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The Expression of Kinetic Isotope Effects during the Time Course of Enzyme-Catalyzed Reactions

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Because of the progressive isotopic enrichment of substrate during the time course of an enzymatic reaction, the kinetic isotope effect calculated from the enrichment in a substrate (the preferred measurement for isotope effects of less than 1.05) or the depletion in a product (the more common measurement) varies with time and thus must be corrected. Bigeleisen and Wolfsberg (*Adv. Phys. Chem.* **1**, 15–76, 1958) derived a correctional equation for irreversible first-order uncatalyzed reactions, whose validity for an enzyme-catalyzed reaction is examined in this work. The primary finding is that, if significant depletion of a second substrate occurs during the course of an enzymatic reaction, then the expression of an isotope effect will change extensively in a manner which is not anticipated by the correctional equation. This was demonstrated in a single time-course assay of the oxidation of tritiated NADH by pyruvate beef heart lactate dehydrogenase, in which the corrected isotope effect varied from 1.39 at the start of the reaction to 1.8 at 95% reaction. High concentrations of pyruvate inhibit the isotope effect with an apparent K_I of 358 ± 24 μM. The inhibition arises because pyruvate is the second substrate to bind and its value agrees with the apparent K_m of 379 ± 88 μM, as expected. Separate tritium isotope effects extrapolate to 2.64 ± 0.05 at zero pyruvate and 1.01 ± 0.05 at infinite pyruvate. When the concentration of pyruvate was well in excess of that of NADH, so as to minimize the effect of depletion of pyruvate, the correctional equation was found to be valid for up to 85% of the oxidation of tritiated NADH. An analysis of standard errors over the complete time course shows a minimum at 50% of reaction and maxima near 0 and 100%. Hence, the precision of competitive isotope effects is greatly increased by choosing conditions under which the correctional equation applies and allowing the reaction to proceed halfway, avoiding the more common practice of restricting measurements to the period of initial velocity. Computer simulations suggest that the correction may remain a reasonable approximation for up to 50% of an approach to equilibrium in reactions more reversible than that of lactate dehydrogenase.

INTRODUCTION

The study of isotope effects is now firmly established as a powerful tool for investigating the mechanism of an enzyme-catalyzed reaction (*1*). For stable isotopes, there are two alternative procedures for determining isotope effects, the competitive and noncompetitive methods (*2*). The latter method involves comparing the initial velocity of the isotopic substrate to that of the normal substrate, measured separately. If initial velocities are collected over a range of concentra-

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tion of substrates, then isotope effects on both V (the maximal velocity) and V/K (K is K_m) can be determined. For radioactive isotopes, only the competitive method is possible. It involves a single reaction mixture containing a trace of the labeled substrate in the presence of normal substrate. Isotope effects are extracted from measurements of isotopic enrichment or depletion in the substrate or product, instead of comparisons of initial velocities. These isotopic discriminations yield only the V/K isotope effect, irrespective of the substrate concentration, and cannot be used to answer questions about the maximal velocity or rate-limiting step.

Given a normal isotope effect in which the isotopic substrate reacts more slowly, there will be a gradual enrichment of the remaining substrate during isotopic discrimination. As the reaction proceeds, this enrichment will tend to compensate for the lower rate of reaction of the isotopic substrate. Thus, the specific radioactivity of the substrate and the formed product will vary as a function of how far the reaction has proceeded, leading to a progressive decrease in the apparent isotope effect as calculated directly from isotopic discrimination. This problem was considered for nonenzymic reactions by Bothner-By and Bigeleisen (*3*) and by Downes and Harris (*4*). This work was reviewed and extended by Bigeleisen and Wolfsberg (*5*), who derived several equations which allow the correct isotope effect to be determined from isotopic discrimination at all stages of a reaction. This was an important advance because it alleviated the problems of determining very small changes in the isotopic ratio of substrates or of isolating very small quantities of product. Bigeleisen and Wolfsberg considered a variety of experimental conditions including cases where either reactant or product was measured and where the reactant was more than trace-labeled.

Although the Bigeleisen and Wolfsberg corrections have been applied to enzymatic reactions, the original derivations addressed only uncatalyzed reactions which were irreversible and followed first-order kinetics. The validity of this extension to enzymatic reactions has not been demonstrated, although it has been questioned to some extent.[1] For example, in a review of heavy-atom isotope effects in enzyme-catalyzed reactions, O'Leary (*7*) states that while no corrections are necessary at low conversions because "even at 10–15% reaction the error is small," the corrections "are not appropriate for use in studies of hydrogen isotope effects," which derives from the popular notion that the enrichment problem is more severe with isotopes of hydrogen than of carbon, oxygen, or nitrogen because of the larger isotope effects of the former. Northrop (*8*) notes the inadequacies of experimental designs while reviewing the highly precise data of Dahlquist *et al.* (*9*), Goitein *et al.* (*10*), and Bull *et al.* (*11*) to the application of the competitive method to hydrogen isotope effects on enzyme-catalyzed reactions and states that it "has been frequently assumed that negligible error ensues from allowing isotopic discrimination to proceed for 5–10% of the reaction, but these more precise studies show considerable error even at low levels of product formation" and "several early determinations must be obtained and extrapolated back

[1] The Bigeleisen and Wolfsberg correction has also been extended to biological systems, namely, isotopic CO_2 fixation in plants (*6*). It was not applicable in its original form and required modification to accommodate the influx of new CO_2 as isotopic CO_2 was depleted. The problem, however, differs from those addressed here.

to 0% of reaction." However, how to accomplish this extrapolation was not obvious as the appropriate correctional equation for enzymatic reactions had not been established. More recently, and at the other extreme, Cleland (*12*) offered the seemingly arbitrary advice that the Bigeleisen and Wolfsberg correction can be applied to enzymes if the fraction of reaction does not exceed 40%.

Because of their familiarity with initial velocities, enzyme kineticists are inclined to restrict their measurements of isotopic discrimination to the first 5–10% of reaction and to ignore possible deviation from a true zero-time measurement. In this paper, we ask whether a correction is necessary during the period of initial velocity measurements and later show that the restriction to the first few percent of reaction results in a maximal expression of experimental error. Secondly, we consider whether the Bigeleisen and Wolfsberg approach has application to enzyme-catalyzed reactions, noting conditions when it does not. We concentrate on experiments in which the isotopic content of the product is measured and where the isotopic substrate is trace-labeled with tritium.

THEORY

Origin and precision of the correctional equation. In this section we review the relevant theory for the specific case of a tritium isotope effect although the treatment is completely general and can be applied without alteration to other isotopes. We use the nomenclature of Northrop (*14*) in which a tritium isotope effect (^{T}k) is the ratio of the rates or reaction of unlabeled (S_H) and tritium-labeled (S_T) substrate, relative to their respective concentrations:

$$^{T}k = \frac{\dfrac{d[S_H]/dt}{[S_H]}}{\dfrac{d[S_T]/dt}{[S_T]}}. \qquad [1]$$

If the rates of disappearance of S_H and S_T are approximated by the accumulated concentrations of the corresponding products (P_H and P_T), then Eq. [1] is easily rearranged to

$$^{T}k = \frac{[S_T]/[S_H]}{[P_T]/[P_H]}, \qquad [2]$$

which states that the tritium isotope effect is equal to the specific radioactivity of the substrate, divided by that of the product. It is important to note that Eq. [2] is an approximation, valid only at the very beginning of the reaction. Unless the isotope effect is unity, the specific radioactivity of the substrate will change as the reaction proceeds and cause the specific radioactivity of the accumulated product to express this progressive change as well. Herein lies the first part of the problem: how far can a reaction be allowed to proceed before the ratio of the initial specific radioactivity of the substrate to the specific radioactivity of the accumulated product becomes significantly different from the correct isotope effect?

The problem is illustrated in Fig. 1 in which the percentage error of the apparent isotope effect as calculated directly from specific activity measurements is plotted

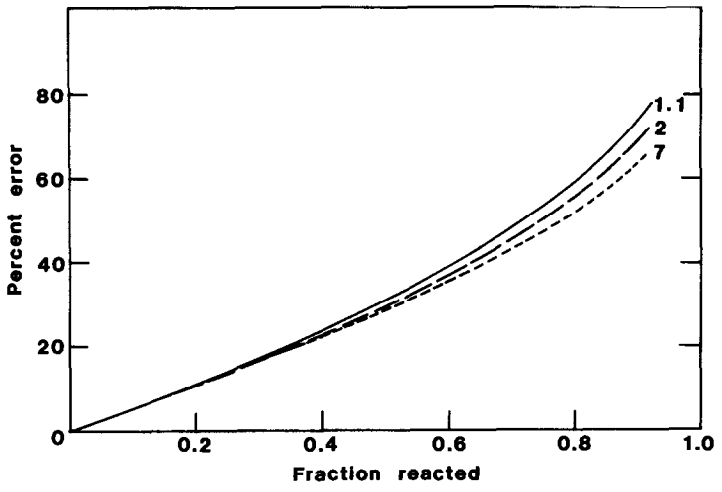


FIG. 1. Predicted error in isotope effects of chemical reactions in which isotopic ratios of products are measured and used directly without correction. The apparent isotope effect is calculated using Eq. [2] and expressed as a percentage error relative to the correct isotope effects of 1.1 (—), 2.0 (– – –), and 7.0 (---).

against the fraction of the reaction. Note first that the error becomes significant at an extremely early stage of the reaction, and second that the error is independent of the magnitude of the isotope effect until late in the reaction. Hence, while the amount of isotopic enrichment is much less with heavy atom isotopes than with isotopic hydrogen, the percentage error on the apparent isotope effect is exactly the same and equally in need of correction.

The errors introduced by Eq. [2] were overcome by Bigeleisen and Wolfsberg (*5*), who simplified and rearranged Eq. [1] to give

$$\frac{d[S_H]}{d[S_T]} = {}^{T}k\,\frac{[S_H]}{[S_T]}\,. \qquad [3]$$

Integration and rearrangement yield

$${}^{T}k = \frac{\ln(1 - [P_H]/[S_H]_0)}{\ln(1 - [P_T]/[S_T]_0)}\,. \qquad [4]$$

At trace levels of tritium, $[P_H]/[S_H]_0$ approximates the total fraction of the substrate which has reacted, and can be replaced by f as in

$${}^{T}k = \frac{\ln(1 - f)}{\ln\{1 - f([P_T]/[P_H])/([S_T]_0/[S_H]_0)\}}\,, \qquad [5]$$

which corresponds to Eq. [V.23] of Bigeleisen and Wolfsberg, and which has been reproduced with slight variations by several others, sometimes erroneously.[2]

[2] For example, see Eq. [4] of O'Leary (*7*) and Eq. [54] of Cleland (*12*), and note errors in Eq. [35] of Northrop (*14*) and Eq. [26] of MacColl (*13*).

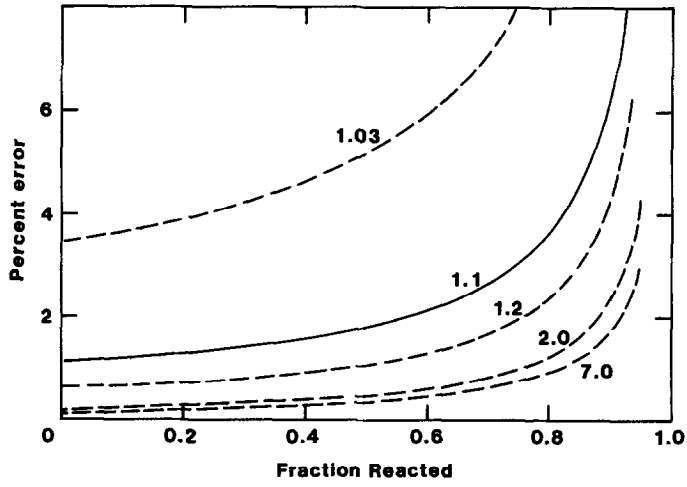


FIG. 2. Effect of experimental error on the corrected isotope effect from product measurements. For a series of correct isotope effects (k), the isotopic ratio of the product was calculated using Eq. [5] and then an error of 0.5% was subtracted from f, while an error of 0.1% was added to the product ratio. The apparent isotope effect (k') was then calculated from the altered values using Eq. [6] and the error $(k - k')/(k - 1)$ expressed as a percentage. The lines shown represent correct values between 1.03 and 7.0 as indicated. The solid line follows that in Fig. 3 of Bigeleisen and Wolfsberg (*5*).

The propagation of experimental error through Eq. [5] to corrected isotope effects was addressed by Bigeleisen and Allen (*15*) by assuming relative experimental errors of 0.1% in measurements of the specific activity of products and 0.5% in the fraction of reaction. Results from their calculations based upon an isotope effect of 1.1 is reproduced as the solid line in Fig. 2 (cf. their Fig. 3). Melander and Saunders (*16*) similarly considered error propagation with a heavy-atom isotope effect of 1.05 and a tritium effect of 20. Neither work, however, drew any clear correlation between the size of the isotope effects and the magnitude of the errors. Therefore, also shown in Fig. 2 are new calculations of the expected error for isotope effects both smaller and progressively larger than 1.1. Note that the experimental errors are greatly magnified for small isotope effects, particularly at very high fractions of reaction, and moderately suppressed with large isotope effects of hydrogen.

Alternatively, in some experiments it may be more convenient to measure the specific activity of the remaining substrate instead of product. Bigeleisen and Wolfsberg gave an expression equivalent to Eq. [5] for this situation (their Eq. [V.22]) and Fig. 3 illustrates the influence of experimental errors on the correction. Again, experimental errors are magnified by the correction, but more so here for large isotope effects instead of small. Moreover, some of the errors are so large even at low fractions of reaction, that this experimental approach is of little practical value for isotope effects of hydrogen. As has been pointed out by Cleland (*17*), this magnification of error is due to the lower reactivity of the labeled substrate; with isotope effects greater than about 2, the labeled substrate is mostly

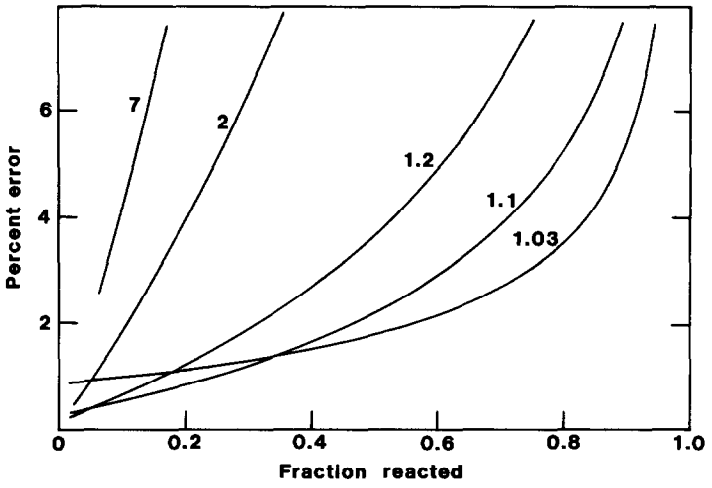


FIG. 3. Effect of experimental error on the corrected isotope effect from reactant measurements. Calculations were as in Fig. 2, except that substrate isotopic ratios were calculated. The lines shown represent correct values between 1.03 and 7.0 as indicated.

left behind, regardless of the exact value of the isotope effect, so that value is indeterminate. The break point between the two figures is approximately 1.05; hence, isotope effects greater than 1.05 should be determined from product ratios, while those less than 1.05 should be determined from substrate ratios.

Equation [5] contains three quantities which must be determined from experimental measurements; the fractional reaction and the specific radioactivity of the product at each point, and the specific radioactivity of the starting substrate (or, as is sometimes preferred, that of the product at infinite time shown below). Often, these three quantities are not measured directly and independently. A typical example is the work of Goitein *et al.* (*10*), who used a double labeled (^{3}H and ^{14}C) substrate to determine both the fraction of reaction and the specific radioactivities from coincident measurements. Since f represents the ratio $[P_H]/[S_H]_0$, a part of the denominator of Eq. [5] cancels out, and what is actually calculated is the expression:

$$^{T}k = \frac{\ln(1 - f)}{\ln(1 - r)}, \qquad [6]$$

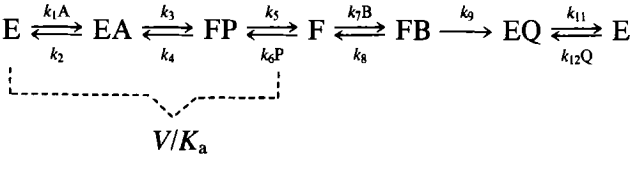
where f is determined from a comparison of the ^{14}C counts at the time of measurements to counts at the completion of reaction, and where r represents the fraction of tritium-labeled substrate which has reacted, determined from a similar comparison of the ^{3}H counts. It is this form which is referred to as the correctional equation in the remainder of the present paper. The isotope effect defined by Eq. [6] is referred to as the corrected isotope effect, while that defined by Eq. [2], equivalent to $^{T}k = f/r$, is termed the cumulative isotope effect.

Validity of the correctional equation in enzyme-catalyzed reactions. The corrections suggested by Bigeleisen and Wolfsberg were derived for chemical reac-

tions where the decrease in the concentrations of the labeled and unlabeled reactant are independent of one another and where both follow first-order kinetics in irreversible reactions. Although Downes and Harris (*4*) had considered more complex kinetic schemes, it is implicit in their work that the reaction has a fixed kinetic order of 1 or more that did not change during the reaction. In marked contrast are enzyme-catalyzed reactions, in which the reaction progress begins as a zero-order reaction at high substrate and gradually changes to first-order as the concentration of substrate drops to below K_m levels. Moreover, since labeled and unlabeled substrate must compete for a common catalyst, the reactions cannot be independent of one another.

The situation is further complicated for enzymatic reactions having two or more substrates. It was implicitly assumed in the integration of Eq. [3] that the isotope effect is independent of $[S_H]$ and $[S_T]$. While this is usually true for nonenzymatic reactions, it frequently does not hold for enzymatic reactions. The isotope effect measured in a competitive experiment is a V/K effect; since the majority of enzymatic reactions involve a second substrate, a decrease in $[S_H]$ and $[S_T]$ will be accompanied by a decrease in the concentration of the cosubstrate, which will alter the expression of isotope effects on V/K in sequential reaction mechanisms (*14*). Furthermore, products will accumulate and these too may affect the expression of an isotope effect on V/K. Competitive product inhibition should not alter the corrected isotope effect because competitive inhibitors simply remove the amount of enzyme available for reaction and will not alter the isotopic discrimination exercised by the uninhibited enzyme. Noncompetitive product inhibition, on the other hand, may change isotopic discrimination if it alters catalysis. Product inhibition is normally competitive for enzymes catalyzing reactions of a single substrate and single product, which are frequently the model for theoretical discussions, but enzymes requiring multiple substrates and products almost always display noncompetitive product inhibition. Thus it is to be expected that for more complex enzyme-catalyzed reactions, the corrections of Bigeleisen and Wolfsberg *should not work* except when applied to measurements made on the early stages of a reaction, before significant accumulation of products and depletion of cosubstrates. The question is, how large is the deviation and how early in the reaction does it become significant?

To illustrate this point, consider the irreversible ping-pong kinetic mechanism shown in Scheme 1. If the conversion of EA to FP is the isotopically sensitive step and A is the labeled substrate, then the V/K_a isotope effect obtained from initial velocity measurements would be independent of the concentration of the substrate B (*14, 22*). However, if the reaction is allowed to proceed to the extent that



SCHEME 1. Ping-pong kinetic mechanism.

significant amounts of P accumulate, the corrected isotope effect from measurements of isotopic discrimination of A might be affected because P is a noncompetitive inhibitor of A and may cause reversal of the first half reaction long before equilibrium is approached. Moreover, the presence of accumulating P will forge a reversible connection between the two halves of the reaction so that V/K_a will become dependent on the concentration of substrate B, which is in competition with P for the F form of enzyme. At high B, P is prevented from binding and is of no effect, while at low B, the second half of the ping-pong reaction becomes rate-determining to V/K_a which in turn will suppress the isotope effect.

EXPERIMENTAL

Enzymatic assays using radioactive isotopes. Incubation mixtures were prepared at 30°C in 160 mM potassium phosphate buffer, pH 7.22 (referred to below as KP_i buffer), and usually contained 5–10 nCi/ml of [^{14}C]pyruvate and 100–150 nCi/ml of [^{3}H]NADH, prepared and purified according to the method of Northrop and Duggleby (*18*), plus unlabeled pyruvate and NADH as needed to produce the desired final concentrations of these substrates. The relative amounts of the two labeled compounds were so chosen that only a small correction was required for spillover of ^{14}C into the ^{3}H channel during scintillation counting.

The reaction was started by addition of beef heart lactate dehydrogenase (Sigma, 0.01–0.05 units/ml) and 1-ml samples were withdrawn at intervals. The reaction was stopped in the samples by rapid removal of NADH through addition of 0.1 ml of a stopping solution containing 5.5 mM acetaldehyde and 4 units/ml of yeast alcohol dehydrogenase. The amounts added were sufficient to remove all of the NADH present within 1 s. (One advantage of this stopping method was that the ^{3}H from NADH was transferred to ethanol which could readily be removed by evaporation; see below.) The stopping solution was freshly prepared in KP_i buffer, stored in a capped tube on ice, and used within 3 h.

Pyruvate and acetaldehyde were converted to their 2,4-dinitrophenyl hydrazones by addition of 1-ml of a solution containing 0.1% (w/v) 2,4-dinitrophenyl hydrazine in 2 N HCl; this reagent was usually added within seconds of adding the stopping solution, although a delay of up to 10 min resulted in no detectable redistribution of radioactivity. After allowing at least 20 min (but less than 2 h) for the reaction to reach completion, 0.02 ml of 100 mM lactic acid was added as a carrier and the sample passed through a 1.5-ml column of AGMP-1 which had been washed with water. Following application of the sample, 2 ml of water was added and all eluted material was combined in a preweighed vial. Carrier ethanol (0.2 ml) was added and the samples placed in an oven at 95°C until the volume was reduced by 45–55% (usually approximately 2 h). Water was added to bring the solution to 2.5 g (determined by reweighing the vial) and a 1-ml sample taken for scintillation counting using 10 ml of Biofluor (New England Nuclear) and a Packard Model 2425 scintillation counter.

In addition to the samples taken throughout the course of the lactate dehydrogenase reaction, several samples were taken before addition of the enzyme and

analyzed for radiochemical impurities. Several samples were also taken after allowing the reaction to go to completion, which was assured by adding more lactate dehydrogenase (0.2–1.0 units/ml).

Determination of radioactivity. Observed counts per minute were first adjusted for quenching and spillover of ^{14}C into the ^{3}H channel to determine the true radioactivity resulting from the two isotopes. The counts were then corrected for small radiochemical impurities by subtracting the mean of the samples taken before adding lactate dehydrogenase. The corrected counts were then expressed as a fraction of the mean of the counts of samples taken when the reaction was complete. These fractions represent f (^{14}C) and r (^{3}H) as defined under Theory.

Derivation of rate equations, regression analysis, and computer simulations. Rate equations were derived with the aid of the computer program described by Canela (*19*). Nonlinear regression analysis was done using the DNRP53 program (*20*). Computer simulations of reversible reactions were performed using a Runge–Kutta–Fehlberg (*21*) method of orders 3 and 4. These programs were run on a Cleveland XT computer (Computer Corp. of Australia).

RESULTS AND DISCUSSION

Cumulative isotope effects of lactate dehydrogenase. A normal tritium isotope effect will result in preferential utilization of the unlabeled substrate. Consequently, the labeled molecules will be more likely to remain unreacted and the specific radioactivity of the residual substrate will rise as the reaction proceeds (Fig. 4A). The isotope effect is the specific radioactivity of the substrate at any instant, relative to that of the product formed at that instant. At very early stages of the reaction, we can approximate the cumulative isotope effect as the specific radioactivity of the initial substrate, relative to that of the accumulated product (Eq. [2]). This approximation becomes progressively worse as the reaction proceeds due to the gradual increase in the specific radioactivity of the substrate and the resulting change in the specific radioactivity of the accumulated product. The result is that the cumulative isotope effect gradually declines (Fig. 4B), approaching unity upon completion of the reaction.

Corrected isotope effects of lactate dehydrogenase. The correction equation allows for the changing isotopic composition of the substrate and, provided that the isotope effect is itself unchanging, should yield the same value no matter how far the reaction has proceeded. The solid lines in Figs. 4A, 4B, and 4C represent theoretical curves based on an unchanging isotopic discrimination of 1.757. The lines clearly describe the data very well, irrespective of the form in which the data are represented. The advantage of applying the correctional equation to the data prior to display as in Fig. 4C is that the constancy of the isotope effect is visually apparent as a horizontal line. Also shown in Fig. 4C is an error envelope, within which data points will fall given reasonable origins of experimental error (see figure legend). The envelope is clearly widest at the very beginning and end of the reaction; hence, these periods—including what is generally regarded as the initial velocity, the most common experimental design for isotopic discrimination in

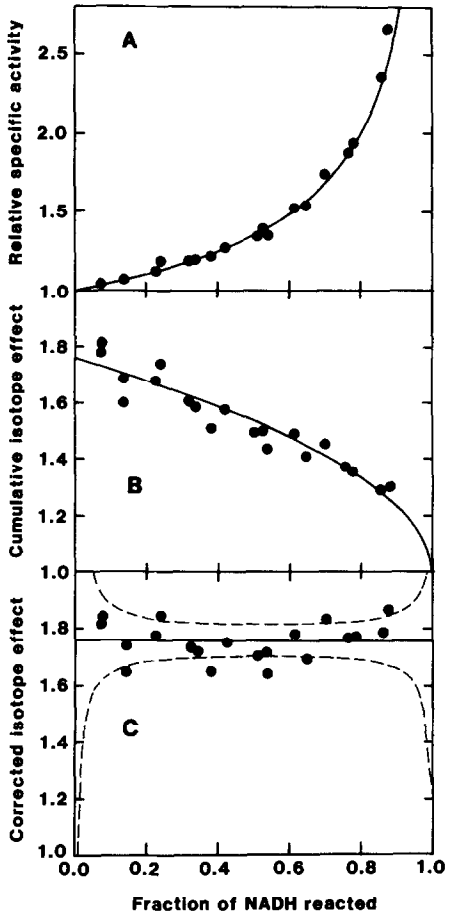


FIG. 4. Correction of the isotope effect for heavy atom enrichment. Muscle lactate dehydrogenase was incubated with 40 μM tritiated NADH and 560 μM [^{14}C]pyruvate, samples removed at intervals and the fractional transfer of ^{14}C (f) and tritium (r) into lactate was determined. This information was used to calculate the specific radioactivity of the residual NADH, relative to the initial specific radioactivity, which is plotted against the fraction of NADH utilized in (A). The cumulative (Eq. [2]) and corrected (Eq. [6]) isotope effects (see Theory) are shown in (B) and (C), respectively. The solid line in all three graphs represents the theoretical expectation, based on a V/K isotope effect of 1.757. The broken lines in (C) represent an error envelope, based upon the combined effects of a sampling error of 0.25% (i.e., 2.5 μl in 1 ml) and the expected counting error of the samples which contained up to 40 cpm of ^{14}C and 31,500 cpm of ^{3}H and which were counted for 5 min.

enzyme-catalyzed reactions—are unfavorable times to collect isotopic data. Instead, data collected between approximately 15 and 85% reaction will be the most reliable in determining an isotope effect.

Effects of changes in the concentration of pyruvate. The experiment shown in Fig. 4 used initial NADH and pyruvate concentrations of 50 and 560 μM, respectively. The calculated isotope effect was found to be altered considerably by

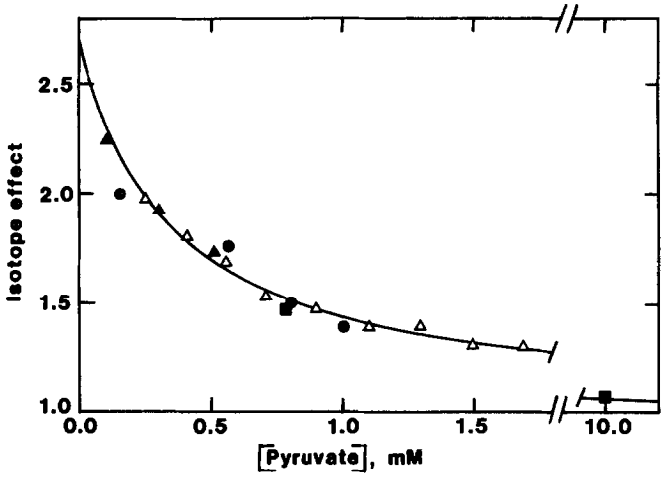


FIG. 5. Effect of pyruvate concentration of the isotope effect for lactate dehydrogenase. Muscle lactate dehydrogenase was incubated with the indicated concentration of pyruvate as described under Experimental; the concentration of [^{3}H]NADH was between one-10th and one-20th of the pyruvate concentration. Samples were taken at intervals and the isotope effect calculated using Eq. [6]. Each experimental point represents the mean value of all determinations from a complete experiment like that depicted in Fig. 4C. The various symbols correspond to different preparations of [^{3}H]NADH.

variation of the initial concentration of pyruvate (Fig. 5). These data were analyzed by fitting the equation (cf. Eq. [41] of Northrop (*14*)):

$$^{T}k = \frac{^{T}k_o - 1}{1 + [\text{pyr}]/K_I} + 1 \qquad [7]$$

to the data to obtain the extrapolated limiting isotope effect ($^{T}k_o$) of 2.64 ± 0.05 at zero pyruvate, and an "inhibition constant" (K_I) of 358 ± 24 μM.

Each of the data points in Fig. 5 represents the mean isotope effect from a complete experiment, such as that shown in Fig. 4C. The NADH concentration was not the same in all experiments but was always one-10th or less of the pyruvate concentration. However, it is expected that the isotope effect would be independent of NADH concentration. This expectation was confirmed experimentally; altering the initial NADH concentration while using a constant pyruvate concentration of 788 μM gave an invariant isotope effect of 1.47 ± 0.04 (data not shown).

This experimental design, with a large excess of pyruvate, was deliverately chosen so that there would be only a small change in the pyruvate concentration from the beginning to the end of the experiment. However, when this design was altered by using 1002 μM pyruvate and 900 μM NADH, the corrected isotope effect was no longer constant (Fig. 6) but rose from an extrapolated initial value of approximately 1.39 to 1.78 after 95% of the NADH had reacted. This increase is a result of the decline in the pyruvate concentration from 1002 to 147 μM; as was shown in Fig. 5, a considerable change in the isotope effect occurs over this range of concentrations of pyruvate.

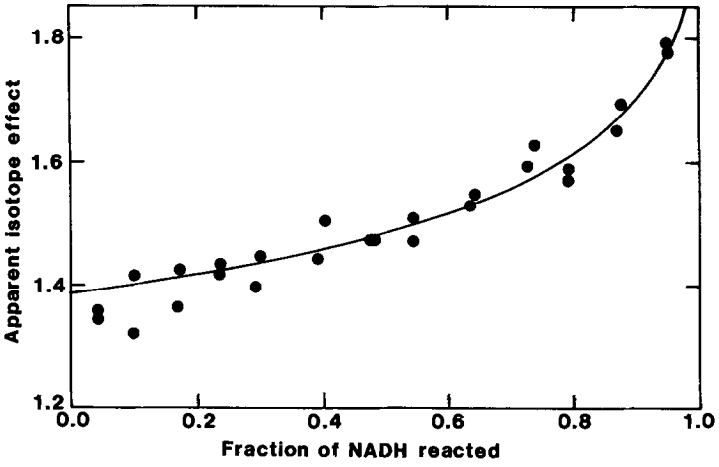


FIG. 6. Effect of pyruvate depletion on the apparent isotope effect for lactate dehydrogenase. The experiment was conducted as described for Fig. 4 except that the concentrations of NADH and pyruvate were 900 and 1002 μM, respectively. The apparent isotope effect is calculated from Eq. [6]; the line was obtained by fitting Eq. [8] to the data, treating f as the independent variable and r as the dependent variable. This nonlinear regression analysis gave ${}^{T}k_0 = 2.70 \pm 0.14$ and $K_I = 296 \pm 38$ μM.

The hyperbolic relationship in Fig. 5 is governed by Eq. [7], which may be integrated to give:

$$\{{}^{T}k_0 \cdot K_I + [\text{pyr}]_0 - [\text{NADH}]_0\}\ln(1 - r) = K_I\{{}^{T}k_0 - 1\}\ln(1 - f[\text{NADH}_0/\{{}^{T}k_0 \cdot K_I + [\text{pyr}]_0\}) + \{K_I + [\text{pyr}]_0 - [\text{NADH}]_0\}\ln(1 - f), \quad [8]$$

which relates f, r, ${}^{T}k_0$, and K_I. Equation [6] was solved for r and fitted to the experimental values depicted in Fig. 6, to yield values for ${}^{T}k_0$ of 2.70 ± 0.14 and for K_I of 296 ± 38 μM. This fit is indicated by the solid line in Fig. 6. The parameters which describe this curve do not differ significantly from those obtained from the analysis of the data in Fig. 5; however, Fig. 5 represents the combined results of 18 separate experiments while Fig. 6 demonstrates that the same two parameters can be determined from a single experiment. Repetition of this experiment using initial concentration of pyruvate and NADH of 622 and 550 μM, respectively, gave ${}^{T}k = 2.62 \pm 0.08$ and $K_I = 302 \pm 70$ μM.

In an ordered reaction mechanism, the isotope effect is predicted to be abolished when the second substrate is saturating (*22*) and, indeed, raising the pyruvate concentration to 10 mM reduced the isotope effect to 1.07 ± 0.01. This is a finite isotope effect but the concentration of pyruvate was not truly saturating, so an extrapolation to truly saturating conditions was performed by fitting an equation similar to Eq. [7] to the data shown in Fig. 5, using the asymptote as a parameter to be estimated rather than a fixed constant of 1.0. This analysis gave an isotope effect 1.01 ± 0.05, thus not only confirming the ordered mechanism of this enzyme, but doing so with much greater precision and certainty than can be obtained by alternative methods.

TABLE 1

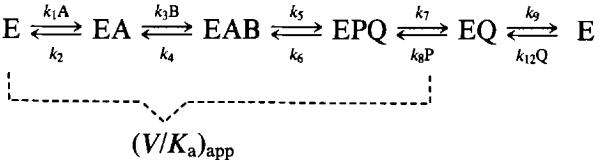
Summary of Experimental Result

Experiment	$^{T}k_o$	K_I (μM)	Data
Pyruvate variation	2.64 ± 0.05	358 ± 24	Fig. 5
Pyruvate depletion	2.70 ± 0.14	296 ± 38	Fig. 6
Pyruvate depletion	2.62 ± 0.08	302 ± 70	Not shown
Initial velocity	Not determined	379 ± 88	Not shown

The apparent K_I for inhibition of the isotope effect should equal the Michaelis constant for pyruvate, extrapolated to an NADH concentration of zero. Although this constant is not easy to determine from initial velocity measurements owing to the low concentrations of NADH required, an approximate value of 379 ± 88 μM for the K_m was determined, in agreement with the values obtained for K_I. The four separate determinations of K_I together with the three estimates of $^{T}k_o$ are summarized in Table 1.

Computer simulation of reversible reactions. The derivation of Eq. [6] and its application to the reaction catalyzed by lactate dehydrogenase depends on the assumption that the overall reaction is irreversible. While this approximation is very accurate in this particular case, we wished to investigate the extent to which Eq. [6] can be used for reversible reactions. The V/K isotope effect and extent to which a reaction is reversible depends upon the combined values of all the individual rate constants in the mechanism; even with the relatively simple ordered bi–bi mechanism shown in Scheme 2, there are unlimited possibilities for choosing combinations of rate constants to yeild an equilibrium constant and V/K isotope effect of any desired magnitude. A thorough examination of all possible variations is beyond the scope of the present work. However, it is instructive to consider a few examples of the effect of reversibility.

We used as a starting point some published values (*23*) for the alkaline branch of beef heart lactate dehydrogenase. These data do not include values for the rate constants for the interconversion of the central complexes (k_5 and k_6), only their ratio. Values for these two rate constants were chosen to give a V/K isotope effect of 1.4 to 1.5 at 1 mM pyruvate (cf. Fig. 5) for an intrinsic tritium isotope effect of 7.4. This latter value is within the range reported for other dehydrogenases (*24*). For the sake of numerical simplicity, a small (less than 0.7%) adjustment was made to k_3 so that equilibrium constant was exactly 2^{16}. A simulation of the



SCHEME 2. Ordered kinetic mechanism.

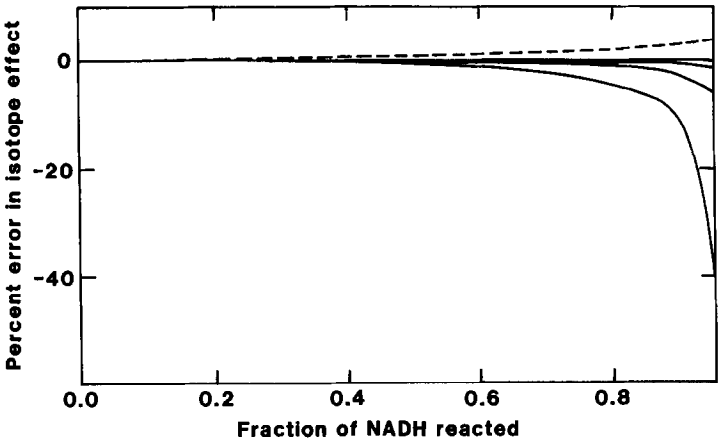


FIG. 7. Computer simulation of the corrected isotope effect for a reversible reaction. The mechanism shown in Scheme 2 was simulated using a program written by RGD. After expansion of the mechanism by including labeled NADH and lactate as a competing substrate/product pair, the distribution equations were determined and the isotope effect expressed as a differential equation. This was solved by numerical integration (keeping the concentration of pyruvate constant) and the corrected isotope effect calculated from Eq. [6]. Data are expressed as the percentage error in the calculated isotope effect, relative to that which would be expected in the absence of reversibility. The rate constants used for the essentially horizontal solid line were $k_1 = 2 \times 10^7$ M^{-1} s^{-1}, $k_2 = 50$ s^{-1}, $k_3 = 5562126.92$ M^{-1} s^{-1}, $k_4 = 540$ s^{-1}, $k_5 = 66$ s^{-1}, $k_6 = 22$ s^{-1}, $k_7 = 4 \times 10^4$ s^{-1}, $k_8 = 9.5 \times 10^5$ M^{-1} s^{-1}, $k_9 = 340$ s^{-1}, $k_{10} = 2.7 < 10^6$ M^{-1} s^{-1}. The intrinsic isotope effect on both k_5 and k_6 was taken to be 7.4 and the initial concentrations of NADH and pyruvate were 3×10^{-5} and 10^{-3} M, respectively. Successive solid lines were obtained by simultaneously decreasing k_1 and increasing k_{10} by factors of 64, 128, and 256, respectively. The broken line was calculated from the same simulation as the lowest of the solid lines, except that f and r of Eq. [6] were defined in terms of equilibrium concentrations of lactate rather than intitial concentrations of NADH.

expected corrected isotope effect is shown in Fig. 7; the results (horizontal line) are indistinguishable from those expected for a irreversible reaction.

The V/K isotope effect in the absence of products is independent of both k_1 and k_{10}. This allowed an examination of the result of a change in the equilibrium constant while maintaining a constant isotope effect. Successive halving of k_1 accompanied by doubling of k_{10} (thereby reducing the equilibrium constant by factors of 4) did not alter the isotope effect calculated using the correction equation until the equilibrium constant was reduced to 16 (Fig. 7). Even then the error was very small; when 93.4% of the NADH had been utilized, the isotope determined by simulation (1.430) differed only slightly from that expected for an irreversible reaction (1.435). Further reduction of the equilibrium constant accentuated the deviation so that when the equilibrium constant was 1.0 (Fig. 7, lowest line), the corrected isotope effect was in error by more than 2% by the time that 70% of the NADH was utilized.

As noted earlier, the extent of reaction can be defined relative to the initial amounts of substrate or the final amounts of products. These two definitions are identical for an irreversible reaction but they differ for a reversible reaction. To a

considerable extent, the errors in calculating the corrected isotope effect for the simulations described above result from the definitions of f or r (Eq. [6]) in terms of initial concentrations of unlabeled and labeled NADH. If the definitions are reexpressed in terms of final concentration of hydrogen- and tritium-labeled lactate, a rather different picture emerges. Recalculating the simulation at an equilibrium constant of 1.0 gave the broken line shown in Fig. 7; although the values for this "equilibrium-corrected isotope effect" do not agree precisely with that expected for an irreversible reaction, the error is quite small and does not exceed 3% until more than 90% of the NADH is utilized. Errors of this magnitude would be insignificant in relation to experimental errors (Fig. 4C).

The particular rate constants chosen for these simulations were based on the known properties of a lactate dehydrogenase. As such, they preserve one of the features of this enzyme; release of lactate, the first product, contributes substantially to the overall irreversibility. This property is unchanged when the equilibrium constant is altered by adjusting k_1 and k_{10}. We were concerned that this might be biasing the results toward a similarity between reversible and irreversible reactions. Irreversible lactate release would tend to prevent the labeled lactate from reentering the catalytic pathway, thereby isolating V/K from rate constants associated with NAD^+ release. The simulation with $K_{eq} = 1.0$ was therefore repeated with the values of k_7 and k_8 interchanged with those of k_9 and k_{10}. The resulting dissociation constants for lactate and NAD^+ were 4.92×10^{-7} and 4.21×10^{-2} M, respectively, providing ample opportunity for the accumulating lactate to influence the distribution of enzyme forms. Despite this, the calculated values for the equilibrium-corrected isotope effect (data not shown) are marginally closer to the expected V/K isotope effect than the broken line shown in Fig. 7.

CONCLUSIONS

The correctional equation derived by Bigeleisen and Wolfsberg (*5*) may be used to determine the V/K isotope effect with respect to isotopic discrimination of tritiated NADH for the reaction catalyzed by muscle lactate dehydrogenase. Within the limits of experimental error, this correction is valid up to at least 85% reaction. The validity of the correction may well extend beyond this extreme but the calculation becomes unreliable due to a sharp increase in the propagation of experimental error. Calculations from the first 15% of reaction are also unreliable, because experimental errors themselves are very large when only small amounts of a labeled product are formed.

The correction may only be applied when the expression of the isotope effect itself is unchanging during the course of reaction.[3] Since the V/K effect of isotopic discrimination of NADH by lactate dehydrogenase depends on the concentration

[3] In general, the expression of an isotope effect should be expected to change during the progress of an enzymatic reaction (a) in ordered mechanisms when the first substrate carries the isotopic label, as in lactate dehydrogenase, and (b) in steady-state random mechanisms with "sticky" substrates. Rapid equilibrium random and irreversible ping-pong half reactions should display isotope effects which are independent of changes in the concentration of cosubstrates.

of pyruvate which inevitably changes as the reaction proceeds, it is essential that the initial pyruvate to NADH ratio is large (e.g., at least 10) in order to make the change minimal.[4] Nevertheless, lower substrate ratios can sometimes be useful since the changing isotope effect as pyruvate declines can be used to estimate the limiting V/K isotope effect at zero pyruvate. The added complexity of the analysis is more than offset by the extra information that the experiment yields, which is a complete description of the dependence of the isotope effect on pyruvate concentration within a single experiment.

The effect of reaction reversibility on the correction appears very late in the time course of a reaction. The results of simulations suggest that equilibrium constants as low as 1.0 give tolerably small errors within the first half of reaction, provided the calculations of the fractional reaction and isotopic transfer are based on the final product at equilibrium rather than initial substrate.

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[4] An alternative experimental design, given a moderate to high Michaelis constant and not investigated here, is to set the initial concentration of second substrate much below its K_m, such that its concentration does not contribute to the commitment to catalysis of the first substrate. With this design, it is possible to preset the fraction of reaction to be undertaken, e.g., to 50% reaction, for example, by simply setting the concentration of the second substrate to half that of the first and allowing the reaction to run to completion (W. W. Cleland, personal communication).

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