 2.20 with an average of 2.16 ± 0.05, and $\alpha\beta$ values were 1.60, 1.65, and 1.67 with an average value of 1.64 ± 0.04.

[3] The concentration of CO_2 used in eq 13 was the total concentration of CO_2 and bicarbonate present, since equilibration between these two is enough faster than the rate of the perturbation so that they must be considered a common pool of material.

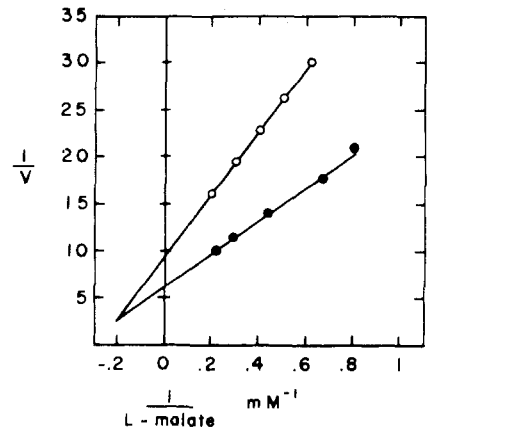

FIGURE 5: Deuterium isotope effects with malate dehydrogenase. Reaction mixtures contained 50 m*M* pyrophosphate (pH 9.3), 1.18 m*M* DPN, 0.1 m*M* EDTA, and either L-malate-*2-d* (○) or L-malate-*2-h* (●) in 3.0-ml volume in 1-cm cuvettes. Reaction was started by addition of 0.38 unit of pig heart malate dehydrogenase, and followed at 340 nm and 25°. Lines drawn from a fit to eq 36.

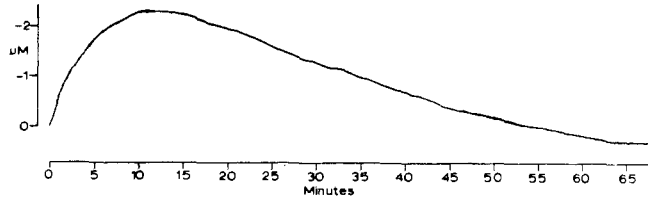

FIGURE 6: Equilibrium perturbation with malate dehydrogenase. The reaction mixture contained 50 m*M* pyrophosphate (pH 9.3), 1.97 m*M* DPN, 0.1 m*M* oxaloacetate, 0.04 m*M* DPNH, 0.1 m*M* EDTA, and 1.14 m*M* L-malate-*2-d* in 3.0-ml volume. Reaction at 25° started with 0.38 unit of malate dehydrogenase. Full scale was 0.05 OD, and chart speed 0.2 in./min. Calculated α = 1.60; $\alpha\beta$ = 1.22.

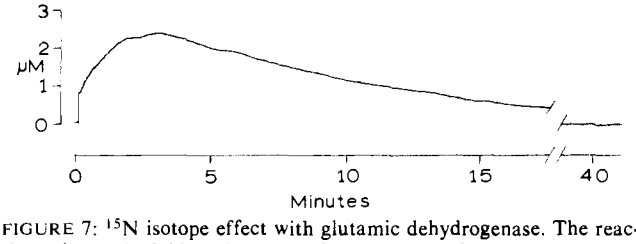

FIGURE 7: ^{15}N isotope effect with glutamic dehydrogenase. The reaction mixture in 2.925 ml contained 15.3 m*M* L-glutamate, 1.12 m*M* α-ketoglutarate, 0.358 m*M* $^{15}NH_4^+$ (as sulfate), 1.09 m*M* TPN, 0.120 m*M* TPNH, and 130 m*M* glycine. Reaction was started by addition of 25 μl (13.8 units) of NH_3-free glutamic dehydrogenase (Sigma type II), and followed at 340 nm. Final pH, 8.1. The initial shape of the recorded trace results from turning on the chart motor as mixing began, and the servo motor only after the cell compartment was closed. Calculated α value from eq 7, 1.048.

^{15}N Isotope Effect with Glutamic Dehydrogenase. Figure 7 shows the 2.38 μM perturbation produced with glutamic dehydrogenase when $^{15}NH_4^+$ and normal glutamate are the perturbing molecules. No change was seen in a comparable system containing $^{14}NH_4^+$. To analyze this perturbation, $[R'_0]$ is evaluated from eq 13 as 0.350 m*M* and

$[A'_0]$ from eq 31 as 0.0986 mM. Then since $[A'_0/[R'_0]$ equals 0.28, which is less than 0.3, $[R''_0]$ was calculated from eq 34 with $y = 2.3$ as 0.1376 mM, and α from eq 17 as 1.048. A similar experiment with 14.8 mM glutamate, 1.34 mM TPN, 0.368 mM $^{15}NH_4^+$, 1.35 M α-ketoglutarate, and 0.112 mM TPNH gave a perturbation of 2.25 μM, corresponding to $\alpha = 1.045$. These values are the same as those seen by Brown and Drury (1965) for the decomposition of phenyl diazonium salts (1.047, calculated for 25° by interpolation between values measured at 7 and 40°), suggesting that iminoglutarate hydrolysis is probably the rate-limiting step for the portion of the reaction sequence prior to product release. No perturbation was observed at pH 9 when norvaline replaced glutamate, which is consistent with the conclusion based on the large deuterium effects seen with norvaline-*2-d* that hydride transfer is rate limiting with this substrate.

Discussion

With malic enzyme, malate-*2-d* and TPNH give a perturbation corresponding to an isotope effect of 1.45 in the forward direction. As expected, this is somewhat smaller than the value of 1.5–1.8 seen on V/K when malate-*2-d* is varied (Schimerlik and Cleland, 1975), since CO_2 and pyruvate are present at finite levels, and thus decrease the observed effect according to eq 35 and 21. With malate dehydrogenase a similar situation exists, since the average α values from the perturbations were 1.70 at 100 μM oxaloacetate and 2.16 at 10.8 μM oxaloacetate. Clearly, higher levels of oxaloacetate decrease α somewhat as predicted by eq 35 (oxaloacetate is released before DPNH); but since the measured value of $(V/K)_H/(V/K)_D$ from Figure 5 is 1.9 (which is not significantly different from the value of 2.16 at 10.8 μM oxaloacetate), it is clear that 10 μM oxaloacetate is low enough not to have an effect.

The results in Figures 4 and 7 show that the equilibrium perturbation method is sensitive enough in favorable cases to detect isotope effects with ^{13}C and ^{15}N. In these two cases the α values should be identical with those obtained from the isotope effects on V/K, since CO_2 and NH_3 are the first molecules released by these enzymes, and thus b in eq 3 is the same for the two types of experiment. Heavy isotope effects have usually been determined by measuring the depletion of the natural abundance of heavy isotope in the first portion of product formed with an isotope ratio mass spectrometer, and this method gives the isotope effect on V/K.

One potential problem with this method, however, is the need for exact temperature control. Since K_{eq} for most reactions varies with temperature, temperature changes of less than a degree can cause very visible perturbations in the equilibrium position. A 4.8 μM (2.7%) per degree change in TPNH was observed under one set of conditions with glutamic dehydrogenase, which has $\Delta H = 15500$ cal/mol for oxidation of glutamate. When we used 1-ml cuvettes in an attempt to conserve material, we discovered that holding the cuvette with the fingers to add enzyme and mix by inverting added sufficient heat to the heavy glass walls of the cuvette so that the equilibrium was rapidly perturbed in one direction as this heat first diffused into the small volume of liquid in the cuvette, and then returned to normal as this heat was dissipated and temperature equilibrium was restored by the thermospacers. The resulting trace looked very much like those produced by isotopic substitution, and for this reason one should always run controls with unlabeled compounds to be sure such problems do not arise. Clearly enzyme should be added in a very small volume, and if possible should be at the temperature of the reaction mixture.

As the examples discussed above clearly show, equilibrium perturbation caused by isotopic substitution is a practical and sensitive method for detecting isotope effects. The purity of the isotopically substituted compound is not critical since impurities can only change the rate of the reaction, and not the shape or size of the perturbation. The exact concentration of the labeled reactant need not be known, since one adjusts conditions until exact equilibrium is reached, and thus a concentration based on enzymatic assay is sufficient. Gradual denaturation of enzyme only slows down the eventual return to equilibrium, but unless it is very rapid, will not affect the maximum size of the perturbation, which is achieved in a short time. Likewise, slow breakdown of TPNH, which occurs at pH's below 8, is easily allowed for by letting the reaction run long enough for this linear breakdown to be extrapolated back to zero time and form a baseline for the perturbation. Because of the form of the equations, the major error is the precision with which the size of the perturbation can be measured, and the percentage error of $\alpha - 1$ will be the same as the percentage error in the perturbation size.

The sensitivity of the method is its major advantage, however. With 220 μM TPNH ($OD_{340} = 1.37$), 2.2 mM malate-*2-d*, and a full scale sensitivity on the 10-in. recorder of 0.05 OD (8.03 μM), an apparent isotope effect (appα) of 1.0074 gives a perturbation of 0.5 μM, or $\frac{5}{8}$ in., as long as other reactants are all above 1 mM (if $\beta = 0.8$, this corresponds to a true α of 1.23). The sensitivity achievable in measuring ^{13}C or ^{15}N effects is comparable, but here the limiting factor will be DPNH or TPNH concentration, which can only be used at levels below 400 μM. Thus $[A'_0]$ cannot be greater than this, so that high levels of the perturbing molecules that determine $[R'_0]$ do not increase the size of the perturbation, but only lower the time needed to reach the maximum. Thus at $[A'_0]$ and $[R'_0]$ values of 400 μM and 4 mM, an isotope effect of 1.002 gives a perturbation of 0.57 μM. With care this technique should approach in accuracy the usual isotope depletion method for ^{13}C and ^{15}N isotope effects, which requires the use of an isotope ratio mass spectrometer. It is particularly useful for mechanisms that are freely reversible, while the isotope depletion method works well only for nearly irreversible reactions. It requires a high percentage of label in one of the reactants, unlike the isotope depletion method which is normally done with the natural abundance of heavy isotope, but does not require any subsequent chemical handling of the products.

The changes in equilibrium constant resulting from deuterium substitution have apparently not been previously noticed by biochemists, although they are predicted by the calculations of Hartshorn and Shiner (1972), which are based on fundamental vibration frequencies. The cause of the change is a different degree of "stiffness" in the C–H bond in substrate and product, with deuterium being enriched in the molecule with the stiffer C–H bond (that is, with the higher zero point energy). These authors have found that the stiffness of C–H bonds depends almost entirely on the nature of the other atoms bonded directly to the carbon, with only small changes resulting from more remote structural differences. While they did not include oxygen-containing molecules in their calculations, Dr. Shiner in a private communication suggests that oxygen should be-

have very much like fluorine in influencing the stiffness of C-H bonds. Accepting this postulate, the data in Table X of Hartshorn and Shiner (1972) show that the fractionation factors for replacing hydrogen in methane with carbon, nitrogen, and oxygen (= fluorine) are about 1.10, 1.15, and 1.18. These factors are multiplicative, so that if more than one atom is changed, the factors are multiplied (or divided) together.

The transferable hydrogen in DPNH or TPNH is bonded to a carbon with two other carbons and a hydrogen attached. In malate, C-2 is attached to two carbons and an oxygen, in addition to the hydrogen. Thus the fractionation factor should be 1.18 in favor of malate, which is in reasonable agreement with the experimental $1/\alpha$ values of 1.2–1.3, especially since the equivalence of oxygen and fluorine may not be exact. Similar effects should be observed with lactate and isocitrate dehydrogenases. With alcohol dehydrogenases, secondary alcohols will show the same fractionation factor, but primary alcohols (if deuterated only in the transferable position; see the comments below for secondary isotope effects) will show a lower factor ($1.18/1.10 = 1.07$), since a carbon in DPNH has also been replaced by hydrogen. For glutamic dehydrogenase the value of 1.18 observed in this laboratory is in good agreement with the calculated value of 1.15 in favor of glutamate.

Glucose-6-P dehydrogenase should show a larger factor ($1.18 \times 1.18/1.10 = 1.27$) since two oxygens have replaced one hydrogen and one carbon, and a similar effect is expected for glyceraldehyde-3-P dehydrogenase, where glyceraldehyde-3-P is almost fully hydrated to the *gem*-diol form. By contrast, a dehydrogenase such as the acyl-ACP dehydrogenase that participates in fatty acid synthesis should show no effect, since the hydrogen is bonded to identical carbons in TPNH and the substrate. It will be interesting to see whether the above predictions are verified experimentally.

The same principles discussed above for equilibrium primary isotope effects also explain the existence of equilibrium secondary isotope effects when a nontransferable deuterium is bonded to a carbon that is altered during reaction. For example, Schmidt et al. (1969) have determined for the fumarase reaction an average equilibrium isotope effect for tritium substitution in fumarate of 1.24 ± 0.03 (since fumarate is symmetrical, the resulting value is the average of the values for tritium substitution at carbons 2 and 3 of malate). The deuterium effect is then $(1.24)^{0.694} = 1.16$ (tritium effects always being the 1.442 power of deuterium effects). The predicted value of this effect computed by Schmidt et al. (1969) is 1.18; an identical value can be calculated from the partition factors given by Hartshorn and Shiner (1972). (The effect for C-3 is that of changing a double bond to single bonds to hydrogen and carbon, or 1.082 by taking the ratio of partition factors from Table X of Hartshorn and Shiner (1972) for ethane and ethylene. For C-2 an oxygen then replaces hydrogen in addition, or $1.18 \times 1.082 = 1.276$. The average of 1.082 and 1.276 is 1.18.)

Acknowledgment

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