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# Deuterium and Tritium Exchange in Enzyme Kinetics[†]

W. John Albery and Jeremy R. Knowles*

ABSTRACT: The theory of the isotopic exchange of deuterium and tritium between an enzyme–substrate complex and the solvent is derived for 16 different types of experiment involving measurements of initial velocities and of the isotopic content of the reactants and products as a function of the extent of reaction. It is shown how the data from these experiments can be analyzed to obtain the rate constants for the individual steps and thereby the Gibbs free energies of the intermediates and transition states in the reaction. The effects of isotopic substitution on each intermediate and transition state are also found and this allows conclusions to be drawn as to the extent to which a proton is in flight in a particular transition state. Neither substrate handling (that is, on-off steps), nor the isotopic exchange with the solvent, is assumed to be rapid.
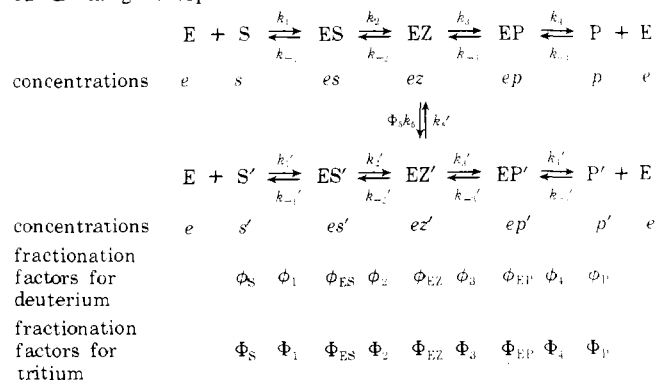
$\mathbf{A}$ very large number of enzyme-catalyzed reactions rely on acid–base catalysis for the movement of protons to and from the substrate(s) in the course of catalysis. In many of these, the substrate protons become partially or completely equilibrated with the solvent protons at an intermediate stage of the reaction. This paper presents the kinetic treatment for such reactions, in which the fate of a hydrogen isotope is followed when either the substrate or the solvent is isotopically labeled.

The present work arose from the investigation of the mechanistic details of the reaction catalyzed by the glycolytic enzyme triose phosphate isomerase. This reaction involves the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate:

$$
\begin{array}{cc}
CH_2OH & CHO \\
| & | \\
C=O & H-C-OH \\
| & | \\
CH_2OPO_3{}^{2-} & CH_2OPO_3{}^{2-}
\end{array}
$$
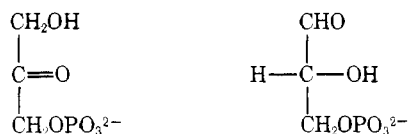
The elegant work of Rose (1962) demonstrated that the triose phosphate isomerase reaction is a particular example of a class of enzyme reactions whose pathway is shown in Scheme I. This pathway is evidently followed by a large number of aldose-ketose isomerases and, with relatively minor modification,

SCHEME I: Pathway for a Reaction That Interconverts S and P, Catalyzed by an Enzyme E, in Which a Reaction Intermediate EZ Can Exchange Isotope with the Solvent.[a]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EZ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} P + E$$

concentrations $\quad e \quad s \quad\quad es \quad\quad ez \quad\quad ep \quad\quad p \quad e$

$$\Phi_s k_5 \big\Updownarrow k_5{'}$$

$$E + S' \underset{k_{-1}{'}}{\overset{k_1{'}}{\rightleftharpoons}} ES' \underset{k_{-2}{'}}{\overset{k_2{'}}{\rightleftharpoons}} EZ' \underset{k_{-3}{'}}{\overset{k_3{'}}{\rightleftharpoons}} EP' \underset{k_{-4}{'}}{\overset{k_4{'}}{\rightleftharpoons}} P' + E$$

concentrations $\quad e \quad s' \quad\quad es' \quad\quad ez' \quad\quad ep' \quad\quad p' \quad e$

fractionation factors for deuterium $\quad \phi_S \quad \phi_1 \quad \phi_{ES} \quad \phi_2 \quad \phi_{EZ} \quad \phi_3 \quad \phi_{EP} \quad \phi_4 \quad \phi_P$

fractionation factors for tritium $\quad \Phi_S \quad \Phi_1 \quad \Phi_{ES} \quad \Phi_2 \quad \Phi_{EZ} \quad \Phi_3 \quad \Phi_{EP} \quad \Phi_4 \quad \Phi_P$

[a] The primes indicate isotopic substitution by either D or T; unprimed rate constants refer to the all-hydrogen system.
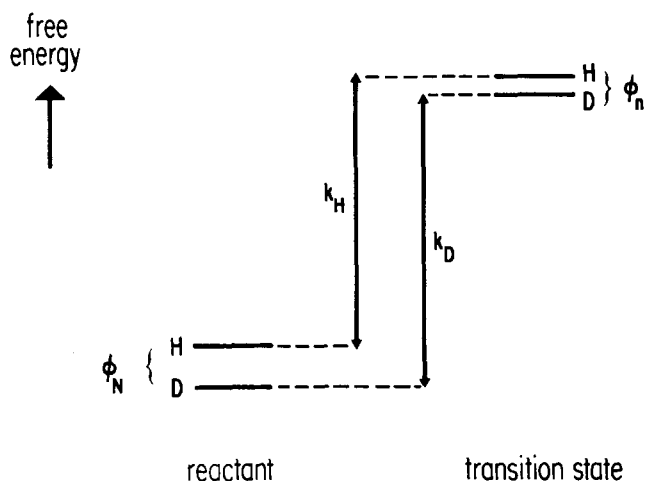
free
energy

FIGURE 1: Illustration of the relationship between rate constants and fractionation factors. k_H and k_D are the rate constants for protonated and deuterated species, respectively. ϕ_N is the reactant fractionation factor and ϕ_n is the transition state fractionation factor.
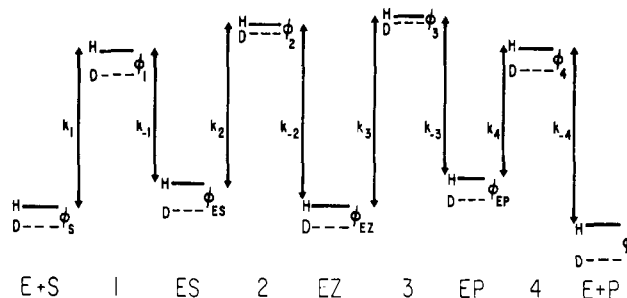

FIGURE 2: Arbitrary free-energy diagram for the reaction of Scheme I. This diagram shows the rate constants and fractionation factors the interrelation of which this paper describes.

represents part of the pathway followed by other enzymes that involve enolization, such as the aldolases, some kinases, carboxylases, etc., which catalyze the exchange of protons between their substrates and the solvent. Application of the equations developed in this paper to such systems will allow some delineation of the energetics of these processes.

What follows is a generalized kinetic treatment for Scheme I. We do not assume that the isotopic exchange is rapid, nor that the substrate-handling steps are fast relative to the catalytic steps.

Fractionation Factors. To describe the effect of isotopic substitution, we use fractionation factor theory (Gold, 1960; Kresge, 1964). The fractionation factor for a site X (which may be in a stable species, in a reaction intermediate, or in a transition state) describes the isotopic "equilibrium" (for deuterium substitution):

$$XH + \tfrac{1}{2}D_2O \rightleftharpoons XD + \tfrac{1}{2}H_2O$$
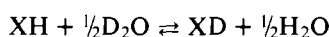
and

$$\phi = \frac{[XD][H_2O]^{1/2}}{[XH][D_2O]^{1/2}} \qquad (1.1)$$

The factor ϕ (for list of symbols, see end of paper) describes the equilibrium isotopic composition of the site X compared with that of water. In particular, in a 50:50 mixture of H_2O and D_2O

$$\phi = [XD]/[XH]$$

If $\phi > 1$, then D concentrates on the site compared with the water; the atom fraction of D on the site is larger than that in the solvent. Conversely if $\phi < 1$, then H prefers the site and the H atom fraction is larger on the site than in the solvent. In particular for a transition state in which the proton is in flight, ϕ is considerably less than unity, usually in the range of 0.15 to 0.30. The factors ϕ_n describe the fractionation for D; the factors Φ_n describe the fractionation for T. The two factors are likely to be connected by the Swain–Schaad relation (Swain et al., 1958):

$$\Phi_n = \phi_n^{1.44} \qquad (1.2)$$

This relation works well when the isotope effect arises solely from the differences in zero-point energies, but if the X–H or X–D vibrations are excited then there may be deviations. In

our proposed analysis of the data we have used this relationship as sparingly as possible; however, separate measurements of ϕ_n and Φ_n which obey eq 1.2 would confirm the correctness of the model and the analysis.

The rate constants k_n' (for the D or T case) are related to k_n (for the H case) by the fractionation factors (Kresge, 1964):

$$k_{|n|}'/k_{|n|} = \phi_{|n|}/\phi_N \text{ for D}$$
$$= \Phi_{|n|}/\Phi_N \text{ for T}$$

where $\phi_{|n|}$ and $\Phi_{|n|}$ are the fractionation factors for the nth transition state and ϕ_N and Φ_N are the fractionation factors of the reactant for that particular step.

This relation between a ratio of rate constants and a ratio of fractionation factors is illustrated in Figure 1. Any kinetic isotope effect is a function of four free energies. A rate constant describes the difference in Gibbs free energy between reactant and transition state for a particular isotope, whereas a fractionation factor, ϕ, describes the difference in Gibbs free energy between H and D compounds for the same species (compared with the standard, H_2O/D_2O). Equilibrium isotope effects are similarly described by a ratio of the appropriate fractionation factors. For the enzyme-catalyzed reaction of Scheme I, we are interested first in knowing the values of all the rate constants $k_{\pm n}$, so that we may construct the free-energy profile for the all-hydrogen reaction, and secondly in describing the effect of isotopic substitution on each intermediate and transition state in terms of its fractionation factor. Figure 2 is an arbitrary free-energy diagram for Scheme I, and we will show that by suitable combinations of experiments we can determine all the parameters in Figure 2 which relate to elementary steps that are kinetically significant. Because a fractionation factor describes the effect of isotopic substitution on a single site at a particular point along the reaction coordinate, this approach gives us a more complete description of the system than we should get if we used crude ratios of rate constants. For instance, in drawing mechanistic conclusions, it is better to use the size of the transition state fractionation factor ϕ_n rather than the rate constant ratio, since the latter may include significant reaction fractionation, ϕ_N (see Figure 1). [Typical values of fractionation factors for many different kinds of site have been collected by Schowen (1972).] Furthermore, for reactions where two transition states are each partially rate determining, we may define a *mixed fractionation factor* (see below) which is a weighted mean of the fractionation in each of the individual transition states.

A particular problem arises with the isotopic exchange reaction described by k_5 (Scheme I). Like all other k_n, k_5 describes the all-H system:

$$EZ(H) + H_2O \underset{k_5}{\overset{k_5}{\rightleftharpoons}} EZ(H) + H_2O$$
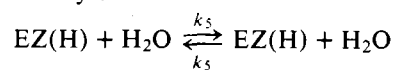
Experiments in which deuterium is picked up from the solvent require such high atom fractions of D that the interpretation of the results is complicated by solvent isotope effects. We therefore will not consider experiments performed in D_2O or in mixtures of H_2O and D_2O. So the step, $EZ \rightarrow EZ'$, is only important when T is being picked up from the solvent:

$$EZ(H) + HTO \xrightarrow{\Phi_5 k_5} EZ'(T) + H_2O$$
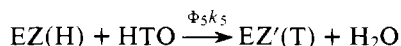
The rate constant for this process is $k_5\Phi_5$ since EZ is not isotopically substituted and the T is coming from a solvent site, where $\Phi = 1$ by definition, to the transition state (Φ_5). Note that when EZ and EZ' are in equilibrium:

$$k_5\Phi_5 x \, ez = k_5(\Phi_5/\Phi_{EZ})(1 - x)ez'$$

where x is the atom fraction of T in the solvent. Hence

$$\Phi_{EZ} = \frac{ez'(1 - x)}{ez \, x}$$

which agrees with eq 1.1 given above.

The factors Φ_S and Φ_P can be measured independently of the kinetic measurements by isotopic analysis of S and P in solvents where the atom fraction of T is x.

$$\Phi_S = \frac{s'(1 - x)}{sx} \simeq \frac{s'}{sx} \quad \text{and} \quad \Phi_P \simeq \frac{p'}{px} \quad (1.3)$$

In order to keep the algebraic expressions concise, it is useful to define the ratios of the forward and backward rate constants for the three intermediates ES, EZ, and EP:

$$\alpha = k_2/k_{-1} \quad (1.4)$$

$$\beta = k_{-2}/k_3 \quad (1.5)$$

$$\gamma = k_{-3}/k_4 \quad (1.6)$$

These ratios, therefore, describe the free-energy differences between transition states 1 and 2, transition states 2 and 3, and transition states 3 and 4 (see Figure 2).

It is also helpful to define the ratio of the effective overall forward and backward rate constants for EZ, going to E + S and going to E + P:

$$\theta = \frac{\beta(1 + \gamma)}{(1 + \alpha)} = \frac{k_{-2}}{[1 + (k_2/k_{-1})]} \frac{[1 + (k_{-3}/k_4)]}{k_3} \quad (1.7)$$

A large value of θ means that EZ goes to S more than to P, and a small value of θ means EZ goes to P more than to S. Since isotopes are exchanged with the solvent at EZ and EZ', the importance of θ is that isotope will appear in S or in P, depending on the size of θ compared with unity.

Mixed Fractionation Factors. If transition states 1 and 2 or transition states 3 and 4 are of comparable free energy, then the effect of isotopic substitution can best be described by the mixed fractionation factors:

$$\phi_{1,2} = \frac{\phi_2(1 + \alpha)}{[1 + (\alpha\phi_2/\phi_1)]} = \frac{\phi_1(1 + \alpha^{-1})}{[1 + (\phi_1/\alpha\phi_2)]} \quad (1.8)$$

and

$$\phi_{3,4} = \frac{\phi_3(1 + \gamma)}{[1 + (\gamma\phi_3/\phi_4)]} = \frac{\phi_4(1 + \gamma^{-1})}{[1 + (\phi_4/\gamma\phi_3)]} \quad (1.9)$$

with similar expressions for $\Phi_{1,2}$ and $\Phi_{3,4}$.

If either of the pair of steps is cleanly rate limiting, then the mixed fractionation factor reduces to the single fractionation factor for the rate-limiting transition state. For instance, if

$$k_2 \ll k_{-1}, \ \alpha \ll 1 \text{ and } \phi_{1,2} \simeq \phi_2$$

TABLE I: Definitions of r, s, and p at Time t.

		Direction of the Reaction	
		$S \longrightarrow P \xrightarrow{fast} P_2$	$S_2 \xleftarrow{fast} S \longleftarrow P$
Fraction of reactant remaining	r	$\dfrac{(s + s')_t}{(s + s')_{t = 0}}$	$\dfrac{(p + p')_t}{(p + p')_{t = 0}}$
Isotopic content of S or S_2	s	$\left(\dfrac{s'}{s + s'}\right)_t$	$\left(\dfrac{s_2'}{s_2 + s_2'}\right)_t$
Isotopic content of P or P_2	p	$\left(\dfrac{p_2'}{p_2 + p_2'}\right)_t$	$\left(\dfrac{p'}{p + p'}\right)_t$

TABLE II: The 16 Different Isotopic Experiments.

Isotope	Starts in	Reactant at $t = 0$	Measure	As a Function of	Title
—	—	S	$v_0/\Sigma e$	s_0	HSv
D	S'	S'	$v_0/\Sigma e$	s_0'	DS'v
D	S'	S'	s	r	DS's
D	S'	S'	p	r	DS'p
T	S'	S + S'	s	r	TS's
T	S'	S + S'	p	r	TS'p
T	Solvent	S	s	r	TSs
T	Solvent	S	p	r	TSp
—	—	P	$v_0/\Sigma e$	p_0	HPv
D	P'	P'	$v_0/\Sigma e$	p_0'	DP'v
D	P'	P'	p	r	DP'p
D	P'	P'	s	r	DP's
T	P'	P + P'	p	r	TP'p
T	P'	P + P'	s	r	TP's
T	Solvent	P	p	r	TPp
T	Solvent	P	s	r	TPs

but if

$$k_2 \gg k_{-1}, \ \alpha \gg 1 \text{ and } \phi_{1,2} \simeq \phi_1$$

However, if both steps are partially rate limiting (α or $\gamma \sim 1$), then the mixed factor will be intermediate between the factors for the two transition states. Thus the mixed fractionation factors describe the effective transition state fractionation for the reaction of EZ to either S or P.

Types of Experiment. Experiments can be carried out either in the S to P direction or in the P to S direction. The reaction of the target enzyme is made irreversible by the rapid conversion of the product to another species: P is converted to P_2 (when the reaction is run in the S $\rightarrow$ P direction) or S is converted to S_2 (when the reaction is run in the P $\rightarrow$ S direction). The species S_2 and P_2 must have the same isotopic composition as S and P from which they are formed. The isotopic compositions of the reactant (S or P) and of the final product (P_2 or S_2) are measured as a function of the extent of consumption (r) of the reactant. In the course of any reaction, r decreases from unity to zero as the reactant is consumed.

Table I gives the definitions of r, and of s and p, where s and p are the experimentally measured isotopic compositions of S (or S_2) and P (or P_2).

There are 16 different types of experiment which are listed in Table II. In order to describe how the results from the different experiments can be combined together, each experiment is given a three-letter description: the first letter gives the isotope; the second letter gives the reactant (the prime again denoting whether the reactant is isotopically labeled or not);

and the third letter describes the type of experiment (v is an initial velocity measurement, v_0, as a function of substrate concentration s_0 or p_0 and total enzyme concentration Σe; **s** and **p** show that the isotopic composition of S or P is measured as a function of the extent of reaction (r)).

The Kinetic Equations

Preview. In this section we derive the kinetic equations for each type of experiment in Table II. Four of these relations are the steady-state Michaelis equations (in Lineweaver–Burk form). Four relate to the measurement of **s** and **p** as a function of r for reactions of unlabeled reactant in tritiated water. Eight relate **s** and **p** as a function of r for reactions of specifically labeled (D or T) reactant in unlabeled water.

Since the system is symmetrical, we derive the equations for the 8 experiments in the S to P direction: the relations for experiments in the P to S direction can then be written down. The algebraic form of the relations between the experimentally measured quantities is relatively simple, and the experimental results can be plotted to yield values for parameters that we have called A_n (for S to P experiments) and B_n (for P to S experiments). Because Scheme I contains nine transition states, the A_n and B_n parameters are complicated functions of the individual rate constants and fractionation factors. The second part of this paper therefore deals with the way to combine the determined values of A_n and B_n so as to extract the individual rate constants and fractionation factors. First, however, we must obtain the equations for the 8 experiments (in the S to P direction) of Table II.

Basic Kinetic Equations. The basic equations are written down in terms of the fluxes f_n and f_n' through each transition state of Scheme I:

$$f_1 = k_1 e \cdot s - k_{-1} es$$

$$f_2 = k_2 es - k_{-2} ez$$

and so on until

$$f_5 = k_5 \phi_5 x\, ez - k_5'(1 - x)ez'$$

We next make the steady-state approximations for the intermediates, giving:

$$f_1 = f_2$$
$$f_2 = f_3 + f_5 \qquad (2.1)$$
$$f_3 = f_4$$
$$f_1' = f_2'$$
$$f_2' = f_3' - f_5 \qquad (2.2)$$
$$f_3' = f_4'$$

Finally the total enzyme concentration is constant, giving:

$$\Sigma e = e + es + ez + ep + es' + ez' + ep' \qquad (2.3)$$

This method of writing down the kinetic equations leads to much simpler algebra than do more traditional methods of writing dx/dt = etc.

We now derive the equations for experiments in the S to P direction. Since P and P' are being rapidly removed (to P_2 and P_2'), we can put p and p' equal to zero. We then obtain by successive substitutions in the equations for the fluxes in the H, and in the D or T systems, the following expressions for the steady-state concentrations of the different intermediates:

for H

$$ep = f_3/k_4 \qquad (2.4)$$
$$ez = [(1 + \gamma)f_3]/k_3 \qquad (2.5)$$
$$es = [f_2 + \theta(1 + \alpha)f_3]/k_2 \qquad (2.6)$$
$$e \cdot s = [(1 + \alpha)(f_2 + \theta f_3)]/(\alpha k_1) \qquad (2.7)$$

for D or T

$$ep' = f_3'/k_4' \qquad (2.8)$$
$$ez' = [(1 + \gamma')f_3']/k_3' \qquad (2.9)$$
$$es' = [f_2' + \theta'(1 + \alpha')f_3']/k_2' \qquad (2.10)$$
$$e \cdot s' = [(1 + \alpha')(f_2' + \theta'f_3')]/(\alpha'k_1') \qquad (2.11)$$

In these equations the primed quantities α', γ', and θ' have the same definitions in terms of the primed rate constants as their unprimed counterparts. Now we consider each type of experiment in the S to P direction given in Table II.

The HSv Experiment. This experiment is the measurement of steady-state initial rates of reaction (v_0) at different initial substrate concentrations (s_0). No isotopes are involved. Since it is an all-H experiment:

$$f_5 = 0$$

and

$$f_2 = f_3 = v$$

Substitution of eq 2.4 to 2.7 in 2.3 gives, as $t \to 0$:

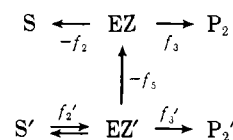$$\Sigma e/v_0 = A_1/s_0 + A_2 \qquad (3.1)$$

where

$$A_1 = \frac{(1 + \alpha)(1 + \theta)}{\alpha k_1} \qquad (3.2)$$

$$A_2 = \frac{1 + \theta(1 + \alpha)}{k_2} + \frac{(1 + \gamma)}{k_3} + \frac{1}{k_4} \qquad (3.3)$$

This equation is the usual Lineweaver–Burk form of the Michaelis equation, where $A_1 = K_m/V_{max}$ and $A_2 = 1/V_{max}$.

The DS'v Experiment. This is the same type of experiment as HSv but we start with the specifically deuterated substrate S', rather than S.

SCHEME II

$$S \xleftarrow{-f_2} EZ \xrightarrow{f_3} P_2$$
$$\Big\uparrow -f_5$$
$$S' \xrightleftharpoons{f_2'} EZ' \xrightarrow{f_3'} P_2'$$

The course of the experiment may be illustrated with respect to Scheme II. The boundary conditions at $t = 0$ are $s = 0$ and $s' = s_0'$. We start by writing:

$$f_3' = A_5 f_2' \qquad (4.1)$$

where

$$A_5 = [1 + (k_5'(1 + \gamma'))/k_3']^{-1} \qquad (4.2)$$

The parameter A_5 describes the fate of EZ'. A low value of A_5 means that the deuterium is washed out and nearly all EZ' goes to EZ. A value of unity means that no deuterium is washed out and EZ' goes to P' and then to P_2'. From eq 2.1, 2.2, and 4.1, the fluxes $-f_2$ and f_3 are given by:

$$f_3 = \frac{f_2'(1 - A_5)}{1 + \theta} \qquad (4.3)$$

and

$$-f_2 = \frac{\theta f_2'(1 - A_5)}{1 + \theta} \tag{4.4}$$

Remember that θ was defined so as to describe the fate of EZ between going to S and going to P. The rate of production of P and P' is given by:

$$v_0 = f_3 + f_3' = f_2' \frac{(1 + A_5\theta)}{(1 + \theta)}$$

Substitution of the fluxes given by eq 4.1 to 4.4 in eq 2.2 to 2.9 for the concentrations, and their substitution in eq 2.10 gives:

$$\frac{\Sigma e}{v_0} = \frac{A_3}{s_0'} + A_4 \tag{4.5}$$

where

$$A_3 = A_1 \frac{\phi_S}{\phi_{1,2}} \left[\frac{1 + A_5\theta'}{1 + A_5\theta} \right] \tag{4.6}$$

and

$$A_4 = A_2 + \frac{(1 + \theta)}{(1 + A_5\theta)} \left[\frac{1}{k_2'} - \frac{1}{k_2} + A_5 \left\{ \frac{\theta'(1 + \alpha')}{k_2'} \right. \right.$$
$$\left. \left. - \frac{\theta(1 + \alpha)}{k_2} + \frac{1 + \gamma'}{k_3'} - \frac{1 + \gamma}{k_3} + \frac{1}{k_4'} - \frac{1}{k_4} \right\} \right] \tag{4.7}$$

Equation 4.5 has the same form as the usual Lineweaver–Burk expression. However, A_3 and A_4 are in general different from A_1 and A_2. Note that, if the isotopic substitution makes no difference, which means that the primed and unprimed quantities are the same and $\phi_{1,2} = \phi_S = 1$, then $A_3 = A_1$ and $A_4 = A_2$. On the other hand, if there is no isotopic exchange at EZ, $A_5 = 1$ and this means that A_3 and A_4 reduce to the primed counterparts of A_1 and A_2.

The DS's and DS'p Experiments. The reaction and the boundary conditions are the same as for DS'v. In these experiments, however, we analyze the isotopic content of the reactants (S' and S) and the products (P$_2'$ and P$_2$) as a function of the extent of the reaction. We are not just interested in the initial rate of the reaction, and the scheme for the experiment is, therefore, as shown in Scheme III. Again the EZ' to EZ step is an irreversible washing out of the isotope.

SCHEME III

$$S \rightleftharpoons EZ \longrightarrow P_2$$
$$\uparrow$$
$$S' \rightleftharpoons EZ' \longrightarrow P_2'$$
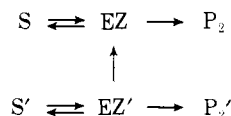
At $t = 0$, $s' = s_0'$ and $s = 0$. The deuterated system behaves the same as for DS'v, while for the hydrogen system we can write from eq 2.1, 2.2, and 4.1:

$$f_3 = f_2 - f_5 = f_2 + f_2'(1 - A_5)$$

Substitution of the fluxes in eq 2.7 and 2.11 gives:

$$\frac{\alpha k_1 s}{\alpha' k_1' s'} = \frac{\alpha k_1}{\alpha' k_1'} (s^{-1} - 1)$$

$$= \frac{(1 + \alpha)[(f_2/f_2')(1 + \theta) + \theta(1 - A_5)]}{(1 + \alpha')(1 + \theta'A_5)} \tag{5.1}$$

But

$$ds/ds' = f_2/f_2'$$

and, from

$$s' = srs_0'$$

and

$$s = rs_0' - s'$$

$$\frac{ds}{ds'} = s_0' \frac{dr}{ds'} - 1$$

and

$$\frac{ds'}{s_0'r} = \frac{1}{1 + ds/ds'} = \frac{1}{1 + f_2/f_2'} = s + \frac{ds}{d \ln r} \tag{5.2}$$

Substitution in eq 5.1 gives

$$s + \frac{ds}{d \ln r} = \frac{A_6}{s^{-1} - 1 + A_1/A_3}$$

where

$$A_6 = \frac{\phi_{1,2}}{\phi_S} \frac{(1 + \theta)}{(1 + A_5\theta')} \tag{5.3}$$

This equation can be integrated to give for $A_6 \neq 1$

$$(A_6 - 1) \ln r =$$

$$\ln (s/s_0) - A_6 \ln \left[\frac{A_6 - 1 + [1 - (A_1/A_3)]s}{A_6 - 1 + [1 - (A_1/A_3)]s_0} \right] \tag{5.4}$$

In the particular case of $A_6 = 1$

$$\ln r = \frac{1}{1 - A_1/A_3} \left(\frac{1}{s_0} - \frac{1}{s} \right) - \ln \left(\frac{s}{s_0} \right) \tag{5.5}$$

In the DS's experiment considered here $s_0 = 1$ since all the starting material is deuterated at $t = 0$, and therefore for $A_6 \neq 1$

$$s^{1/A_6} r^{(1/(A_6 - 1))} = 1 - \left[\frac{1 - (A_1/A_3)}{A_6 - (A_1/A_3)} \right] (1 - s) \tag{5.6}$$

or for $A_6 = 1$

$$\ln sr = \frac{1}{1 - (A_1/A_3)} \left(1 - \frac{1}{s} \right) \tag{5.7}$$

For the DS'p experiment we have from eq 4.1:

$$\frac{dp_2'}{ds'} = -\frac{f_3'}{f_2'} = -A_5 \tag{5.8}$$

Integration gives:

$$p_2' + A_5 s' = A_5 s_0'$$

and

$$p_2' = A_5 s_0'(1 - sr)$$

but

$$p_2 + p_2' = s_0'(1 - sr)$$

Hence

$$p = \frac{p_2'}{p_2 + p_2'} = \frac{A_5(1 - sr)}{(1 - r)} \tag{5.9}$$

Note that when $r \rightarrow 0$, and the reaction is complete:

$$p_\infty \rightarrow A_5$$

This must be correct since the partitioning between k_3' and k_5' as EZ' goes to P$_2'$ or EZ determines the overall isotopic content of the product at the end of the reaction. All the material that has the isotope washed out will eventually end up as P$_2$. But

at the start of the reaction, by expanding eq 5.9 with the binomial theorem, we may show that

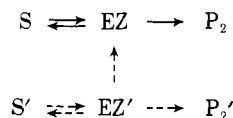$$\mathbf{p}_0 \rightarrow A_5 A_6 A_3 / A_1 \qquad (5.10)$$

where

$$\frac{A_6 A_3}{A_1} = \frac{1 + \theta}{1 + A_5 \theta} \lessgtr 1$$

Thus the product is richer in deuterium at the start of the reaction than at the end, especially if a lot of S′ is converted to S. This happens when the isotope is washed out ($A_5 \ll 1$) and when EZ goes to S rather than P ($\theta \gg 1$). The step EZ′ to P_2' forms product immediately, but the washing out process forms S rather than P_2; the material accumulates as S and then is subsequently converted to P_2 diluting the isotopic content of the product from $\mathbf{p}_0$ to $\mathbf{p}_\infty$.

The TS′s and TS′p Experiments. In these experiments the tritium content of the reactant S or the product P_2 is found as a function of the extent of reaction. The reactant is S, of which a small fraction is tritiated so the boundary conditions at $t = 0$ are $s = s_0$ and $\mathbf{s} = \mathbf{s}_0 \ll 1$. The scheme for this experiment is as shown in Scheme IV.

SCHEME IV

$$
\begin{array}{ccccc}
S & \rightleftharpoons & EZ & \longrightarrow & P_2 \\
 & & \uparrow & & \\
 & & \vdots & & \\
S' & \rightleftharpoons & EZ' & \dashrightarrow & P_2'
\end{array}
$$

In this case since $s_0 \ll 1$ we may, providing $A_6' \neq 1$, ignore the third term in eq 5.4. Hence:

$$\ln (\mathbf{s}/\mathbf{s}_0) = (A_6' - 1) \ln r \qquad (5.11)$$

or

$$\mathbf{s} = \mathbf{s}_0 r^{A_6'-1}$$

We have introduced the prime on A_6 to indicate that the fractionation factors must refer to T and not D.

If $A_6' = 1$, from eq 5.5

$$\mathbf{s}_0/\mathbf{s} = 1 + \mathbf{s}_0[1 - (A_1'/A_3')] \ln (s_0/sr)$$

$$\simeq 1$$

because $\mathbf{s}_0 \ll 1$. This is the same result as given by eq 5.11. In eq 5.11 since r goes from 1 to 0 as the reaction proceeds, if $A_6' > 1$, the isotopic content of the reactant decreases; if $A_6' = 1$, the isotopic content of the reactant remains constant; and if $A_6' < 1$, the isotopic content of the reactant increases. Now from eq 5.3

$$A_6' = \frac{\Phi_{1,2}(1 + \theta)}{\Phi_S(1 + A_5 \theta')}$$

We consider two special cases of the expression for A_6'. First, if the step ES → EZ is the only rate-limiting step, then $\Phi_{1,2} \simeq \Phi_2$ and θ is much less than one, since EZ goes to P rather than S, and so $A_6' \simeq \Phi_2/\Phi_S$. Then for a rate-determining proton transfer, $\Phi_2 < \Phi_S$ and $A_6' < 1$. The isotopic content of the reactant increases because the tritiated reactant, S′, is consumed more slowly than S.

Secondly, if the isotopic exchange between EZ′ and EZ is rapid, then $A_5 \ll 1$. Now, if either of the steps after the exchange (EZ → EP → P) are rate determining, $\theta \gg 1$ and $A_6' > 1$. Here the slow step from EZ to P and the rapid isotope exchange means that the isotope is washed out by the route S′ → EZ′ → EZ → S early on in the reaction and the isotopic content decreases.

For the tritium content of the product, by similar arguments to those used for DS′p:

$$p_2' = a_5's_0(s_0 - sr)$$

$$p_2 + p_2' = s_0(1 - r)$$

and

$$\mathbf{p} = \mathbf{s}_0 A_5'(1 - r^{A_6'})/(1 - r) \qquad (5.12)$$

As in eq 5.9 at long times when r is small:

$$\mathbf{p}_\infty \rightarrow \mathbf{s}_0 A_5'$$

Again, as in DS′p, A_5' describes the partition between the washing out of the isotope into the medium and its retention in the product.
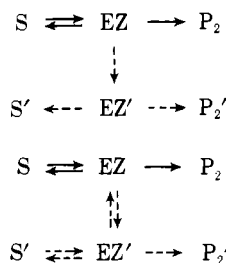
At the start of the reaction

$$\mathbf{p}_0 \rightarrow \mathbf{s}_0 A_5' A_6' \qquad (5.13)$$

Depending on whether A_6' is greater or smaller than 1, $\mathbf{p}_0$ will be larger or smaller than $\mathbf{p}_\infty$. The reason why $\mathbf{p}$ might decrease during the course of the reaction is the same as for the deuterium case; as discussed above, A_6' is greater than 1 when S′ is converted to S rather than reacting to P′. In the first case discussed above where $A_6' < 1$, the fractionation factor ratio (Φ_S/Φ_2) means that S′ reacts more slowly than S. Hence to start with, the [T]/[H] ratio in the product is smaller than when S′ has eventually had time to react.

The TSs and TSp Experiments. In these experiments the reactant is initially unlabeled S with a tritium atom fraction in the solvent of x. The uptake of T from the solvent into the reactant and the product is measured as a function of the extent of reaction. The scheme for this experiment is, therefore, as shown in Scheme V. The boundary conditions at $t = 0$ are $s = s_0$ and $s' = 0$.

SCHEME V
at start:

$$
\begin{array}{ccccc}
S & \rightleftharpoons & EZ & \longrightarrow & P_2 \\
 & & \downarrow & & \\
S' & \longleftarrow\!\!- & EZ' & \dashrightarrow & P_2'
\end{array}
$$

later on:

$$
\begin{array}{ccccc}
S & \rightleftharpoons & EZ & \longrightarrow & P_2 \\
 & & \updownarrow & & \\
S' & \rightleftharpoons & EZ' & \dashrightarrow & P_2'
\end{array}
$$

From eq 2.1 and 2.2

$$f_5 = f_3' - f_2' = k_5 \Phi_5 x \, ez - k_5' ez' = f_2 - f_3$$

Since $x \ll 1$, $|f_3'| \ll f_3$, $|f_2'| \ll f_2$

and

$$f_2 \simeq f_3 \qquad (6.1)$$

Substitution of eq 2.5, 2.9, and 4.2 gives:

$$f_3' = A_5'[f_2' + k_5 \Phi_5 x f_2(1 + \gamma)/k_3] \qquad (6.2)$$

Then from eq 2.7 and 2.11 we obtain:

$$\frac{\alpha' k_1' s'}{\alpha k_1 s} =$$

$$\frac{(1 + \alpha')[\theta' A_5' \Phi_5 x k_5(1 + \gamma)/k_3 + (1 + \theta' A_5')f_2'/f_2]}{(1 + \alpha)(1 + \theta)}$$

Since $s' \ll s$, from eq 5.2

$$\frac{ds'}{ds} = \frac{f_2'}{f_2} = \mathbf{s} + \frac{d\mathbf{s}}{d \ln r} \qquad (6.3)$$
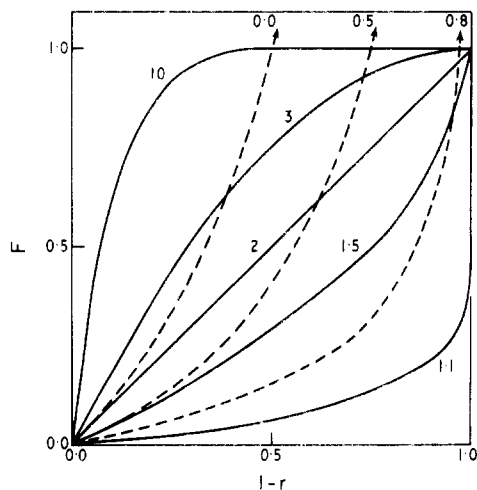
FIGURE 3: Plots of eq 6.8 showing isotopic content of reactant during the course of reaction as a function of the fraction of substrate remaining (r) for the different values of A_6' given by each line. Full lines refer to $A_6' > 1$ scale; broken lines to $A_6' < 1$.

and

$$s' \simeq ss$$

Hence

$$\frac{d(s/x)}{d \ln r} = (A_6' - 1)\frac{s}{x} - A_7' \tag{6.4}$$

where

$$A_7' = \frac{\theta \Phi_{1,2}(1 - A_5')}{1 + A_5'\theta'} \tag{6.5}$$

Integration gives for $A_6' \neq 1$

$$\frac{s}{x} = \frac{A_7'}{A_6' - 1}(1 - r^{A_6'-1}) \tag{6.6}$$

For $A_6' = 1$ we obtain

$$s/x = -A_7' \ln r \tag{6.7}$$

For different values of A_6', Figure 3 shows the behavior of the function F plotted against the course of the reaction given by $1 - r$ where

$$F = |(1 - r^{A_6'-1})| \tag{6.8}$$

Three types of behavior may be distinguished depending on the size of A_6':

"equilibration" $A_6' > 2$

"accumulation" $A_6' < 1$

and "intermediate" $2 > A_6' > 1$

where from eq 5.3

$$A_6' = \frac{\Phi_{1,2}(1 + \theta)}{\Phi_S(1 + A_5\theta')}$$

For "equilibration" the curves flatten out at the end of the reaction and the isotopic content of the reactant reaches a steady value which will be close to its equilibrium value. These curves arise when $A_6' > 2$. This condition is found if there is fast isotopic exchange ($A_5' \ll 1$) and if either of the last two steps (EZ → EP → P) is rate determining ($\theta \gg 1$). The rapid isotopic exchange coupled with the slow final steps allows the reactant time to equilibrate isotopically with the solvent before it is converted to product. By contrast for "accumulation" the

isotopic content of the reactant never reaches a steady value but the tritium content of the reactant grows steadily greater until the last molecule of reactant decomposes and it will be tritiated. These curves for which $A_6' < 1$ are shown by the broken lines in Figure 3. This condition is found where the second step (ES → EZ) is both rate determining ($\theta \ll 1$ and $\Phi_{1,2} \simeq \Phi_2$) and a proton transfer ($\Phi_2 < \Phi_S$). The rate-determining proton transfer means that any tritiated reactant that is formed lingers on while the H reactant is preferentially consumed. The "intermediate" curves for $2 > A_6' > 1$ have the same curvature as the "accumulation" curves but like the "equilibration" curves they tend to a finite value at the end of the reaction.

Although the parameter A_6' describes the shape of the curve as shown in Figure 3 the actual magnitude of s/x is also determined by A_7'. In particular at the start of the reaction when $r \simeq 1$ expanding eq 6.6 by the binomial theorem gives:

$$s/x \simeq A_7'(1 - r)$$

Thus the initial gradient of s/x against r is determined entirely by A_7'. From eq 6.5

$$A_7' = \frac{\theta \Phi_{1,2}(1 - A_5')}{1 + A_5'\theta'}$$

$$\simeq \theta \Phi_{1,2} \text{ for } A_5' \to 0$$

The condition $A_5' \to 0$ corresponds to fast isotopic exchange. A_7' is mainly determined by θ. If $\theta \gg 1$ then both $A_7' \gg 1$ and $A_6' \gg 1$ giving us "equilibration"; s/x rises rapidly to a steady value of:

$$\frac{s}{x} \simeq \frac{A_7'}{A_6'} \simeq \Phi_S$$

The isotopic content of the reactant does indeed reach its equilibrium value as given by the fractionation factor Φ_S. At the other extreme for "accumulation" $A_7' \ll 1$ and the initial uptake of isotope is slow compared with conversion.

For the product (TS'p) from eq 6.1 and 6.2:

$$\frac{dp_2'}{dp_2} = \frac{f_3'}{f_3} = A_5'\frac{f_2'}{f_2} + \left[\frac{k_5\Phi_5 x(1 + \gamma)}{k_3}\right]$$

$$= A_5'\left[\frac{ds'}{ds} + \frac{\Phi_5 k_5 x(1 + \gamma)}{k_3}\right]$$

From eq 6.3, 6.4, and 6.6:

$$\frac{dp_2'}{x dp_2} = A_5'\left[A_6'\frac{s}{x} - A_7'\frac{(1 + \gamma)\Phi_5 k_5}{k_3}\right]$$

$$= A_5'\left[\frac{A_7'}{A_6' - 1} + \frac{(1 + \gamma)\Phi_5 k_5}{k_3} - \frac{A_6'A_7'}{A_6' - 1}r^{A_6'-1}\right]$$

Since $dp_2 = -s_0 dr$, this equation can be integrated, with the boundary condition $p_2' = 0$ when $r = 1$, to give:

$$\frac{p_2'}{x s_0} = A_5'\frac{A_7'}{A_6' - 1} + \frac{(1 + \gamma)\Phi_5 k_5}{k_3}(1 - r)$$

$$- \frac{A_7'}{A_6' - 1}(1 - r^{A_6'})$$

but, since for tritium $x \ll 1$, $p_2 = s_0(1 - r)$, and so

$$\frac{p}{x} = A_8' - \frac{A_5'A_7'}{A_6' - 1}\left(\frac{r - r^{A_6'}}{1 - r}\right) \tag{6.9}$$

where

$$A_8' = \Phi_{3,4}(1 - A_5') \tag{6.10}$$

TABLE III: Equations for the Experiments in Table II.

Expt	Eq ($S \rightarrow P \rightarrow P_2$)	Eq No.	Expt	Eq ($S_2 \leftarrow S \leftarrow P$)
HSv	$\Sigma e/v_0 = A_1/s_0 + A_2$	(3.1)	HPv	$\Sigma e/v_0 = B_1/p_0 + B_2$
DS'v	$\Sigma e/v_0 = A_3/s_0' + A_4$	(4.5)	DP'v	$\Sigma e/v_0 = B_3/p_0' + B_4$
DS's	$A_6 \ln\left[\dfrac{A_6 - 1 + (1 - A_1/A_3)s}{A_6 - A_1/A_3}\right] =$ $\ln s - (A_6 - 1)\ln r$ for $A_6 \neq 1$	(5.6)	DP'p	$B_6 \ln\left[\dfrac{B_6 - 1 + (1 - B_1/B_3)p}{B_6 - B_1/B_3}\right] =$ $\ln p - (B_6 - 1)\ln r$ for $B_6 \neq 1$
	$\ln(sr) = (1 - s^{-1})/(1 - A_1/A_3)$ for $A_6 = 1$	(5.7)		$\ln(pr) = (1 - p^{-1})/(1 - B_1/B_3)$ for $B_6 = 1$
DS'p	$p = A_5(1 - sr)/(1 - r)$ $p_0 = A_5 A_6 A_3/A_1$ $p_\infty = A_5$	(5.9) (5.10)	DP's	$s = B_5(1 - pr)/(1 - r)$ $s_0 = B_5 B_6 B_3/B_1$ $s_\infty = B_5$
TS's	$\ln(s/s_0) = (A_6' - 1)\ln r$	(5.11)	TP'p	$\ln(p/p_0) = (B_6' - 1)\ln r$
TS'p	$p/s_0 = A_5'(1 - rA_6')/(1 - r)$ $p_0/s_0 = A_5'A_6'$ $p_\infty/s_0 = A_5'$	(5.12) (5.13)	TP's	$s/p_0 = B_5'(1 - rB_6')/(1 - r)$ $s_0/p_0 = B_5'B_6'$ $s_\infty/p_0 = B_5'$
TSs	$s/x = A_7'(1 - rA_6'^{-1})/(A_6' - 1)$ for $A_6' \neq 1$ $s/x = -A_7' \ln r$ for $A_6' = 1$	(6.6) (6.7)	TPp	$p/x = B_7'(1 - rB_6'^{-1})/(B_6' - 1)$ for $B_6' \neq 1$ $p/x = -B_7' \ln r$ for $B_6' = 1$
TSp	$p/x = A_8' - \dfrac{A_5'A_7'}{A_6' - 1}\left(\dfrac{r - rA_6'}{1 - r}\right)$ $p_0/x = A_8' - A_5'A_7'$ $p_\infty/x = A_8'$	(6.9) (6.11)	TPs	$s/x = B_8' - \dfrac{B_5'B_7'}{B_6' - 1}\left(\dfrac{r - rB_6'}{1 - r}\right)$ $s_0/x = B_8' - B_5'B_7'$ $s_\infty/x = B_8'$

Note that, if $A_5' = 0$, which corresponds to fast isotopic equilibration of EZ, $p/x = \Phi_{3,4}$. This is the correct answer since the isotopic content of the product is then determined by the fractionation in the steps after EZ. At long times, when $r \rightarrow 0$

$$\left(\frac{p}{x}\right)_\infty \rightarrow A_8' = \Phi_{3,4}(1 - A_5')$$

The term in A_5' describes the reduced isotopic content of the product because of slow equilibration at EZ. At short times:

$$\left(\frac{p}{x}\right)_0 \rightarrow A_8' - A_5'A_7'$$

$$= \frac{\Phi_{3,4}(1 - A_5')}{(1 + A_5'\theta')} \tag{6.11}$$

The term in A_5' describes the diversion of some of the tritiated material at the start of the reaction to form reactant S' rather than product P_2'; this leads initially to a smaller tritium content in the product. Eventually, however, this material decomposes and (p/x) increases to its final value.

Experimental Equations. We have collected together in Table III the equations describing the experimental data for the different types of experiment in Table II. Experiments in the P to S direction are described by a similar set of equations with A_n replaced by B_n, s replaced by p, S by P, s by p, and p by s. In Table IV we have collected together the definitions of A_n and B_n. The equations for B_n are derived from the symmetry of the system. This completes the first stage of the analysis.
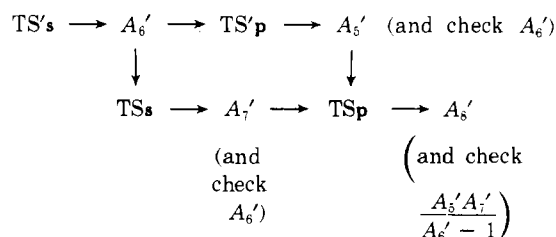
Derivation of Rate Constants and Fractionation Factors from Data

The equations presented in Table III allow one to determine from the experimental data values of the parameters A_n and B_n; however, the definitions in Table IV show that in general

A_n and B_n are functions of a number of rate constants and fractionation factors. The purpose of this section is to show how, by doing the right group of experiments and obtaining values for different A_n and B_n, one can then derive values for the individual rate constants and fractionation factors without assuming simplified forms of the definitions in Table IV for A_n and B_n.

We first discuss the best procedures for obtaining reliable values of A_n and B_n.

Tritium Experiments. We start with the experiments involving T and indicate schematically how the parameters A_n' (and B_n') can be found from the experiments. From Table III

$$\text{TS's} \longrightarrow A_6' \longrightarrow \text{TS'p} \longrightarrow A_5' \text{ (and check } A_6')$$
$$\downarrow \qquad\qquad\qquad\qquad \downarrow$$
$$\text{TSs} \longrightarrow A_7' \longrightarrow \text{TSp} \longrightarrow A_8'$$
$$\text{(and check } A_6') \qquad \left(\text{and check } \dfrac{A_5'A_7'}{A_6' - 1}\right)$$

The scheme shows that TS's gives a value of A_6'; this with TS'p yields a value of A_5' and may provide a check on A_6', and so on. For B_n, replace S with P and A with B, and interchange s and p. In this scheme each type of experiment has only to yield a value for one parameter. The linearity of the plots and the values of the intercepts would provide confirmation of the model and the values of the parameters already determined.

From the eight pieces of data A_5' to A_8' and B_5' to B_8' one can calculate four relations between the various A_n' and B_n' and four useful pieces of kinetic information:

relations between A_n and B_n:

$$\frac{B_5'}{A_5'} = \frac{\Phi_S}{\Phi_P}\frac{A_6'}{B_6'} = \frac{B_8'}{B_7'}\frac{[1 - B_5']}{[1 - A_5'B_5']} = \frac{A_7'}{A_8'}\frac{[1 - A_5'B_5']}{[1 - A_5']} \tag{7.1}$$

TABLE IV: Definitions of A_n and B_n.[a]

A_n	Eq	B_n
$A_1 = \dfrac{(1 + \alpha)(1 + \theta)}{\alpha k_1}$	(3.2)	$B_1 = \dfrac{(1 + \gamma)(1 + \theta)}{\gamma \theta k_{-4}}$
$A_2 = \dfrac{1 + \theta(1 + \alpha)}{k_2} + \dfrac{(1 + \gamma)}{k_3} + \dfrac{1}{k_4}$	(3.3)	$B_2 = \dfrac{1 + \theta^{-1}(1 + \gamma)}{k_{-3}} + \dfrac{1 + \alpha}{k_{-2}} + \dfrac{1}{k_{-1}}$
$A_3 = A_1 \dfrac{\phi_S(1 + A_5\theta')}{\phi_{1,2}(1 + A_5\theta)}$	(4.6)	$B_3 = B_1 \dfrac{\phi_P}{\phi_{3,4}} \dfrac{(1 + B_5/\theta')}{(1 + B_5/\theta)}$
$A_4 = A_2 + \dfrac{(1 + \theta)}{(1 + A_5\theta)}\left[\dfrac{1}{k_2'} - \dfrac{1}{k_2} + A_5A_9\right]$	(4.7)	$B_4 = B_2 + \dfrac{(1 + \theta)}{(\theta + B_5)}\left[\dfrac{1}{k_{-3}'} - \dfrac{1}{k_{-3}} + B_5B_9\right]$
$A_5 = (1 + k_5'(1 + \gamma')/k_3')^{-1}$	(4.2)	$B_5 = (1 + k_5'(1 + \alpha')/k_{-2}')^{-1}$
$A_6 = \dfrac{\phi_{1,2}}{\phi_S} \dfrac{(1 + \theta)}{(1 + A_5\theta')}$	(5.3)	$B_6 = \dfrac{\phi_{3,4}}{\phi_P} \dfrac{(1 + \theta^{-1})}{(1 + B_5/\theta')}$
$A_7' = \dfrac{\theta\Phi_{1,2}(1 - A_5')}{1 + A_5'\theta'}$	(6.5)	$B_7' = \dfrac{\Phi_{3,4}(1 - B_5')}{\theta(1 + B_5/\theta')}$
$A_8' = \Phi_{3,4}(1 - A_5')$	(6.10)	$B_8' = \Phi_{1,2}(1 - B_5')$
$A_9 = \dfrac{\theta'(1 + \alpha')}{k_2'} - \dfrac{\theta(1 + \alpha)}{k_2} + \dfrac{1 + \gamma'}{k_3'} - \dfrac{1 + \gamma}{k_3} + \dfrac{1}{k_4'} - \dfrac{1}{k_4}$	(4.7)	$B_9 = \dfrac{1 + \gamma'}{\theta'k_{-3}'} - \dfrac{1 + \gamma}{\theta k_{-3}} + \dfrac{1 + \alpha'}{k_{-2}'} - \dfrac{1 + \alpha}{k_{-2}} + \dfrac{1}{k_{-1}'} - \dfrac{1}{k_{-1}}$

[a] Notes: $A_n' = A_n$ with ϕ replaced by Φ; $\Phi_{1,2} = [\phi_2(1 + \alpha)]/(1 + \alpha')$; $\Phi_{1,2} = [\Phi_2(1 + \alpha)]/(1 + \alpha')$; $\alpha = k_2/k_{-1}$; $\alpha' = k_2'/k_{-1}'$; $\theta = (k_{-2}/k_3) \times [(1 + \gamma)/(1 + \alpha)]$; $B_n' = B_n$ with ϕ replaced by Φ; $\phi_{3,4} = [\phi_3(1 + \gamma)]/(1 + \gamma')$; $\Phi_{3,4} = [\Phi_3(1 + \gamma)]/(1 + \gamma')$; $\gamma = k_{-3}/k_4$; $\gamma' = k_{-3}'/k_4'$; $\theta' = (k_{-2}'/k_3')[(1 + \gamma')/(1 + \alpha')]$.

$$\frac{A_7'}{\Phi_S A_6'(1 - A_5')} + \frac{B_7'}{\Phi_P B_6'(1 - B_5')} = 1 \qquad (7.2)$$

kinetic information:

$$\Phi_{1,2} = B_8'/(1 - B_5') \qquad (7.3)$$

$$\Phi_{3,4} = A_8'/(1 - A_5') \qquad (7.4)$$

$$\theta = \frac{A_7'}{\Phi_S A_6'(1 - A_5') - A_7'} = \frac{\Phi_P B_6'(1 - B_5') - B_7'}{B_7'} \qquad (7.5)$$

$$\frac{k_3'}{k_5'(1 + \gamma')} = \frac{A_5'}{1 - A_5'}$$

or

$$\frac{k_{-2}'}{k_5'(1 + \alpha')} = \frac{B_5'}{1 - B_5'} \qquad (7.6)$$

Both Φ_S and Φ_P can be measured in separate exchange experiments at equilibrium as described by eq 1.3.

Equations 7.1 and 7.2 can be used to check the consistency of the model and data or to calculate any A_n' and B_n' not determined experimentally; the use made of these equations will depend on the number of different types of experiment that can be or have been actually carried out. Notice that even if it is possible only to measure A_5' to A_8' one can still calculate B_5' to B_8' (and vice versa).

In many systems the k_5 step, exchanging the isotopes at EZ, may be very rapid and A_5' and B_5' may then be much smaller than 1. Equations 7.1 to 7.5 all simplify and we have three relations between A_n' and B_n' and three pieces of kinetic information.

The value of θ may be combined with the data from the Lineweaver-Burk plots from HSv and HPv giving:

$$\frac{\alpha k_1}{1 + \alpha} = \frac{1 + \theta}{A_1} \qquad (7.7)$$
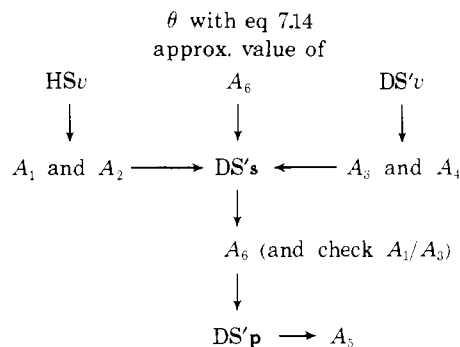
$$\frac{1}{k_2} - \frac{1}{k_{-3}} = \frac{A_2 - B_2\theta}{1 + \theta} \qquad (7.8)$$

and

$$\frac{\gamma k_{-4}}{1 + \gamma} = \frac{1 + \theta^{-1}}{B_1} \qquad (7.9)$$

Deuterium Experiments. We now turn to the experiments with D. The routes for finding the experimental parameters may be expressed schematically (see Scheme VI). For B_n again

SCHEME VI

replace S with P and A with B and interchange s and p. The six pieces of data, A_n and B_n where $n = 3, 5$, and 6, give two relations between the parameters and four pieces of kinetic information:

relations between A_n and B_n:

$$\frac{B_5}{A_5} = \frac{\phi_S A_6}{\phi_P B_6} \qquad (7.10)$$

$$\frac{A_1/A_6A_3 - A_5}{1 - A_5} + \frac{B_1/B_6B_3 - B_5}{1 - B_5} = 1 \qquad (7.11)$$

kinetic information:

$$\phi_{1,2} = \frac{A_1\phi_S(1 - A_5B_5)}{A_3(1 + A_5\theta)(1 - B_5)} \qquad (7.12)$$

$$\phi_{3,4} = \frac{B_1\phi_P}{B_3} \frac{(1 - A_5B_5)}{(1 + B_5\theta^{-1})(1 - A_5)} \qquad (7.13)$$

$$\theta = \frac{B_1/B_6B_3 - B_5}{1 - B_1/B_6B_3} = \frac{1 - A_1/A_6A_3}{A_1/A_6A_3 - A_5} \qquad (7.14)$$

and

$$\frac{k_3'}{k_5'(1 + \gamma')} = \frac{A_5}{1 - A_5}$$

or

$$\frac{k_{-2}'}{k_5'(1 + \alpha')} = \frac{B_5}{1 - B_5} \qquad (7.15)$$

Again if A_5 and B_5 are small (rapid isotopic exchange at EZ) the equations simplify to give one check (eq 7.11) and three pieces of information (eq 7.12 to 7.14). As in the T case eq 7.10 and 7.11 can be used to check the consistency of the data and the model or to calculate B_5 and B_6 if, for instance, only one isotopic dilution–conversion experiment has been carried out. The deuterium experiments give the equivalent pieces of kinetic information to the tritium experiments, namely $\phi_{3,4}$, $\phi_{1,2}$, θ, and a ratio containing k_5. The measurement of θ would also allow eq 7.7 to 7.9 to be used. By doing experiments with both T and D, one can also check whether the same value of θ is obtained from eq 7.5 and 7.14.

Conclusions from the Mixed Fractionation Factors. Although $\Phi_{1,2}$, $\phi_{1,2}$, $\Phi_{3,4}$, and $\phi_{3,4}$ are mixed fractionation factors interesting information can be obtained from them. For instance taking $\phi_{1,2}$, we first assume that

$$\phi_1 \simeq \phi_S \qquad (8.1)$$

We can make this assumption because the first step is the binding of the enzyme to the substrate and it is unlikely that this will cause any change in the fractionation factor. Then we can compare $\phi_{1,2}$ with ϕ_S and, if $\phi_{1,2}$ is significantly less than ϕ_S, then the second step is a proton transfer and the free energy of this transition state is comparable to or higher than that of the "substrate-on" step. On the other hand if $\phi_{1,2}$ is equal to ϕ_S, then we can conclude either that the second step is not a proton transfer or that the free energy of the proton transfer transition state is lower than that of the "substrate-on" step. Similar conclusions can be reached from the other mixed factors.

When experiments have been carried out with both T and D, we can compare the values for the mixed fractionation factors from eq 7.3 and 7.12 or from eq 7.4 and 7.13. If one of the two steps is cleanly rate limiting, then the factors should obey the Swain–Schaad relationship (Swain et al., 1958)

$$\Phi = \phi^{1.44}$$

On the other hand if both steps are partially rate limiting, then the Swain–Schaad relationship will not be obeyed exactly. Taking $\phi_{1,2}$ as an example, we define the quantity c as the percentage deviation from the Swain–Schaad relation:

$$1 + c/100 = \frac{(\phi_{1,2})}{(\Phi_{1,2})^{0.69}}$$

$$= \frac{(\alpha + 1)^{0.31}[\alpha + (\phi_1/\phi_2)^{1.44}]^{0.69}}{\alpha + (\phi_1/\phi_2)} \qquad (8.2)$$

where $0.69 = 1.44^{-1}$, and the right hand side of eq 8.2 derives from the definition of the mixed fractionation factors in eq 1.8 and 1.9. Figure 4 shows values of c for different values of α and of the ratio ϕ_1/ϕ_2. We assume that ϕ_2 describes a proton transfer and ϕ_1 does not, so $\phi_1/\phi_2 > 1.0$. It is evident from Figure 4 that deviations from the Swain–Schaad relation are only significant when ϕ_1/ϕ_2 is greater than 5, *and* when the
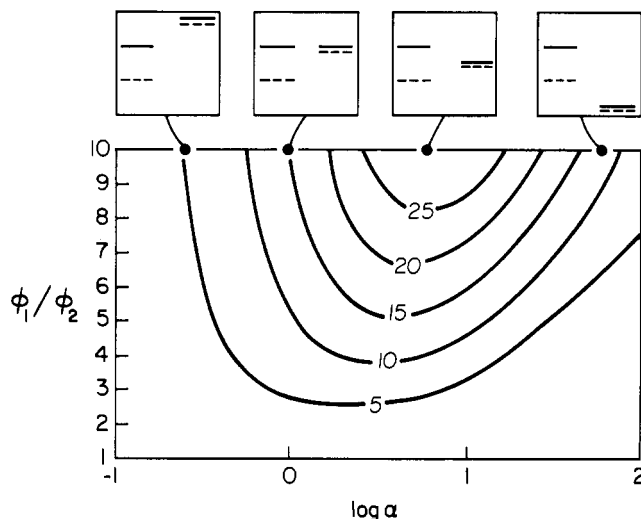

FIGURE 4: Contour diagram showing breakdown of the Swain–Schaad relation. Contour diagram for c, the percentage deviation from the Swain–Schaad relation for a mixed fractionation factor (see eq 8.2). Each contour is labeled with its value of c. The ordinate is the ratio of fractionation factors for the two steps, ϕ_1/ϕ_2, and since ϕ_2 describes a proton transfer step this ratio is always greater than 1. The abscissa (log α, where $\alpha = k_2/k_{-1}$) describes the difference in free energy between the two transition states for the all-H system. The square boxes illustrate these free-energy differences schematically. In the square boxes the free-energy levels of the H (full lines) and D or T (dashed lines) transition states are shown for the two steps. At the left of the diagram α is small and the second transition state (that involves proton transfer) is cleanly rate limiting, whereas at the right of the diagram the first transition state (that does not involve proton transfer) is rate limiting.

free energy of the H-transfer transition state is somewhat below the free energy for H in the first transition state. Under these circumstances, the change of isotope actually changes the rate-limiting step.

For most cases, however, the deviations from the Swain–Schaad relation are less than 20%, and Figure 4 shows how precise the experimental results have to be before any conclusions can be drawn from this approach. In general it is unlikely that a measured value of c will be known with sufficient precision to deduce values of α and ϕ_1/ϕ_2. Outside the relatively narrow range of α and ϕ_1/ϕ_2 where significant deviations are found, the Swain–Schaad relation can be applied to mixed fractionation factors and can therefore be used to check the consistency of the results.

The Exchange Process between EZ and EZ'. In order to find out the details of the exchange process between EZ and EZ', it is necessary to do experiments with both T and D. Assuming that the Swain–Schaad relation connects the unmixed factors ϕ_5 and Φ_5, we obtain from eq 1.8, 1.9, 7.6, and 7.15:

$$\phi_5 = \left[\left(\frac{1 - A_5'}{A_5'}\right) \left(\frac{A_5}{1 - A_5}\right) \frac{\Phi_{3,4}}{\phi_{3,4}} \right]^{2.3}$$

$$= \left[\left(\frac{1 - B_5'}{B_5'}\right) \left(\frac{B_5}{1 - B_5}\right) \frac{\Phi_{1,2}}{\phi_{1,2}} \right]^{2.3} \qquad (8.3)$$

and either

$$\frac{k_5}{k_3}(1 + \gamma) = \left(\frac{A_5'}{1 - A_5'}\right)^{2.3} \left(\frac{1 - A_5}{A_5}\right)^{3.3} \frac{(\phi_{3,4})^{3.3}}{(\Phi_{3,4})^{2.3}} \qquad (8.4)$$

or

$$\frac{k_5}{k_{-2}}(1 + \alpha) = \left(\frac{B_5'}{1 - B_5'}\right)^{2.3} \left(\frac{1 - B_5}{B_5}\right)^{3.3} \frac{(\phi_{1,2})^{3.3}}{(\Phi_{1,2})^{2.3}} \qquad (8.5)$$

These equations can be somewhat simplified if it is found (or

if it is assumed; see above) that the Swain–Schaad relation holds for the mixed fractionation factors, since in that case:

$$\left(\frac{\Phi_{m,n}}{\phi_{m,n}}\right)^{2.3} = \phi_{m,n}$$

and

$$\frac{(\phi_{m,n})^{3.3}}{(\Phi_{m,n})^{2.3}} = 1$$

Evaluation of the Thermodynamic Parameters. We are now in a position to construct the free-energy profile of the reaction, and start by deriving the relative free energies of the substrate S, the intermediates ES, EZ, and EP, and the product P.

The parameters A_4 and B_4 have not yet been used and, although these are rather complicated functions (see Table IV), in many systems A_5 and B_5 are small since there is often rapid isotopic exchange between EZ and the solvent.

Hence from eq 4.7 we may write:

$$\frac{1}{k_2}\left(\frac{\phi_{ES}}{\phi_2} - 1\right) = \left(\frac{1 + A_5\theta}{1 + \theta}\right)(A_4 - A_2) - A_5A_9$$

$$\simeq \left(\frac{1 + A_5\theta}{1 + \theta}\right)(A_4 - A_2) \quad (9.1)$$

and similarly from the equation for B_4 (Table IV):

$$\frac{1}{k_{-3}}\left(\frac{\phi_{EP}}{\phi_3} - 1\right) \simeq \left(\frac{1 + B_5\theta^{-1}}{1 + \theta^{-1}}\right)(B_4 - B_2) \quad (9.2)$$

The small terms A_5A_9 and B_5B_9 have been neglected, although their value can be found when the analysis is complete, and if necessary they can be included by iteration.

Next, we make the reasonable assumption that the binding of the substrates S and P to the enzyme is unaffected by isotope substitution, i.e.:

$$\phi_{ES} \simeq \phi_1 \simeq \phi_S \quad (9.3)$$

and

$$\phi_{EP} \simeq \phi_4 \simeq \phi_P \quad (9.4)$$

To simplify the algebra, we define two new parameters A_{10} and B_{10}, which can be derived from quantities already evaluated:

$$A_{10} = \frac{\phi_{1,2}(A_4 - A_2)(1 + A_5\theta)}{(\phi_S - \phi_{1,2})} \quad (9.5)$$

$$B_{10} = \frac{\phi_{3,4}(B_4 - B_2)(1 + B_5\theta^{-1})}{(\phi_P - \phi_{3,4})} \quad (9.6)$$

The various equilibrium constants that define the thermodynamic part of the free-energy profile, that is, the free energies of the intermediates, are then given by:

$$\frac{k_1}{k_{-1}} = \frac{A_{10}}{A_1} \quad (9.7)$$

$$\frac{k_2}{k_{-2}} = \frac{A_2 + B_2 - A_{10} - B_{10}}{A_{10}} \quad (9.8)$$

$$\frac{k_3}{k_{-3}} = \frac{B_{10}}{A_2 + B_2 - A_{10} - B_{10}} \quad (9.9)$$

and

$$\frac{k_4}{k_{-4}} = \frac{B_1}{B_{10}} \quad (9.10)$$

Note that the usual Haldane relationship (relating the individual kinetic constants to the overall equilibrium constant)

is obeyed, and

$$\frac{k_1k_2k_3k_4}{k_{-1}k_{-2}k_{-3}k_{-4}} = \frac{B_1}{A_1}$$

It may be that in the reaction, one or more of the intermediates is "kinetically insignificant";[1] a kinetically insignificant intermediate is so unstable with respect to the reactants and other intermediates that material does not accumulate at that point in the reaction, and the reaction rate is unaffected by the free energy of that intermediate. In the present approach, we do not assume which intermediates are kinetically insignificant, but we may deduce the following:

ES will be kinetically insignificant if:

$$A_{10} = 0 \quad (9.11)$$

EZ will be kinetically insignificant if

$$A_2 + B_2 - A_{10} - B_{10} = 0 \quad (9.12)$$

and EP will be kinetically insignificant if

$$B_{10} = 0 \quad (9.13)$$

Evaluation of the Kinetic Parameters. To derive the individual kinetic parameters, we need the following equations:

$$\alpha = \frac{\phi_2^{-1} - \phi_{1,2}^{-1}}{\phi_{1,2}^{-1} - \phi_S^{-1}} \quad (10.1)$$

$$\gamma = \frac{\phi_3^{-1} - \phi_{3,4}^{-1}}{\phi_{3,4}^{-1} - \phi_P^{-1}} \quad (10.2)$$

$$k_1 = \frac{(1 + \theta)(1 + \alpha^{-1})}{A_1} \quad (10.3)$$

$$k_2 = \frac{(1 + \theta)(1 + \alpha)}{A_{10}} \quad (10.4)$$

$$k_{-3} = \frac{(1 + \theta^{-1})(1 + \gamma)}{B_{10}} \quad (10.5)$$

$$k_{-4} = \frac{(1 + \theta^{-1})(1 + \gamma^{-1})}{B_1} \quad (10.6)$$

and from eq 7.8

$$\frac{A_{10}}{1 + \alpha} - \frac{B_{10}}{1 + \gamma} = A_2 - B_2\theta \quad (10.7)$$

To fix the free energies of the transition states, we only need four rate constants; the other rate constants will be given by eq 9.7 to 9.10. Unfortunately, we now have eight unknowns (α, γ, ϕ_2, ϕ_3, and the four rate constants) and only seven equations (eq 10.1 to 10.7). Thus a complete solution is impossible, though we can proceed if one of the transition states is kinetically insignificant. (A kinetically insignificant transition state is one of such low free energy that it is in no way rate determining.) As with the evaluation of the thermodynamic situation in the previous section, we may deduce from the experimental results whether this is so:

if $\phi_{1,2} = \phi_2$, transition state 1 is kinetically insignificant, and

$$\alpha \simeq 0 \quad (10.8)$$

if $\phi_{1,2} = \phi_S$, transition state 2 is kinetically insignificant, and

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[1] The concept of "kinetic significance" was suggested by R. M. Noyes in a series of lectures in Oxford in 1972.
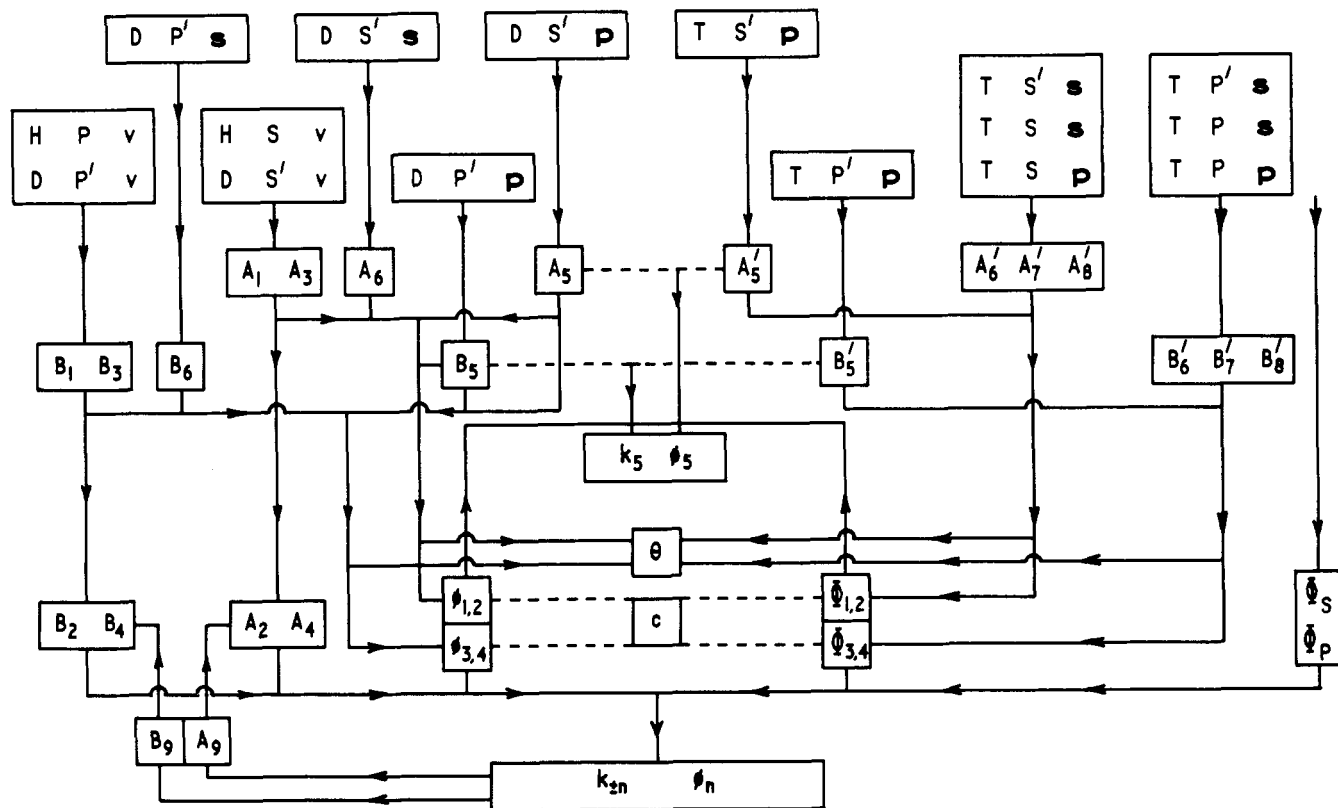
FIGURE 5: Relationships amongst experiments and derived parameters. The purpose of this diagram is to show how to proceed from the experiments to the intermediate parameters A_n and B_n, and thence to the rate constants and fractionation factors. Broken lines indicate connections depending upon the Swain–Schaad relation.

$$\alpha^{-1} \simeq 0 \tag{10.9}$$

if $\phi_{3,4} = \phi_P$, transition state 3 is kinetically insignificant, and

$$\gamma^{-1} \simeq 0 \tag{10.10}$$

if $\phi_{3,4} = \phi_3$, transition state 4 is kinetically insignificant, and

$$\gamma \simeq 0 \tag{10.11}$$

Once a solution for either α or γ is found, then the other parameter may be deduced from eq 10.7. Of course, if the nth transition state is kinetically insignificant, k_n and k_{-n} will be too large to be determined.

If no transition state is kinetically insignificant, then one must proceed by assuming either a plausible value of ϕ_2, or of ϕ_3, or that $\phi_2 = \phi_3$. This last assumption may only be made when both the reactions (involving transition states 2 and 3) are proton transfers of a similar type, as for instance in isomerases. In any case, using different plausible values of ϕ_n, one can find out how sensitive the other values are to the exact choice of ϕ_2 or ϕ_3.

General Conclusions

Figure 5 shows the connections between the various experiments, the parameters A_n and B_n that are found from each experiment, and the intermediate quantities (the mixed fractionation factors and θ) that are required to give the individual rate constants and fractionation factors. Broken lines are used to indicate connections that depend on the Swain–Schaad relation. Note that the intermediate quantities can be obtained

by three different groups of experiments (D experiments, the TS group, or the TP group). It is a pity that a complete analysis cannot be made when all four transition states are kinetically significant. But this is a general model involving three intermediates and five transition states and few real systems are likely to have as many kinetically significant intermediates and transition states. The general case presented in this paper allows one to deduce which special case is appropriate for a particular reaction.

A great advantage of the isotopic experiments described in this paper is that, in all the experiments, except for the initial rate experiments (HSv, HPv, $DS'v$, and $DP'v$), time is not used as an experimental variable. This means that the activity of the enzyme need not remain constant, and inhibition (for instance by the products) is unimportant. This is because in deriving equations such as 4.5 and 5.1, e, the concentration of the free enzyme, cancels out top and bottom on the left-hand side. Hence the results from the isotopic content of the reactant and the product as a function of the conversion are not dependent on the enzyme activity remaining constant.

Finally, it must be emphasized that the *only* approximations and assumptions made in the derivation of the equations and in the analysis of the data to yield the free energy profile are:

(1) The steady state approximation for the intermediates;

(2) The atom fraction of T is much less than unity;

(3) The binding of the enzyme to the substrates does not alter the fractionation factors, so that $\phi_{ES} = \phi_1 = \phi_S$ and $\phi_{EP} = \phi_4 = \phi_P$

(4) To obtain k_5 and ϕ_5, the Swain–Schaad relation holds for ϕ_5 and Φ_5;

(5) If all the transition states 1 to 4 are kinetically signifi-

cant, then one has to assume a plausible value of either ϕ_2 or ϕ_3.

Apart from these approximations, the free energies of the intermediates and transition states and the isotopic effects on the transition states are obtained directly from the data with no further assumptions. Particularly important in our view is the fact that assumptions are not made concerning the speed of substrate handling (on-off) steps compared with steps involving covalency changes, nor is it assumed, as is quite common practice, that the observed effects of isotopic substitution relate to a single step in the reaction pathway.

List of Symbols

A_n	parameter determined from experimental data without T, see Table IV
B_n	parameter determined from experimental data without T, see Table IV
A_n'	parameter determined from experimental data without D, see Table IV
B_n'	parameter determined from experimental data without D, see Table IV
c	percentage breakdown of Swain–Schaad relation for a mixed fractionation factor.
D	^{2}H
e	concentration of free enzyme
Σe	total concentration of enzyme
ep	concentration of EP
ep'	concentration of EP'
es	concentration of ES
es'	concentration of ES'
ez	concentration of EZ
ez'	concentration of EZ'
E	an enzyme that interconverts S and P
EP	P bound to E
EP'	P' bound to E
ES	S bound to E
ES'	S' bound to E
EZ	an enzyme-bound reaction intermediate that exchanges H with solvent water
EZ'	EZ substituted with D or T
f_n	flux through nth transition state with H
f_n'	flux through nth transition state with D or T
$k_{\pm n}$	rate constants for the nth step with H
$k_{\pm n}'$	rate constants for the nth step with D or T
n	an integer
N	is S, ES, EZ, EP, or P
p	concentration of P
$\mathbf{p}$	atom fraction of D or T in P or P$_2$ (see Table I)
p'	concentration of P'

p_2	concentration of P$_2$
p_2'	concentration of P$_2'$
P	a substrate or product of the enzyme reaction
P'	P substituted with D or T
P$_2$	a final product formed rapidly from P
P$_2'$	P$_2$ substituted with D or T
r	fraction of reactant remaining (see Table I)
s	concentration of S
$\mathbf{s}$	atom fraction of D or T in S or S$_2$ (see Table I)
s'	concentration of S'
s_2	concentration of S$_2$
s_2'	concentration of S$_2'$
S	a substrate or product of the enzyme reaction
S'	S substituted with D or T
S$_2$	a final product formed rapidly from S
S$_2'$	S$_2$ substituted with D or T
t	time
T	^{3}H
v	velocity of reaction
$\mathbf{x}$	atom fraction of D or T in solvent
α	k_2/k_{-1}
α'	k_2'/k_{-1}'
β	k_{-2}/k_3
β'	k_{-2}'/k_3'
γ	k_{-3}/k_4
γ'	k_{-3}'/k_4'
θ	$(k_{-2}/k_3)[(1+\gamma)/(1+\alpha)]$
θ'	$(k_{-2}'/k_3')[(1+\gamma')/(1+\alpha')]$
ϕ_n	fractionation factor for substitution by D in nth transition state
$\phi_{1,2}$	$(1+\alpha)/(\phi_2^{-1}+\alpha\phi_1^{-1})$
$\phi_{3,4}$	$(1+\gamma)/(\phi_3^{-1}+\gamma\phi_4^{-1})$
ϕ_N	fractionation factor for substitution by D in S, ES, EZ, EP, or P
Φ_n	fractionation factor for substitution by T in nth transition state
$\Phi_{1,2}$	$(1+\alpha)/(\Phi_2^{-1}+\alpha\Phi_1^{-1})$
$\Phi_{3,4}$	$(1+\gamma)/(\Phi_3^{-1}+\gamma\Phi_4^{-1})$
Φ_N	fractionation factor for substitution by T in S, ES, EZ, EP, or P

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