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REVIEW

The Use of Isotope Effects in the Detailed Analysis of Catalytic Mechanisms of Enzymes

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Serious use of isotope effects to determine the catalytic mechanisms of enzymes began only a decade ago with the discovery of Northrop (*1*) that comparison of deuterium and tritium isotope effects on V/K could be used to calculate the intrinsic isotope effect on the bond-breaking step. Northrop's discovery led to development of the theory for isotope effects on enzyme-catalyzed reactions (*2–6*), including even better ways to determine intrinsic isotope effects (*7*) and a number of experimental studies that have provided information that would have been difficult to obtain any other way.

In addition to the expected ability to determine which step in a reaction is rate limiting, it has proven possible to tell stepwise from concerted reactions and, for the former, to tell which step comes first and thus the nature of the intermediate (*7*). The nature of catalytic groups on enzymes can be deduced from their fractionation factors, especially if they are thiols (*8, 9*). The variation of intrinsic isotope effects with redox potential of the substrates has been used to deduce transition state structures (*10, 11*) and the natures of transition states deduced from intrinsic isotope effects have been used to determine chemical mechanisms. While space limitation precludes an exhaustive review, we will give selected examples of each of these applications to illustrate the power of isotope effects in deducing mechanisms.

ISOTOPE EFFECT THEORY

We will give only a short introduction to isotope effect theory; the reader is referred to earlier articles for more thorough treatments, plus a discussion of the use of isotope effects to determine kinetic mechanisms and rate-limiting steps in enzyme-catalyzed reactions (*2–6, 12*). We will use the notation of Northrop (*13*), in which isotope effects are shown by a leading superscript (D, T, 13, 14, 15, and 18 corresponding to deuterium, tritium, ^{13}C, ^{14}C, ^{15}N, and ^{18}O).

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An isotope effect is caused by substitution of a heavy atom for a light one (deuterium or tritium for hydrogen; ^{13}C or ^{14}C for ^{12}C; ^{15}N for ^{14}N; ^{18}O for ^{16}O, etc.). *Kinetic* isotope effects are the ratio of reaction rates for molecules containing light and heavy isotopes such as k_H/k_D (written as ^Dk), while *equilibrium* isotope effects are the ratio of equilibrium constants. Isotope effects are *primary* when they involve cleavage or synthesis of a bond to the isotopic atom, and *secondary* when they do not. Values of kinetic or equilibrium isotope effects greater than unity are called *normal,* and ones less than unity are called *inverse.*

The *fractionation factor* of an atom in a molecule is the tendency of the heavy isotope to enrich in this position relative to a reference molecule (liquid water is the usual reference for deuterium, tritium, or ^{18}O isotope effects, while dissolved CO_2 or NH_3 are the usual references for ^{13}C or ^{15}N (*14*)). The fractionation factor is thus an equilibrium isotope effect. A value above unity shows that the heavy atom enriches in the compound relative to the reference molecule, while a value less than unity shows that it becomes depleted. The heavy atom becomes enriched in the molecule where it is bound more stiffly in terms of vibrational modes. Since fractionation factors depend largely on the local structure of the molecule, and atoms more than two bonds removed usually have no effect, it is practical to have tables of fractionation factors for common structures, and empirical rules for supplementing values based on experiments (*14*). For example, thiols have low fractionation factors relative to water (~0.5), and the deuterium content of an SH group in aqueous solution will be only half that in each end of the water molecules. The ^{15}N fractionation factor of NH_4^+ is 1.9% higher than that of NH_3, so ^{15}N enriches in NH_4^+. Fractionation factors for most molecules of interest are now available (*14, 15*).

An equilibrium isotope effect is the ratio of the fractionation factors of substrate and product, while a kinetic isotope effect is the ratio of the fractionation factors of substrate and transition state. Since a similar situation prevails in the reverse reaction, the ratio of kinetic isotope effects in the two directions is the equilibrium isotope effect for the overall reaction, because the transition state is the same regardless of the direction from which it is approached. Transition states generally have low fractionation factors for primary isotope effects because the vibrational motion that accomplishes the reaction has an imaginary frequency, that is, a negative force constant, and the isotopic atom is thus more weakly bonded in the transition state than in the substrate.

For secondary isotope effects, however, the bonding of the isotopic atom in the transition state is likely to be intermediate between that in substrate and that in the product, so that the kinetic isotope effect is normal one way and inverse in the other direction, with the value a guide to actual structure. Secondary isotope effects are known, however, where the bonding is stiffer in the transition state because of steric crowding than in either reactant, so that the isotope effect is inverse in both directions (*16*). By contrast, when a bending mode of an isotopic secondary hydrogen is coupled to the translation of a primary hydrogen, these vibrational modes couple and have no restoring force in the transition state, so that the secondary deuterium isotope effect is normal in both directions. This occurs commonly with dehydrogenases (*6, 11, 17*).

ENZYME KINETIC THEORY

For an enzymatic reaction there are two fundamental kinetic parameters: V (or V_{max}), the maximum velocity at extrapolated infinite substrate concentration, and V/K, the apparent first-order rate constant for reaction at low substrate concentrations. Most enzymatic reactions obey the rate law: $v = VA/(K + A)$, where v is observed velocity, A is substrate concentration, and K is the Michaelis constant (often written K_m), which is the substrate level giving a velocity half of V. This equation can also be written: $v = (V/K)VA/(V + (V/K)A)$ to emphasize that V and V/K are the fundamental constants, with K their ratio. For a detailed discussion of kinetic theory, see (*12*).

Isotope effects, if large enough, can be measured on both V and V/K separately (these are written ^{D}V and $^{D}(V/K)$) by varying either the labeled or unlabeled substrate concentrations and determining V and V/K separately for each from reciprocal plots in which $1/v$ is plotted vs $1/A$:

$$1/v = (K/V)(1/A) + 1/V.$$

In such a plot V/K is the reciprocal of the slope and V the reciprocal of the vertical intercept. This is the *only* way to determine isotope effects on V, and unless the isotope effects are equal on V and V/K (in which case the value can be determined with care to 1–2%), it is difficult to measure a value with precision unless it is at least 1.05 or larger. The direct comparison method is thus most useful for measuring deuterium isotope effects. We should mention that actual statistical analysis is carried out by nonlinear least-squares methods, using combined data for deuterated and unlabeled substrates (*18*).

The other major method for determining isotope effects is the internal competition method, where one follows the ratio of heavy to light atoms in either residual substrate or product or both as the reaction proceeds. This method of measurement gives only a V/K isotope effect, and further it is the V/K for the labeled compound (in direct comparison experiments one measures V/K for the substrate whose concentration is varied, regardless which one is labeled). This method must be used for tritium or ^{14}C isotope effects, since the labeled molecules are present at very low ratios compared with the unlabeled ones. It is also used with ^{13}C, ^{15}N, or ^{18}O isotope effects where the 1.1, 0.37, and 0.2% natural abundance levels of the heavy atoms can be used as the label. In this case one must isolate CO_2 (for ^{13}C or ^{18}O) or N_2 (by hypobromite oxidation of NH_3 for ^{15}N) and use an isotope ratio mass spectrometer to determine the mass ratio (*19*). The result obtained is, however, very precise (the value can be determined to 0.0001).

For ^{15}N isotope effects the nitrogen is easily removed by Kjeldahl digestion if it is the only one in the molecule. For ^{18}O the CO_2 must of course not be allowed to equilibrate with water, but if the reaction is run under high vacuum in aqueous solution, CO_2 that is formed enzymatically leaves rapidly enough not to exchange (*20*). The other approach used for ^{18}O (it will work for any label in an inaccessible position) is the remote label method in which one heavy atom is used as a correlated label with another (*21*). Thus to measure secondary ^{18}O isotope effects on phosphoryl transfer, glucose 6-phosphate was prepared with ^{13}C at C-1 and three

^{18}Os in the phosphoryl group (*22*). This was mixed with 99 times as much glucose 6-phosphate containing ^{12}C (that is, carbon depleted by two orders of magnitude below natural abundance in ^{13}C) at C-1. The final glucose 6-phosphate had the natural abundance of ^{13}C at C-1, but with every ^{13}C accompanied by ^{18}Os in the phosphoryl group. The isotope effect caused by ^{18}O was measured by following the change in mass ratio at C-1, which is easily removed by enzymatic oxidation to give CO_2 (*22*). Use of the remote label method greatly broadens the scope of the isotope ratio mass spectrometer, which is important because the precision obtained in these experiments is often needed for analysis of mechanisms.

The equations used to calculate the isotope effect in an internal competition experiment (here used for ^{13}C) are

$$^{13}(V/K) = \log(1 - f)/\log(1 - fR_p/R_0),$$

when the mass ratio in product (R_p) is compared with that in initial substrate (R_0), and

$$^{13}(V/K) = \log(1 - f)/\log[(1 - f)(R_s/R_0)],$$

when the mass ratio in residual substrate (R_s) is compared with R_0. The parameter f is the fractional reaction at the time of measurement. R_0 can be determined by running the reaction to completion and measuring the ratio in product if this is more convenient. For maximum information one measures mass ratios in both product and residual substrate in the same experiment. One thus has two independent determinations of the isotope effect, and if they agree, one can be confident of the results. It is possible, in fact, to determine the isotope effect solely from mass ratios in residual substrate and product by the equation

$$^{13}(V/K) = \log(1 - f)/\log[(1 - f)/(1 - f + fR_p/R_s)].$$

EQUATION FOR THE OBSERVED ISOTOPE EFFECT

It is rare in an enzymatic reaction for the isotope-sensitive step to be completely rate limiting, and when only one step is isotope sensitive, the observed V/K isotope effect is given by (*3*)

$$^{D}(V/K) = (^{D}k + c_f + {}^{D}K_{eq}c_r)/(1 + c_f + c_r),$$

where ^{D}k is the intrinsic isotope effect on the bond-breaking step, $^{D}K_{eq}$ is the equilibrium isotope effect on the overall reaction, and c_f and c_r are forward and reverse commitments. A commitment is the ratio of the rate constant for the isotope-sensitive step to the net rate constant for dissociation from the enzyme of (a) the varied substrate for c_f in a direct comparison experiment, (b) the labeled substrate for c_f in an internal competition experiment, or (c) the first product released (or first irreversible step, if this comes first) for c_r. Slow release of the substrate commits the reaction to take place, thus suppressing the isotope effect; while slow product release, or slow steps after the isotope-sensitive one but prior to product release, brings the chemical step to equilibrium, so that one observes the equilibrium isotope effect.

The equation for the isotope effect on V is similar, except that c_f is replaced by c_{Vf}, a constant made up of the sum of the ratios of the rate constants for the isotope-sensitive step and each other forward unimolecular net rate constant (*3*). Slow steps that follow first product release thus elevate c_{Vf} and suppress the isotope effect on V, while having no effect on $^D(V/K)$.

It is clear that for analysis of the mechanism it is preferable to minimize commitments. Slow alternate substrates are best for this because release of reactants is unlikely to be slow enough to give finite commitments. However, there may still be internal commitments which lead to finite values of c_f and/or c_r. Enzymatic reactions involve conformation changes following substrate binding that set the stage for catalysis. The minimum mechanism is thus

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA^* \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EPQ^* \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EPQ \xrightarrow{k_9} EQ + P,$$

where k_5 and k_6 are for the chemical step which is isotope sensitive, and the steps isomerizing EA and EPQ are conformation changes in the enzyme. The subsequent dissociation of EQ will affect V, but not V/K. In this mechanism,

$$c_f = (k_5/k_4)(1 + k_3/k_2) \qquad \text{and} \qquad c_r = (k_6/k_7)(1 + k_8/k_9).$$

While k_3/k_2 and k_8/k_9 will be small for slow alternate substrates (they can also be minimized by operating off of the pH optimum when catalysis, but not binding, is pH dependent (*4*)), k_5/k_4 and k_6/k_7 are often still finite.

As a result of commitments, most observed isotope effects are less than intrinsic ones, and ways must be found to estimate the intrinsic isotope effects and commitments. The first such method was that of Northrop (*1*), in which the fact that intrinsic tritium isotope effects are the 1.44 power of deuterium ones (*23*) was exploited by the use of the equation

$$[^D(V/K) - 1]/[^T(V/K) - 1] = [^Dk - 1]/[(^Dk)^{1.44} - 1].$$

This equation is exact only when c_r is zero, or $^DK_{eq} = 1.0$, and is not well conditioned unless $[^D(V/K) - 1]$ is 10–30% of $[^Dk - 1]$ (*24*), but has been useful with alcohol dehydrogenases where c_r is near zero (*4, 5*). It predicted a value of 5–8 for ^Dk for malic enzyme (*25*), while the exact value later turned out to be 5.7 (*26*).

More powerful methods of determining intrinsic isotope effects involve measurement of ^{13}C isotope effects with deuterated and unlabeled substrates when the same step is both deuterium- and ^{13}C sensitive (*7*). The equations for the two ^{13}C isotope effects and for the deuterium one contain four unknowns (^{13}k, ^Dk, c_f, and c_r), but the limits on possible values of ^Dk, and in particular ^{13}k, tend to be narrow and the solution becomes more exact the smaller the commitments become. In addition, the high precision available for ^{13}C isotope effects determined by the isotope ratio mass spectrometer leads to accurate values. In favorable cases measurement of a secondary deuterium isotope effect on the same step, plus the ^{13}C isotope effect with secondary deuterated substrate gives five equations in five unknowns, and thus an exact solution to all parameters (*7*).

DISTINGUISHING STEPWISE AND CONCERTED REACTIONS

One of the most powerful applications of isotope effects has been the distinction between stepwise and concerted reactions by measuring the effect of deuteration in one position on the size of a ^{13}C (or ^{15}N) isotope effect at another position (*7*). If the isotope effects are on the same step, that is, the mechanism is concerted, deuteration will slow down this step and make it more rate limiting; that is, c_f and c_r for measurement of the ^{13}C isotope effect are both decreased. The result will be a higher observed ^{13}C isotope effect or no change if commitments were zero to start with.

On the other hand, in a stepwise mechanism deuteration slows down a step *other* than the ^{13}C-sensitive one, and thus *increases* one of the commitments and necessarily reduces the size of the observed ^{13}C isotope effect. However, the equations for the ^{13}C isotope effect with deuterated and unlabeled substrates and for the deuterium isotope effect are now no longer independent, but are related in the following way when the deuterium-sensitive step comes first,

$$[^{13}(V/K)_H - 1]/[^{13}(V/K)_D - 1] = {}^D(V/K)/{}^DK_{eq},$$

and by the following equation in the reverse direction when the ^{13}C-sensitive step comes first,

$$[^{13}(V/K)_H - {}^{13}K_{eq}]/[^{13}(V/K)_D - {}^{13}K_{eq}] = {}^D(V/K).$$

Since ${}^DK_{eq}$ values usually differ from unity far more than ${}^{13}K_{eq}$ ones, these equations are sufficiently different to tell which one is fitted by the experimental data.

Attempts have been made to use the effect of deuteration in one position on another deuterium isotope effect to distinguish stepwise from concerted mechanisms (*27*). This requires a knowledge of the fractionation factors of the enzymic bases involved in the reaction, and is subject to the uncertainty caused by coupled hydrogen motions in concerted reactions (*17*), and is thus of more limited application than the effect of deuteration on ^{13}C or ^{15}N isotope effects.

Malic Enzyme

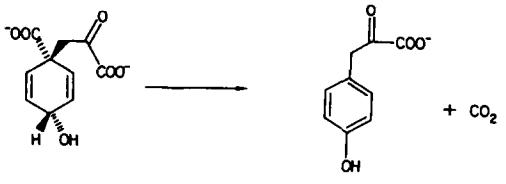
To no one's great surprise, chicken liver malic enzyme was shown to catalyze dehydrogenation prior to decarboxylation when the ^{13}C isotope effect at C-4 was measured with 2-deuterated and unlabeled malate (*7*), and similar results have been obtained with 6-phosphogluconate dehydrogenase (*28*) and isocitrate dehydrogenase (*29*). With chicken malic enzyme it was possible to add TPNH and oxaloacetate and regenerate an active intermediate complex that partitioned to both pyruvate by decarboxylation and to malate by reduction (*26*). The pyr/mal ratio was 0.47 with unlabeled TPNH and 0.81 with TPND. Together with the ^{13}C and primary deuterium and tritium isotope effects, these partition ratios permitted an exact solution for the intrinsic isotope effects and partition ratios in this system (see (*26*) for the equations used in this analysis). The ^{13}k value was 1.044 or 1.045 when calculated from the partition ratios with TPNH or TPND, respectively, while the ${}^Dk$ value was 5.7 ± 0.3, the forward commitment to hydride transfer was

3.3 ± 0.4, and reverse hydride transfer was 10 ± 1 times the rate of decarboxylation, which was assumed to be irreversible and not have a reverse commitment.

With acetylpyridine-TPN, which has a higher redox potential than TPN so that the equilibrium is more favorable for hydride transfer, the ^{13}k value was 1.040, and the pyr/mal ratio from the partitioning experiment was 9.9 (*26*). Clearly the transition state for decarboxylation of the oxaloacetate intermediate is very similar to that with TPN, as one might expect, but partly because of the redox potential change, reverse hydride transfer is now only ~40% as fast as decarboxylation. While decarboxylation is much more rate limiting than hydride transfer with TPN, the opposite is true with acetylpyridine-TPN.

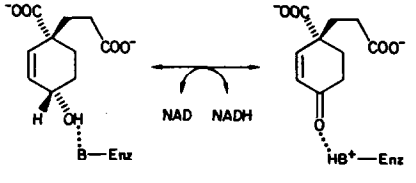
Prephenate Dehydrogenase

A real eye opener was the result with prephenate dehydrogenase, which catalyzes the oxidative decarboxylation of prephenate to *p*-hydroxyphenylpyruvate (*30*):



Deoxoprephenate, which lacks the keto group in the side chain, has a V value 80% that of prephenate, and a Michaelis constant only four times greater. When ^{13}C isotope effects in the CO_2 product were measured with 4-deuterated and unlabeled deoxoprephenate, the values of 1.0103 and 1.0033, together with the $^{D}(V/K)$ value of 2.34, showed (a) that the reaction is *concerted* and (b) that ^{D}k was 7.3, ^{13}k was 1.0155, and c_f was 3.7 (a zero reverse commitment was assumed, because of the irreversibility of the reaction).

When one double bond was removed from the ring, the enzyme simply oxidized the resulting substrate reversibly without decarboxylation:



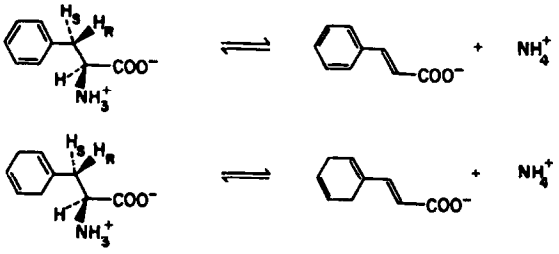
Although the concerted oxidative decarboxylation of prephenate violates all the rules, since the hydride and CO_2 leave from different sides of the ring, the energy released by aromatization is so great that C–C bond cleavage begins before hydride transfer is complete. The decarboxylation is thus enzyme catalyzed only in the sense that the enzyme provides a nonaqueous pocket for the carboxyl group to sit in. It is the inherent instability of the dehydrogenated substrate that causes decarboxylation, and the partially hydrogenated substrate is not decarboxylated

(the fully hydrogenated molecule is also a very slow substrate that is not decarboxylated).

The size of the isotope effects with deoxoprephenate, however, suggests that while the reaction is concerted, it is not synchronous, and that in the transition state C–H bond cleavage is much farther advanced than C–C cleavage. It is interesting to contrast the concerted enzymatic reaction with the nonenzymatic acid-catalyzed decarboxylation of prephenate or deoxoprephenate to phenylpyruvate or phenylpropionate. These reactions occur by a stepwise mechanism with a finite rate of return of the carbonium ion intermediate (*30*). The difference here may lie in hydration of the carboxyl group in water; decarboxylation presumably requires breaking these hydrogen bonds, and thus gives the carbonium ion a finite lifetime in the nonenzymatic reaction.

Phenylalanine Ammonia Lyase

Another surprise concerning mechanism was provided by ^{15}N and deuterium isotope effect studies on phenylalanine ammonia lyase (*15*), which catalyzes the elimination of ammonia and the pro-3*S* hydrogen from phenylalanine or its dihydro analog to give *trans*-cinnamate or dihydrocinnamate:



This enzyme contains a dehydroalanine prosthetic group to which the monoanionic form of the substrate binds covalently to give a protonated secondary amine (*31*). The first product released is cinnamate, and ammonia release from dehydroalanine completes the reaction.

Phenylalanine was found to be a sticky substrate with a finite external commitment, that is, $k_3 > k_2$ in mechanism 1, and the $^{D}(V/K)$ value was only 1.15. The dihydro analog gave a $^{D}(V/K)$ value of 2.0 and was not sticky (V was 7% that of phenylalanine). ^{15}N isotope effects were pH dependent with both substrates because the monoanion was the active substrate, and $^{15}K_{eq}$ for deprotonation is 1.016. Extrapolated $^{15}(V/K)$ values for the monoanion were 1.0047 and 0.9921 for unlabeled and deuterated dihydrophenylalanine, while the corresponding values for phenylalanine were 1.0021 and 1.0010.

These data are consistent only with a stepwise mechanism with a carbanion intermediate (see (*15*) for the equations for concerted, carbanion, and carbonium ion mechanisms). Further, they require that $^{15}K_{eq}$ for addition of substrate to dehydroalanine to give the secondary amine be 0.979 and suggest that intrinsic ^{15}k values for C–N cleavage are 1.03–1.04, and ^{D}k values for C–H cleavage are 4–6. The sum of commitments is ~3.2 with dihydrophenylalanine, and an order of

magnitude higher, including an external portion greater than 3, for phenylalanine.

This study was undertaken because it was felt likely that the mechanism was *not* a carbanion one because of the absence of obvious ways to delocalize the electrons in the carbanion intermediate; for elimination reactions like those catalyzed by fumarase, enolase, and aconitase, strong inhibition by ionized nitronate analogs of the substrates suggests that the carbanion intermediates assume aci-carboxylate structures (*32–34*). Apparently the phenyl ring or the single conjugated double bond in the dihydro analog is adequate to permit electron delocalization in the carbanion. It is intriguing to note that the fully saturated analog of phenylalanine is not a substrate, but is bound in a way that suggests that a secondary amine adduct may form. The total absence of ways to delocalize electrons in a carbanion from this intermediate presumably prevents reaction in this case.

Liver Alcohol Dehydrogenase

An interesting example of a stepwise mechanism deduced from isotope effects other than combinations of heavy atom and deuterium ones is that of alcohol dehydrogenases. For most dehydrogenases the observed isotope effects stay the same or increase (when external commitments are eliminated) as one moves off of the pH optimum (*4*). With liver alcohol dehydrogenase, however, V/K isotope effects with deuterated cyclohexanol were 2.5 up to pH 8, but decreased to a limiting value of 1.0 above a pK of 9.4 (*5*). The values of $^{D}(V/K)$ for cyclohexanone were lower by a factor of 1.18 (the equilibrium isotope effect) at each pH value, and thus became 0.85 at high pH, despite the fact that V/K for cyclohexanone decreases sharply above a pK of 8.8. These data show that hydride transfer and proton transfer are separate steps in the mechanism, unlike the case for most dehydrogenases, where the two are concerted processes (*4*).

If one starts with cyclohexanone and DPNH, hydride transfer occurs and comes to equilibrium at high pH, thus, $^{D}(V/K) = 0.85$, because the catalytic base (probably His-51) is not protonated and thus the Zn-alkoxide intermediate formed by hydride transfer cannot obtain a proton and dissociate. At neutral pH where His-51 is protonated, the Zn-alkoxide intermediate undergoes hydride transfer 2.5 times faster than protonation and subsequent release of alcohol, and this ratio constitutes the only visible commitment in the system with cyclohexanol as substrate (*5*).

DEDUCING THE NATURE OF CATALYTIC GROUPS FROM FRACTIONATION FACTORS

An important part of a chemical mechanism is the acid–base chemistry involved. pH profiles have been used to deduce the existence of groups having required protonation states (*35*), but it is easier to demonstrate the existence of such groups than identify them, especially when no X-ray structure of the enzyme is available. One interesting application of isotope effects is their use to prove the nature of acid–base catalytic groups on enzymes by measuring their fractionation

factors. This works particularly well when the groups are thiols because of their low fractionation factor of ~0.5, but the method can of course be applied in reverse to eliminate SH groups from consideration when the fractionation factor is ~1.0 or higher. We will discuss several examples where the existence of thiols has been demonstrated in this way.

Proline Racemase

This enzyme which catalyzes the interconversion of D- and L-proline has been subjected to intensive study by Knowles and co-workers (*8, 27, 36–40*). The free enzyme exists in two states, one of which can combine with D-proline and the other with L-proline, and it appears that the difference is simply the protonation state of the two catalytic bases on the enzyme, one of which must be protonated and the other unprotonated for catalytic activity. Interconversion of the free enzyme forms is normally 40 times faster than V, but in the presence of high levels of substrates the levels of free enzyme forms are so reduced that their interconversion can be made rate limiting. During the interconversion, tritium is not transferred between the two catalytic bases, but is lost to the solvent.

Deuterium and tritium isotope effects were measured by a variety of methods and the deuterium isotope effect was shown to be 3.1 for L-proline and 2.7 for D-proline; that is, the ratio is 0.86 (*40*). This appears at first to be impossible, since ${}^{D}K_{eq} = 1.0$ and the ratio of ${}^{D}(V/K)$ values has to equal ${}^{D}K_{eq}$. However, *the isotope effects do not relate to opposite directions of the same reaction.* Starting with deuterated L-proline one makes unlabeled D-proline and HDO, while starting with deuterated D-proline one makes unlabeled L-proline and HDO. Both reactions are irreversible as far as the label goes, because of the large concentration of H_2O.

A very clever isotope effect experiment was then used to determine the fractionation factors of the enzymic bases that remove the proton from proline or donate it to the carbanionic intermediate in the reverse reaction. The system was set up with high levels of reactants so that free enzyme interconversion was rate limiting. Equimolar D- and L-proline were present, but one enantiomer was partly or wholly deuterated at C-2, so that its reaction was slower, and the reaction initially was perturbed from equilibrium. The time course of the reaction showed that the rate of achievement of the maximum perturbation was slower when the labeled proline enantiomer was only partly deuterated than when it was fully substituted. This difference shows that the fractionation factor of the enzymic base which became deuterated in the reaction was *less* than that of carbon-bound hydrogen in the substrate bound to the enzyme, so that with D- and L-proline interconversion at equilibrium on the enzyme (free enzyme interconversion from E′ back to E was rate limiting), deuterium was enriching in the substrate in the E–S complex, rather than in the enzymic base in the E′–P and E′ enzyme forms. When D- or L-proline was originally fully deuterated, all of E′–P and E′ were of course deuterated. The calculated fractionation factors of the enzymic bases were ~0.55, which is only consistent with their being sulfhydryl groups (*8*).

A similar low fractionation factor in free enzyme was deduced from the failure to see a solvent isotope effect (H_2O vs D_2O) when low nonsaturating levels of L-

proline were used (*8*). This experiment shows that there is no isotope effect on the transfer of hydrogen from the catalytic base (which is deuterated in D_2O) to proline. Since the reverse step gives an isotope effect of 2.7, the fractionation factor of the catalytic base is ~0.44 (that of proline is 1.17, and 1.17/2.7 = 0.44). While this value of ~0.44 for the free enzyme may not be significantly different from that of 0.55 ± 0.10 for enzyme complexed with D- or L-proline, it is possible that torsional restriction of rotation of the SH group around the C–S bond may raise the fractionation factor in the presence of D- or L-proline as the result of steric crowding.

To assess the importance of this effect, we have calculated the result of varying the torsional force constant on the fractionation factor of the SH hydrogen in methanethiol (Fig. 1). These calculations were made with the BEBOVIB-IV program of Sims *et al.* (*41*) using the force constants for methanethiol (*42*). Changing the torsional force constant over the range shown in Fig. 1 (0.006 to 0.4 mdyn Å rad^{-2}) altered the torsional frequency from 166 to 1355 cm^{-1}, but had little effect on other vibrational frequencies of the molecule. The calculated fractionation factors are for methanethiol relative to water in the gas phase at 25°C. Values relative to liquid water will be lower by a factor of 1.079 (*43*).

There should be little torsional restraint to an SH group in free enzyme, since there is not appreciable hydrogen bonding between this group and water, which is all that the active site contains. The presence of D- or L-proline, however, may lead to torsional restriction, and this would have the advantage of keeping the hydrogen of the SH group in the correct position for reaction. An increase in the torsional force constant from ~0.006 to ~0.2 mydn Å rad^{-2} would match the experimentally observed values.

Torsional restriction has been considered in the acetylcholinesterase-catalyzed hydrolysis of acetylcholine (*44*), but because of the absence of an isotope effect with methylene-deuterated substrates, it was concluded not to be important in that case. Torsional constraint may, however, be the cause of the high fraction-

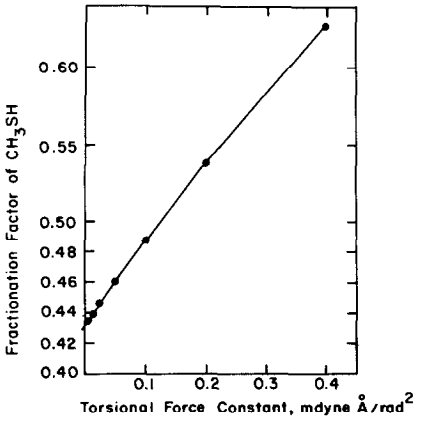


FIG. 1. Fractionation factor of the SH hydrogen of methanethiol relative to water as a function of torsional force constant. The calculations are for the gas phase at 25°C.

ation factor of the catalytic carboxyl group of fumarase (~1.2) which is protonated in the E–carbanion intermediate (*45*).

When the interconversion of free enzyme forms of proline racemase was made rate limiting by high D- and L-proline levels, the deuterium isotope effect on enzyme form isomerization was 2.4–2.8, suggesting a transition state structure very much like that for the racemization of proline (*37*). Free enzyme isomerization (rate $\sim 10^5$ s^{-1}) is catalyzed by small buffers with p*K*s in the range of 5–11 such as ammonia, hydrazine, hydrogen sulfide, and hydroxylamine, but not by bulky ones that could not fit in the active site. It thus appears that both D- and L-proline isomerization and free enzyme isomerization involve reactions in which one thiol removes a proton from the bound reactant in the active site (proline, water, or one of the small buffers) and the other thiol donates a proton to the resulting intermediate (carbanion of proline, hydroxide, or the deprotonated form of the buffer). On the basis of the isotope effects, it appears that in the transition states for these transfers the hydrogens are very nearly on the thiols, with very little S–H bond breaking. This is what one expects on the basis of the p*K*s of the groups involved.

Adenosine Deaminase

An interesting application of isotope effects in deducing the nature of catalytic groups on enzymes is a recent study of adenosine deaminase in this lab (*9*). The *V*/*K* profile for adenosine drops both at low and high pH, suggesting that two catalytic groups on the protein are involved in the reaction. The chemistry almost certainly involves tetrahedral adduct formation at C-6, either by addition of water or of a group such as cysteine from the enzyme. Elimination of ammonia then gives either inosine directly, or via another cycle of water addition and enzyme group release. Since 1-deazaadenosine is tightly bound (K_i, 2 μM) but not a substrate, while 3-deazaadenosine is not bound at all at 100 μM levels (*46*), it appears that N-1 is involved in catalysis (it is protonated in inosine) and N-3 in binding.

The enzyme is unusual in showing an inverse D_2O solvent isotope effect (reported as 0.86 (*47*); 0.77 in our hands), while ^{15}N isotope effects were 1.0040 in water and 1.0023 in D_2O. This *decrease* in the ^{15}N isotope effect in D_2O at the same time that the reaction is going faster puts tight restraints on the mechanism. A solution to the equations for the isotope effects does exist; however, if ^{15}k for C–N cleavage to eliminate NH_3 is 1.026, the partition ratio of the tetrahedral intermediate toward NH_3 release as opposed to back reaction to the original binary complex of enzyme and adenosine is 1.4, and the product of the $^{D}K_{eq}$ value for forming the tetrahedral adduct and the ^{D}k value for its breakdown by C–N cleavage is 0.45. The ^{15}k value is a little less than expected, since $^{15}K_{eq}$ for C–N cleavage will be 1.0333 (*15*), and ^{15}k in the reverse direction for C–N bond formation should be slightly normal, since reaction coordinate motion of the nitrogen is involved. In view of the errors involved, however, it is not clear that the value of 1.026 is significantly less than 1.0333.

A value of 0.45 for the product of $^{D}K_{eq}$ for tetrahedral adduct formation and the ^{D}k value for its breakdown are surprises. The value of ^{D}k certainly should be

somewhat normal, since the secondary deuterium isotope effect on the hydrogens of the $-NH_3^+$ group is normal, as would be any primary deuterium isotope effect if other proton motions are involved. While conversion of the $-NH_2$ group of adenine into a $-NH_3^+$ group elevates the fractionation factors of the protons from 0.95 to 1.06 (*48*) and conversion of water to an –OH group (if the adduct involves water addition) will add a factor of 1.12 (*49*), these effects alone could not produce a $^DK_{eq}$ value lower than 0.68. The enzymic group donating the additional proton needed for tetrahedral adduct formation (1 proton on N-1, 3 on the $-NH_3^+$ group, 1 on the –OH group, compared to 2 on the $-NH_2$ group of adenine and 2 in H_2O) thus must have a very low fractionation factor. The only such group on proteins is the sulfhydryl group of cysteine, with a fractionation factor of ~0.5. If such a group donates the extra proton needed for tetrahedral adduct formation, $^DK_{eq}$ for this step could be as low as 0.34, which is consistent with the observed value.

These conclusions were reinforced when isotope effects were measured with the slow substrate 8-oxoadenosine. The D_2O solvent isotope effect here was 0.45(!), while the ^{15}N isotope effects were 1.0150 and 1.0131 in H_2O and D_2O. These numbers give $^{15}k = 1.0335$ for C–N cleavage, 0.10 for the partition ratio of the tetrahedral intermediate, and 0.39 for the product of $^DK_{eq}$ on adduct formation and ^Dk for its breakdown. These parameters are probably not significantly different from those of adenosine except for the partition ratio, which is 14-fold lower, showing that C–N bond cleavage is now much more rate limiting.

These results clearly confirm the conclusions concerning the involvement of a sulfhydryl group in the reaction. It is not clear whether the role of the sulfhydryl group is to donate a proton to N-1 or to form a covalent adduct at C-6 during the reaction, but the fact that the enzyme catalyzes the reversible hydration of pteridine suggests that the former mechanism is more likely. The tight binding of 1-deazaadenosine suggests that a hydrophilic group such as a carboxyl or histidine is not involved in protonating N-1, but replacing a nitrogen with a carbon next to a sulfhydryl group which does not form hydrogen bonds would lead to tighter binding, as observed. Finally, in the V/K pH profile for 8-oxoadenosine the displacement in D_2O of the pK of the enzyme group with pK 8.45 is only 0.15 pH units, while that of the group with pK 5.24 is 0.75 units. These values are consistent with a sulfhydryl group with pK 8.45 having to be protonated for reaction, and a carboxyl or histidine group of pK 5.24 having to be ionized, presumably to provide general base assistance to addition of water at C-6.

Biotin-Containing Carboxylases

Isotope effects have recently been used to study the mechanism of biotin-containing enzymes. The ^{13}C isotope effect in the carboxylation of pyruvate to oxaloacetate by transcarboxylase was measured by converting the oxaloacetate product to malate with malate dehydrogenase, and later isolating it and decarboxylating it with malic enzyme to give CO_2 for isotope ratio mass spectrometry (*50*). The other subunit reaction (malonyl-CoA and acetyl-CoA, which react 70% as fast as methylmalonyl-CoA and propionyl-CoA, were used) had a rate at least 10 times as great as the rate of production of oxaloacetate, and so was nearly at equilibrium

during the experiments. The $^{13}(V/K)$ value was 1.023 with unlabeled, and 1.014 with trideuterated pyruvate, while the $^{D}(V/K)$ value was 1.4. These data show that the mechanism must be stepwise and cannot be concerted as had been postulated on the basis that the reaction showed retention of configuration (*51*).

Similar studies have been carried out with pyruvate carboxylase, using the decarboxylation of oxaloacetate catalyzed in the presence of oxamate (*52*). This is a ping-pong reaction occurring solely at one subunit of the enzyme in which carboxybiotin formed from oxaloacetate is induced to decarboxylate by oxamate (*53*). While the decarboxylation of carboxybiotin is slower than its formation from oxaloacetate, this has no effect on the V/K isotope effects, since slow steps after pyruvate release do not affect the V/K for oxaloacetate.

$^{13}(V/K)$ values were 1.0323 in H_2O and 1.0252 in D_2O (*54*). The D_2O solvent isotope effect was 2.1 with unlabeled oxaloacetate, while the secondary deuterium isotope effect with dideuterated oxaloacetate was 1.7. These values agree well with the $^{D}(V/K)$ value of 2.8 with trideuteropyruvate in the reverse reaction, after allowance for $^{D}K_{eq}$.

These isotope effects show that the reaction must be stepwise. Further, the large secondary deuterium isotope effect, which exceeds the equilibrium isotope effect for enolpyruvate formation, suggests that enolpyruvate is an intermediate, and that during the protonation of enolpyruvate to give ketopyruvate the bending motion of the secondary hydrogens is coupled to the translation of the primary hydrogen and becomes part of the reaction coordinate motion. As noted earlier, this coupling of hydrogen motions appears to be a common phenomenon.

Three basic stepwise mechanisms were considered for the carboxylation of biotin by oxaloacetate (*54*). The first involved decarboxylation of oxaloacetate to CO_2 and enolpyruvate, followed by proton transfer from N-1 of biotin to enolpyruvate, and finally carboxylation of enolbiotin to give carboxybiotin. The second involved decarboxylation of oxaloacetate and transannular C–S bond formation in biotin to make N-1 tetrahedral and nucleophilic, as has been postulated to occur in acid-catalyzed proton exchange at N-1 (*55*), so that it would react with CO_2 to form a carbamate. Proton transfer to enolpyruvate completes the reaction. The third mechanism involves proton removal from N-1 of biotin to enolize it by a base on the enzyme, followed by decarboxylation of oxaloacetate and carboxylation of biotin, and finally reprotonation of enolpyruvate by the enzymic base. These three mechanisms show (a) ^{13}C-sensitive steps flanking a central deuterium-sensitive one, (b) ^{13}C-sensitive steps preceding the deuterium-sensitive one, and (c) deuterium-sensitive steps flanking central ^{13}C-sensitive ones.

The first mechanism predicts a much lower $^{13}(V/K)$ value in D_2O than observed. The second also predicts such a low value unless the ^{13}C fractionation factor of the zwitterionic carbamate intermediate is 0.5% less than that of CO_2. The measured fractionation factor of a carbamate is 1.2% *higher* than that of CO_2, however (*54*). The third mechanism can be made to fit the data only if the fractionation factor of the enzymic base is ~0.6 or less. This suggests a SH group, but since the group starts out ionized prior to reaction and the V/K is pH independent, the pK of the SH group would have to be <5. Such values are known (~4 in papain (*56*)), but only when the cysteine is in an ion pair with a protonated group. Thus a Cys–Lys

ion pair in the free enzyme which was pulled apart by biotin binding to give an ionized thiolate as the base to remove the proton from N-1, and a protonated lysine to assist in the enolization by protonating the ureido oxygen fits the data. Pyruvate carboxylase does contain an SH group specifically alkylated by bromopyruvate (*58*), but further evidence is needed to confirm this mechanism suggested by the isotope effects.

DETERMINATION OF TRANSITION STATE STRUCTURE

An ultimate understanding of the chemical mechanism of a reaction includes a description of the transition state. While physical organic chemists have been deducing transition state structures for some time (*58*), only recently have serious attempts been made to do this for enzymatic reactions. One needs, of course, to have intrinsic isotope effects to work with, so one is limited either to reactions where these can be deduced by the methods described above or to cases where commitments are low enough that intrinsic isotope effects can be observed directly. One also needs isotope effects for all of the atoms in the vicinity of the chemical changes in order to do a complete job. While this approach should prove very powerful in the future, only a few cases have been studied to date. Proline racemase is one, and we have discussed above the transition state structure deduced for this reaction (*36*). Other examples are discussed below.

Catechol-O-Methyltransferase

This enzyme catalyzes the transfer of methyl groups from *S*-adenosylmethionine to various acceptors. Commitments are thought to be low, so that intrinsic isotope effects are observed. In the reaction with 3,4-dihydroxyacetophenone the secondary deuterium isotope effect on V for a trideuterated methyl group was 0.83 ± 0.05, while the primary ^{13}C isotope effect on V was 1.09 ± 0.05 (*16*). In similar nonenzymatic reactions, however, the secondary deuterium isotope effects ranged from 0.97 (methoxide as the acceptor) to 1.17 (water), while the primary ^{13}C isotope effect was still 1.08 with methoxide (*59, 60*). These data have been compared with extensive calculations for various transition state structures (*61*), and it was concluded that the transition states for enzymatic and nonenzymatic reactions differed in that those of the former were more compressed (bond orders close to 0.5 along the reaction coordinate, with a C–S distance of ~2.0 Å and a C–O bond distance of ~1.6 Å), while the latter (for transfer to water) had bond lengths ~0.15 Å longer (bond orders ~0.3 to the methyl group being transferred). A major portion of the rate acceleration by the enzyme thus may result from such compression of the reaction coordinate.

Transition States for Dehydrogenases

For several of these enzymes sufficient isotope effects are available for deductions to be made about transition state structure. Intrinsic isotope effects and commitments have been determined for glucose-6-phosphate dehydrogenase by

the use of ^{13}C isotope effects with primary and secondary deuterated substrates in both H_2O and D_2O (*17*). While the ^{13}k value at C-1 of glucose 6-phosphate (1.041) was not appreciably changed in D_2O, the primary ^{D}k value was 5.3 in H_2O and 3.7 in D_2O, and the secondary ^{D}k value for deuteration at C-4 of the nucleotide substrate dropped from 1.05 to 1.0 ($^{D}K_{eq}$ is 0.89 for the secondary position). These data were interpreted as showing simultaneous coupled motion of three hydrogens in the transition state (translation of the hydride transferred to the nucleotide from C-1, movement of the proton transferred from the 1-hydroxyl to the catalytic base on the enzyme, and the bending motion of the secondary hydrogen at C-4 of the nucleotide). Further, tunneling must be involved to see the decrease in deuterium isotope effects in D_2O where the OH group is an OD group (*62, 63*).

Transition state structures have been investigated as a function of the redox potential of the nucleotide substrate with liver alcohol (*10*) and formate dehydrogenases (*11*). For both enzymes the transition state appeared late with DPN, but became earlier as the redox potential of the nucleotide became more positive. Thus with benzyl alcohol and liver alcohol dehydrogenases, ^{13}k values dropped from 1.025 to 1.012 and ^{D}k values increased from 4 to 6.3 in going from DPN ($E^{\circ\prime} = -0.320\ v$) to acetylpyridine-DPN ($E^{\circ\prime} = -0.258\ v$) (*10*). With formate dehydrogenase, ^{13}k values dropped from 1.042 to 1.036, and ^{D}k values increased from 2.17 to 3.32 in changing from DPN to acetylpyridine-DPN (*11*). In addition, the secondary deuterium isotope effect at C-4 of the nucleotide was 1.23 for DPN, but only 1.06 with the acetylpyridine analog, but in both cases the value dropped half way to the equilibrium isotope effect (0.89) when deuterated formate was the substrate, showing the coupling of hydrogen motions in the transition state, along with tunneling.

The nonenzymatic oxidation of formate by I_2 in dimethyl sulfoxide (DMSO) shows an interesting and related series of changes in transition state structure as the mole fraction of water increases (*11*). ^{13}k rises from 1.0159 to 1.036 in going from DMSO to water; ^{D}k rises from 2.2 to 3.8, and the rate decreases by seven orders of magnitude. The transition state is clearly very early in DMSO and gets later and more symmetrical in water, and the values in water are remarkably close to the enzymatic ones with acetylpyridine-DPN. What is not clear in these studies is how the transition state structures for the enzymatic reactions can change as much as they appear to without the predicted changes in rates. Further work is clearly needed on these systems.

Mechanism of Phosphoryl Transfer

Arguments have raged for years about the mechanism of phosphoryl group transfer in enzymatic reactions. In nonenzymatic systems, phosphoryl transfer reactions occur only from monoprotonated phosphate esters, unless the leaving groups have particularly low p*K*s (*64*), and the mechanisms are thought to be dissociative, although whether free metaphosphate is an intermediate or the reaction involves an expanded transition state with low axial bond order is still being investigated.

In enzymatic systems, however, dianions are normally the substrates, and for

kinases at least the transferred phosphoryl group is coordinated to Mg^{2+}, which does not appreciably catalyze nonenzymatic reactions. Thus it has been postulated that enzymatic reactions are associative, or S_N2, in character. Recent studies show that phosphorylated thiols, which hydrolyze very readily when monoprotonated by a dissociative mechanism, are good substrates for alkaline phosphatase and phosphoglucomutase, but not for kinases, suggesting that there may be different mechanisms for the two classes of enzymes (*65*).

This problem is being investigated by the use of secondary ^{18}O isotope effects in the transferred phosphoryl group. Calculations show that dissociative mechanisms will have slightly inverse or very small normal isotope effects, while associative ones will have larger normal ones (*22*). The nonenzymatic hydrolysis of glucose 6-phosphate at 100°C, pH 4.5, which should have a dissociative mechanism, gave an isotope effect of 1.0004 per ^{18}O, using remote labeled material in which the mass ratio at C-1 was used as an indicator for discrimination caused by ^{18}O in the phosphoryl group (*22*).

The same remote labeled glucose 6-phosphate has been used with alkaline phosphatase at pH 8, which is on the pH optimum, and at pH 6, which is below the pK of the V/K profile. After correction for the ^{18}O isotope effects on deprotonation to the dianion (*66*), which is the actual substrate, the isotope effects were 0.9943 at pH 6 and 0.9983 at pH 8 (*9*). Both values may contain a contribution from an equilibrium isotope effect on binding to the enzyme, but the ratio between the two values (0.996) should reflect the effect of making catalysis more rate limiting by changing the pH. The fact that this ratio is inverse strongly supports a dissociative mechanism for phosphoryl transfer catalyzed by alkaline phosphatase, and such a conclusion is consistent with the fact that phosphorylated thiols like 6-thioglucose 6-phosphate are excellent substrates.

Similar ^{18}O isotope effect experiments are now underway with hexokinase, which does not use 6-thioglucose 6-phosphate as a substrate well at all (rate down by 10^5). Calculations suggest that an associative, or S_N2 mechanism for phosphoryl transfer might give secondary ^{18}O isotope effects as large as 1.04 (for three ^{18}O), in contrast to the inverse or very slightly normal values with dissociative mechanisms. This method, which looks directly at transition state structure, should prove very useful in determining the mechanisms of enzymatic phosphoryl transfer mechanisms.

AMP Nucleosidease

This enzyme catalyzes the hydrolysis of AMP to adenine and ribose 5-phosphate and shows allosteric activation by MgATP, with the V 200 times higher in its presence than in its absence, although the K_m for AMP is not much changed. Commitments are thought to be low, so that intrinsic isotope effects are observed. Isotope effects have been measured for the acid-catalyzed hydrolysis of AMP and the enzymatic reaction without and with MgATP (*67*). All were measured by the internal competition method using 5′-tritium or ^{14}C labels as markers for labeled or unlabeled species, and measuring changes in tritium/^{14}C ratios. The ^{14}C isotope effect at C-1′ of ribose was 1.044 in acid, but 1.035 and 1.032 without and with

ATP in the enzymatic reaction. The α-secondary deuterium isotope effects at C-1′ of ribose were 1.123, 1.045, and 1.030 in the same series, while the primary ^{15}N isotope effects at N-9 of adenine were 1.030, 1.030, and 1.025, and the β-secondary deuterium isotope effects at C-2′ of ribose were 1.077, 1.061, and 1.043.

An extensive series of calculations was carried out to match these experimental isotope effects and thus deduce transition state structures (*68*). The transition states were concluded to have considerable oxycarbonium ion character at C-1′ of ribose, but to be S_N2 in nature with water as a nucleophile, but with low bond order along the reaction coordinate. The bond order between C-1′ and the ring oxygen of ribose was concluded to be 1.91 for acid hydrolysis, but 1.73 or 1.77 in the enzymatic reaction without or with MgATP. The degree of hyperconjugation varied somewhat (5, 4, or 3% increase in bond order between C-1′ and C-2′ in the series). The bond order to the water from C-1′ of ribose was small (0.02 in acid; 0.03 in the enzymatic reactions), but there was considerable variation in C-1′ to N-9 bond order. This was 0.02 in acid (thus almost a fully dissociative mechanism), but 0.16 without ATP and 0.21 in its presence for the enzymatic case.

The enzyme clearly leads to an earlier transition state, postulated to result partly from the presence of negatively charged groups to stabilize the positive charges on the ring oxygen of ribose and N-7 of adenine. The allosteric activation leads to a still earlier transition state, possibly as the result of movement of the negatively charged groups stabilizing the transition state.

CONCLUSIONS

Space has only allowed a sampling of the many isotope effect studies on enzymes which are now appearing, but hopefully the reader will take home the lesson that isotope effects can be a very powerful tool for the study of enzyme mechanisms. In fact, because of the controlled geometry and lack of side reactions, we may eventually understand transition state structures for enzymatic reactions better than for nonenzymatic reactions in solution. For the present, the theory is developed, the methods are at hand, and it only remains for workers to exploit them.

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