Journal of Labelled Compounds and Radiopharmaceuticals
J Label Compd Radiopharm 2007; **50**: 1006–1015.
Published online 19 June 2007 in Wiley InterScience
(www.interscience.wiley.com). DOI: 10.1002/jlcr.1390

JLCR

Review Article

Use of isotope effects to determine enzyme mechanisms†

W. W. CLELAND*

Institute for Enzyme Research and Department of Biochemistry, University of Wisconsin, 1710 University Avenue, Madison, WI 53726, USA

Received 29 March 2007; Accepted 18 April 2007

Abstract: Isotope effects are a powerful tool for determining the mechanisms of enzymatic reactions. Methods for measurement of isotope effects are reviewed and the equations that describe observed isotope effects and permit determination of intrinsic isotope effects on the isotope-sensitive step are presented. Aspartate transcarbamoylase is used as an example for how the kinetic mechanism can be determined by observing the size of an isotope effect as a function of the concentration of another substrate. Cytidine, adenosine and AMP deaminases are used to illustrate determining the relative rates of steps in the mechanism. Determination of chemical mechanism is illustrated by data for malic enzyme, OMP decarboxylase, aspartate transcarbamoylase, L-ribulose-5-P 4-epimerase, oxalate decarboxylase, tryptophan 2-monooxygenase and the K58A mutant of aspartate aminotransferase when rescued by ammonia. Determination of transition state structure is illustrated by data for formate dehydrogenase, prephenate dehydrogenase, chorismate mutase, as well as for enzymes that catalyze phosphoryl and acyl transfer. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: isotope effects; enzyme mechanisms; isotope ratio mass spectrometry

Substitution of a H, C, N or O atom with a heavy isotope (deuterium, tritium, ^{13}C, ^{14}C, ^{15}N or ^{18}O) causes isotope effects on rates and equilibrium constants. These arise from the fact that the heavier atom prefers a more stiffly bonded environment, while the lighter one prefers looser bonding. We express isotope effects as the ratio of the parameter for the light isotope divided by that for the heavy atom. The nature of the isotopic atom is indicated by a leading superscript (D, T, 13, 14, 15, 18 for deuterium, tritium, ^{13}C, ^{14}C, ^{15}N, ^{18}O). Thus,

$$^{D}k = k_H/k_D, \quad ^{D}K_{eq} = K_{eq\,H}/K_{eq\,D},$$
$$^{13}(V/K) = (V/K)_{C\text{-}12}/(V/K)_{C\text{-}13} \qquad (1)$$

For enzymatic reactions, the intrinsic isotope effect on a rate constant is ^{D}k, while the observed isotope effects will be ^{D}V on the maximum velocity, and $^{D}(V/K)$ on the V/K for a substrate.

Primary isotope effects on rates (ones where bonds are made or broken to the isotopic atom) are almost always normal (greater than unity), since the vibration corresponding to the reaction coordinate motion does not have a restoring force at the transition state. Secondary isotope effects (ones where bonding to the isotopic atom changes during the reaction, but no bonds are made or broken to it) may be either normal or inverse (less than unity), depending on the stiffness of bonding in the transition state relative to that in the substrate. Equilibrium isotope effects also can be either normal, if the isotopic atom is more stiffly bonded in the substrate, or inverse if it is in a stiffer environment in the product. The equilibrium isotope effect in the reverse direction is the reciprocal of that in the forward direction.

Equilibrium isotope effects can be measured by comparing the equilibrium constant values with deuterated and unlabeled reactants. For heavy atom equilibrium isotope effects, one can determine mass ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$ or $^{18}O/^{16}O$) in substrate and product at equilibrium by the use of an isotope ratio mass spectrometer and take the ratio of these. For small molecules where vibrational frequencies can be determined by IR and Raman and used to calculate a force field, it is possible to calculate equilibrium isotope effects.

Measurement of isotope effects

For deuterium isotope effects greater than 1.05, one can compare reciprocal plots with deuterated and

*Correspondence to: W. W. Cleland, Institute for Enzyme Research and Department of Biochemistry, University of Wisconsin, 1710 University Avenue, Madison, WI 53726, USA. E-mail: cleland@enzyme.wisc.edu
Contract/grant sponsor: NIH; contract/grant numbers: GM18938, GM70455
†Paper presented as part of a special issue on 'Recent Developments in the Use of Isotopically Labelled Molecules in Chemistry and Biochemistry'.

unlabeled substrates. The ratio of the slopes is $^{D}(V/K)$, the isotope effect on V/K, and the ratio of the vertical intercepts is ^{D}V, the effect on the maximum velocity. This is the only method that can determine isotope effects on V; the other methods determine only the effect on V/K, or a parameter similar to it. Accurate determination of $^{D}(V/K)$ requires accurate concentration calibration, while the purity is not important. For determination of ^{D}V purity is critical, as competitive inhibitors decrease V, but accurate concentrations are not important since one is extrapolating to infinite concentration.

Equilibrium perturbation

The next most sensitive method for measuring isotope effects is equilibrium perturbation. In this method a reaction mixture is made up close to equilibrium, but with a labeled substrate and unlabeled product, or vice versa. When enzyme is added, the reaction is perturbed towards the labeled reactant (for a normal isotope effect), and then returns to equilibrium as isotopic mixing occurs. The size of the perturbation determines the isotope effect, which is similar to one on V/K (see below for the differences). For a full description of the method, see Reference 1. The method can determine isotope effects to ± 0.05 and is most useful for deuterium isotope effects. It has been used to determine a ^{13}C isotope effect of 1.03, however, and gave the same value as that determined by the much more accurate internal competition method that uses the isotope ratio mass spectrometer. The only drawback to the method is that it only works for reversible reactions that can be studied at equilibrium.

Internal competition

For tritium or ^{14}C isotope effects, or when the natural abundance of ^{13}C (1.1%), ^{15}N (0.37%) or ^{18}O (0.2%) is used as the label, one uses the internal competition method by measuring changes in specific activity or mass ratio in substrate or product as the reaction proceeds. The radioactive isotopes require careful counting, but can determine isotope effects to ± 0.002. With the isotope ratio mass spectrometer used to analyze mass ratios in N_2 or CO_2, one can get values accurate to ± 0.0002. To use the isotope ratio mass spectrometer, however, one must isolate the atom of interest in N_2 or CO_2 by combustion, decarboxylation or other degradation. The isotope effect determined by internal competition is one on V/K for the labeled substrate. If R_0, R_p and R_s are mass ratios in initial substrate (or product at 100% reaction if more convenient to measure), or in product or substrate at fraction of reaction f, then

$$^{x}(V/K) = \log(1-f)/[\log(1-fR_p/R_0)] = \log(1-f)/[\log(1-f)(R_s/R_0)] \quad (2)$$

where x denotes the isotopic atom.

Remote label method

When it is not convenient or possible to isolate the isotopic atom in N_2 or CO_2, one must resort to the remote label method to measure an isotope effect. This is particularly true for measurement of ^{18}O isotope effects on reactions of phosphate esters. We will illustrate by the hydrolysis of *p*-nitrophenyl phosphate (PNPP).[2] One synthesizes a molecule of PNPP with ^{15}N in the nitro group and ^{18}O in either the phenolic or non-bridge oxygens of the phosphate. One also makes a larger amount of PNPP that contains depleted ^{14}N in the nitro group (one can buy NH_4NO_3 containing ^{14}N with a very low ^{15}N content). The two species of PNPP are mixed, 0.37% of the double-labeled species and 99.63% of the depleted ^{14}N species. This gives a substrate with the natural abundance of ^{15}N in the nitro group, but with every ^{15}N accompanied by one or three ^{18}O's. One follows the change in the ^{15}N mass ratio during the reaction and the calculated isotope effect is the product of the ^{15}N and ^{18}O isotope effects. One then runs the reaction with normal PNPP, and this determines the ^{15}N isotope effect alone. Dividing the $^{15}N,^{18}O$ isotope effect by the ^{15}N one gives the desired ^{18}O one. See Reference 2 for the details of the corrections needed for this method.

A number of remote labels have been used. The nitro group has been used in *p*-nitrophenol or *m*-nitrobenzyl alcohol. The exocyclic $-NH_2$ group of adenine is readily inserted by reaction of NH_3 with Cl–purine riboside and removed by adenosine deaminase. Amides and amines are convenient remote labels, and C-1 of glucose is readily inserted by cyanohydrin synthesis and removed as CO_2 by treatment with hexokinase, glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase. In practice, one can determine almost any isotope effect with the remote label method. Only if a molecule does not contain carbon or nitrogen is the method unavailable.

Equations for isotope effects

In the following mechanism:

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA^{*} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EPQ^{*} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EPQ \overset{k_9}{\rightarrow} EQ \overset{k_{11}}{\rightarrow} E \quad (3)$$

the isotope-sensitive step is the conversion of EA^{*} to EPQ^{*} (k_5 and k_6), while the rate constants k_3 and k_4 are

DOI: 10.1002.jlcr

for the conformation change that converts the initial EA complex to the activated EA* one. The rate constants k_7 and k_8 are for the conformation change that converts the EPQ* complex to the more open form from which the first product, P, can dissociate. If there is only one isotope-sensitive step in the reaction, mechanism 3 is as complicated as necessary to interpret experimental results.

The observed isotope effects on V/K and V in mechanism (3) are

$$^{x}(V/K) = [^{x}k + c_f + c_r\,{}^{x}K_{eq}]/[1 + c_f + c_r] \quad (4)$$

$$^{x}V = [^{x}k + c_{vf} + c_r\,{}^{x}K_{eq}]/[1 + c_{vf} + c_r] \quad (5)$$

In Equation (4), c_f and c_r are forward and reverse commitments, and represent the ratio of the rate constant for the isotope-sensitive step and the net rate constant for release of the substrate or first product from the enzyme. In mechanism (3), these are

$$c_f = (k_5/k_4)(1 + k_3/k_2), \quad c_r = (k_6/k_7)(1 + k_8/k_9) \quad (6)$$

Note that the equation for ^{x}V contains c_r, but has a different constant, c_{vf}, in place of c_f. c_{vf} is the sum of the ratios of the rate constant for the isotope-sensitive step (multiplied by the proportion of the enzyme in the EA* form at equilibrium prior to this step) to each unimolecular net rate constant that limits formation of EA*, or is involved in conversion of EPQ* back to free E. A small value of k_{11}, for example, will make c_{vf} large so the rate-limiting release of the second product makes ^{x}V close to unity.

Since isotope effects on V/K are the only ones obtained by the internal competition method, these are the ones usually determined and in this article we will limit our discussion to these.

In Equation (4) c_f is calculated for the substrate A. If there are two or more substrates the value of c_f will differ depending on which V/K is considered. In an internal competition experiment one has no choice; the isotope effect is on V/K for the labeled substrate. When one uses the direct comparison method, one can vary the concentration of either substrate and the $^{D}(V/K)$ for the last substrate to add to the enzyme will give the largest isotope effect. c_r in either a direct comparison or internal competition experiment will be for release of the first product (actually the first irreversible step; if product P is present, it will be for release of the second product).

Isocitrate dehydrogenase

As noted above, the equilibrium perturbation method gives an isotope effect that resembles a V/K one. Equation (4) is valid, but c_f and c_r are figured for the perturbants, the molecules between which the label is exchanged. The difference can be seen with isocitrate dehydrogenase.[3] $^{D}(V/K_{isocitrate})$ is close to unity, since isocitrate is sticky and c_f is large ($k_3 \gg k_2$ in mechanism 3). c_r is for CO_2, the first product released, and is small. In an equilibrium perturbation experiment with deuterated isocitrate, however, c_r is for NADPH which is released slowly so that $c_r \gg c_f$ in this case. As a result, one sees a value of 1.15, close to the equilibrium isotope effect for the reaction.

Determining intrinsic isotope effects

It is clear from Equation (4) that only when the commitments are very small is the intrinsic isotope effect observed. This is sometimes the case with slow alternative substrates, or with slow mutant enzymes, but usually the commitments are finite. In this case one needs a method for determining intrinsic isotope effects and commitments. Two methods have been used. Northrop's method involves comparison of deuterium and tritium isotope effects on V/K[4]:

$$[^{D}(V/K) - 1]/[^{T}(V/K) - 1] = (^{D}k - 1)/(^{T}k - 1) \quad \text{where } {}^{T}k = (^{D}k)^{1.44} \quad (7)$$

This method assumes either that c_r is zero or that $^{D}K_{eq}$ is unity. When this is not the case one can divide $^{D}(V/K)$ by $^{D}K_{eq}$ and $^{T}(V/K)$ by $^{T}K_{eq}$, which is $(^{D}K_{eq})^{1.44}$, to get experimental isotope effects in the reverse direction to use in Equation (7). Comparison of the results using forward and reverse isotope effects puts limits on the intrinsic isotope effects. With malic enzyme this gave 5–8 in the forward direction and 4–6.5 in the back reaction.[5]

The other method for determining intrinsic isotope effects involves measuring the effect of deuteration on the ^{13}C or other isotope effect on the same step.[6] The equations for $^{13}(V/K)_H$ and $^{D}(V/K)$ will be Equation (4) with 13 or D as the superscript. In the equation for $^{13}(V/K)_D$, c_f is divided by ^{D}k and c_r by $^{D}k/{}^{D}K_{eq}$. This gives three equations in the four unknowns ^{D}k, ^{13}k, c_f and c_r. If c_r is very small, as in decarboxylations, or the equilibrium isotope effects are close enough to unity so that c_f and c_r can be added together, solution of the equations gives values for ^{D}k, ^{13}k, and the sum of the commitments. If one also determines a secondary deuterium isotope effect on the same step and the ^{13}C isotope effect with secondary deuterated substrate, one now has five equations in the five unknowns, and a direct solution is possible.

DOI: 10.1002.jlcr

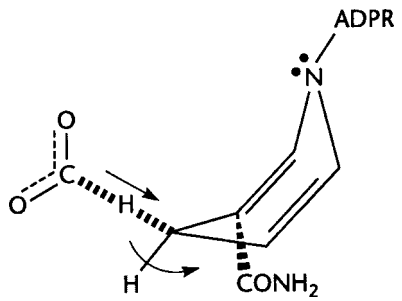
Intrinsic isotope effects for glucose-6-P dehydrogenase

When the five equations described in the previous paragraph were used with glucose-6-P dehydrogenase, ^{13}k was 1.041 ± 0.002, ^{D}k was 5.3 ± 0.3, $^{sec\text{-}D}k$ was 1.054 ± 0.035, c_f was 0.75 ± 0.26 and c_r was 0.49 ± 0.27.[6] The commitments are not well determined, but their sum of 1.23 ± 0.14 is. In D_2O ^{D}k was reduced to 3.7, but the sum of the commitments increased to 2.5 ± 0.3.[7] Thus, the major effect of the solvent was on the conformation changes before and after catalysis, rather than on the chemistry.

The decrease in ^{D}k in D_2O is the result of decreased tunneling. The motion of three hydrogens is coupled in the transition state. The hydrogen at C-1 of glucose-6-P is transferred to NADP as a hydride, while the proton from the 1-hydroxyl group is moved to the aspartate general base on the enzyme. The hydrogen at C-4 of the nicotinamide ring of NADP is moving from in plane to out of plane and its motion is coupled into the reaction coordinate, thus producing the secondary isotope effect greater than unity, although $^{sec\text{-}D}K_{eq}$ is 0.89 for this reaction.

Formate dehydrogenase and coupled hydrogen motions

The coupled motion of primary and secondary hydrogens is characteristic of dehydrogenase reactions where tunneling is involved.



With formate dehydrogenase, which catalyzes the conversion of formate to CO_2 by hydride transfer to NAD, the transition state is late with NAD and $^{sec\text{-}D}(V/K)$ is 1.23 as the result of tight coupling of the hydrogen motions.[8] As the redox potential of the nucleotide is increased, making the reaction more spontaneous and the transition state earlier, the $^{sec\text{-}D}(V/K)$ decreases to 1.18 with thioNAD and 1.06 with acetylpyridine-NAD. In each case when deuterated formate was used, the $^{sec\text{-}D}(V/K)$ decreased half way to 0.89, the value of $^{sec\text{-}D}K_{eq}$. This study nicely showed the variation in the degree of coupling of hydrogen motions with how early or late the transition state is, as well as the effect of the first deuterium substitution in eliminating the tunneling and thus decreasing the coupled motion effect.

Determination of kinetic mechanism

Kinetic studies of enzymatic reactions involve four steps. First, one determines the kinetic mechanism, which involves the order in which substrates and products bind to and are released from the enzyme. Second, one tries to determine the relative rates of the steps in the kinetic mechanism. Third, one determines the chemical mechanism, and fourth, one determines the transition state structure. Isotope effects are useful in all four steps of the analysis and we will give examples of each.

The kinetic mechanism describes the order in which substrates combine with the enzyme and the products dissociate. Isotope effects are very useful in determining the order of combination of substrates. Consider a mechanism with two substrates:

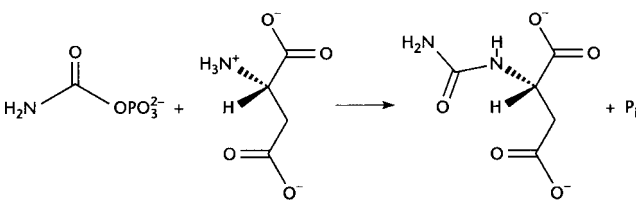
$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} EAB \overset{k_5}{\rightarrow} E + \text{products} \qquad (8)$$

We have lumped all steps for further reaction of EAB into one step and k_5 is the apparent rate constant for these steps. The isotope effect on k_5 will not be an intrinsic isotope effect, as there will likely be an internal forward commitment as well as a reverse one. The external forward commitment for the isotope effect on V/K_b will be k_5/k_4, while that for V/K_a will be $(k_5/k_4)(1 + k_3B/k_2)$. Thus, c_f for $^{x}(V/K_a)$ will vary from k_5/k_4 at low B to infinity at infinite B. Suppression of the isotope effect on V/K_a as the level of B is raised is thus a characteristic of an ordered mechanism.

If the mechanism is random and A can be released from the EAB complex at a finite rate (k_7), c_f for $^{x}(V/K_a)$ will vary from $k_5/(k_4 + k_7)$ at low B to k_5/k_7 at infinite B. c_f for $^{x}(V/K_b)$ will vary from $k_5/(k_4 + k_7)$ at low A to k_5/k_4 at infinite A.

Aspartate transcarbamoylase

The isotope effects on this enzyme provide an excellent example of the application of this method.



With wild-type holoenzyme, the ^{13}C isotope effect in carbamoyl-P was 1.022 at low aspartate, but unity at infinite aspartate, showing ordered addition of carbamoyl-P before aspartate.[9] With isolated catalytic sub-

DOI: 10.1002.jlcr

units, the value ranged from 1.024 at low aspartate to 1.004 at infinite aspartate. In this case the mechanism has become partly random and the presence of aspartate slows down, but does not prevent the release of carbamoyl-P from the ternary complex. With a slow alternative substrate to aspartate, cysteine sulfinate (ß-carboxyl now an SO_2^-), the ^{13}C isotope effect in carbamoyl-P was 1.039 and independent of the level of cysteine sulfinate. The kinetic mechanism is thus now fully random. With a H134A mutant of the holoenzyme, the isotope effect was 1.04 and independent of aspartate level, showing that the kinetic mechanism was also random in this case.[10]

Determination of the relative rates of steps in the mechanism

Isotope effects have been used to determine the degree to which a given step is rate limiting and we will illustrate this by several examples.

Cytidine deaminase

This enzyme catalyzes the elimination of ammonia from cytidine to give uridine. C-4 of the pyrimidine ring is attacked by Zn-bound OH to give a tetrahedral adduct, and N-3 is protonated in the same step. The ^{15}N isotope effect in the exocyclic amino group was 1.0109 with wild-type enzyme at neutral pH and was only increased to 1.0123 at pH 4.2 where the rate of the reaction was reduced by a factor of 18.[11] Mutants that slowed the reaction increased the value at neutral pH, with a H102N mutant giving 1.0158. The ^{15}N isotope effect at N-3 at neutral pH was 0.988, which is consistent with protonation prior to the rate-limiting C–N cleavage. It is likely that the value of 1.0158 for the H102N mutant is close to the intrinsic isotope effect and that the reduced value at pH 7.3 is the result of an internal forward commitment (there is little external commitment since the reaction is independent of viscosity).

Adenosine deaminase catalyzes a similar reaction, and the ^{15}N isotope effect in the exocyclic amino group was 1.0041 with adenosine, but 1.0150 with 7,8-dihydro-8-oxoadenosine, a slow substrate.[12] C–N cleavage appears to be rate limiting with the slow substrate, while only partly so with adenosine. AMP deaminase showed ^{15}N isotope effects ranging from 1.010 (without the ATP activator) to 1.016 with ATP present.[13] In all of these cases, the observed ^{15}N isotope effect is the product of an equilibrium isotope effect for formation of a tetrahedral intermediate (which is inverse) and the kinetic isotope effect on C–N cleavage, which probably is at least 1.03.

Urease

An enzyme where the full ^{15}N isotope effect for C–N cleavage is seen is urease with the slow substrate formamide as substrate. The ^{15}N isotope effect is 1.033,[14] while that for urea is 1.015[15] (assuming the observed value to be only for the nitrogen that is leaving as ammonia). The ^{13}C isotope effect is 1.021 with urea, and 1.024 with formamide. Clearly breakdown of the tetrahedral intermediate formed by Ni-bound OH attack is rate limiting with formamide, but only partly so with urea. ^{18}O isotope effects with formamide are 0.996 in the carbonyl oxygen and 0.978 in the attacking OH. The formyl hydrogen $^{D}(V/K)$ value was 0.95, showing the stiffening effect of tetrahedral adduct formation. All of these isotope effects are consistent with tetrahedral adduct formation coming essentially to equilibrium and rate-limiting C–N cleavage.

Determination of chemical mechanism

Isotope effects are very useful in determining the chemical mechanisms of enzymatic reactions. We discussed above the effects of deuteration on a ^{13}C isotope effect when both effects were on the same step. When the isotope effects are on different steps, the following equations apply:

D-sensitive step first :

$$(^{13}(V/K)_H - 1)/(^{13}(V/K)_D - 1) = {}^{D}(V/K)/{}^{D}K_{eq} \quad (9)$$

^{13}C-sensitive step first :

$$(^{13}(V/K)_H - {}^{13}K_{eq})/(^{13}(V/K)_D - {}^{13}K_{eq}) = {}^{D}(V/K) \quad (10)$$

These are really the same equation, but the second is expressed in terms of the parameters for the reverse reaction of the first. Since $^{D}K_{eq}$ is often different from unity, experimental data will fit one equation and not the other.

Malic enzyme

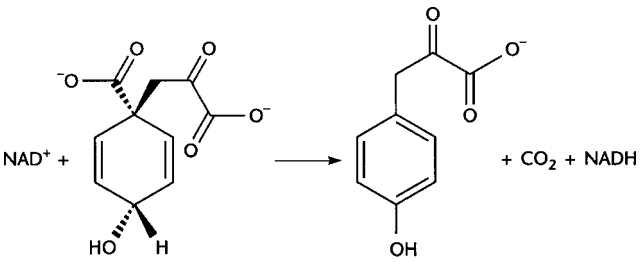
For malic enzyme with NADP as substrate, deuterium isotope effects were determined at C-2 and ^{13}C ones at C-4.[6] The first equation gave $1.21 \pm 0.05 = 1.25 \pm 0.03$, while the second gave $1.20 \pm 0.05 \neq 1.47 \pm 0.03$. Thus, malate is dehydrogenated to oxaloacetate, which is then decarboxylated.

When the nucleotide was changed to one with a higher redox potential, which makes the reaction more spontaneous, the ^{13}C isotope effects got larger, rather than smaller with deuterated malate.[16,17] This indicates a change from a stepwise to a concerted mechanism.

DOI: 10.1002.jlcr

Prephenate dehydrogenase

Another enzyme catalyzing a concerted oxidative decarboxylation is prephenate dehydrogenase, which converts prephenate into *p*-hydroxyphenylpyruvate using NAD as the oxidant.[18]



The ^{13}C isotope effect in the carboxyl that leaves as CO_2 was 1.0033 with unlabeled substrate and 1.0103 with 4-deuterated substrate (the substrate used lacked the keto group in the side chain but showed a similar V_{max}). The $^{D}(V/K)$ was 2.34, and these three values were used to calculate $^{D}k = 7.3$, $^{13}k = 1.0155$ and $c_f = 3.7$. Although the reaction is concerted, it has an asynchronous transition state, with C–H cleavage well advanced, but C–C cleavage only just starting.

OMP decarboxylase

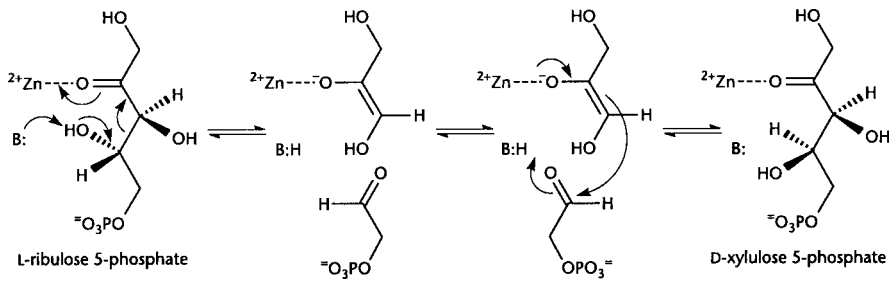
In order to determine the mechanism of this enzyme, the ^{15}N isotope effect at N-1 of the pyrimidine ring was determined to be 1.0036.[19] This eliminates the ylide mechanism that applies to the non-enzymatic reaction of similar molecules. The normal value of the ^{15}N isotope effect rules out formation of a positive charge at N-1 that would stabilize the formation of a carbanion at C-6 (enolization to give such a positive charge increases bond order to N-1 and would give an inverse isotope effect). In place of a positive charge at N1 the enzyme has a positively charged lysine next to C-6 which plays the same role in stabilizing the transient carbanion intermediate formed during decarboxlation and protonates it when it forms.

Aspartate transcarbamoylase

There has been controversy about the chemistry of this reaction. While most workers have believed that aspartate attacks carbamoyl-P to give a tetrahedral intermediate that decomposed to phosphate and carbamoylaspartate, others proposed that carbamoyl-P decomposed to cyanic acid, an electrophile that then reacted with aspartate. The question was solved by measuring the ^{15}N isotope effect in carbamoyl-P.[20] The dianion of carbamoyl-P decomposes by internal general base catalysis and C–O cleavage to give cyanic acid, and the ^{15}N isotope effect for this is 1.0114. The monoanion decomposes by P–O cleavage with an ^{15}N isotope effect of 1.0028. In the enzymatic reaction, the isotope effects were 1.0014 with holoenzyme and low aspartate, 1.0024 with cysteine sulfinate, and 1.0027 with the H134A mutant. Since the latter two give apparently intrinsic ^{13}C isotope effects (see above), it appears that the reaction does not involve cyanic acid as an intermediate, since that should give a primary ^{15}N isotope effect greater than 1.01, as in the non-enzymatic case.

L-Ribulose-5-P 4-epimerase

This enzyme converts L-ribulose-5-P to D-xylulose-5-P.



To establish the mechanism, ^{13}C isotope effects were determined at each carbon, and deuterium ones at C3 and C4.[21] The ^{13}C isotope effects at C3 and C4 were over 1.02, showing that cleavage takes place between these atoms. The deuterium isotope effects at C3 and C4 were only 1.04 and 1.19, which are secondary isotope effects, ruling out C–H cleavage in the mechanism. The enzyme catalyzes a retro-aldol reaction in which a zinc atom polarizes the carbonyl group to allow cleavage to the enolate of dihydroxyacetone and

DOI: 10.1002.jlcr

glycolaldehyde-P. Rotation of the latter and condensation completes the reaction.

Oxalate decarboxylase

This enzyme catalyzes the conversion of the monoanion of oxalate to CO_2 and formate. The enzyme contains Mn and requires catalytic levels of oxygen, which is not used up. Since the sodium salts of oxalate and formate can be oxidized to CO_2 by I_2 in dimethylsulfoxide without exchange of the oxygens, one can determine both the ^{13}C and ^{18}O isotope effects for formation of CO_2 or formate by analysis of the ^{13}C and ^{18}O mass ratios in residual oxalate and the formate formed, plus the ^{13}C mass ratio in the CO_2 product. At pH 5.7 where the chemistry is rate limiting, the ^{13}C isotope effects were 1.019 going to formate and 1.008 going to CO_2.[22] The ^{18}O isotope effects were 1.010 in formate and 0.993 in CO_2. The small ^{13}C isotope effect in CO_2 shows that decarboxylation is not the major rate-limiting step, while the large value going to formate shows that the C–O bond order in the end of oxalate going to formate is reduced in a step prior to decarboxylation. Steps after decarboxylation will not affect V/K isotope effects.

A model which fits the data is for oxalate to coordinate to Mn, which with oxygen has formed a Mn[III] superoxide, or some similar structure. Electron transfer to Mn, along with proton transfer to Glu333, gives a radical intermediate with ~1.15 C–O bond order in the coordinated end of the radical. This leads to ~70% positive charge on this carbon, which facilitates decarboxylation. The resulting species is a formate radical anion, which picks up an electron from Mn and a proton from Glu333 to give formate. The radical intermediate decarboxylates four times faster than it reverts to oxalate.

Tryptophan 2-monooxygense

This enzyme catalyzes the oxidative deamination of amino acids via a ping-pong mechanism with FAD being reduced by the substrate and being reoxidized by oxygen. In a study with alanine, a slow substrate showing a pH-independent primary deuterium isotope effect of 6, the ^{15}N isotope effect was 0.992 after correction for deprotonation of the amino group (the ^{15}N equilibrium isotope effect for deprotonation is 1.0233).[23] This value is inconsistent with mechanisms that involve direct attack of the amino group on the flavin, but supports a hydride transfer mechanism.

Aspartate aminotransferase

The K258A mutant of this transaminase is essentially inactive, since this lysine is required to form an imine with pyridoxal phosphate and during the reaction with the substrate. However the reaction can proceed in the presence of ammonia, which replaces the lysine as general acid and base during the reaction. In this case, pyridoxal-P is in the aldehyde form prior to addition of the aspartate, and ammonia catalyzes the removal of the proton to give the intermediate that reprotonates on the aldehyde carbon of pyridoxal-P to give the ketimine which later hydrolyzes to oxaloacetate. In the second part of the reaction, pyridoxamine-P reacts with ketoglutarate to regenerate an aldimine of aspartate and pyridoxal-P.

When the primary deuterium isotope effect was determined in a single turnover experiment, a value of 5.2 was seen.[24] However, in multiple turnover studies this isotope effect was near unity. The ^{13}C isotope effects at C-2 and C-3 of aspartate were 0.993 and 1.0007, while the ^{15}N isotope effect was 1.0168 corrected to the unprotonted amino group.[25] Eliminating the internal aldimine of lysine and pyridoxal-P makes the initial combination of aspartate and pyridoxal-P more spontaneous than the normal transimination reaction by 10^5. The lack of a deuterium isotope effect results from the reaction between the aldimine of aspartate and the ketimine of oxaloacetate coming to equilibrium, and since this reaction washes the label out of aspartate, it is the reaction of unlabeled aspartate that is actually observed in multiple turnover experiments. Since the observation of the ^{15}N isotope effect requires that aspartate can be released from the enzyme faster than ketimine hydrolysis, it was possible to estimate that the forward commitment for the proton shift was at least 400 and the reverse commitment at least 90. The equations for the model suggest that the intrinsic ^{15}N isotope effect on ketimine hydrolysis is 1.034, while the ^{13}C isotope effects at C-2 and C-3 are small, in agreement with proton transfer steps being close to equilibrium. It appears that collapse of the carbinolamine during ketimine hydrolysis is the rate-determining step in this system.

Determination of transition state structure

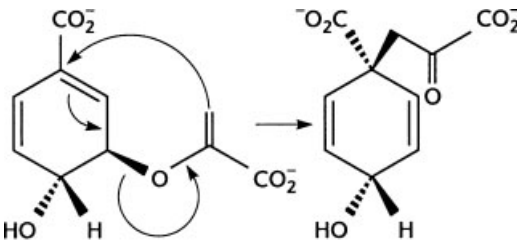
Isotope effects can give useful information on transition state structure. Observation of a sizeable primary isotope effect shows that the chemistry is at least partly rate limiting, and then secondary isotope effects

DOI: 10.1002.jlcr

give clues as to the bonding in the transition state. The case of formate dehydrogenase is discussed above, where raising the redox potential of the nucleotide substrate made the transition state become earlier and decreased the coupling between the secondary hydrogen and the primary hydride motions. Also discussed above, prephenate dehydrogenase shows a concerted but asynchronous reaction, with C–H cleavage well advanced and C–C cleavage just beginning in the transition state.

Chorismate mutase

This enzyme catalyzes the conversion of chorismate to prephenate.



chorismate prephenate

The bond between the bridge oxygen of the enolpyruvate side chain and C-5 breaks and a new bond is made between C-1 of the ring and C-3 of the enolpyruvate. The ^{18}O isotope effect in the ether oxygen was 1.045, showing that C–O cleavage is well advanced in the transition state.[26] The ^{13}C isotope effect at C-1 of the ring was 1.005, while that at C-3 of the enolpyruvate side chain was 1.013. These data show that the reaction is a concerted pericyclic one with C–C bond formation lagging behind C–O cleavage.

Phosphoryl transfer reactions

These reactions have been studied with ^{18}O isotope effects, using the nitrogen of *p*-nitrophenol, *m*-nitrobenzyl alcohol, *p*-carbamoylphenol or choline as the remote label. The primary isotope effect for P–O cleavage tells whether the chemistry is rate limiting, and if it is, the secondary ^{18}O isotope effect in the non-bridge oxygens indicates the degree of associative or dissociative character of the transition state. Reactions of phosphate monoesters normally have concerted mechanisms with low axial P–O bond order; this corresponds to a dissociative or loose transition state. The secondary ^{18}O isotope effect will be slightly inverse or slightly normal. Reported values vary from 0.998 to 1.0019, the latter for a D92N mutant of a human VHR phosphotyrosine phosphatase.[27,28]

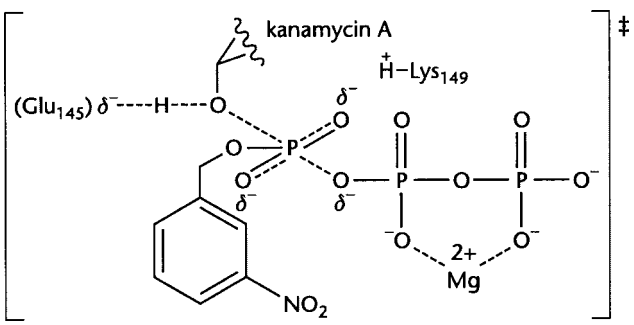
The secondary ^{15}N isotope effects when *p*-nitrophenol is the leaving group indicate the degree of electron delocalization into the nitro group in the transition state. If the enzyme protonates the leaving group, which occurs with phosphotyrosine phosphatases, the value is near unity, since protonation prevents the electron delocalization. But when the catalytic aspartates are mutated, the ^{15}N isotope effects range from 1.0019 to 1.0030, showing considerable electron delocalization, with the higher values indicating later transition states.[27,28] The primary ^{18}O isotope effects with *p*-nitrophenol as leaving group usually range from 1.012 to 1.015, since protonation of the leaving group decreases the primary ^{18}O isotope effect. But the aspartate mutants, where *p*-nitrophenolate ion is the leaving group, show values of 1.027–1.029, consistent with later transition states.

Phosphate diester reactions

These reactions are S_N2 ones, and the secondary ^{18}O isotope effects are usually slightly above unity. For ribonuclease with the slow substrate uridine 3′-*m*-nitrobenzyl phosphate, the primary ^{18}O isotope effect was pH independent at 1.016, while the secondary one in the non-bridge oxygens was 1.005 at the pH optimum of 5.[29] These data support a concerted displacement, with His12 acting as a general base to remove the proton from the attacking 2′-oxygen of the ribose, and His119 protonating the leaving group (in this case, *m*-nitrobenzyl alcohol). The size of the primary isotope effect is consistent with the leaving group being protonated during the reaction.

Kanamycin nucleotidyltransferase

This enzyme normally reacts MgATP with kanamycin to form kanamycin-4′-AMP and Mg pyrophosphate, but the chemistry is not rate limiting with MgATP as substrate. With *m*-nitrobenzyl triphosphate, however,



which reacts two orders of magnitude more slowly, the primary ^{18}O isotope effect in the α–ß bridge was 1.016 and the secondary ^{18}O isotope effect in the α-non-bridge

DOI: 10.1002.jlcr

oxygens was 1.0033.[30] The reaction appears to be concerted with a slightly associative transition state.

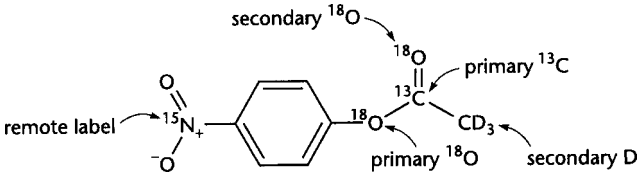
Phosphate triesters

These substrates react with associative transition states, although phosphorane intermediates form only where pseudorotation is necessary to place a leaving group in an axial position. With phosphotriesterase, diethyl-*p*-nitrophenyl phosphate gives small primary (1.0020) and secondary (1.0021) ^{18}O isotope effects, so it appears that the chemistry is not fully rate limiting (the values are three times larger in the non-enzymatic hydrolysis by hydroxide).[31] With diethyl-*p*-carbamoylphenyl phosphate, however, the primary isotope effect was 1.036 and the secondary one 1.018. This is a more associative and later transition state than with the better leaving group (p*K* of 8.6 vs 7.0), and the value of 1.018 indicates considerable single bond character in the non-bridge oxygen in the transition state.

A recent review on phosphoryl transfer includes more examples of the application of isotope effects.[32]

Acyl transfer reactions

For the hydrolysis of *p*-nitrophenyl acetate, five isotope effects have been determined.[33] These are secondary ^{15}N (the remote label), primary ^{18}O in the phenolic oxygen, secondary ^{18}O in the carbonyl group, ^{13}C in the carbonyl carbon and three deuteriums in the methyl group.



Except for acid protease, where the leaving group is protonated by the enzyme, the other enzymes studied (chymotrypsin, carbonic anhydrase and papain) all showed primary ^{18}O isotope effects of 1.020–1.033, and secondary ^{15}N values of 1.0005–1.0011, showing electron delocalization into the nitro group in the transition state. The large primary ^{18}O isotope effects show that the reactions are concerted, with tetrahedral transition states, but not intermediates. Only when the p*K* of the leaving group is above 15 do true intermediates form and ^{18}O exchange with the solvent occur in non-enzymatic reactions. Acid protease gave a primary ^{18}O isotope effect of 1.0141 and no ^{15}N effect, as expected.

All four enzyme showed secondary ^{18}O isotope effects of 1.0064–1.0075, which is consistent with reduced C–O bond order in the transition state, and all showed ^{13}C isotope effects of 1.028–1.036 in the carbonyl carbon, suggesting that the chemistry was totally rate limiting. The ß-deuterium isotope effects in the methyl group were all inverse (from 0.982 to 0.999), showing loss of hyperconjugation in the transition state. However, the values for the enzymatic reactions were not as inverse as for non-enzymatic ones (~0.96 for attack by oxygen nucleophiles). The reason is that when the substrate is bound to the enzyme the carbonyl group is polarized to *increase* hyperconjugation and lead to more rapid attack on the carbonyl carbon. Approach to the transition state then decreases hyperconjugation, but from an increased level, so comparison with unbound substrate gives a smaller isotope effect.

Conclusion

This review has focused mainly on work from the author's laboratory or from his collaborations. Hopefully the reader will be left with a feeling for how powerful isotope effect studies can be for study of enzymatic reactions. For further coverage of the topic, the reader is referred to other books.[34–36]

REFERENCES

1. Cleland WW. *Methods Enzymol* 1980; **64**: 104–125.
2. Hengge AC, Edens WA, Elsing H. *J Am Chem Soc* 1994; **116**: 5045–5049.
3. Cook PF, Cleland WW. *Biochemistry* 1981; **20**: 1797–1805.
4. Northrop DB. *Biochemistry* 1975; **14**: 2644–2651.
5. Schimerlik MI, Grimshaw CE, Cleland WW. *Biochemistry* 1977; **16**: 571–576.
6. Hermes JD, Roeske CA, O'Leary MH, Cleland WW. *Biochemistry* 1982; **21**: 5106–5114.
7. Hermes JD, Cleland WW. *J Am Chem Soc* 1984; **106**: 7263–7264.
8. Hermes JD, Morrical SW, O'Leary MH, Cleland WW. *Biochemistry* 1984; **23**: 5479–5488.
9. Parmentier LE, O'Leary MH, Schachman HK, Cleland WW. *Biochemistry* 1992; **31**: 6570–6576.
10. Waldrop GL, Turnbull JL, Parmentier LE, O'Leary MH, Cleland WW, Schachman HK. *Biochemistry* 1992; **31**: 6585–6591.
11. Snider MJ, Reinhardt L, Wolfenden R, Cleland WW. *Biochemistry* 2002; **41**: 415–421.
12. Weiss PM, Cook PF, Hermes JD, Cleland WW. *Biochemistry* 1987; **26**: 7378–7384.
13. Merkler DJ, Kline PC, Weiss P, Schramm VL. *Biochemistry* 1993; **32**: 12993–13001.
14. Marlier JF, Cleland WW. *Biochemistry* 2006; **45**: 9940–9948.
15. Schmidt HL. In *Stable Isotopes, Analytical Chemistry Symposia Series*, Schmidt HL, Forstel H,

Heinzinger K (eds), vol. 11. Elsevier: Amsterdam, 1982; 77–82.
16. Weiss PM, Gavva SR, Harris BG, Urbauer JL, Cleland WW, Cook PF. *Biochemistry* 1991; **30**: 5755–5763.
17. Edens WA, Urbauer JL, Cleland WW. *Biochemistry* 1997; **36**: 1141–1147. DOI: 10.1021/bi962128s
18. Hermes JD, Tipton PA, Fisher MA, O'Leary MH, Morrison JF, Cleland WW. *Biochemistry* 1984; **23**: 6263–6275.
19. Rishavy MA, Cleland WW. *Biochemistry* 2000; **39**: 4569–4574. DOI: 10.1021/bi000376p
20. Waldrop GL, Urbauer JL, Cleland WW. *J Am Chem Soc* 1992; **114**: 5941–5945.
21. Lee LV, Vu MV, Cleland WW. *Biochemistry* 2000; **39**: 4808–4820. DOI: 10.1021/bi992894+
22. Reinhardt LA, Svedruzic D, Chang CH, Cleland WW, Richards NGJ. *J Am Chem Soc* 2003; **125**: 1244–1252. DOI: 10.1021/ja0286977
23. Ralph EC, Anderson MA, Cleland WW, Fitzpatrick PF. *Biochemistry* 2006; **45**: 15844–15852. DOI: 10.1021/bi061894o
24. Toney MD, Kirsch JF. *Protein Sci* 1992; **1**: 107–109.
25. Wright SK, Rishavy MA, Cleland WW. *Biochemistry* 2003; **42**: 8369–8376. DOI: 10.1021/bi030092f
26. Wright SK, DeClue MS, Mandal A, Lee L, Wiest O, Cleland WW, Hilvert D. *J Am Chem Soc* 2005; **127**: 12957–12964. DOI: 10.1021/ja052929v
27. Hengge AC, Sowa GA, Wu L, Zhang Z-Y. *Biochemistry* 1995; **34**: 13982–13987.
28. Hengge AC, Denu JM, Dixon JE. *Biochemistry* 1996; **35**: 7084–7092. DOI: 10.1021/bi960255i
29. Sowa GA, Hengge AC, Cleland WW. *J Am Chem Soc* 1997; **119**: 2319–2320. DOI: 10.1021/ja963974t
30. Gerratana B, Frey PA, Cleland WW. *Biochemistry* 2001; **40**: 2972–2977. DOI: 10.1021/bi002557x
31. Caldwell SR, Raushel FM, Weiss PM, Cleland WW. *Biochemistry* 1991; **30**: 7444–7450.
32. Cleland WW, Hengge AC. *Chem Rev* 2006; **106**: 3252–3278. DOI: 10.1021/cr050287o
33. Hengge AC, Hess RA. *J Am Chem Soc* 1994; **116**: 11256–11263.
34. Cook PF, Cleland WW. *Enzyme Kinetics and Mechanism*. Garland Science: New York, 2007.
35. Cook PF. *Enzyme Mechanism from Isotope Effects*. CRC Press: Boca Raton, FL, 1991.
36. Kohen A, Limbach H-H. *Isotope Effects in Chemistry and Biology*. CRC Press: Boca Raton, FL, 2006.