

Transition States of Biochemical Processes

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Preface

The transition-state theory has been, from the point of its inception, the most influential principle in the development of our knowledge of reaction mechanisms in solution. It is natural that as the field of biochemical dynamics has achieved new levels of refinement its students have increasingly adopted the concepts and methods of transition-state theory. Indeed, every dynamical problem of biochemistry finds its most elegant and economical statement in the terms of this theory. Enzyme catalytic power, for example, derives from the interaction of enzyme and substrate structures in the transition state, so that an understanding of this power must grow from a knowledge of these structures and interactions. Similarly, transition-state interactions, and the way in which they change as protein structure is altered, constitute the pivotal feature upon which molecular evolution must turn. The complete, coupled dynamical system of the organism, incorporating the transport of matter and energy as well as local chemical processes, will eventually have to yield to a description of its component transition-state structures and their energetic response characteristics, even if the form of the description goes beyond present-day transition-state theory. Finally, the importance of biochemical effectors in medicine and agriculture carries the subject into the world of practical affairs, in the use of transition-state information for the construction of ultrapotent biological agents.

Because there has been no general treatment of transition-state approaches to mechanistic biochemistry, we thought it worthwhile to compile this volume, in which the principles and some of the findings and prospects are set forth by leading scientists in the field. We hope the volume will serve several purposes. It ought to introduce the advantages and disadvantages of these techniques to mechanistic biochemists who are not now using them. It should be a useful textbook for students and for researchers in other fields who may be planning work in mechanistic biochemistry. We are particularly optimistic that it may encourage biochemists to use methods traditionally associated with physical organic chemistry while at the same time alerting other mechanisms scientists to the difficult and exciting unsolved problems of mechanistic biochemistry.

The book has four parts. In the first, the basic principles of transition-state

theory are reviewed and set in a biochemical context. In the second part there are fairly detailed presentations of the major techniques in current use. In the third part, transition-state information about several important processes is discussed. Finally a brief consideration is given to the use of transition-state concepts in inhibitor and drug design.

The authors have aimed in all cases to write chapters accessible to scientists inexperienced in the subject, capable of bringing the reader up to the level of current research, but nevertheless containing much of interest to the specialist as well. We believe they have all succeeded. Our principle of editing was to persuade, in each case, a scientist whose knowledge and ideas we knew to be thorough and valuable to write about his subject. Thereafter, we took a *laissez-faire* attitude. The reader will therefore detect a certain amount of repetition, as the same matters are taken up by several authors. A Russian proverb says, "Repetition is the mother of learning," in agreement with which we take the view that many will find successive encounters with the same subject, seen from various angles, as helpful and welcome. Indeed, in a not inconsiderable number of cases, direct conflicts between the views of different authors will be found. This is quite in accord with the mode of operation of science and gives an accurate picture of opinion in the field. We hope the reader will consider this feature an advantage.

Naturally the selection of subjects to be treated reveals various idiosyncracies of the editors. We have tried to be as broad as possible in the selection, without duplicating extensively material easily available elsewhere. Because of the importance of enzyme mechanisms as a field for the application of transition-state theory to biochemistry at the current time, there is a certain concentration on methods especially adaptable to enzyme systems. Introductory notes are supplied at the beginning of each of the four parts of the book to show how the chapters fit together with each other and with the general shape of thought in the field.

The task of persuading overworked scientists to write contributions for this volume and the companion task of extracting the latest chapter from its author before the earliest one received had become thoroughly outdated have had their challenging aspects. Nevertheless, it is hard to imagine a more cooperative and patient group than our colleagues in this enterprise, whom we offer our cordial thanks.

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Transition States of Biochemical Processes

Solvent Hydrogen Isotope Effects

Katharine B. J. Schowen

1. SOLVENT ISOTOPE EFFECTS AND BIOLOGICAL SYSTEMS

Biological and biochemical processes are affected by the partial or complete replacement of solvent protium oxide (H_2O) by deuterium oxide (D_2O)⁽¹⁾ Although some very simple organisms (certain algae, bacteria, etc.) have survived complete replacement of protium by deuterium, differences in morphology and metabolism are very apparent. Not surprisingly, deuterium substitution affects virtually all aspects of metabolism and physiological function. Higher plants, after experiencing impaired chlorophyll synthesis and inhibition of cell division, will cease to grow after $\sim 70\%$ D exchange. Mammals (unable to survive much more than 25% D incorporation) show evidence of severe difficulty with protein synthesis, decreased enzyme levels, decreased erythrocyte production, impaired carbohydrate metabolism, hormone imbalance, central nervous system disturbance, difficulty with mitosis, and increasingly impaired reproductive capability. Most of these consequences are directly or indirectly attributable to changes in the *rates* of biochemical reactions.

A number of such individual biochemical processes, isolated from the organism for *in vitro* laboratory study, have shown pronounced changes in rate with a change in solvent from H_2O to D_2O . These rate differences are conventionally expressed and reported as the ratio $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$ of the respective rate constants for the reaction in light and heavy water; this ratio defines the term *kinetic solvent (hydrogen) isotope effect*.* In Table I are some recently

* Hydroxylic solvents other than water also give rise to kinetic solvent isotope effects. Studies with these other solvents are relatively rare and will be mentioned infrequently in this chapter; the general principles discussed, however, will apply.

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Table I
Solvent Isotope Effects for Some Biochemical Reactions^a

	Isotope effect ^b	Reference
Monovalent-cation-induced tetramerization of formyltetrahydrofolate synthetase subunits	~ 3	2
Polymerization of beef brain tubulin	5	3
Conformational isomerization of ribonuclease	4.3; 4.8	4, 5
Deacetylation of acetyl- α -chymotrypsin	2.4; 2.7	6, 7
Acylation of α -chymotrypsin by <i>N</i> -acetyl-L-tryptophanamide	1.9; 2.0	8, 7
Deacetylation of acetyltrypsin	1.4	8
Deacylation of α - <i>N</i> -benzoyl-L-arginyltrypsin	2.6	8, 9, 10
Acylation of trypsin by α - <i>N</i> -benzoyl-L-phenylalanyl-L-valyl-L-arginyl <i>p</i> -nitroanilide	3.9	10, 11
Deacylation of α - <i>N</i> -benzoyl-L-arginylthrombin	2.9	8b, 12
Deacetylation of acetylastase	2.2	8
Acylation of elastase by α - <i>N</i> -carbobenzyloxy-L-alanyl <i>p</i> -nitrophenyl ester	1.8	8b, 12
Acylation of elastase by <i>N</i> -acetyl-L-alanyl-L-prolyl-L-alanyl <i>p</i> -nitroanilide	2.1	8b
Acylation of α -lytic protease by <i>N</i> -acetyl-L-alanyl-L-prolyl-L-alanyl <i>p</i> -nitroanilide	2.8	8b
Glutaminase-catalyzed hydrolysis of glutamine	1.8 ^c	8a, 10, 13
Asparaginase-catalyzed hydrolysis of asparagine	2.9 ^c ; 2.6 ^d	8a, 10, 13
Inorganic pyrophosphatase-catalyzed hydrolysis of inorganic pyrophosphate	1.9	14

^a Reactions for which complete proton inventories have been carried out.

^b Ratio of rate constants ($k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$) or of velocities ($v_{\text{H}_2\text{O}}/v_{\text{D}_2\text{O}}$).

^c Enzyme obtained from *E. coli*.

^d Enzyme obtained from *Erwinia carotovora*.

available values for the kinetic solvent isotope effects (KSIE's) observed for a variety of biochemical reactions (enzyme-catalyzed hydrolyses, enzyme-substrate reactions, protein conformation changes, and protein subunit associations), some of which will be discussed in detail in Section 8. Systems chosen for inclusion in Table I are limited to those for which a proton inventory (Section 5) has been carried out. The list is meant to be illustrative and not an exhaustive collection of solvent isotope effects reported for biological reactions.

2. ISOTOPE EFFECTS AND TRANSITION-STATE STRUCTURE

The magnitude of an experimentally observed kinetic solvent isotope effect should, in principle, provide information about the transition state for the particular reaction under investigation. Since the rate constant of a chemical reaction is determined by the free energy of activation [$\Delta G^\ddagger$, the difference in free energy between the transition-state-activated complex and the reactant ground state, Eq. (3)], the isotope effect $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$ is an expression involving the difference between the free energy of activation for the reaction in light solvent and heavy solvent:

$$k = \frac{kT}{h} e^{-\Delta G^\ddagger/RT} \quad (1)$$

$$k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = e^{(\Delta G_0^\ddagger - \Delta G_{\text{H}}^\ddagger)/RT} = e^{\delta\Delta G^\ddagger/RT} \quad (2)$$

where

$$\Delta G^\ddagger = G^\ddagger - G_r \quad (3)$$

and

$$\delta\Delta G^\ddagger = \Delta G_D^\ddagger - \Delta G_H^\ddagger = (G^\ddagger - G_r)_D - (G^\ddagger - G_r)_H \quad (4)$$

Illustrated by means of a very simple energy diagram (Fig. 1) is a possible situation where the overall reactant-state *zero-point energy difference* in the two solvents *alters* (decreases in this case) on going to the transition state, a situation which arises when the bonding to an isotopic hydrogen atom becomes weaker or “looser.” If the bonding of more than one hydrogen atom is changed on activation, a common situation in solvent isotope-effect studies, the overall isotope effect will be a net reflection of all the bonding changes, some perhaps “tighter,” some “looser”; it will be the product of the isotope effects for all the bonds to hydrogen that undergo change on activation. For the situation illustrated in Fig. 1, $\Delta G_D^\ddagger > \Delta G_H^\ddagger$ and $k_{H_2O}/k_{D_2O} > 1$ (reaction faster in light solvent). The conclusion is that at least one bond to a hydrogen atom (in water or in the reactant as a result of hydrogen exchange) is looser or undergoing transfer in the transition state of the rate-determining step of the reaction. It is important to realize that it is only when the isotopic free-energy difference is changed on going from ground state to transition state that an isotope rate effect is observed. Since this change will only occur when a bond to an isotopic atom undergoes some change between ground state and transition state, isotope effects (provided we have independent knowledge about reactant-state structure) can provide information about transition-state structure in the neighborhood of the isotopic atom(s).

Certainly it is true that isotopic substitution is the least disturbing structural change that can be made in a system in order to gain mechanistic information. Nuclear charge, electron distribution, and potential energy remain un-

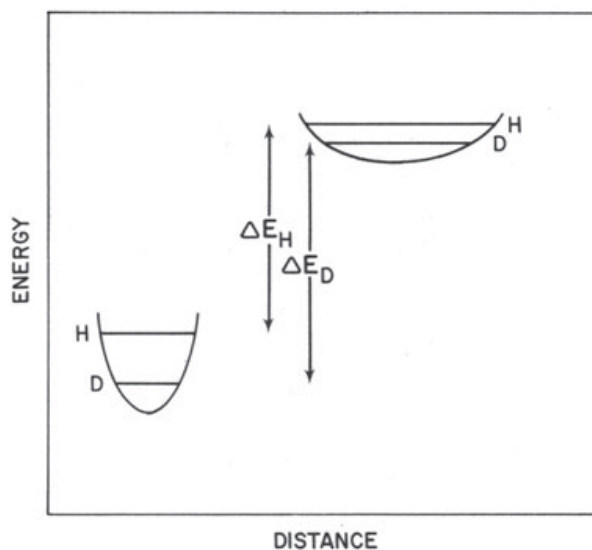


Fig. 1. Diagram showing that the zero-point energy difference for bonds to H and to D decreases on going to the transition state for a reaction in which bonding to hydrogen becomes looser, resulting in $E_H < E_D$ and a normal isotope effect $k_H/k_D > 1$.

changed; reactions of isotopically substituted substances take place on the same potential surface. Since the only change introduced upon isotopic substitution is one of mass, the distinguishing properties of isotopic species are those consequently affected: translational energy of the molecule as a whole and the rotational and vibrational energies of the bonds to the isotopic atoms. As the translational and rotational effects can be expressed in terms of the vibrational effects according to the Redlich–Teller rule, the entire kinetic isotope effect can be expressed by the Bigeleisen⁽¹⁵⁾ equation,

$$\frac{k}{k'} = \frac{v_L}{v'_L} \left\{ \prod_i^{\text{real transition-state frequencies}} \left(\frac{v_i}{v'_i} \right) e^{-\Delta u_i/2} \frac{1 - e^{-u_i}}{1 - e^{-u'_i}} \right\} \left\{ \prod_j^{\text{reactant frequencies}} \left(\frac{v_j}{v'_j} \right) e^{-\Delta u_j/2} \frac{1 - e^{-u'_j}}{1 - e^{-u_j}} \right\} \quad (5)$$

in terms of changes in the vibrational energies alone. In Eq. (5), $\mu = hv/kT$, $\Delta u = u - u'$ (where the prime refers to the heavy isotope), and v_L/v'_L is the imaginary frequency ratio for the reaction coordinate of the activated complex. The last term is factored out because it has no corresponding zero-point energy ($e^{-\Delta u/2}$) or excitation factor $[(1 - e^{-\mu'})/(1 - e^{-\mu})]$. If the appropriate reactant- and transition-state frequencies are available (from infrared or Raman spectra for the former, from estimation by vibrational analysis of possible transition-state structures, a computationally complex undertaking, for the latter), an isotope effect can be calculated. Equation (5) can be expressed in terms of the reactant-state contribution (RSC) and the transition-state contribution (TSC) to the isotope effect,

$$k/k' = \text{TSC/RSC} \quad (6)$$

Rearrangement to

$$\text{TSC} = (k/k')(\text{RSC}) \quad (7)$$

emphasizes the point of this and earlier chapters, namely, that information about the transition state is available from experimental isotope-effect measurements. Because of the dependence of TSC on vibrational frequencies, the information should be expressible in terms of transition-state force constants, bond lengths, and bond angles. It is our purpose in this chapter to show how transition-state structures and reaction mechanisms can be deduced from those isotope effects that arise when reactions of biochemical interest are carried out in isotopic solvents.

3. THE ORIGIN OF KINETIC SOLVENT ISOTOPE EFFECTS

Solvent isotope-effect theory has been presented in a number of excellent reviews by Laughton and Robertson,⁽¹⁶⁾ Arnett and McKelvey,⁽¹⁷⁾ Gold,^(18,19)

Schowen,⁽²⁰⁾ Thornton and Thornton,⁽²¹⁾ Kresge,⁽²²⁾ and Alberty⁽²³⁾ and earlier by Wiberg,⁽²⁴⁾ Gold and Satchell,⁽²⁵⁾ and Bader.⁽²⁶⁾ Consequently only the briefest outline will be attempted here. It must of course first be recognized that a number of different phenomena contribute to produce a net observed solvent isotope effect. Changing the hydroxylic hydrogen atoms of water from protium (^1H) to deuterium (^2H) will bring about rate differences in reactions occurring in those solvents if any or all of the following undergo *change* on going from reactant to transition state: (1) differences in bulk solvent properties, (2) differences in solute–solvent interactions, (3) differences in zero-point energy of O—L bonds (where L is an unspecified isotope of hydrogen) of reacting solvent water molecules, or (4) differences in zero-point energy of solute bonds to (exchangeable) H which have become labeled by rapid exchange with solvent. The first two arise from changes in the medium and may be termed “transfer” effects and “solvation” effects, respectively. The latter two arise from actual bond changes in reacting molecules. These may be further classified as *primary effects* if the reaction involves rate-determining proton (deuteron) transfer from either reactant water or solute and as *secondary effects* if the exchangeable H in either water or solute is involved in the reaction but not undergoing rate-determining transfer. Explanations of these various contributions to the overall solvent isotope effect will generally involve either (1) the effect of isotopic substitution on internal (that is, pertaining to the reacting molecules) vibration (stretching, bending) frequencies of bonds to H (D) and the changes in these frequencies on going from reactant state to transition state or (2) the effect of isotopic substitution on the librational (that is, the hindered rotational) frequencies of external solvent water molecules and the changes in these frequencies on activation.⁽²⁷⁾

The overall observed KSIE is the *product* of each of these various isotope-effect contributions, that is,

$$\left(\frac{k_{\text{H}_2\text{O}}}{k_{\text{D}_2\text{O}}} \right)_{\text{total}} = \left(\frac{k_{\text{H}}}{k_{\text{D}}} \right)_{\text{pri}} \left(\frac{k_{\text{H}}}{k_{\text{D}}} \right)_{\text{sec}} \left(\frac{k_{\text{H}_2\text{O}}}{k_{\text{D}_2\text{O}}} \right)_{\text{medium}} \quad (8)$$

recognizing that $(k_{\text{H}}/k_{\text{D}})_{\text{sec}}$ may itself be the product of more than one secondary isotope effect. One can then, as shown below, examine the transition state for a proposed reaction mechanism, estimate the primary and secondary SIE contributions, and compare the product with the experimentally determined value.

3.1. Transfer and Solvation Isotope-Effect Contributions

Heavy water is not the same solvent as its light counterpart; there are differences in bulk structure (partly reflecting differences in intermolecular hydrogen bonding) and, of course, in molecular weight and, consequently, in physical properties. At a given temperature, for example, the isotopic solvents differ in viscosity, density, and dielectric constant and in their ability to solvate

ionic and nonpolar solutes. In Table II are given some important physical properties of light and heavy water.

It would be interesting to know what effect a change of solvent from light to heavy water would have on the rate of a reaction *per se* (because of a change in bulk dielectric constant, for example) and *in the absence of any specific solute-solvent interaction*. Such information is difficult to obtain; the effects most certainly are small, and it is generally assumed that such “transfer” effects (resulting from the intact transfer of a reaction from one solvent to another) on reaction rates are safely neglected. These effects and their possible contribution to the net solvent isotope effect have been discussed in some detail by Gold.⁽¹⁸⁾

The contribution from the medium to the overall isotope effect that is observed results from solute-solvent interactions, that is, from solvation of the reacting solutes by the solvent water molecules. Such “solvation” isotope effects are estimated to be modest, ranging from 1.2 to 1.3.⁽²¹⁾ The solute-solvent interactions giving rise to these purely “solvation” isotope effects are a consequence of the structure of water. Although the precise structure of water is not exactly known, a degree of understanding sufficient to provide workable models for the calculation and prediction of solvation isotope effects exists. Water is known^(1,17) to have a great deal of internal three-dimensional structure arising from intermolecular hydrogen bonding; its properties depend to a large extent on the degree and nature of this structure. At least up to 35°C heavy water appears to have more structure, i.e., more intermolecular hydrogen bonding than light water. Evidence for the preceding is provided by the facts of poorer solubility of ions in D₂O,⁽¹⁷⁾ the SIE's on ionic conductivity,⁽²⁸⁾ the greater temperature of maximum density, and the much greater viscosity of D₂O. The structure of water decreases with increasing temperature; the rate of this decrease is greater with D₂O. Solution of nonpolar nonelectrolytes

Table II
Some Physical Properties of Light and Heavy Water^a

Property (unit)	H ₂ O	D ₂ O
Molecular weight (¹² C scale)	18.015	20.028
Melting point (°C)	0.00	3.81
Boiling point (°C)	100.00	101.42
Density at 25° (g/cm ³)	0.9970	1.1044
Temperature of maximum density (°C)	3.98	11.23
Dielectric constant, 25°	78.39 ^b	78.06 ^b
Viscosity at 25° (cP)	0.8903	1.107
Viscosity at 20° (cP)	1.005 ^c	1.25 ^c
Ionization constant at 25°	1×10^{-14}	1.95×10^{-15}

^a Data taken from more extensive tables in Reference 17, pp. 392–394.

^b Reference 57.

^c Reference 17, p. 347.

in water is accompanied by a large loss in entropy, evidence for an increase in water structure in the neighborhood of the solute. This structure-making effect is somewhat greater in D_2O than H_2O (presumably because of the greater strength of the H bond in the former solvent). Hydrophobic bonding in biological polymers should, as a consequence, be favored in D_2O . Ionic substances when dissolved in water appear to effect a slight net decrease in the overall solvent structure. This structure-breaking effect is greater in D_2O (presumably because there is more structure to begin with).

Based on this understanding of the structure of water and its observed effect on the solvation of various kinds of solutes, two fairly detailed physical models to explain solvation isotope effects have been proposed. That of Bunton and Shiner⁽²⁹⁾ emphasizes changes in internal stretching frequencies of bonds to hydrogen in water molecules or labeled solutes as the basicity or acidity of solutes change on activation, while Swain *et al.*⁽³⁰⁾ have favored the librational (hindered external rotational) degrees of freedom of the water molecules as the major origin of solvent isotope effects of this type. According to the latter theory, water molecules are treated as hindered rotors 4-coordinated by hydrogen bonding to four adjacent water molecules in the ground state. Most of the solvation isotope effect arises from changes in the librational frequencies (as opposed to internal vibrations and translations) of the water molecules solvating each reactant molecule upon activation. Derivation from this theory of a relationship that can be used to calculate the isotope effect for simple ionic solubilities is provided by Thornton and Thornton.⁽²¹⁾ Isotope effects for solubilities of salts range from 1.01 to 1.33.⁽¹⁷⁾ From such treatments it is expected that ion-forming reactions will have a solvation isotope-effect contribution to the overall solvent isotope effect greater than 1, consistent with a net decrease in librational frequencies due to a net increase in structure breaking on going from reactants to transition state. k_{H_2O}/k_{D_2O} contributions of less than 1 are expected for ion-destroying reactions. Unfortunately, in spite of the importance of understanding the role of the solvent in contributing to solvent isotope effects, there is a regrettable lack of data allowing for the separation of solvation effects from specific effects where water is a reactant or exchangeable proton transfer is occurring. Experimental solvent isotope effects for the solvolysis of alkyl halides vary from around 1.2 to 1.3 at temperatures between 80° and 90°C.⁽²¹⁾ Both the secondary isotope effect associated with nucleophilic attack and the solvation isotope effect (since the reaction is ion forming and ions are structure breaking) should be greater than 1, implying that the solvation isotope effect is a small but not negligible contributor to the overall observed solvent isotope effect. Thus it is clear that the contribution of the structural differences of solvent light and heavy water to the stability of reactants and activated complexes by virtue of their different solvating ability should be considered, specifically the thermodynamics of transfer (ΔH_i° , ΔS_i°) from light to heavy water. It is at present not easy to make safe predictions about the relative magnitude of these effects.

3.2. Primary Isotope-Effect Contributions

A primary kinetic hydrogen isotope effect is observed for a particular reaction when an isotopically substituted hydrogen involved in the reaction (that is, one whose bond is being made or broken) is *in motion* along the reaction coordinate at the transition state. The theory of primary isotope effects and their relation to transition-state structure have been discussed in detail by Westheimer,⁽³¹⁾ Bigeleisen,⁽³²⁾ Bell,⁽³³⁾ Willi and Wolfsberg,⁽³⁴⁾ More O'Ferrall and Kouba,⁽³⁵⁾ Albery,⁽²³⁾ Thornton and Thornton,⁽²¹⁾ Schowen,⁽²⁰⁾ and More O'Ferrall.⁽³⁷⁾ Treatment particularly relevant to the elucidation of biochemical transition states appears in Chapter 4 in this volume. A simple treatment using Westheimer's three-atom transition-state model⁽³¹⁾ has been applied by Schowen⁽²⁰⁾ to make quick approximate primary isotope-effect calculations and will be summarized in Section 4.3.

3.3. Secondary Isotope-Effect Contributions

Secondary hydrogen isotope effects are observed when an isotopically substituted hydrogen atom undergoes some change in bonding on going from reactant to transition state but is not actually moving or in the process of transfer. Excellent theoretical treatments of the origin of such isotope effects have been made available by Thornton and Thornton⁽²¹⁾ and in Chapter 5 in this volume. Secondary isotope effects originate mainly from the difference in zero-point energy as a result of changes in force constants upon going from reactants to the transition state. Smaller force constants for bonds to isotopic atoms in the transition state than in the reactant state (bond "loosening"), decreasing the zero-point energy difference, tend to make the secondary contribution, $k_H/k_D > 1$ and vice versa. Secondary isotope effects can be calculated using a computer to arrive at moments of inertia and normal vibrations for assumed geometries and various force constants.⁽³⁸⁻⁴²⁾ They can also be estimated less rigorously but more rapidly using fractionation factors, as described in the following sections.

4. CALCULATION OF SOLVENT ISOTOPE EFFECTS

The ultimate purpose of determining a KSIE is usually to gain information about the transition state for the rate-determining step of the reaction in question. The way to relate an experimentally determined KSIE to a transition-state structure is to propose a number of reasonable structures, assign to each an expected isotope effect, and compare the actual result to the model. Any method of isotope-effect estimation will usually suffice: rigorous calculation from theory, approximations using simplifying assumptions, or simply by analogy with known reaction mechanisms.

To rigorously calculate the solvent isotope effect expected for a given

transition state a complete vibrational analysis is needed, a difficult and time-consuming process. A more approximate but simpler method has been described by Schowen⁽²⁰⁾ using isotopic fractionation factors to obtain the secondary isotope-effect contribution and the simple triatomic proton-transfer transition state of Westheimer⁽³¹⁾ to obtain the primary contribution.

4.1. Ground-State Fractionation Factors^(18,22,43,44)

Placement of a molecule SH with one exchangeable hydrogen atom in a solvent mixture of ROH and ROD results in an equilibrium described by



with an equilibrium constant given by

$$K = \frac{[\text{SD}][\text{ROH}]}{[\text{SH}][\text{ROD}]} = \frac{[\text{SD}]/[\text{SH}]}{[\text{ROD}]/[\text{ROH}]} = \phi \quad (10)$$

This equilibrium constant is identical with and defines the term ϕ , the isotopic fractionation factor for the hydrogenic position of SL. ϕ is an expression of the preference of the hydrogenic position in SL for deuterium over protium relative to the preference of the hydrogenic position of ROL for deuterium over protium. In more general terms, an isotopic fractionation factor for any particular site in a molecule is defined as the ratio of its preference for deuterium over protium relative to the similar preference of a single site in a solvent molecule. For water, it is assumed that the deuterium preference of one of the sites is independent of whether the other site is occupied by H or D, i.e., that the "rule of the geometric mean" holds. Although the rule does not hold entirely, any errors so introduced have generally been considered to be slight.^(45,46) Albery⁽²³⁾ discusses this point more fully, implying that corrections may on occasion be required. It is likewise assumed that for substrates with more than one exchangeable site the fractionation factor for a given position is independent of the H or D content at any other sites. It is generally true that the heavier isotope will accumulate in those sites of an equilibrium such as Eq. (9) in which bonds with the larger force constants (i.e., stronger, tighter bonds) are found. For a fractionation factor greater than 1, Eq. (10) implies that D prefers the particular solute site relative to a solvent site, that is, that D will preferentially accumulate in the *i*th solute site, indicating that hydrogens are more tightly bound in SL than in the solvent. Likewise, for ϕ values less than 1, the implication is that H prefers the particular solute site relative to solvent and that the particular SL bond is weaker than the solvent bonds to hydrogen. Not only do we assume that fractionation factors are independent of isotopic substitution at other positions in the same molecule but also that they remain the same for the same bond types, i.e., that they are unaffected by the particular molecular environment.⁽⁴⁷⁾ A number of fractionation factors have been experimentally determined and are collected in Table III. It will be noted that

Table III
Isotopic Fractionation Factors
Relative to Water^a

Functional group	Fractionation factor, ϕ
$\diagup\text{O-L}$	1.0
$-\text{CO}_2\text{-L}$	1.0
$\begin{array}{c} \diagup \quad \diagdown \\ \text{C} \\ \diagdown \quad \diagup \\ \text{O} \\ \\ \text{O-L} \end{array}$	1.23–1.28
$\text{>}\overset{+}{\text{O}}\text{-L}$	0.69
$\overset{-}{\text{O}}\text{-L}$	0.47–0.56
>N-L	0.92
$\text{>}\overset{+}{\text{N}}\text{-L}$	0.97
$\diagup\text{S-L}$	0.40–0.46
>C-L	0.62–0.64 (sp) 0.78–0.85 (sp^2) 0.84–1.18 (sp^3)

^a Data are taken from similar tables in Schowen⁽²⁰⁾ and Schowen.⁽¹⁰⁾ Original sources and discussion of the values reported are contained in those references.

the isotopic fractionation factor for an $-\text{O}-\text{L}$ position is 1.0; i.e., deuterium or hydrogen is randomly distributed among such bonds.

To see how fractionation factors may be used in isotope-effect calculations, consider a situation in which protiated and deuterated reactants RH and RD undergo chemical reaction to give protiated and deuterated products PH and PD,



It is likely that the reactant fractionation factor ϕ^R and the product fractionation factor ϕ^P are different. It is readily seen that the equilibrium isotope effect, $K_{\text{H}}/K_{\text{D}}$, for this process is given by the fractionation factor ratio ϕ^R/ϕ^P :

$$\begin{aligned}\frac{K_H}{K_D} &= \frac{[\text{PH}]/[\text{RH}]}{[\text{PD}]/[\text{RD}]} = \frac{[\text{RD}]/[\text{RH}]}{[\text{PD}]/[\text{PH}]} \\ &= \frac{[\text{RD}]/[\text{RH}]}{[\text{ROD}]/[\text{ROH}]} \bigg/ \frac{[\text{PD}]/[\text{PH}]}{[\text{ROD}]/[\text{ROH}]} = \frac{\phi^R}{\phi^P}\end{aligned}\quad (13)$$

For substrates with multiple exchangeable hydrogenic positions, the equilibrium isotope effect is given by

$$\frac{K_H}{K_D} = \frac{\prod_{\text{reactant sites } i} \phi_i^R}{\prod_{\text{product sites } j} \phi_j^P} \quad (14)$$

where a ϕ value is included for every hydrogenic site, even if identical with another. Thus, using the ground-state fractionation factors as given in Table III, one can easily calculate equilibrium isotope effects. As an example, consider the equilibrium



The isotope effect K_H/K_D roughly calculated from fractionation factors should be

$$\begin{aligned}K_H/K_D &= (\phi)_{\text{O-L}}^4 / (\phi)_{\text{O-L}}^3 (\phi)_{\text{O-L}} \\ &= 1.0 / (0.69)^3 (0.5) \simeq 6.1\end{aligned}\quad (16)$$

Experimental values range from 5.2 to 7.4.⁽⁴⁸⁻⁵²⁾ The necessary ϕ values can be obtained experimentally most conveniently by NMR techniques.⁽⁵³⁻⁵⁵⁾

4.2. Transition-State Fractionation Factors and Calculation of Approximate Secondary Solvent Isotope Effects

By the reasoning outlined in the foregoing section, one should be able to define a transition-state fractionation factor which could then be used to calculate kinetic secondary hydrogen isotope effects. If PH in Eq. (11) were a transition state, TH, the kinetic isotope effect would be given by the ratio of reactant- and transition-state fractionation factors,

$$k_H/k_D = \phi^R / \phi^T \quad (17)$$

For substrates with multiple exchangeable hydrogenic positions the kinetic isotope effect is given by

$$\frac{k_H}{k_D} = \frac{\prod_{\text{reactant sites } i} \phi_i^R}{\prod_{\text{transition-state sites } j} \phi_j^T} \quad (18)$$

in terms of the reactant- and transition-state isotopic fractionation factors for every separate exchangeable hydrogenic site.

The problem of obtaining ϕ^T values now arises. In particular, considering secondary effects, how do we obtain fractionation factors for those bonds that are intermediate in character to those given in Table III (H—O $^{\delta+}$, for example). (Partially formed or broken bonds where the hydrogen is being transferred give primary isotope effects and can be so treated.)

Assume for the process



that the isotope effect on the transition-state free energy is a weighted average of the isotope effects on reactant and product free energies.⁽⁵⁶⁾ Then, where χ is a weighting factor describing the transition-state structure ($\chi = 0$ for an exactly reactant-like transition state and $\chi = 1$ for an exactly product-like transition state) and $\delta\Delta G^0$ is the double difference in zero-point energy between reactant SL and product PL and given by [see Eq. (2)]

$$K_{\text{H}}/K_{\text{D}} = \phi^{\text{R}}/\phi^{\text{P}} = e^{\delta\Delta G^0/RT} \quad (20)$$

then

$$\delta\Delta G^\ddagger = \chi \delta\Delta G^0 \quad (21)$$

and from Eqs. (2) and (20)

$$k_{\text{H}}/k_{\text{D}} = \phi^{\text{R}}/\phi^{\text{T}} = e^{\delta\Delta G^\ddagger/RT} = e^{\chi \delta\Delta G^0/RT} = (\phi^{\text{R}}/\phi^{\text{P}})^\chi \quad (22)$$

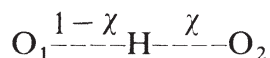
The transition-state fractionation factor is then given by

$$\phi^{\text{T}} = (\phi^{\text{R}})^{1-\chi} (\phi^{\text{P}})^\chi \quad (23)$$

If fractionation factors ϕ^{R} and ϕ^{P} are known for the substances on either side of a transition state, ϕ^{T} can then be calculated provided an estimate of χ is available, from Brønsted α or β values, for example.⁽²⁰⁾ Also, knowing an experimental value for $k_{\text{H}}/k_{\text{D}}$ in addition to the ground-state fractionation factors, estimates for χ can be obtained—and thus information about the transition-state structure.

4.3. Calculation of Approximate Primary Solvent Isotope Effects

Approximate values of primary solvent isotope effects for hydrogen transfer may be obtained by the method described by Schowen⁽²⁰⁾ and briefly recapitulated here. Consider a transition state for the transfer of a proton from O₁ to O₂



where χ defines the bond order for the forming H—O bond. For $\chi = 0$, the transition state is like reactants, and there is no isotope effect; i.e., $k_{\text{H}}/k_{\text{D}} = 1$. For $\chi = 1$, the transition state is like products, and the isotope effect is equal to the equilibrium isotope effect,

$$k_{\text{H}}/k_{\text{D}} = K_{\text{H}}/K_{\text{D}} = \phi^R/\phi^P \quad (24)$$

where R and P refer to the immediate reactants and products of the rate-determining step. A third value of χ is χ_{max} , defined as the bond order which gives a maximum isotope effect. This occurs when the force constants on the two partial transition-state $\text{O} \cdots \cdots \text{H}$ bonds are equal. An expression for χ_{max} is

$$\chi_{\text{max}} = 1/[1 + (v_{\text{P}}^{\text{H}}/v_{\text{R}}^{\text{H}})^2] \quad (25)$$

where v_{P}^{H} and v_{R}^{H} are the stretching frequencies in reciprocal centimeters for the reactant and product bonds in question (OH in this case).

The idea is to construct a plot of isotope effect, $k_{\text{H}}/k_{\text{D}}$, vs. χ for the three cases $\chi = 0$, $\chi = 1$, and $\chi = \chi_{\text{max}}$. The maximum isotope effect is roughly given by

$$(k_{\text{H}}/k_{\text{D}})_{\text{max}} = e^{-\Delta u_{\text{R}}/2} \quad (26)$$

resulting in, assuming $v_{\text{R}}^{\text{H}}/v_{\text{R}}^{\text{D}} \sim \sqrt{2}$,

$$\log(k_{\text{H}}/k_{\text{D}}) = (3.2 \times 10^{-4}) v_{\text{R}}^{\text{H}} \quad (27)$$

From the resulting plot one can read off values for the primary isotope effect, $(k_{\text{H}}/k_{\text{D}})_{\text{pri}}$. Alternatively, one can use the relationship

$$\left(\frac{k_{\text{H}}}{k_{\text{D}}}\right)_{\text{pri}} = 1 + \left[\left(\frac{k_{\text{H}}}{k_{\text{D}}}\right)_{\text{max}} - 1\right] \left(\frac{\chi}{\chi_{\text{max}}}\right) \quad (28)$$

for values of $\chi \leq \chi_{\text{max}}$, and

$$\left(\frac{k_{\text{H}}}{k_{\text{D}}}\right)_{\text{pri}} = \left(\frac{\phi_{\text{R}}}{\phi_{\text{P}}}\right) + \left[\left(\frac{k_{\text{H}}}{k_{\text{D}}}\right)_{\text{max}} - \left(\frac{\phi_{\text{R}}}{\phi_{\text{P}}}\right)\right] \left(\frac{1 - \chi}{1 - \chi_{\text{max}}}\right) \quad (29)$$

for $\chi \geq \chi_{\text{max}}$ to arrive at an estimate of the primary isotope effect.

Occasionally it is true that proton transfer occurs late in a reaction sequence. This means that the actual proton-donating reactant is different from the initial reactant with consequent differences in fractionation factors. The isotope effect calculated by means of Eqs. (28) and (29) must be corrected by a term $\phi_{\text{R}}/\phi'_{\text{R}}$, where ϕ'_{R} is the fractionation factor for the site in the species immediately preceding the rate-determining proton-transfer step:

$$\left(\frac{k_{\text{H}}}{k_{\text{D}}}\right)_{\text{pri}} = \left(\frac{\phi_{\text{R}}}{\phi'_{\text{R}}}\right) \left(\frac{k_{\text{H}}}{k_{\text{D}}}\right)_{\text{pri}} \text{ [from Eqs. (28) and (29)]} \quad (30)$$

4.4. Estimation of the Overall KSIE

We have seen that the major contributions to a solvent isotope effect are either primary (from transferring hydrogen) or secondary (from hydrogens not undergoing transfer but experiencing some change in bonding). The overall

solvent isotope effect can be expressed as

$$\frac{k_{\text{H}_2\text{O}}}{k_{\text{D}_2\text{O}}} = \left(\frac{k_{\text{H}}}{k_{\text{D}}} \right)_{\text{pri}} \left(\frac{k_{\text{H}}}{k_{\text{D}}} \right)_{\text{sec}} \quad (31)$$

Its magnitude can be approximated using fractionation factors in Table III and Eq. (22) and Eq. (28) or (29) as described in the preceding sections to obtain estimates for the secondary and primary contributions, respectively. A procedure to follow is:

1. Postulate a transition-state structure, indicating bond orders.
2. Estimate or look up fractionation factors for all exchangeable hydrogens not being transferred.
3. Calculate $(k_{\text{H}}/k_{\text{D}})_{\text{sec}}$ from Eq. (22).
4. Estimate ϕ'_R for the transferring hydrogen in the species just prior to the rate-determining step, and calculate ϕ_R/ϕ'_R .
5. Calculate $(k_{\text{H}}/k_{\text{D}})_{\text{pri}}$ from Eq. (28) or (29) and then Eq. (30).
6. Calculate $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$ from Eq. (31).

The calculated isotope effect can then be compared to the experimental value, and necessary modifications in the structure of the transition state proposed in step 1 can be made. Numerous examples illustrating the ease, accuracy, and utility of this method are given by Schowen.⁽²⁰⁾

It should be noted that the method just outlined is but one way to estimate KSIE's. As stated in the beginning of Section 4, any method used to predict an isotope effect for a proposed mechanism, including simple analogy with a known reaction, is usually acceptable. The estimate may then be compared with the experimental KSIE and the proposed transition-state structure confirmed or modified as necessary.

In summary, then, we have seen that kinetic solvent isotope effects are determined by the double difference in zero-point energy as a result of bonding changes to H (D) on going from reactant to transition state, that the magnitude of these effects is determined by and provides information about the structure of the transition state (insofar as it involves bonds to hydrogen) and to some extent about its solvational environment, and that values for these effects can be estimated from theory, fractionation factors, and hypothetical transition-state models. These calculated values can then be compared to those obtained after experimentally obtaining the rate constants for the reaction in each of two solvents, pure H_2O and pure D_2O , and the transition-state model accepted or rejected. The technique is generally rapid, simple, and reliable. While a degree of ambiguity will exist for more complex systems, i.e., those with numerous exchangeable hydrogens (see below), the isotope effects obtained from the pure isotopic solvents alone can always be used to gain rough initial transition-state information.

5. SOLVENT ISOTOPE EFFECTS IN MIXTURES OF LIGHT AND HEAVY SOLVENTS: THE PROTON INVENTORY TECHNIQUE

The foregoing method for deducing transition-state structures from solvent isotope-effect calculations and experimental data is most useful, accurate, and unambiguous when the system is relatively simple and there are few exchangeable hydrogens. As the number of exchangeable hydrogens in the reactants increases so does the number of possible transition-state structures. The origin of the isotope effect now becomes less certain. To what extent, for example, is the net effect now the result of rate-determining proton transfer (primary effect) or of many many smaller secondary changes in bonding to H multiplying together to give the observed effect?

This is, of course, precisely the situation we face in studying biochemical reactions. Biological molecules have abundant exchangeable hydrogens, those bonded to oxygen, nitrogen, and sulfur atoms, to carbon atoms located in the α position relative to a carbonyl group, etc. The number of such exchangeable sites on proteins and other biopolymers is enormous. Not only may a very large number of protons be undergoing bonding changes on activation but conformation changes (thus altering reactant shape, energy, etc.) may be induced on dissolution in D_2O as a result of exchange of H for D at sites throughout the molecule. Solvent isotope-effect studies of enzyme-catalyzed reactions or reactions otherwise involving biological macromolecules are then a particular problem, although the determination of their reaction mechanisms and transition-state structures is crucially important for fundamental and practical (see Part IV) reasons.

What is clearly needed for these biochemical reactions is (1) a “list” of all hydrogens undergoing a change of state on going to the transition state, i.e., of those generating the net isotope effect, and (2) the isotope effect for each. Such a list is known as a *proton inventory* and is obtained from reaction-rate studies in mixtures of light and heavy water. A complete proton inventory should give information about the number of hydrogens undergoing transfer or secondary bond changes in the transition state. One can then apply methods similar to those outlined in the previous sections to arrive at reasonable transition-state structures.

5.1. The Gross–Butler Equation

Forty years ago LaMer and Chittum⁽⁵⁸⁾ and then Gross *et al.*⁽⁵⁹⁾ and Butler⁽⁶⁰⁾ reported that the solvent isotope effect, k_0/k_n , for a variety of proton-transfer reactions in mixtures of light and heavy water did not vary linearly with n , the atom fraction of D in the mixture. The reason for this behavior rests in the fact that not all exchangeable protons responsible for the isotope

effect have the same isotopic composition as the solvent; i.e., their fractionation factors are not necessarily equal to unity. Theoretical treatments of this phenomenon led to a mathematical formulation known as the Gross–Butler equation. The form of this equation currently is that given by

$$K_n = K_0 \prod_i^v (1 - n + n\phi_i^P) \bigg/ \prod_j^v (1 - n + n\phi_j^R) \quad (32)$$

$$k_n = k_0 \prod_i^v (1 - n + n\phi_i^T) \bigg/ \prod_j^v (1 - n + n\phi_j^R) \quad (33)$$

for equilibrium and rate processes, respectively. The ϕ 's are the fractionation factors defined in Sections 4.1 and 4.2 and n is the atom fraction D in the solvent and varies from 0 (pure protiated solvent) to 1 (pure deuterated solvent). K_0 and k_0 are the respective equilibrium and rate constants in pure protiated solvent ($n = 0$); K_n and k_n are the corresponding constants obtained for a solvent in which the atom fraction D is equal to n . A simple derivation of the Gross–Butler relationship for rate processes follows.⁽⁶¹⁾

5.2. Simple Derivation of the Gross–Butler Relation

The rate constant k_n for a reaction carried out in a mixture of D₂O (mole fraction n) and H₂O (mole fraction $1 - n$) will equal that for a reaction in pure water, k_0 , multiplied by a series of correction factors, J_i^n ,

$$k_n = k_0 \prod_i^v J_i^n \quad (34)$$

one for each of the v exchangeable hydrogenic positions which contribute to the solvent isotope effect. The correction factor for a particular hydrogen will depend on the value of n , i.e., the amount of D₂O relative to H₂O in the solvent mixture, and on the differences in bonding of the particular hydrogen atom in the reactant and transition state. This correction factor will equal the equilibrium fraction (χ) of protium present in the particular hydrogenic site of the reactant divided by the fraction for that particular site in the transition state.

$$J_i^n = \left[\frac{\chi_H^R/(\chi_H^R + \chi_D^R)}{\chi_H^T/(\chi_H^T + \chi_D^T)} \right]_i^n = \frac{1 + (\chi_D^T/\chi_H^T)_i^n}{1 + (\chi_D^R/\chi_H^R)_i^n} \quad (35)$$

It can be seen that if the particular (i th) site being considered prefers deuterium more in the reactant than in the transition state, $J_i^n < 1$ and $k_n < k_0$. This is the situation encountered when bonding to hydrogen is weakened on going to the transition state, resulting in “normal” isotope effects, $k_0/k_n > 1$. Deuterium, it will be recalled, prefers that side of an equilibrium in which bonding to hydrogen is tighter.

For a given hydrogenic site i and a particular solvent composition n , the fraction χ_D^R/χ_H^R , for example, in Eq. (35), is equivalent to the concentration

ratio $[\text{SD}]/[\text{SH}]$ for the equilibrium of Eq. (9) and is thus related to the isotopic fractionation factor, as shown

$$\phi^R = \frac{[\text{SD}]/[\text{SH}]}{[\text{D}_2\text{O}]/[\text{H}_2\text{O}]} = \frac{\chi_D^R/\chi_H^R}{n/(1-n)} \quad (36)$$

$$(\chi_D^R/\chi_H^R)_i = \phi_i^R n/(1-n) \quad (37)$$

The expression for J_i^n in Eq. (35) can thus be written in terms of these fractionation factors,

$$J_i^n = \frac{1 + [\phi_i^T n/(1-n)]}{1 + [\phi_i^R n/(1-n)]} = \frac{1-n + \phi_i^T n}{1-n + \phi_i^R n} \quad (38)$$

and Eq. (34) becomes

$$k_n = k_0 \prod_i^v (1-n + n\phi_i^T) \bigg/ \prod_i^v (1-n + n\phi_i^R) \quad (39)$$

This, then, is the Gross-Butler equation [cf. Eq. (33)] relating rate constants to mole fraction D_2O for reactions in mixtures of H_2O and D_2O . Equation (33) is the more general form of the equation, showing that the number of hydrogenic sites in the reactant and transition states need not be counted as equal.

Equation (33) or (39) can be expressed in terms of a reactant-state contribution (RSC) and a transition-state contribution (TSC),

$$k_n = k_0 [(\text{TSC})_n/(\text{RSC})_n] \quad (40)$$

and, as has been argued earlier, since k_n and k_0 are accessible from experiment and RSC can be evaluated from known or estimated reactant-state fractionation factors, the TSC can be determined

$$(\text{TSC})_n = (k_n/k_0) (\text{RSC})_n \quad (41)$$

and information about the number of ϕ 's and their values obtained. A list of all the ϕ_i^T obtained by fitting $k_n(n)$ with known or assumed ϕ_j^R gives an inventory of transition-state protons and the isotope effect associated with each one.

In Section 6 we shall discuss some of the experimental considerations necessary for proton inventory work, and in Section 7 we shall discuss how the rate data so generated may be analyzed so as to provide mechanistic information.

6. EXPERIMENTAL PROCEDURES⁽⁶²⁾

6.1. Preparation and Analysis of Mixtures of Light and Heavy Water

Heavy water is readily available from commercial sources. The usual 99.7–99.8% purity is entirely adequate for proton inventory work since the

deuterium content of the experimental solutions will generally be independently determined. Distillation over barium nitrate is recommended if the pD is greater than 7; otherwise the D₂O may be used directly. Ordinary distilled water may be passed through an ion-exchange column and/or boiled to remove CO₂. Manipulations involving D₂O should normally be carried out in a dry box under dry nitrogen. Solutions can be stored in flasks equipped with serum caps. Transfer from one container to another can then be effected by means of a syringe. Contamination of D₂O solutions upon *brief* (3–4 min) exposure to atmospheric moisture has been demonstrated to be negligible.⁽⁶³⁾

Mixtures of H₂O and D₂O can be prepared gravimetrically, or somewhat less accurately, volumetrically, using the molecular weights and densities given in Table II to obtain the desired values of *n*, mole fraction D₂O. The various H₂O–D₂O mixtures thus obtained may then be used to prepare solutions of the required ionic strength (by dilution of KCl, for example) or of the required pH (by dilution of various buffer pairs). (In the latter case account must be taken of any exchangeable protons which will dilute the mixture with protium and decrease the atom fraction of D.) Such preparation of salt or buffer solutions directly from the mixed solvent tends, however, to be wasteful of D₂O, still relatively expensive. One can therefore make each solution directly each time from weighed amounts of buffers and/or salt, diluting to volume by the addition of successive, weighed amounts of D₂O and H₂O. Again account must be taken of exchangeable hydrogen in the buffer. As this procedure tends to be tedious, alternative methods using stock H₂O solutions containing buffer and/or salt and dilution of these with the proper amount of pure H₂O and pure D₂O are therefore preferable. In calculating the final atom fraction of D and of H, the actual weight of these substances must be computed and account taken of any H added via buffer. Section 6.2 contains more information about buffers.

Experimental verification of these calculations is essential. It is desirable to have this verification for each solvent mixture used and preferably both at the start and at the end of an experimental run. Selected solutions may most conveniently be sent off for deuterium analysis* or analyzed by means of NMR techniques, one of which is described in the following paragraph. Selected references to other analytical methods are given in Reference 62.

Small amounts of H₂O and D₂O may be determined⁽⁶²⁾ by adding small weighed amounts of pure H₂O and pure *p*-dioxane (or acetonitrile, etc.) to a weighed quantity of H₂O–D₂O mixture, the composition of which is to be determined, and determining the NMR spectrum of the resulting solution. The amount of dioxane and total H₂O should be adjusted so that the NMR peak heights will be similar. The ratio of the integrated peak heights for H₂O and dioxane is related to the total number of moles of H₂O present:

* Joseph Nemeth, 303 W. Washington, Urbana, Illinois 61801 performs deuterium analyses using the falling drop method.

$$\frac{8 \text{ (integrated H}_2\text{O peak height)}}{2 \text{ (integrated dioxane peak height)}} = \frac{\text{moles H}_2\text{O total}}{\text{moles dioxane}} \quad (42)$$

Simple subtraction from the moles of H₂O added will give the moles of H₂O in the original H₂O–D₂O mixture.

6.2. *pL Measurements and Preparation of Buffer Solutions in Light and Heavy Water*

In the course of a kinetic investigation, particularly of acid- or base-catalyzed or enzyme-catalyzed reactions, it will become necessary to know the exact pH dependence of the particular reaction under consideration. If a reaction rate is pH controlled and solvent isotope or proton inventory studies are to be made, it is, of course, important not only to be able to measure the pL accurately in each solvent mixture but also to be able to stay at the same relative position on the pH rate curve from solvent mixture to solvent mixture. The former is accomplished by means of a pH meter and the latter usually by using appropriate and correctly prepared buffer solutions as the reaction medium.

Accurate measurement of pL using a pH meter is described in Sections 6.2.1 and 6.2.2. Establishment and maintenance of a desired pL during a reaction is discussed in Sections 6.2.3–6.2.5.

6.2.1. *Interpretation of pH-Meter Readings*

The apparent pH of an aqueous solution of given [L⁺] or [OL[−]], as determined with a glass electrode and read on a pH meter, is dependent on the mole fraction of D₂O. Thus, for example, a solution known to be 0.001 *M* in L⁺ may give a reading on a pH meter of 3.00 in pure H₂O, 2.85 when the mole fraction of D₂O is 0.5, and 2.61 in pure D₂O.

Glasoe and Long,⁽⁶⁴⁾ in their empirical approach to the standardization of a pK scale for solution in D₂O, found that when a pH meter with a glass and a calomel electrode is calibrated with standard pH solutions in H₂O and is then used for the measurement of the pD of solutions of known DCl (molar) concentration in pure D₂O a constant difference of 0.4 pH unit is observed at 25°C:

$$\text{pD} = (\text{meter reading}) + 0.4 \quad (43)$$

or

$$(\Delta\text{pH})_{n=1} = \text{pD} - (\text{meter reading}) = 0.4 \quad (44)$$

Numerous independent investigations have demonstrated the essential correctness of the value 0.4 (± 0.02 pH unit) and that it holds for different electrolytes at various pH's and temperatures as well as for different commercial glass electrodes from different manufacturers.^(48,63-66)

In a similar manner ΔpH_n values have been obtained for different values of n . From their data, obtained at 25° from pH meter readings on solutions 0.01 *M* and 0.1 *M* in LCl and 0.01 *M* in LClO₄ using mixed H₂O–D₂O solvents, Salomaa *et al.*⁽⁴⁸⁾ derived by the method of least squares the empirical equation

$$\Delta\text{pH}_n = 0.076n^2 + 0.3314n \quad (45)$$

which can then be used to obtain the true pL value for any H₂O–D₂O solvent mixture of known mole fraction of D₂O, n . A similar relationship which holds at both 25° and 37° was derived by Schowen *et al.*⁽⁶⁶⁾ from data using 0.001- to 0.0007-*M* LCl solutions in mixtures of light and heavy water:

$$\Delta\text{pH}_n = (0.17324)n^2 + (0.22076)n + 0.00009 \quad (46)$$

$$\pm 0.01298 \quad \pm 0.01216$$

The difference in ΔpH as calculated from either Eq. (45) or (46) is at most 0.03 pH unit.

It may be deemed desirable to prepare a calibration curve of meter reading vs. n for the particular system, pH, instrument, and electrodes to be used in a given investigation. The measurements can easily be made following a procedure similar to that described in the following section.

6.2.2. Experimental Determination of Relationship between pL and $n_{\text{D}_2\text{O}}$

The pH meter is calibrated with standard aqueous buffers and adjusted to the desired temperature controlled to $\pm 0.1^\circ\text{C}$. The electrodes are immersed in a known volume of solvent (pure H₂O, pure D₂O, and at least five mixtures of known mole fraction of D₂O) contained in a thermostatted vessel. The solution and electrodes are allowed to reach thermal equilibrium (about 5 min) at which point a known volume (the same for each solvent mixture) of standard aqueous HCl is added. It is unnecessary to soak or equilibrate the electrode in the particular solvent being used for a longer period of time.^(64,66) After stirring, the pH meter is read. In this fashion a calibration curve of pH meter reading vs. $n_{\text{D}_2\text{O}}$ (corrected for the added titrant) is obtained as shown in Fig. 2, and ΔpH_n for any mole fraction D₂O can thus be read with a good degree of confidence from such a curve. If desired, ΔpH_n vs. n values obtained from this curve can be subjected to polynomial regression analysis^(62,67) to obtain values for the coefficients for the quadratic equation

$$\Delta\text{pH}_n = an^2 + bn + c \quad (47)$$

6.2.3. Ionization of Weak Acids in Light and Heavy Water

The ionization of weak acids decreases by a factor of 3–6 in D₂O.⁽¹⁶⁾ The difference $\text{pK}_\text{D} - \text{pK}_\text{H}$ or ΔpK_a for most acids is about 0.5 to 0.7 unit and has been shown by Rule and LaMer⁽⁶⁸⁾ and Bell⁽⁶⁹⁾ to increase with pK_a

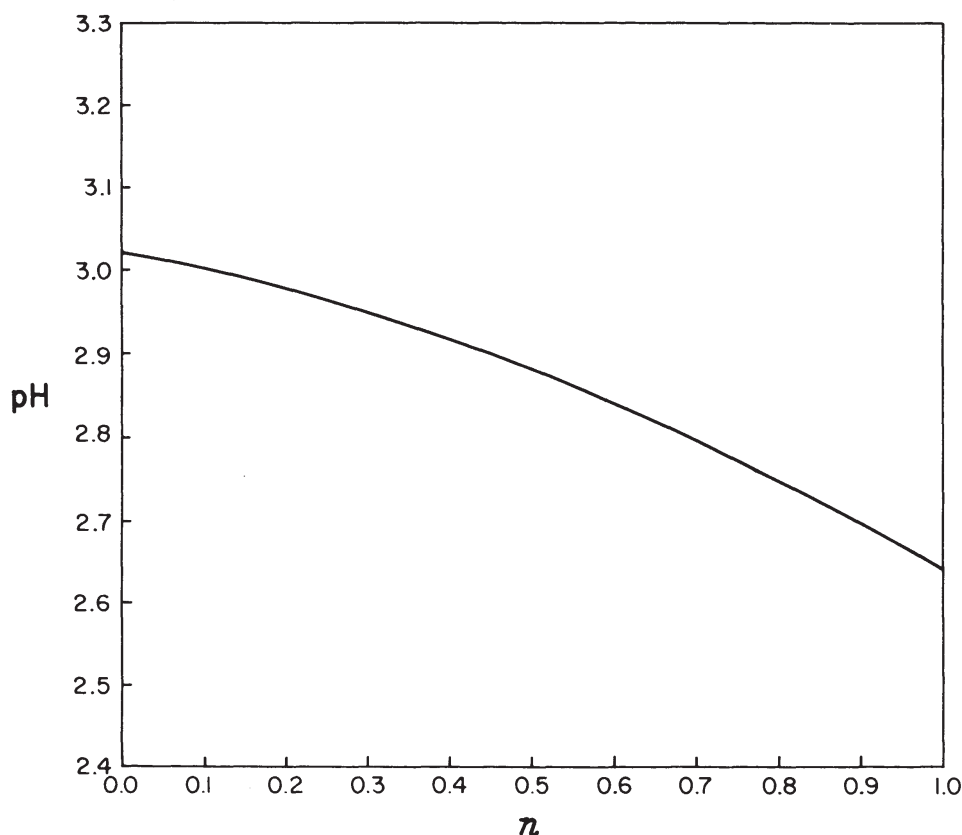


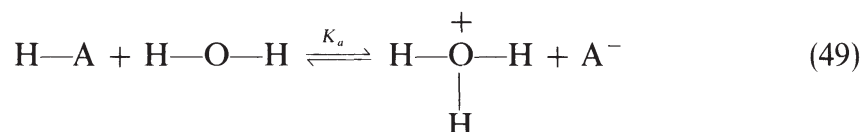
Fig 2. Experimental pH meter reading vs. atom fraction deuterium, n , in mixtures of H_2O and D_2O at 25° .

according to the relationship

$$\Delta pK_a = 0.41 + 0.020pK_H \quad (48)$$

Note that this predicts that acids of pK_a 3–10 should all have ΔpK_a in the range 0.54 ± 0.07 . An excellent collection of pK_H and ΔpK_a values for a large number of weak acids has been compiled by Laughton and Robertson,⁽¹⁶⁾ and in fact at least 70% of the acids listed with pK_a 's between 3 and 10 have ΔpK_a values between 0.46 and 0.61.

In mixtures of H_2O and D_2O , the change in ionization constant of a weak acid follows a nonlinear relationship with the mole fraction of D_2O .^(16,18,22) The particular form of this relationship can be derived by considering the equilibrium for the ionization of a weak acid,



and using the Gross–Butler equation given in Section 5.1 [Eq. (32)] for equi-

librium processes:

$$K_n = K_0 \left[\frac{\prod_{i=1}^v \text{product sites} (1 - n + n\phi_i^P)}{\prod_{j=1}^v \text{reactant sites} (1 - n + n\phi_j^R)} \right] \quad (50)$$

There are on each side of Eq. (49) three hydrogens undergoing a change of state: on the reactant side, the acidic hydrogen of the weak acid and the two (identical) hydrogens of water, and on the product side, the three identical $\text{O}^+\text{—H}$ hydrogens of the hydronium ion. Equation (50) then becomes

$$K_n = K_0 \frac{(1 - n + nl)^3}{(1 - n + n\phi_{\text{H-A}})(1 - n + n\phi_{\text{H}_2\text{O}})^2} \quad (51)$$

where K_n is the acid dissociation constant in an H_2O – D_2O mixture in which the mole fraction D_2O is equal to n ; K_0 is the corresponding constant in pure H_2O , i.e., K_{H} ; and l is the fractionation factor for the $\text{O}^+\text{—H}$ hydrogens of the hydronium ion, 0.69. Since the fractionation factor for O—H bonds is equal to 1.0 (Table III), the squared term in the denominator of Eq. (51) disappears. Setting $n = 1$ (pure D_2O), we see that

$$K_n/K_0 = K_{\text{D}}/K_{\text{H}} = l^3/\phi_{\text{H-A}} \quad (52)$$

and

$$\phi_{\text{H-A}} = l^3/(K_{\text{D}}/K_{\text{H}}) \quad (53)$$

where K_{H} and K_{D} are the acid dissociation constants for the weak acid in pure H_2O and pure D_2O , respectively. Equation (51) then becomes, for all values of n ,

$$K_n = K_0 \frac{(1 - n + nl)^3}{1 - n + nl^3 K_{\text{H}}/K_{\text{D}}} \quad (54)$$

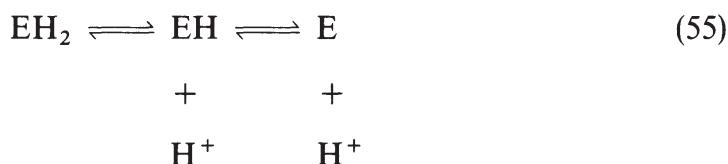
Thus if one has experimentally determined $\text{p}K_{\text{H}}$ and $\text{p}K_{\text{D}}$ in the pure solvents, the value K_{H}/K_n and therefore $\Delta\text{p}K_a$ can be calculated from Eq. (54) for any value of n . Equation (54) assumes that K_{H} and K_{D} are independent of the medium, i.e., independent of changes in the standard states for the ionization process. It also assumes that the proton is monosolvated—hence the term l^3 . If nonspecific solvation of the proton is assumed, an equation linear in n follows. Gold and Lowe⁽⁵⁰⁾ and Salomaa *et al.*⁽⁴⁸⁾ concluded that the cubic equation gave better agreement with experimental K_{H}/K_n values than either assuming the proton was nonspecifically solvated or attributing the change in K_{H}/K_n to medium effects, although Halevi *et al.*⁽⁷⁰⁾ found little to distinguish between the two. For further discussion of this equation, its assumption, limitations, derivation, and alternate forms, see References 16 and 18.

6.2.4. Selection of a Suitable Buffer

To study most conveniently a pH-dependent reaction under the same conditions from solvent to solvent, a buffering system can be selected such that the ΔpK_a of the acidic buffer component is the same as that (or those) for the reagent, catalyst, enzyme, etc., responsible for the pH dependence. In the following paragraphs we shall attempt to rationalize this general statement.

If not already available, a pH-rate profile for the desired reaction should be determined experimentally in both light and heavy water; profiles in H_2O – D_2O mixtures are not required. The results when plotted may appear as sketched in Fig. 3. pD values must be calculated before plotting by adding 0.4 to the experimental “pH” readings, as discussed in Section 6.2.1. As shown in Fig. 4, the pH-rate curves are simply segments of “titration” curves, i.e., plots of concentration (measured here by the rate) of reactive form (protonated, nonprotonated, etc.) of the pH-determining reaction component vs. pH.

Since the existence of a rate variation with pH for a reaction may imply that different concentrations of the reactive form of a reagent are present at different pH's, curvature such as in Fig. 3 may be observed because of the presence of an ionizable group at, for example, an enzyme active site, one form of which is more catalytically active than another. If the following equilibria are possible for an enzyme, with form EH being catalytically active,



curves such as those in Figs. 3 and 4 are expected.

The displacement of the curve in D_2O is observed because the ionization constants are different in light and heavy water (see Section 6.2.3). The values for ΔpK_1 and ΔpK_2 differ very slightly (refer to the Bell equation in Section 6.2.3 and to the fact that ΔpK_a values of almost all acids with pK's between

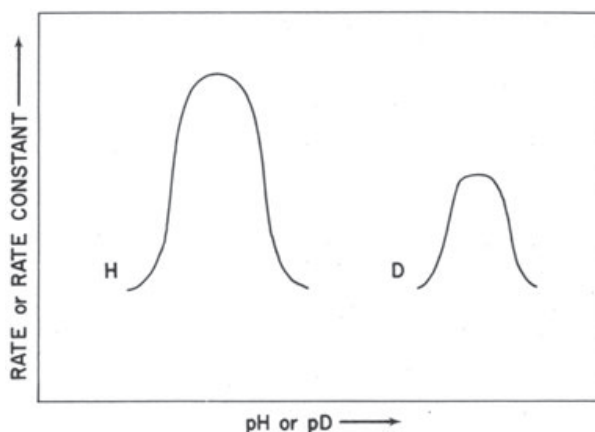


Fig. 3. Hypothetical pH-rate profile for a reaction in H_2O (curve H) or D_2O (curve D).

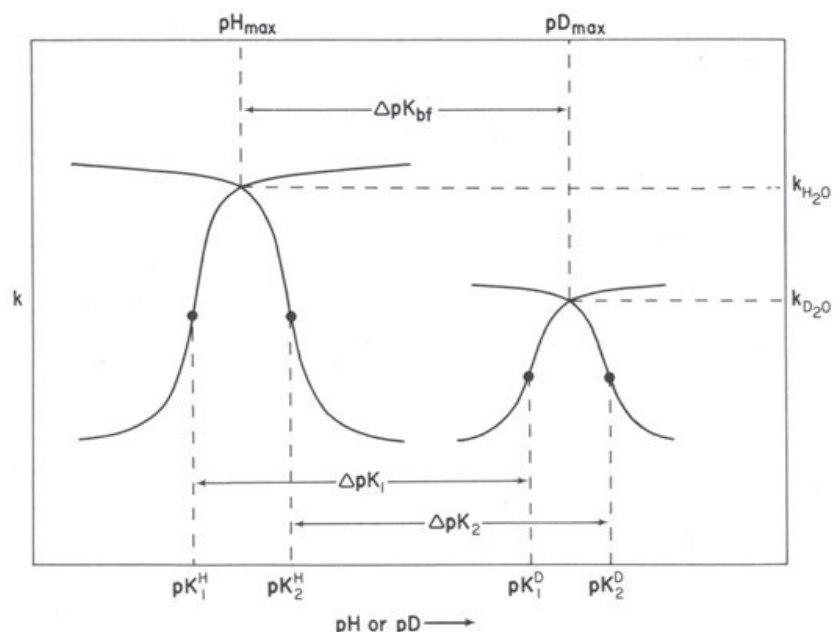


Fig. 4. pH vs. rate constant curves for a hypothetical reaction in H_2O and in D_2O showing differences in pK_a 's.

3 and 10 are in the range 0.54 ± 0.07); the difference between the pH and the pD values at the maximum of each curve, $\text{pD}_{\text{max}} - \text{pH}_{\text{max}}$, will be approximately equal to ΔpK_1 , and ΔpK_2 , or, more exactly, a weighted average of ΔpK_1 and ΔpK_2 . We are particularly interested in the pH (or pD) values at the maxima of these pH-rate curves since it will be at these pH's that the reaction should be studied. At those pH's the reaction rate is least sensitive to small deviations in pH, and errors of measurement will be minimized. Consider that a buffer is chosen for which the acid component has, referring to Fig. 4, a pK_a equal to pK_1 , pK_2 , or, preferably, pH_{max} for the reaction in question and that the reaction will be studied at a pH equal to pH_{max} . Then the change in buffer pK_a , $\Delta\text{pK}_{\text{bf}}$, is such that (assuming always the same buffer ratio) a shift to pure D_2O or any H_2O – D_2O mixture will ensure that one will always be at the same relative point, in this case the maximum, of the pH-rate curve for the solvent mixture.

In practice, then, one selects a buffer (from Laughton and Robertson's table, for example⁽¹⁶⁾) whose acid component has a ΔpK_a close to the difference $\text{pD}_{\text{max}} - \text{pH}_{\text{max}}$ ($\Delta\text{pK}_{\text{bf}}$ of Fig. 4) obtained from experimental pH-rate data for the reaction in light and heavy water. Use of the same concentration and ratio (i.e., the same amounts) of such a buffer acid and its conjugate base for each solvent mixture will assure that one is always working at the same region of the pH-rate curve. The value of pL will be different from solution to solution, but the *relative proportion of reactive species will be the same*.

It should be noted that it is not necessary to determine the pH-rate profile in D_2O . One can simply choose a buffer with a $\text{pK}_{\text{bf}}^{\text{H}}$ close to pK_1^{H} or pK_2^{H} for the reaction, or, lacking even that information, with a $\text{pK}_{\text{bf}}^{\text{H}}$ in the usual 0.54 ± 0.07 region. This will include most common buffering systems, includ-

ing the frequently used Tris buffers. A table in Bates,⁽⁷¹⁾ giving pD values for three buffers (citrate, phosphate, and carbonate, prepared by dissolving the protiated salts in pure D₂O) at various temperatures, may be useful in providing an independent check of the expected pD in D₂O when these buffers are used.

6.2.5. pH Stat Method for Maintaining Constant pH

In some cases constant pH is maintained during the course of a reaction in the absence of buffers by the pH Stat method. The desired pH is set on the instrument and titrant is automatically dispensed according to the appearance–disappearance of acid or base as the reaction proceeds. The rate of production of acetic acid in the acetylcholinesterase-catalyzed hydrolysis of acetylcholine, for example, may be followed by recording the rate of addition of standard NaOH needed to keep the pH at the constant preset value.^(72,73) To do a proton inventory study using the pH Stat technique one would need to determine experimentally at least a section of the pH rate profile of the reaction in *each* H₂O–D₂O mixture in order to find the pH reading for the same region on the curve.

6.3. Determination of Ground-State Fractionation Factors^(55,62)

An experimental technique using nuclear magnetic resonance spectrometry for the determination of ground-state fractionation factors has been used and described by Gold and Kessick,^(53,74) Kresge and Allred,⁽⁵⁴⁾ and Mata-Segreda.⁽⁵⁵⁾ The technique is based on the fact that hydrogen exchange between a hydroxylic solvent and many solutes is rapid relative to the NMR time scale. Consequently the only observed “exchangeable-H” signal has a chemical shift equal to the weighted (according to mole fraction) average chemical shift of *each* solute and solvent exchangeable-proton signal⁽⁷⁵⁾:

$$\delta_{\text{obs}} = \frac{\delta_1\chi_1 + \delta_2\chi_2 \cdots}{\chi_1 + \chi_2 \cdots} = \frac{\sum \delta_i\chi_i}{\sum \chi_i} \quad (56)$$

Here χ_i is the mole fraction of the *i*th kind of exchangeable hydrogen and δ_i is the NMR chemical shift for the *i*th proton when not exchanging with other kinds of protons. The term $\sum \chi_i$ will equal 1 in pure protiated solvent. Separation of solvent from solute contributions in Eq. (56) gives

$$\delta_{\text{obs}} = \delta_{\text{ROH}}\chi_{\text{ROH}} / \sum \chi_j + \sum_i^s \delta_i\chi_i / \sum \chi_j \quad (57)$$

where the first term gives the contribution from the solvent exchangeable protons, *s* means the number of exchangeable substrate hydrogenic sites, and $\sum_i^s$ means a sum over all *s* exchangeable substrate protons. For dilute solutions, in which nonideal behavior of the solution such as specific solvent–solute interactions (e.g., protonation) or solute–solute interactions (e.g.,

dimerization) is at a minimum, the mole fraction of substrate exchangeable protons is negligible compared to solvent; thus $\chi_{\text{ROH}} \simeq \sum \chi_j$, and the relationship becomes

$$\delta_{\text{obs}} = \delta_{\text{ROH}} + \sum_i^s \delta_i \chi_i \bigg/ \sum \chi_j \quad (58)$$

For mixed H_2O – D_2O solvents, and again for very dilute solutions, the total atom fraction of exchangeable protium is essentially that for the solvent H_2O , that is $1 - n$, where n is the mole fraction of D_2O . Equation (58) thus becomes

$$\delta_{\text{obs}} = \delta_{\text{H}_2\text{O}} + \sum_i^s \delta_i \chi_i^{\text{H}} \bigg/ (1 - n) \quad (59)$$

Recalling that the isotopic fractionation factor for the i th hydrogenic site in the solute is given by [see Eq. (36)],

$$\phi_i = (\chi_i^{\text{D}}/\chi_i^{\text{H}})/[n/(1 - n)] \quad (60)$$

and defining the total mole fraction of hydrogens of the i th kind as χ_i^t , where

$$\chi_i^t = \chi_i^{\text{D}} + \chi_i^{\text{H}} \quad (61)$$

then algebraic manipulation of Eq. (60) will give an expression for the mole fraction of the i th exchangeable protium:

$$\chi_i^{\text{H}} = (1 - n)\chi_i^t/(1 - n + \phi_i n) \quad (62)$$

Substituting the right-hand side of Eq. (62) into Eq. (57) gives an expression for the observed chemical shift in terms of the total mole fraction of exchangeable substrate hydrogen of the i th type:

$$\delta_{\text{obs}} = \delta_{\text{H}_2\text{O}} + \sum_i^s \delta_i \chi_i^t \bigg/ (1 - n + \phi_i n) \quad (63)$$

Since the total mole fraction of the i th kind of exchangeable substrate hydrogen, χ_i^t , is equal to the number of positions of the i th type, μ_i , times the mole fraction of solute, χ_s , we can write a final expression for the chemical shift expected for dilute solutions of a solute with i types of hydrogens in mixtures of light and heavy water:

$$\delta_{\text{obs}} = \delta_{\text{H}_2\text{O}} + \sum_i^s \frac{\delta_i \mu_i}{1 - n + \phi_i n} \chi_s \quad (64)$$

According to this equation, for low concentrations of solute in a particular H_2O – D_2O mixture, the slope of a plot of the observed chemical shift vs. mole fraction of solute should be linear. For two experiments using two different solvents (but otherwise identical) the ratio of slopes is given by

$$\frac{\text{slope 1}}{\text{slope 2}} = \frac{(1 - n + \phi_i n)_2}{(1 - n + \phi_i n)_1} \quad (65)$$

If experiment 1 is carried out in pure H_2O , $n = 0$ and Eq. (65) becomes

$$\frac{\text{slope (H}_2\text{O)}}{\text{slope (L}_2\text{O)}} = 1 - n + \phi_i n = 1 - n + \Phi_s n \quad (66)$$

The term Φ_s is defined as the overall fractionation factor for a particular solute and is thus readily available from NMR experiments. If the solute has one or more *identical* exchangeable hydrogenic sites, Φ is the fractionation factor, ϕ , for each site. If a given solute has two or more different exchangeable sites, Φ is an overall or “average” fractionation factor for that solute.

In practice, then, one measures the observed chemical shift for at least three, but preferably four or more, solutions of pure H₂O and for which the solute mole fraction is in the range 0.01–0.1. Lower χ_s may result in difficulty in correctly determining the position of the NMR signal; higher χ_s may introduce undesired complications resulting from solute–solute or solute–solvent interaction. Chemical shift readings should be reproducible to ± 0.1 Hz. The temperature is generally maintained constant, although it appears that ϕ values remain relatively unaffected over a small range in temperature. This procedure may then be repeated for mixed H₂O–D₂O solutions, prepared gravimetrically, for which n , the mole fraction of D₂O, is quite high, about 0.95. The experimental δ_{obs} vs. χ_s data may then be plotted or submitted for linear least-squares analysis and Φ calculated from Eq. (66). The percent error in Φ is given by

$$\begin{aligned} \% \text{ error in } \Phi = [(\% \text{ error in slope H}_2\text{O})^2 + (\% \text{ error in slope L}_2\text{O})^2 \\ + (\% \text{ error in } n)^2]^{1/2} \end{aligned} \quad (67)$$

It should be noted that the technique just described for determining reactant-state (average) fractionation factors has not been used for proteins or other macromolecules. It is essential for this NMR method to be reliable that the solute be concentrated enough to effect an observable change in the chemical shift from that of pure solvent yet not so concentrated that chemical shift changes due to solute–solvent or solute–solute interactions will occur. It is not certain that these conditions can be met in the case of macromolecules, but perhaps more experimentation is justified. (See Section 6.4.2 for further discussion.)

6.4. Macromolecular Systems: Special Considerations⁽⁶²⁾

Enzymes, proteins, and biological macromolecules in general present some special problems when involved in proton inventory studies. Transfer of such substances from protium oxide to deuterium oxide may induce a number of changes in behavior. Some of these changes may arise from the difference in the physical properties of the two solvents (see Table II) but more noticeably from exchange of labile protium for deuterium. An average-sized protein, for example, will have hundreds of exchangeable hydrogens including those of the backbone —NH— and the side-chain functional groups contain-

ing bonds to oxygen, nitrogen, and sulfur. These hydrogens play an important role in maintaining the secondary and tertiary structure and the state of ionization necessary for catalytic action (in the case of enzymes), self-association, binding to small molecules, etc. Replacement of these hydrogens by deuterium will alter the lengths and strengths of hydrogen bonds, pK_a values, and the extent of ionization, with possible resultant changes in conformation, extent of association, binding equilibria, catalytic power, etc. It is important to recognize that all of the above are examples of isotope effects and as such can be analyzed and understood. The following considerations are important in connection with the proton inventory technique.

6.4.1. Rate of Hydrogen Exchange

In carrying out a proton inventory study it is necessary to know the atom fraction of deuterium in the system, n , and how it is distributed, i.e. the reactant-state fractionation factors, ϕ_i^R , at the time the reaction is taking place. It is desirable, therefore, that all exchange of protein labile protons take place instantly or within a few minutes. Hvidt and Nielsen⁽⁷⁶⁾ review in detail methods for determining these exchange rates; Richards and Wyckoff⁽⁷⁷⁾ review results for ribonuclease. In most cases it appears that while exchange of backbone amide N—H may take place over several hours or more,⁽⁷⁸⁾ exchange of side-chain hydrogens is complete within a few minutes.

In the absence of precise exchange-rate information for the various kinds of exchangeable sites for a given protein a series of simple experiments such as those described by Bender and Hamilton⁽⁷⁹⁾ for α -chymotrypsin can be performed. In their case it was found that the same kinetic results were obtained in D_2O if either a stock solution of enzyme in D_2O or a stock solution of enzyme in H_2O was used to initiate the reaction under investigation. Similarly in H_2O , the kinetics was unaffected by use of either stock enzyme solution. Such results would allow one to conclude that the exchange of (at least) those labile hydrogens *involved in the reaction* was very rapid. It was also found in their case that the reaction rate was the same when using freshly prepared D_2O enzyme solutions or similar solutions which had been prepared several days previously. Such results would suggest that there are no very slowly exchanging hydrogens involved in the reaction. Hydrogen exchange occurring at intermediate rates, i.e., during the course of a reaction being studied, will show up as departure from the expected kinetic order, again, if those hydrogens are involved in the reaction under investigation.

6.4.2. Macromolecular Ground-State Fractionation Factors

Average fractionation factors for the exchangeable protons of macromolecules have been obtained from hydrogen-exchange studies in the form of the equilibrium isotope effect used to correct experimental deuterium or tritium exchange data to the numbers of exchangeable hydrogens. The general

subject of hydrogen exchange in proteins has been reviewed by Hvidt and Nielsen⁽⁷⁶⁾ and the equilibrium isotope effect for exchange determined for ribonuclease, human serum albumin and many other proteins, insulin and synthetic polypeptides, small peptides, and nucleic acids (see Reference 5, footnote 3, for many specific references). It was found in these cases that the average fractionation factor (that is, the inverse equilibrium isotope effect for converting macromolecular exchangeable protium to deuterium from the solvent) was either unity (no discrimination) or small, ~ 1.14 (deuterium favored). Of course, since what is required in proton inventory work are the reactant-state fractionation factors, ϕ_i^R , for the specific sites *which change on conversion of reactants to the transition state*, and since proteins and other biomacromolecules have so many exchangeable sites, such average values are of much less use than for substrates with very few and often identical hydrogenic sites. There is at present no straightforward technique that may be used to determine fractionation factors for individual specific sites in macromolecules. Protons in the active site of an enzyme are in principle more accessible than others, and NMR techniques such as those described by Robillard and Shulman⁽⁸⁰⁾ for studying the active-site exchanging protons of α -chymotrypsin may find use for this purpose.

What is generally done at present is to assume that all protein ϕ_i^R 's are equal to unity or do not change on activation. Since most N—H and O—H sites are known to have fractionation factors close to 1.0 (see Table III) and since it is further known that fractionation factors for protein side-chain exchangeable sites are 1.0 except for sulfhydryl (0.40–0.46), the assumption should be justified. *Small* departures from this assumption will affect the results quantitatively but not qualitatively (i.e., conclusions regarding the number of protons undergoing change in the transition state should remain unchanged).

6.5. Precision Required in Rate Measurements

In a proton inventory study rates are measured in pure protiated solvent, in pure deuteriated solvent, and in solvents of mixed isotopic composition. The rate constants are then plotted against atom fraction deuterium and the resulting curve examined for linearity or curvature (see Fig. 5 and the discussion in Section 7). The minimum number of points (solvent compositions) needed to define the curve will be three, most conveniently those for the pure solvents ($n = 0$, $n = 1$) and for an intermediate case, $n = 0.5$, for example. For confidence, depending on the difficulty involved and the precision of the data, more points for different values of n are desirable. Most studies in the literature⁽²⁻¹⁴⁾ report the use of at least three and more often six to nine different solvent compositions, although Alberly⁽²³⁾ has argued for the use of only the one intermediate point where $n = 0.5$. It is, of course, essential that the precision or reproducibility of the experimental rate constant at a given n be accurately known (by performing replicate experiments).

Table IV
Approximate Precision in Rate Constants Required for Proton Inventory Work^a

Solvent isotope effect, k_0/k_1	Number of protons	Gross-Butler equation, k_n/k_0	$k_{0.5}/k_0$	Precision required ^b
1.5	1	$1 - n + n/1.5$	0.833	1 ^o _o
	2	$[1 - n + n/(1.5)^{1/2}]^2$	0.825	0.4 ^o _o
	3	$[1 - n + n/(1.5)^{1/3}]^3$	0.822	
2.0	1	$1 - n + n/2.0$	0.750	2.8 ^o _o
	2	$[1 - n + n/(2.0)^{1/2}]^2$	0.729	1.1 ^o _o
	3	$[1 - n + n/(2.0)^{1/3}]^3$	0.721	
4.0	1	$1 - n + n/4.0$	0.625	10.4 ^o _o
	2	$[1 - n + n/(4.0)^{1/2}]^2$	0.563	4.0 ^o _o
	3	$[1 - n + n/(4.0)^{1/3}]^3$	0.541	
10	1	$1 - n + n/10$	0.55	23.7 ^o _o
	2	$[1 - n + n/(10)^{1/2}]^2$	0.433	10.1 ^o _o
	3	$[1 - n + n/(10)^{1/3}]^3$	0.393	
25	1	$1 - n + n/25$	0.52	36 ^o _o
	2	$(1 - n + n/5)^2$	0.36	18 ^o _o
	3	$[1 - n + n/(25)^{1/3}]^3$	0.302	
60	1	$1 - n + n/60$	0.508	46 ^o _o
	2	$[1 - n + n/(60)^{1/2}]^2$	0.319	25 ^o _o
	3	$[1 - n + n/(60)^{1/3}]^3$	0.248	

^a The numerical percentages calculated assume no reactant-state terms in the Gross-Butler equation and no uncertainty in k_0 and k_1 and are, therefore, only approximate.

^b The first number in this column for a given isotope effect is the precision required to distinguish a two-proton from a one-proton mechanism; the second number is that precision required to distinguish a three-proton from a two-proton mechanism.

To distinguish a linear (one proton in the transition state) plot from a curved (two-proton) plot from an even more curved (three-proton) plot, etc., the required precision in experimental data must be possible. In Table IV is listed the precision necessary in the rate constant at $n = 0.5$ to distinguish one-proton, two-proton, and three-proton mechanisms for different overall solvent isotope effects. Two conclusions are clear from examining the table. (1) The precision required goes down as the isotope effect, k_0/k_1 , goes up. (2) The precision required goes up as one tries to distinguish one- and two-, two- and three-, three- and four-proton, etc., mechanisms. Greater precision will be required for values of $0 < n < 0.5$ and $0.5 < n < 1.0$. The exact numerical percentage values in Table IV were calculated assuming no reactant-state terms in the Gross-Butler equation and no uncertainty in k_0 and k_1 and will therefore not apply to all cases with the same isotope effects. It is obvious that extremely precise data are necessary to draw mechanistic conclusions from a proton inventory for reactions with small solvent isotope effects ($< \sim 2$). Fortunately most enzymic solvent isotope effects encountered lie between 2 and 5, and some very interesting ones are very, very large.

7. ANALYSIS AND INTERPRETATION OF RESULTS

After carrying out a proton inventory study, the data that are obtained and which must be interpreted are a series of rate constants as a function of n , $k_n(n)$. From these data it will be possible to determine the form of the Gross–Butler equation

$$k = k_0 \frac{\text{TSC}}{\text{RSC}} = k_0 \frac{(1 - n + n\phi_1^T)(1 - n + n\phi_2^T) \cdots (1 - n + n\phi_v^T)}{(1 - n + n\phi_1^R)(1 - n + n\phi_2^R) \cdots (1 - n + n\phi_v^R)} \quad (68)$$

for the particular reaction under investigation. It will be recalled that there is a $1 - n + n\phi$ term in the numerator and in the denominator of Eq. (68) for every exchangeable proton in the system. Since most of these protons are not changing state on activation, their reactant- and transition-state terms will cancel. Terms for which $\phi = 1$ will likewise disappear. What remains, then, is an equation from which the number of $1 - n + n\phi$ terms and the corresponding ϕ values can be discovered. For the frequently encountered cases for which $\text{RSC} = 1$ the number of terms in the numerator gives the number of protons involved in the transition state, while the precise ϕ^T values provide information about the nature of these protons. For example,

$$k_n = k_0(1 - n + 0.3n)(1 - n + 0.7n) \quad (69)$$

is for a two-proton reaction for which $\phi_1^T = 0.3$, $\phi_2^T = 0.7$, and $\phi_1^R = \phi_2^R = 1$. In this section we shall attempt to describe just how one can obtain other such equations from $k_n(n)$ data and then how they may be interpreted in terms of transition-state structures.

7.1. Preliminary Examination of Data: Shape of k_n vs. n Plots

The data to be examined consist of a series of rate constants for different mixtures of isotopic solvents. The overall solvent isotope effect k_H/k_D (or k_0/k_1) can be calculated immediately. It can be noted whether it is normal or inverse, and preliminary mechanistic deductions can be made as suggested in Section 4. Following this, all the rate constants should be plotted (ordinate) against their corresponding values of n (abscissa) and the shape of the resulting function examined. The appearance of this plot can immediately provide a clue as to the general form of the Gross–Butler equation for the reaction. It can suggest, for example, whether there is one or more than one TSC term in the numerator and whether RSC terms are present or absent in the denominator.

Let us consider some of the possibilities sketched in Fig. 5 for a hypothetical series of reactions (1–6), each of which has a solvent isotope effect, k_0/k_1 , of 2.5. The isotope effect is normal ($k_0 > k_1$), $\text{TSC} < \text{RSC}$, so all plots go down toward the right. One, however, appears linear, while others are bent upward or downward.

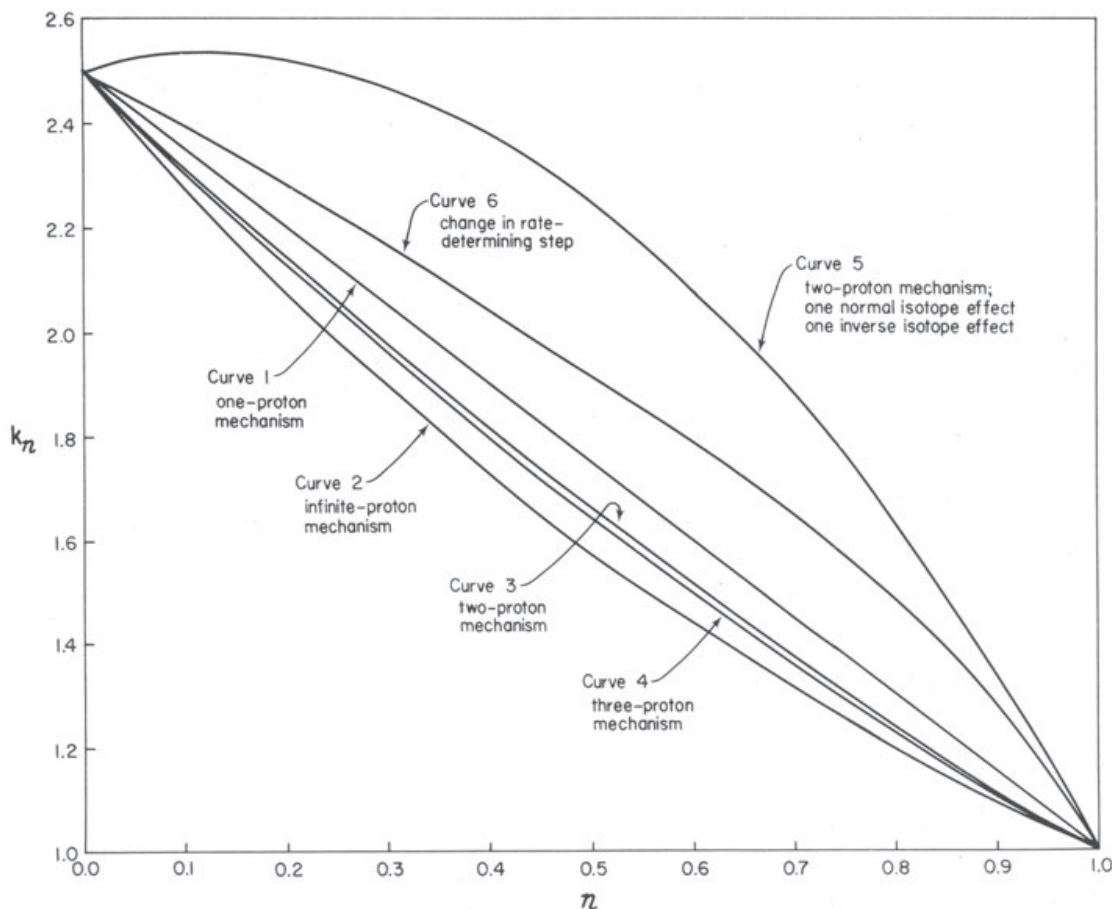


Fig. 5. General shape of k_n (rate constant) vs. n (atom fraction deuterium) plots for some hypothetical reactions possessing the same kinetic solvent isotope effect but proceeding by different mechanisms. Curve 1: a one-proton transition state, normal isotope effect; curve 2: an infinite number of transition-state protons, each contributing a very small normal isotope effect; curve 3: a two-proton transition state, two identical normal isotope effects; curve 4: a three-proton transition state, three identical normal isotope effects; curve 5: a two-proton transition state, one normal and one inverse isotope effect; curve 6: two one-proton transition states in sequence, normal isotope effects, rate-determining step changes with n .

7.1.1. Linear Plot, Curve 1

This is by far the easiest situation for interpretation. There is only one simple or practical explanation for such a curve, namely, that the solvent isotope effect arises from a single hydrogenic site in the transition state. In other words, it means that one proton only is undergoing change in the rate-determining step of the reaction. It is reasonable that such a one-proton mechanism should give a linear plot; the rate constant k_n will be the mole-fraction-weighted average of k_0 (for pure H_2O) and k_1 (for pure D_2O) as in

$$k_n = k_0(1 - n) + k_1(n) = k_0 \left(1 - n + n \frac{k_1}{k_0} \right) = k_0 \left(1 - n + n \frac{k_D}{k_H} \right) \quad (70)$$

This is equivalent to the linear form of the Gross–Butler equation:

$$k_n = k_0(1 - n + n\phi_1^T) \quad (71)$$

The reactant-state fractionation factor ϕ_1^R for the hydrogenic site in question is equal to 1.0, and ϕ_1^T is given by the inverse isotope effect for the reaction. A rapid check for linearity or the absence thereof can be made early in a proton inventory study. Since the degree of curvature of a $k_n(n)$ plot will be greatest where $n = 0.5$, the general shape of the curve becomes most apparent after only three rate constants have been determined, those for $n = 0, 0.5$, and 1. Thus a fair amount of mechanistic information is available once rates have been measured in the pure solvents and a 50:50 mixture of the two. The observed value of $k_{0.5}$ should coincide with the arithmetic mean of k_0 and k_1 . This is easily shown by evaluating Eq. (70) at $n = 0.5$:

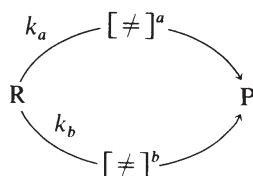
$$k_n = k_0 \left(1 - n + n \frac{k_1}{k_0} \right) \quad (72)$$

$$k_{0.5} = k_0 \left(0.5 + 0.5 \frac{k_1}{k_0} \right) = \frac{1}{2} (k_0 + k_1) \quad (73)$$

If the experimental $k_{0.5} = \frac{1}{2}(k_0 + k_1)$ (or if all the experimental points fall on a line), a linear dependence of k_n on n is indicated.

It is worthwhile to be aware that at least two additional, more complicated kinds of interpretations of linear $k_n(n)$ dependences are possible.

1. If there are two or more parallel reaction routes, at least one of which proceeds via a one-proton transition state and none of which proceeds via a ($n > 1$)-proton transition state, a linear $k_n(n)$ relationship will apply. Consider, for example, a system with two parallel pathways going through two one-proton transition states:



Consider, too, the following equations which describe this system,

$$k_n = k_n^a + k_n^b \quad (74)$$

$$k_n = k_0^a(1 - n + n\phi_a) + k_0^b(1 - n + n\phi_b) \quad (75)$$

$$\frac{k_n}{k_0} = \frac{k_0^a}{k_0}(1 - n + n\phi_a) + \frac{k_0^b}{k_0}(1 - n + n\phi_b) \quad (76)$$

$$= f_0^a(1 - n + n\phi_a) + f_0^b(1 - n + n\phi_b) \quad (77)$$

$$= (f_0^a + f_0^b)(1 - n) + n(f_0^a\phi_a + f_0^b\phi_b) \quad (78)$$

$$= 1 - n + n\Phi \quad (79)$$

where k_0^a and k_0^b are the rate constants for reactions a and b in pure H_2O , respectively; where f_0^a and f_0^b are the fractions of the total reaction that proceed via $[\neq]^a$ and $[\neq]^b$, respectively; where other terms have their usual meanings; and where Φ , the inverse observed solvent isotope effect, is the average of the two “actual” ϕ ’s for the two transition-state protons, weighted according to the fraction of reaction proceeding through those transition states:

$$\Phi = (f_0^a\phi_a + f_0^b\phi_b) \quad (80)$$

Equation (79) is obviously linear. Φ is the fractionation factor for the proton in the “average” or *virtual transition state* for the reaction.⁽⁸¹⁾ Since a virtual transition state for a multipathway process is indistinguishable from a single transition state for a single pathway process, it is best to interpret the data in the simplest way possible.

2. The possibility of linear dependences resulting from a fortuitous cancellation of terms in the Gross–Butler equation was recognized by Kresge.⁽⁸²⁾ If there are $1 - n + n\phi$ terms in the numerator and the denominator of the actual Gross–Butler equation describing a system which by coincidence cancel each other and therefore disappear, a linear dependence may result. One can imagine, for example, a two-proton reaction correctly described by

$$k_n = k_0 \frac{(1 - n + n_1^T)(1 - n + n\phi_2^T)}{(1 - n + n_1^R)(1 - n + n\phi_2^R)} \quad (81)$$

If $\phi_2^R = 1.0$ and if $\phi_1^R = \phi_2^T \neq 1.0$, the expression will reduce to Eq. (71). One can likewise imagine a large number of very small effects in the reactant state combining to offset and cancel one transition-state term (five ϕ^R ’s of 0.95 will multiply to cancel one ϕ^T of 0.77, for example). Such coincidental exact cancellation of terms is conceivable but not highly probable. Since by these arguments there is an infinite number of alternative interpretations of this plot, or indeed one of any other shape, it again seems best in the absence of contrary information to invoke the simplest interpretation.^(6,10)

There exists a further possibility for a one-proton reaction for which $\text{TSC} < \text{RSC}$. If a reactant-state proton of $\phi^R > 1$ undergoes change on activation to give a transition-state proton of $\phi^T = 1$ and all other ϕ^R ’s and ϕ^T ’s are unity, then

$$k_n^{-1} = k_0^{-1}(1 - n + n\phi_1^R) \quad (82)$$

and a plot of k_n^{-1} vs. n will be linear, going down toward the right (normal isotope effect).

7.1.2. “Bulging Down” Plots: Curves 2–4

Plots of this shape almost always indicate the existence of a multi-proton transition state. The Gross–Butler equation is usually of the form of

$$k_n = k_0 \prod_i^v (1 - n + n\phi_i^T) \quad (83)$$

with all $\phi_i^T < 1$ and all RSC terms equal to 1. The $k_n(n)$ plots are polynomials in n of order v :

$$k = k_0 + c_1 n + c_2 n^2 + \cdots + c_v n^v \quad (84)$$

It is also possible for multiproton transition states to have TSC = 1 (all ϕ_i^T 's equal to unity) and RSC $\neq$ 1. For such cases

$$k_n = k_0 \left/ \prod_j^v (1 - n + n\phi_j^R) \right. \quad (85)$$

and it is $1/k_n$ which is a polynomial in n :

$$k_0^{-1} = k_0^{-1} \prod_j^v (1 - n + n\phi_j^R) \quad (86)$$

Thus k_n^{-1} vs. n plots should routinely be constructed and analyzed as described below, especially if it is suspected that TSC = 1 or known that RSC $\neq$ 1.

The order v of the polynomials of Eqs. (84) and (86) gives the number of hydrogenic sites involved on activation and may be any integral number between 2 and ∞ . Before engaging in polynomial regression analysis (Section 7.2) to determine v , a little preliminary data plotting can provide clues as to its magnitude.

A downward-bowing curve could indicate the other extreme of the linear curve, namely, a reaction involving an infinite number of protons all contributing very small, approximately equal normal isotope effects. The ϕ_i^T values will be less than but very nearly equal to 1. If this is in fact the case, (1) a plot of $\ln k_n$ vs. n will be linear and (2) the $k_{0.5}$ value will be given by $\sqrt{k_0 k_1}$, that is, the geometric mean of k_0 and k_1 . The following arguments justify these last two statements. If there is a very large number of protons, m , all contributing about equally to an observed solvent isotope effect, k_0/k_1 , we have

$$k_1/k_0 = \phi^m \quad (87)$$

and

$$k_n = k_0 (1 - n + n\phi)^m \quad (88)$$

Since the value of ϕ will be less than but very nearly equal to 1, $\phi = 1 - \chi$, where χ is very small. Equation (88) now becomes

$$k_n = k_0 [1 - n + n(1 - \chi)]^m = k_0 (1 - n\chi)^m \quad (89)$$

and

$$\ln(k_n/k_0) = m \ln(1 - n\chi) \quad (90)$$

Since $n \leq 1$ and χ is small, $n\chi$ is a small number and $\ln(1 - n\chi) \simeq -n\chi$.

Equation (90) can therefore be written

$$\ln(k_n/k_0) \simeq -mn\chi \quad (91)$$

or

$$\ln k_n \simeq (-m\chi)n + \ln k_0 \quad (92)$$

Thus, as m becomes very large, $\ln k_n(n)$ becomes linear. The rate constant $k_{0.5}$ is evaluated as follows. From Eq. (87) and the relationship $\phi = 1 - \chi$ we can write

$$\ln(k_1/k_0) = m \ln \phi = m \ln(1 - \chi) = m(-\chi) \quad (93)$$

Combining Eq. (93) with Eq. (91), we have

$$\ln(k_n/k_0) \simeq (-m\chi)n = [\ln(k_1/k_0)]n \quad (94)$$

Evaluating Eq. (94) for $n = 0.5$ gives

$$\ln(k_{0.5}/k_0) = \frac{1}{2} \ln(k_1/k_0) \quad (95)$$

$$k_{0.5}/k_0 = (k_1/k_0)^{1/2} \quad (96)$$

$$k_{0.5} = k_0 \sqrt{k_1/k_0} = \sqrt{k_1 k_0} \quad (97)$$

demonstrating that the value of $k_{0.5}$ is the geometric mean of the k_1 and k_0 values if the solvent isotope effect arises from the small equivalent contributions of a very large number of protons in the transition state. Curve 2 shows the appearance of a $k_n(n)$ plot for just such a situation.

In summary, then, one can easily differentiate between the two mechanistic extremes (one active proton vs. a very large number of active protons) by means of two simple tests:

$$1 \text{ H: } \quad k_n \text{ vs. } n \text{ linear, } k_{0.5} = \frac{k_1 + k_0}{2}$$

$$\infty \text{ H: } \quad \ln k_n \text{ vs. } n \text{ linear, } k_{0.5} = \sqrt{k_1 k_0}$$

The degree of departure from these two extremes can be helpful in qualitatively estimating the number of TSC terms.

Data generating plots which bulge downward but lie between the line (curve 1, 1 H) and the extreme (curve 2, ∞ H) are consistent with a mechanism in which two or three or some reasonably small number of protons have undergone change on activation. [At this point one can carry out a rapid check for the possibility of a *two-proton* mechanism in which each proton contributes the same isotope effect, i.e., the case where

$$k_n = k_0(1 - n + n\phi_1^T)(1 - n + n\phi_2^T) \quad (98)$$

and $\phi_1^T = \phi_2^T$, and therefore

$$k_n = k_0(1 - n + n\phi_1^T)^2 \quad (99)$$

A plot of $(k_n/k_0)^{1/2}$ vs. n will be linear for such a reaction, and ϕ^T will be given

by the square root of the inverse solvent isotope effect, $(k_1/k_0)^{1/2}$. If this procedure does not give a linear plot, the data must be subjected to polynomial regression analysis as described in Section 7.2 to obtain an accurate proton count and individual ϕ_i^T values.] Curve 3 in Fig. 5 has been calculated for a two-proton mechanism assuming the overall solvent isotope effect to be 2.5 and both ϕ^T 's equal to $(1/2.5)^{1/2} = 0.63$, and curve 4 for a three-proton reaction, ϕ^T 's = $(1/2.5)^{1/3} = 0.74$. As may be seen in Fig. 5, the curvature will be more pronounced as the number of protons increases until the limit is reached. It is also clear from Fig. 5 that it is very difficult to distinguish a two-proton mechanism from a three-proton mechanism, pointing out the need for precision in kinetic measurements (see also Table IV). The downward curvature implies that the multiple-site effects are all in the same direction (all ϕ^T 's < 1, a normal isotope effect contribution by each "active" H). The curve bows down since even at small n more than one site can be deuterated and the rate will drop off faster than for a one-proton case. The RSC for reactions generating such plots may (initially at least) be assumed to be unity; that is, hydrogen equally stable in H or D solvent, ϕ^R 's = 1.0.

There are other less common but conceivable types of mechanisms which could account for bulging down $k_n(n)$ plots. One of these will be mentioned here. A mechanism involving two or more parallel competing pathways will generate $k_n(n)$ data resulting in "bulged down" plots if at least one of the transition states involves at least two protons. The polynomial [Eq. (84)] will be quadratic if none of the competing transition states has more than two protons, cubic if three is the highest number of protons in any one transition state, etc. In such cases, the "apparent" ϕ values obtained by fitting the data to the appropriate (quadratic, cubic, etc.) form of the Gross-Butler equation, as in

$$k_n = k_0(1 - n + n''\phi_a^T)(1 - n + n''\phi_b^T) \quad (100)$$

may turn out to be complex numbers (real and imaginary). It is also possible to get complex ϕ 's from poor data or experimental error. If the " ϕ " values are not complex, the situation cannot be distinguished from a single-pathway mechanism.

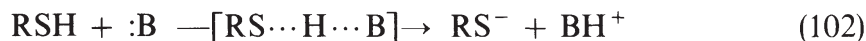
7.1.3. "Bulging Up" Plots, Curves 5-6

Bowing upward when the net SIE is normal is caused when (1) a large normal effect is partially offset by a smaller inverse effect or (2) a change in rate-determining step from one sequential step to another occurs as n is altered. These two different interpretations are not visually distinguishable.

A situation described by the first category is one in which one proton has become "looser" and a second "tighter" on activation. The one will give rise to a normal isotope effect ($\phi_1^T = 0.2$, say) with linear falloff of rate with increasing n , and the other to an inverse isotope effect ($\phi_2^T = 2$, for example):

$$k_n = k_0(1 - n + 0.2n)(1 - n + 2n) \quad (101)$$

The second effect will partially offset the first, thus decreasing the falloff in rate with increasing n and generating an upward bulge in the $k_n(n)$ plot. Since the second effect is smaller than the first, the overall SIE will remain normal and the plot will still go down to the right. Curve 5 in Fig. 5 has been calculated for this example. A second situation that may be considered in this same category is one arising from opposing contributions from transition-state sites and reactant-state sites. An example of such a reaction is



This is a one-proton reaction with $\phi_{\text{SH}}^{\text{R}} \simeq 0.5$ and, say, $\phi^{\text{T}} = 0.2$. One then has

$$k_n = k_0 \frac{1 - n + 0.2n}{1 - n + 0.5n} \quad (103)$$

and the net solvent isotope effect is 2.5. If ϕ^{T} had not been partially offset by $\phi^{\text{R}} = 0.5$, the net solvent isotope effect would have been larger than observed, $k_0/k_1 = 1/0.2 = 5$; if ϕ^{R} had not been cancelled by ϕ^{T} , i.e., if $\phi^{\text{T}} = 1$, the net solvent isotope effect would have been inverse, $k_0/k_1 = 0.5/1 = 0.5$. Thus, because of the TSC, increasing D in the solvent reduces the rate, but the opposing effect of the RSC term causes it to be reduced more slowly than otherwise—hence upward bowing. (The example just cited serves to illustrate another point: ϕ_j^{R} values of less than unity will cause an SIE to appear smaller than otherwise.)

Let us consider a hypothetical example from the second category of “bulging up” plots: a change in rate-determining step from one sequential reaction step to another as n is changed. Consider a reaction with an overall SIE of 2.5 and two steps in sequence: step (a) and step (b). Step (a) is 10% rate determining in H_2O with an isotope effect $k_{\text{H}}/k_{\text{D}} = 10$; step (b) is 90% rate determining in H_2O , and the isotope effect is 1.67. The system is represented by



$$k_n = k_n^{\text{a}} k_n^{\text{b}} / (k_n^{\text{a}} + k_n^{\text{b}}) \quad (105)$$

$$1/k_n = 1/k_n^{\text{a}} + 1/k_n^{\text{b}} \quad (106)$$

$$\frac{1}{k_n} = \frac{1}{k_0^{\text{a}}(1 - n + n/10)} + \frac{1}{k_0^{\text{b}}(1 - n + n/1.67)} \quad (107)$$

$$\frac{k_0}{k_n} = \frac{k_0}{k_0^{\text{a}}(1 - n + 0.1n)} + \frac{k_0}{k_0^{\text{b}}(1 - n + 0.6n)} \quad (108)$$

Since both k_0^{a} and k_0^{b} are larger than the overall rate in H_2O (k_0), k_0/k_0^{a} and k_0/k_0^{b} represent the fraction of the total that that step is rate determining. Thus

$$\frac{k_0}{k_n} = \frac{f_0^{\text{a}}}{1 - n + 0.1n} + \frac{f_0^{\text{b}}}{1 - n + 0.6n} \quad (109)$$

Evaluation of k_n at various values of n will result in a bulged up plot, as shown by curve 6 in Fig. 5.

Analogous information about transition-state structure is conveyed by the shapes of $k_n(n)$ plots for reactions with net *inverse* solvent isotope effects (where $\text{TSC} > \text{RSC}$). Again k_n^{-1} vs. n should be plotted for occasions when TSC might be unity and $\text{RSC} < 1$.

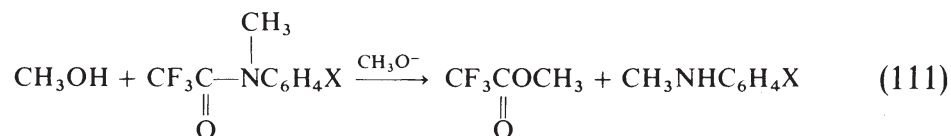
1. A *linear* plot indicates a “one-proton” transition state.
2. Plots *bulging upward* indicate transition states with *two or more protons* each contributing an inverse effect. A linear $\ln k_n$ vs. n plot means small *inverse* contributions from a very large number of protons; a linear $\sqrt{k_n/k_0}$ vs. n plot is evidence for a two-proton mechanism, $\phi_1^T = \phi_2^T$. Plots of this shape can also point to a change in rate-determining step.
3. Plots *bulging downward* indicate a transition state where larger inverse isotope effects are being partially offset by smaller normal isotope effects. As before, the partial cancellation can come from RSC terms, TSC terms, or both. Cautions and the alternative, less common explanations mentioned for normal isotope effects will also apply here.

7.2. Determination of the Number of Active Protons: Polynomial Regression⁽⁶²⁾

For any nonlinear $k_n(n)$ plot the polynomial regression procedure is recommended. For this procedure to be useful either RSC or TSC must be known. In a large number of cases one has good reason to suppose that the reactant state does not contribute to the isotope effect, that is, that all $\phi_j^R = 1$. One then has a situation described by Eq. (83). The common examples of such cases are (1) hydrolysis reactions involving substrates with no exchangeable hydrogens or hydrogens with fractionation factors of 1 and (2) reactions involving enzymes whose exchangeable protons (except those of sulfhydryl groups) are expected to have reactant fractionation factors and hence RSC values of unity (see the discussion of this point in Section 6.4.2). Where RSC is known or suspected to be other than unity, it must be evaluated from knowledge of the individual ϕ_j^R values. This is generally not difficult: The reactant-state structure is known and the appropriate fractionation factors are available, e.g., from Table III. If not, they can be independently determined (see Section 6.3). The appropriate equation then is

$$k_n(\text{RSC}) = k_0 \prod_i^v (1 - n + n\phi_i^T) \quad (110)$$

Care should be taken to consider various perhaps unexpected $\phi^R \neq 1$ contributions to RSC particularly if the data are not easily fit assuming unit RSC. For example, in the methoxide-catalyzed methanolysis reaction



the reactant state consists of the triply solvated methoxide ion $\text{CH}_3\text{O}^-(\text{CH}_3\text{OH})_3$ with $\phi_{\text{CH}_3\text{OH}} = 0.74$, giving an RSC term of $(1 - n + 0.74n)^3$.⁽⁶¹⁾

Occasionally, as mentioned in Section 7.1, another situation is encountered: The isotope effect arises from reactant protons of $\phi_j^R \neq 1$ becoming transition-state protons of $\phi_j^T = 1$. In this case TSC is unity, and one has a system described by Eq. (85) or (86). An example of such a reaction is



where $\phi^R \simeq 0.45$ and $\phi^T = 1.0$.

The three equations [Eqs. (83), (110), and (86)] become, respectively,

$$k_n = k_0 + c_1n + c_2n^2 + c_3n^3 + \cdots + c_vn^v \quad (113)$$

$$k_n(\text{RSC}) = k_0 + c_1n + c_2n^2 + c_3n^3 + \cdots + c_vn^v \quad (114)$$

$$k_n^{-1} = k_0^{-1} + c_1n + c_2n^2 + c_3n^3 + \cdots + c_vn^v \quad (115)$$

in which k_n , $k_n(\text{RSC})$, or k_n^{-1} is a polynomial in n , the order of which (v) specifies the number of protons whose state has been altered on going from ground state to transition state. The coefficients of this polynomial in principle allow for the calculation of ϕ values, i.e., the isotope effect for each such proton. In this way the experimental $k_n(n)$ data, along with some measure or hypothesis concerning RSC(n), can give the proton inventory.

A polynomial regression computer program (available at most computation centers; one such is BMDO5R of the UCLA Health Sciences Computing Facility⁽⁶⁷⁾) is now used to fit the appropriate left-hand side of Eq. (113), (114), or (115) to polynomials in n of ever-increasing order. Each succeeding term in the polynomial is tested for significance, for example, by the F test.⁽⁸³⁾ The exponent of n in the last statistically significant term gives the number of active protons consistent with the experimental data. The results, of course, depend not only on the system studied but also on the number and precision of the measurements. One should recognize, therefore, that hidden in the "noise" of the data may be additional active hydrogens. As always, as previously mentioned and as discussed by Kresge,⁽⁸²⁾ the (slight but real) possibility exists that small RSC contributions are exactly cancelling and thus concealing an additional TSC term and proton. Such a possibility can best be discarded if similar results are obtained for the same reaction with somewhat different substrates or conditions.^(8a)

7.3. Extraction of Fractionation Factor Values

The isotope-effect contribution, or ϕ values, for each active proton could be found from the coefficients c_i of the best-fit polynomial. However,

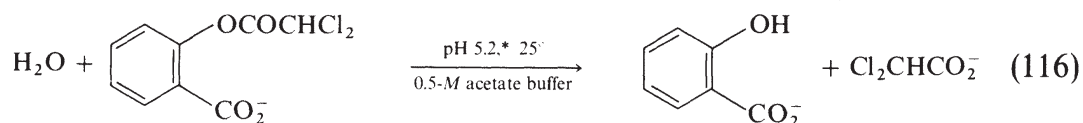
since the polynomial-fitting program pivots around the c_i and not the ϕ values, the procedure does not give desirable results. The preferred procedure is to construct a reasonable model of the transition state based on the results of the polynomial regression analysis. Reasonable ϕ^T values can be postulated for each active proton and the data fitted by any least-squares method directly to the appropriate equation [Eq. (113), (114), or (115)].

8. EXAMPLES FROM THE RECENT LITERATURE

In this section we shall give some examples, chosen for their variety and illustrative character, showing how proton inventory and solvent isotope effect data may be used to gain information about transition-state structures. The format will be to give (1) an equation or other description of the particular reaction; (2) the experimental rate data and/or the net KSIE; (3) a description or actual plot of the $k_n(n)$ function, together with conclusions drawn from its general appearance; (4) a structure for the proposed transition state; (5) the final form of the Gross–Butler equation with the fractionation factor values resulting from data fitting; and (6) comments and remarks about the chosen system as necessary. Further details may be obtained from the original references cited.

8.1. Example I⁽⁸⁴⁾

The reaction is the intramolecular carboxylate-catalyzed hydrolysis of *o*-dichloroacetylsalicylic acid anion:



From the rate data presented in Table V the KSIE is seen to be normal: $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = 2.17$; thus $\text{TSC} < \text{RSC}$. Small KSIE's of around 1.5–3 are generally considered to be inconsistent with a primary isotope-effect contribution; the proton is thus not “in flight” but more likely engaged in “solvation catalysis” and therefore not on the reaction coordinate.⁽⁴⁷⁾ Figure 6 is a plot of k_n vs. n and shows clearly that the experimental points fall on a line sloping down to the right, with the conclusion that a single proton is undergoing change in the transition state of the reaction. The dashed line calculated for a two-proton mechanism for the same KSIE of 2.17 is seen to be outside of experi-

* This and other pH values given in this section are for $n = 0$, pure H_2O ; it is understood that the equivalent pH was used for solvent mixtures (see Section 6.2).

Table V
First-Order Rate Constants for Hydrolysis
of 2×10^{-4} M *o*-Dichloroacetylsalicylate Anion
in Sodium Acetate–Acetic Acid Buffers in Mixtures
of H₂O and D₂O at 25[°](⁸⁴)

n	$10^5 k_n (\text{sec}^{-1})$
0.0000	7510 ± 129^a
0.0955	6994 ± 135
0.1984	6622 ± 61
0.2991	6173 ± 24
0.4012	5882 ± 38
0.5020	5438 ± 26
0.5981	4949 ± 29
0.6945	4699 ± 16
0.8009	4281 ± 54
0.8890	3886 ± 60
0.9964	3355 ± 26

^a Error limits are standard deviations. Rate constants were obtained by a least-squares fit of absorbance-time data.

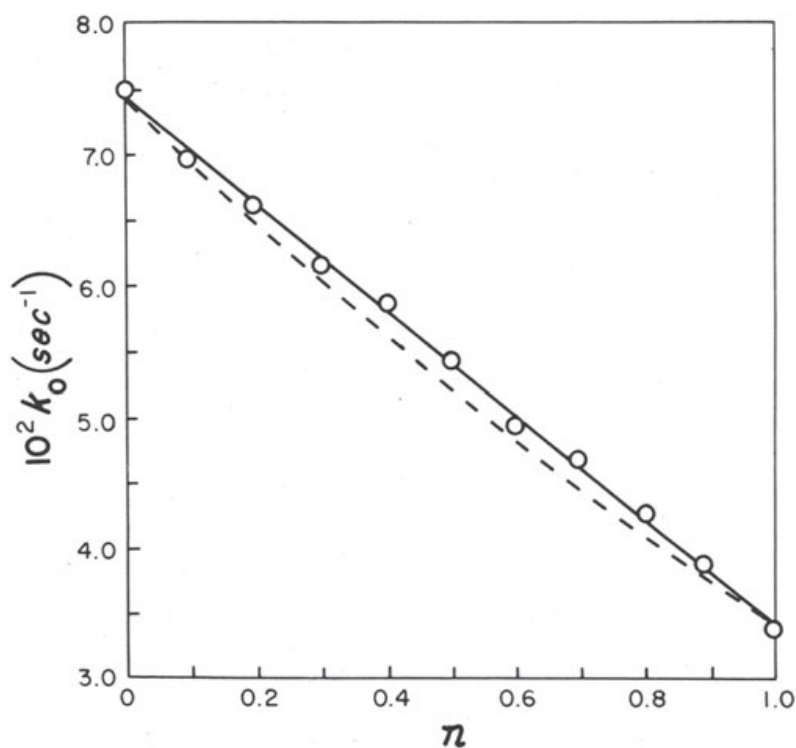


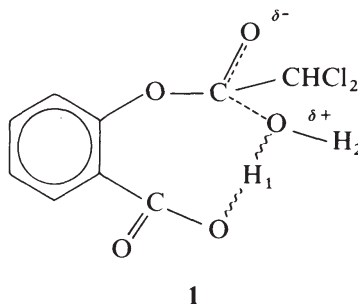
Fig. 6. Observed first-order rate constants vs. n for the hydrolysis of dichloroacetylsalicylate ion. The solid line is a plot of Eq. (117); the dashed line is a plot assuming two protons each contributing the same isotope effect. (Reproduced with permission from S. S. Minor and R. L. Schowen.⁽⁸⁴⁾)

mental error. When subjected to linear least-squares fitting the rate data give

$$10^5 k_n = (7432 \pm 33) [1 - n + (0.460 \pm 0.008) n] \quad (117)$$

which when plotted on Fig. 6 gives the solid line through the experimental points.

The proposed transition-state structure **1** has the bridging proton H_1 responsible for the isotope effect. H_2 , giving no isotope effect, is assumed to be undergoing a smooth transition from a water proton ($\phi_w^R = 1.0$) to a hydroxyl proton (also with $\phi = 1.0$).



The final form of the Gross-Butler equation which results from the experimental data [Eq. (117)] must therefore have been reduced from

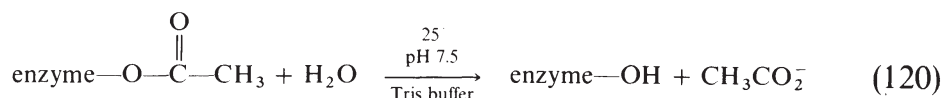
$$k_n = k_0 \frac{(1 - n + n\phi_1^T)(1 - n + n\phi_2^T)}{(1 - n + n\phi_w^R)^2} \quad (118)$$

where $\phi_w = 1.0$, $\phi_2^T = 1.0$, and $\phi_1^T = 0.46$, thus giving the linear relationship

$$k_n = k_0(1 - n + 0.46n) \quad (119)$$

8.2. Example II⁽⁶⁾

The reaction is the deacetylation of acetyl- α -chymotrypsin that occurs during the α -chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate:



The experimental rate data (Table VI) give a net normal KSIE, $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = 2.4$, TSC < RSC. A plot of zero-order rate constants (velocities) vs. n is linear (Fig. 7), suggesting a single-proton mechanism. Since the reactant fractionation factors are all expected to be unity and the $v_n(n)$ plot is linear, the system must be described by

$$k_n = k_0(1 - n + n\phi_1^T) \quad (121)$$

which, when fitted to the data, gives a value for ϕ_1^T of 0.42.

Table VI
Zero-Order Rate Constants for Deacetylation of
Acetyl- α -chymotrypsin at pH 7.5 and Equivalent
in Mixtures of H₂O and D₂O at 25^o(6)

n	$10^{11}v_n(M \text{ sec}^{-1})$
0.000	2649 ± 3
0.175	2425 ± 11
0.261	2239 ± 4
0.398	2126 ± 11
0.485	1878 ± 7
0.497	2064 ± 32
0.583	1791 ± 14
0.745	1479 ± 13
0.765	1459 ± 10
0.995	1102 ± 24

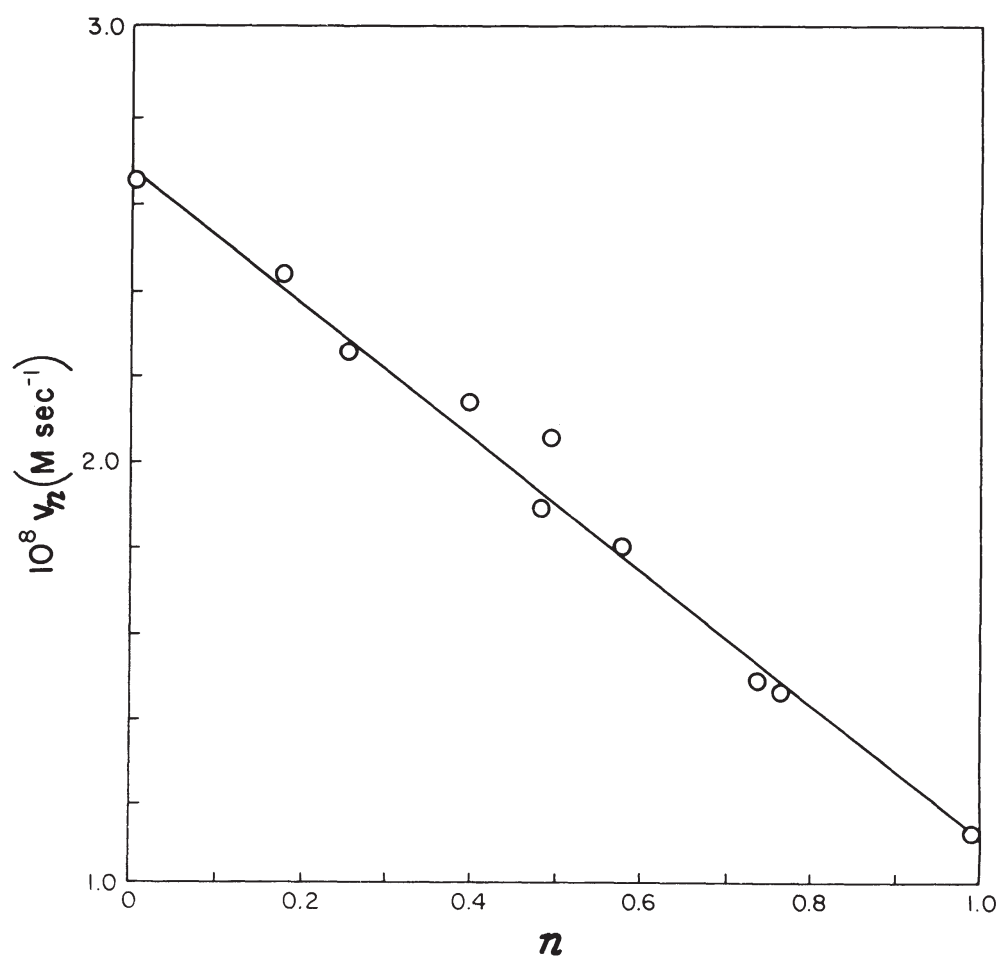
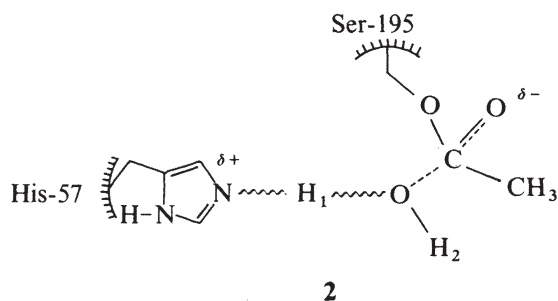
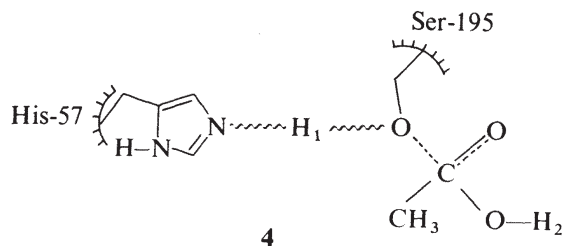
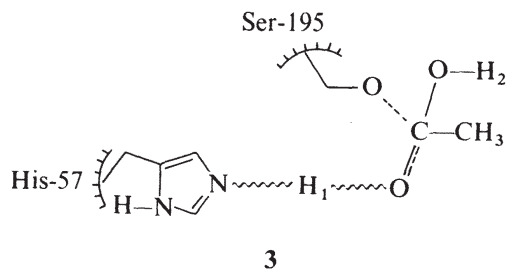


Fig. 7. Velocities of deacetylation of acetyl- α -chymotrypsin vs. the atom fraction of deuterium in the solvent. The data are from Table VI. (Reproduced with permission from E. Pollock *et al.*⁽⁶⁾)

By analogy with Example I and other base-catalyzed ester hydrolyses⁽⁸⁵⁾ where $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} \simeq 2-3$ and $\phi^T \simeq 0.45$, and taking into account what is known about the active site of this⁽⁸⁶⁾ and similar serine proteases, transition state **2**, representing the formation of the tetrahedral intermediate, is reasonable. Again solvation catalysis rather than proton-transfer catalysis by H_1 is as-



sumed to give rise to the isotope effect. Other transition-state structures, for example, **3** or **4**, representing decomposition of the tetrahedral intermediate, are not ruled out by the data, nor is a rate-determining one-proton conformation change on the part of the acyl enzyme.



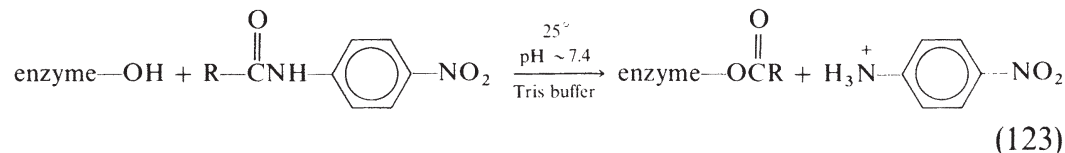
For acetyl enzyme and water going to transition state **2** (or **3** or **4**) the Gross-Butler equation would have the form

$$k_n = k_0 \frac{(1 - n + n\phi_1^T)(1 - n + n\phi_2^T)}{(1 - n + n\phi_w')^2} \quad (122)$$

assuming the fractionation factors for the exchangeable protons in the enzyme all to be unity. With $\phi_w = 1$ and $\phi_2^T = 1$, the final form is that of Eq. (121).

8.3. Example III

Three closely related systems will be considered here. The first reaction is the acylation step,



in the trypsin-catalyzed hydrolysis of *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginyl *p*-nitroanilide, an analog of a tetrapeptide studied by Quinn *et al.*^(10,11) Trypsin, like α -chymotrypsin, is a serine hydrolase with an active site consisting of serine, histidine, and aspartate residues. Both are believed to use a charge-relay mechanism⁽⁸⁶⁾ involving coupled transfer of two protons when acting on their physiological polypeptide substrates.



(R_1 , R_2 , and R_3 are the side-chain groups of the amino acids arginine, valine, and phenylalanine, respectively.)

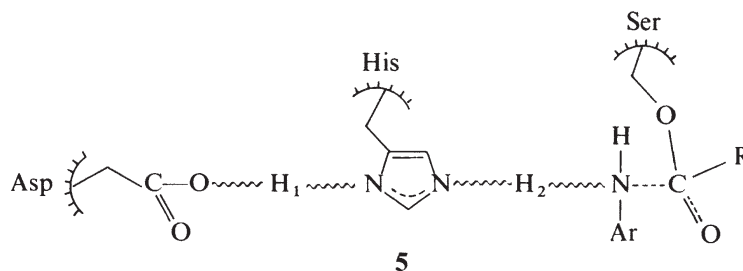
Under saturation conditions $V_{\text{H}_2\text{O}}/V_{\text{D}_2\text{O}} = 4$, and a nonlinear bowed-down k_n vs. n plot is obtained. When $k_n^{1/2}$ is plotted against n a straight line results, implying that the transition state involves two protons, each with an identical fractionation factor. From the slope of the line a value of $\phi_1^T = \phi_2^T = 0.5$ was obtained. Each proton is thus contributing an isotope effect, $k_{\text{H}}/k_{\text{D}}$, of 1/0.5 or 2, consistent with solvation catalysis by each. The Gross-Butler equation for this two-proton acylation should be

$$k_n = k_0 \frac{(1 - n + n\phi_1^T)(1 - n + n\phi_2^T)}{(1 - n + n\phi_w)^2} \quad (124)$$

which is reduced to

$$k_n = k_0(1 - n + 0.5n)^2 \quad (125)$$

The results of this experiment are consistent with a charge-relay mechanism as in transition-state structure **5**, involving coupled solvation catalysis by protons 1 and 2. It is of great interest to note that when R is smaller, i.e., less “physiological,” for both acylation and deacylation steps of serine hydrolase reactions, the two-proton process uncouples and the mechanism becomes that



of one-proton catalysis [see Example II and entries 5–7 and 9–11 in Table I, all of which give linear $k_n(n)$ plots and KSIE's of ~ 1.5 –3].

A recently published report by Hunkapiller *et al.*⁽⁷³⁾ for a similar substrate, acetyl-L-alanyl-L-prolyl-L-alanyl *p*-nitroanilide, and the serine proteases elastase and α -lytic protease at 25° and pH 8.75 gave entirely analogous results. They report normal net solvent isotope effects of $k_{\text{H}_2\text{O}}^{\text{cat}}/k_{\text{D}_2\text{O}}^{\text{cat}}$ of 2.8 (α -lytic protease) and 2.1 (elastase), with bulging down $k_n(n)$ plots, and linear $(k_n^{\text{cat}})^{1/2}$ vs. n plots giving values of $\phi_1^T = \phi_2^T = 0.60$ (α -lytic protease) and 0.68 (elastase). See Fig. 8 for the α -lytic protease data.

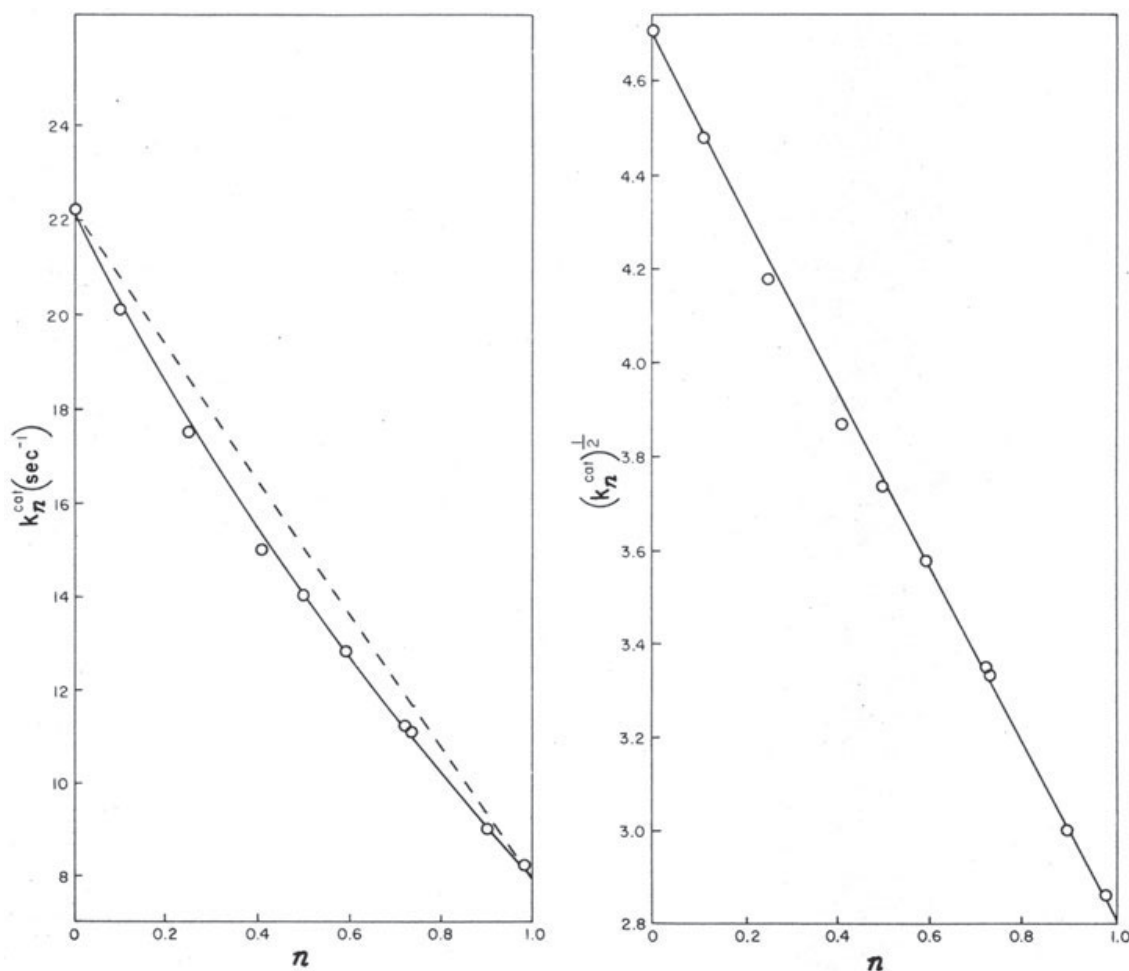
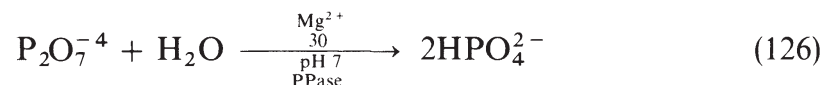


Fig. 8. Plots of k_n^{cat} and $(k_n^{\text{cat}})^{1/2}$ vs. n for the α -lytic protease-catalyzed hydrolysis of Ac-L-ala-L-pro-L-ala *p*-nitroanilide (acylation step). (Reproduced with permission from M. W. Hunkapiller.⁽⁷³⁾)

Their stopped flow kinetic data provide evidence that, for the substrate chosen, the tetrahedral intermediate for the acylation step accumulates, that its decomposition is the rate-limiting step, and that the transition-state structure is analogous to structure 5.

8.4. Example IV⁽¹⁴⁾

The hydrolysis of inorganic pyrophosphate catalyzed by magnesium ion and inorganic pyrophosphatase, represented most simply by



has been shown by Konsowitz and Cooperman to generate a most interesting example of a nonlinear bowed-up $k_n(n)$ plot (Fig. 9) and a net $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$ of 1.90. The appearance of Fig. 9 implies that the transition state contains more than one proton and (see Section 7.1) either that (1) a large normal isotope effect generated by one of the protons is being offset by a smaller inverse isotope effect or (2) one of the protons has a ϕ^R of < 1 . (The possibility of a change in rate-determining step with increasing n was not considered here.) In fact the data fit two two-proton equations about equally well:

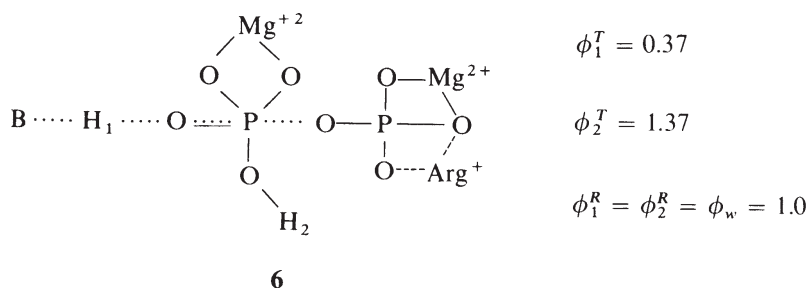
$$k_n = k_0(1 - n + 0.37n)(1 - n + 1.37n) \quad (127)$$

describes situation 1 and

$$k_n = k_0 \frac{1 - n + 0.4n}{1 - n + 0.9n} \quad (128)$$

describes situation 2.

Three transition-state structures were proposed consistent with either Eq. (127) or (128) and with present understanding about the enzyme- and phosphoryl-transfer reactions. It was assumed that the isotope effect arises solely from the two water protons. Structure 6 corresponds to Eq. (127). The reactant-state water protons each have the usual $\phi_w = 1.0$. One transition-state proton is transferring and one is attached to an oxygen atom which, because of its ability to delocalize electrons toward the phosphorus atom and to restrict rotation about the P—O bond, should have a ϕ value similar to that for gem-diols and hemiacetals (~ 1.25 ; see Table III). The value $\phi_2^T = 1.37$ for



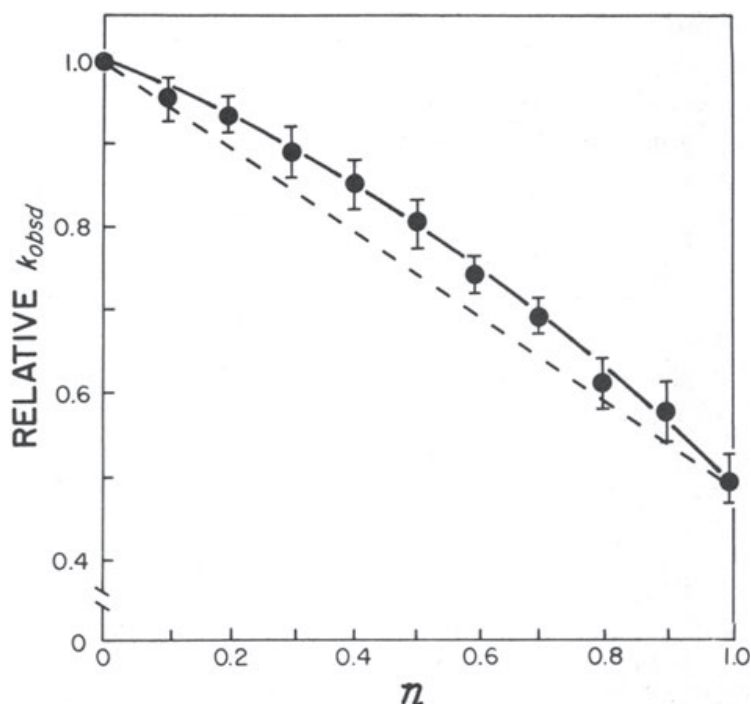
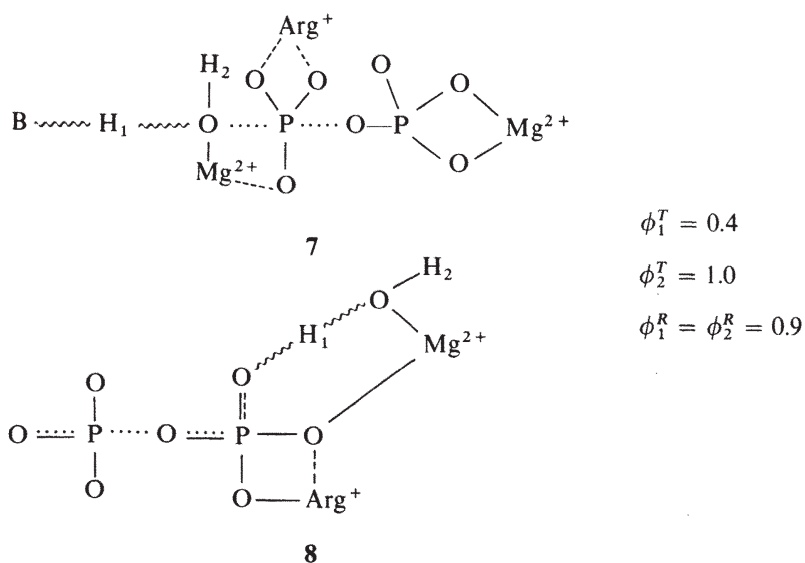
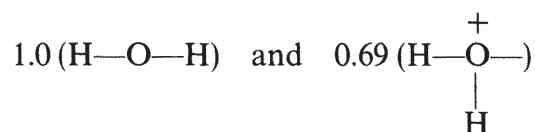


Fig. 9. Relative k_n vs. n for the hydrolysis of pyrophosphate by inorganic pyrophosphatase. (Reproduced with permission from L. M. Konsowitz and B. S. Cooperman.⁽¹⁴⁾)



a “tighter-than-bulk-solvent” H_2 is quite plausible. Structures **7** and **8** are each consistent with Eq. (128). The reactant water molecule is assumed to be coordinated to Mg^{2+} . The resulting partial positive charge on oxygen should give ϕ^R values for the water protons somewhere between



The value of 0.9 for ϕ_1^R and ϕ_2^R is reasonable. One of the water protons is seen in both transition states to remain fully attached to the oxygen with little or no charge, thus allowing a value of unity to be assigned to ϕ_2^T . The other proton is seen as a solvation-catalytic bridge, and the value of $\phi_1^T = 0.4$ ($k_H/k_D = 2.5$) that emerges is most satisfying.

8.5. Example $V^{(5)}$

This example is the isomerization of ribonuclease A, the conversion of the E_1H form of the protein (with $pK_a > 8$ in H_2O) to the conformationally isomeric E_2H form ($pK_a = 6.1$ in H_2O):



The solvent isotope effect is 4.7 ± 0.4 as measured by Wang *et al.*⁽⁵⁾ The magnitude of this normal effect suggests a primary isotope-effect contribution. The values of k (obtained from relaxation times measured by the temperature-jump method) when plotted against n give a bulging down curve (Fig. 10). Such a

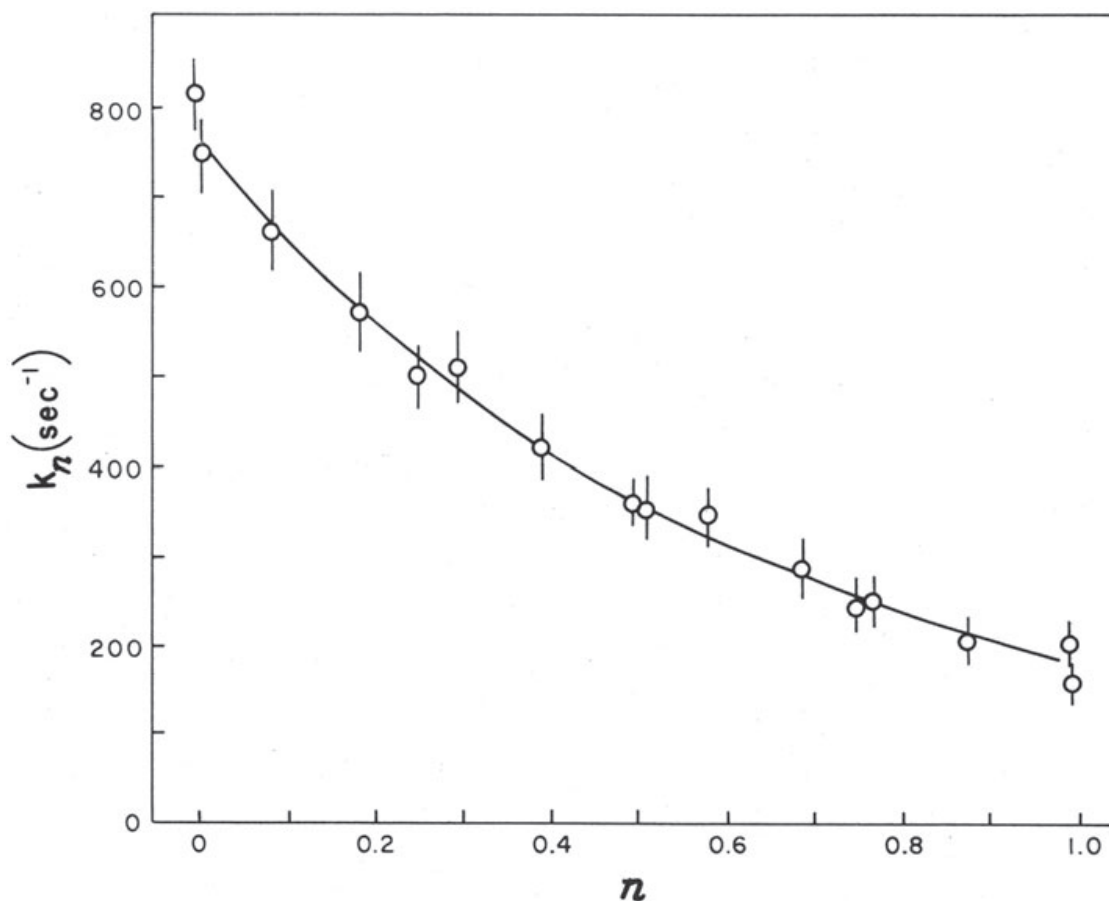
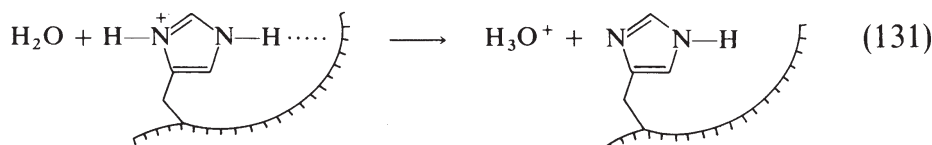


Fig. 10. Values of k_n (sec^{-1}) as a function of n for the isomerization of ribonuclease A. (Reproduced with permission from M.-S. Wang *et al.*⁽⁵⁾)

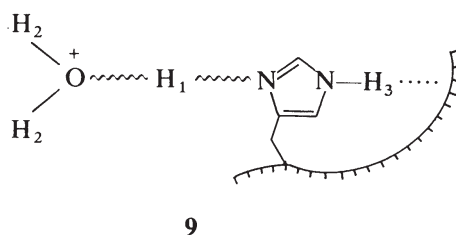
curve is clearly indicative of a multiproton transition state. In treating the data it was first assumed that the ϕ_j^R 's are equal to unity or do not change on activation. The 16 data points of Fig. 10 were then subjected to polynomial regression and nonlinear least-squares fits to polynomials [Eq. (113)] of linear, quadratic, cubic, and higher order. The best fit of the data is to the cubic polynomial:

$$k_n(\text{sec}^{-1}) = 772 + (-1248 \pm 144)n + (1082 \pm 354)n^2 + (-440 \pm 234)n^3 \quad (130)$$

The linear and quadratic terms were shown to be statistically significant at the 99.9% confidence limit by the *F* test, and the cubic terms at the 90% confidence limit. Arguments based on the magnitude of the KSIE, data from the earlier work on this system by French and Hammes,⁽⁴⁾ and other considerations led the authors to conclude that the most reasonable rate-limiting step is the ionization of a histidinium residue,



prior to a conformational change. The transition state for this ionization would be expected to resemble structure **9**. The Gross-Butler equation for



such a process should take the form

$$k_n = k_0 \frac{(1 - n + n\phi_1^T)(1 - n + n\phi_2^T)^2(1 - n + n\phi_3^T)}{(1 - n + n\phi_1^R)(1 - n + n\phi_w)^2(1 - n + n\phi_3^R)} \quad (132)$$

For the transfer of a proton between bases of very different basicity, the transition state is expected to be asymmetric, with the transferring proton closer to the weaker base.⁽⁸⁷⁾ For transition state **9**, then, the hydronium ion is expected to be almost fully formed, with $\phi_2^T \simeq 0.69$ (for H_3O^+). Still assuming all $\phi_j^R = 1.0$ and setting $\phi_3^T = 1.0$ [since $\phi(\text{N}^+-\text{H}) = \phi(\text{N}-\text{H}) = 1.0$, Table III], we obtain a simplified equation:

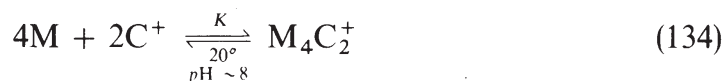
$$k_n = k_0(1 - n + n\phi_1^T)(1 - n + 0.69n)^2 \quad (133)$$

A test for the correctness of this equation was performed by plotting

$k_n/(1 - n + 0.69n)^2$ vs. n . The resulting plot was linear, giving a value of k_0 consistent with experiment and a ϕ_1^T value of 0.46 for the transferring proton H_1 in **9**. This corresponds to a k_H/k_D value of 2.2 for that proton, precisely what is expected for such an asymmetric transition state. The data thus are consistent with transition-state structure **9**, and the most likely fractionation factor values are $\phi_1^T = 0.46$, $\phi_2^T = 0.69$, $\phi_3^T = 1.0$, and $\phi_1^R = \phi_w = \phi_3^R = 1.0$.

8.6. Example VI⁽²⁾

This example deals with rate and equilibrium effects in subunit association of formyltetrahydrofolate synthetase. Contain monovalent cations (K^+ , Cs^+ , NH_4^+) bind to the inactive monomeric form of formyltetrahydrofolate synthetase (from *Clostridium cylindrosporium*), resulting in the formation of an active tetramer containing two of the cations:



Both rate and equilibrium constants of association were found in the studies of Harmony and Himes to be increased in D_2O with $k_{D_2O}/k_{H_2O} = 3.3$ and $K_{D_2O}/K_{H_2O} \simeq 50$. It was shown that the rate effect came solely from a step in which cation and monomer rapidly and reversibly combine:



[The rates were measured at the optimum pL by using a buffer (Tris) with a ΔpK value close to the ΔpH of ~ 0.4 found from the experimental pH-rate profiles shown in Fig. 11. See Section 6.2.] When plotted vs. n the rate data (Table VII) give a curve (Fig. 12) going up to the right (inverse isotope effect,

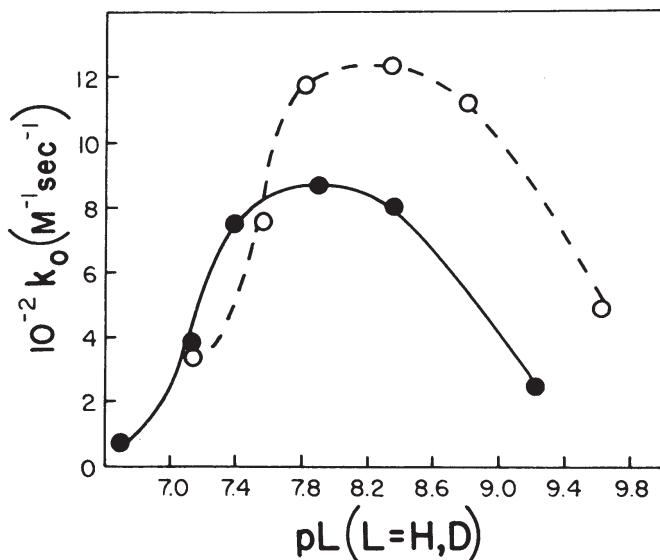


Fig. 11. Dependence on pL in H_2O (solid line) and in 90% D_2O (dashed line) of the rate of K^+ -induced tetramerization of formyltetrahydrofolate synthetase monomers. (Reproduced with permission from J. A. K. Harmony *et al.*⁽²⁾)

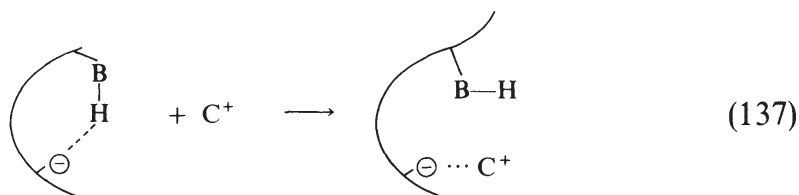
Table VII
Rate and Equilibrium Constants for the
Potassium-Ion-Induced Tetramerization of
Formyltetrahydrofolate Synthetase
Subunits at 20° and pH 7.9 (or Equivalent)
in Mixtures of H₂O and D₂O⁽²⁾

n	$k_n (M^{-1} \text{ sec}^{-1}) \times 10^{-3}$	$K (M^{-3}) \times 10^{-19}$
0.00	2.45	0.04
0.10		0.05
0.20	2.90	0.07
0.25		0.08
0.35	3.35	0.10
0.40		0.12
0.50	3.88	0.16
0.60		0.27
0.70	4.51	0.32
0.80	5.60	0.66
0.90	7.11	1.25
0.99	8.60	1.60

TSC > RSC) and bulging down, thus suggesting a multiproton mechanism. Any of the following multiproton possibilities (it will be recalled from Section 7.1) can give rise to curves of the shape of Fig. 12: (1) large inverse isotope effect(s) partially offset by smaller normal effect(s), (2) opposing reactant- and transition-state contributions (for this case of an inverse isotope effect both ϕ^T and ϕ^R would be > 1 with $\phi^T > \phi^R$), and (3) a change in rate-determining step with n . A fourth possibility, always to be considered, is that the entire contribution may come from one or more reactant-state protons such that all ϕ_j^T 's are unity and that (for this case of a net inverse isotope effect where RSC < TSC) one or more $\phi_i^R < 1$. It was found that a plot of $1/k_n$ vs. n was linear (see Fig. 12) with $\phi^R = 0.33$,

$$k_n^{-1} = k_0^{-1}(1 - n + n\phi_1^R) \quad (136)$$

clear evidence for a one-proton mechanism corresponding to the fourth possibility just cited. Thus the simplest (but in the absence of more information about the enzyme not necessarily the only or the correct) mechanistic model is one involving a single loosely bound reactant proton of $\phi_1^R = 0.33$ being expelled from a cationic binding site and changing in the transition state to a more normal proton of $\phi_1^T = 1.0$ as the cation binds, for example,



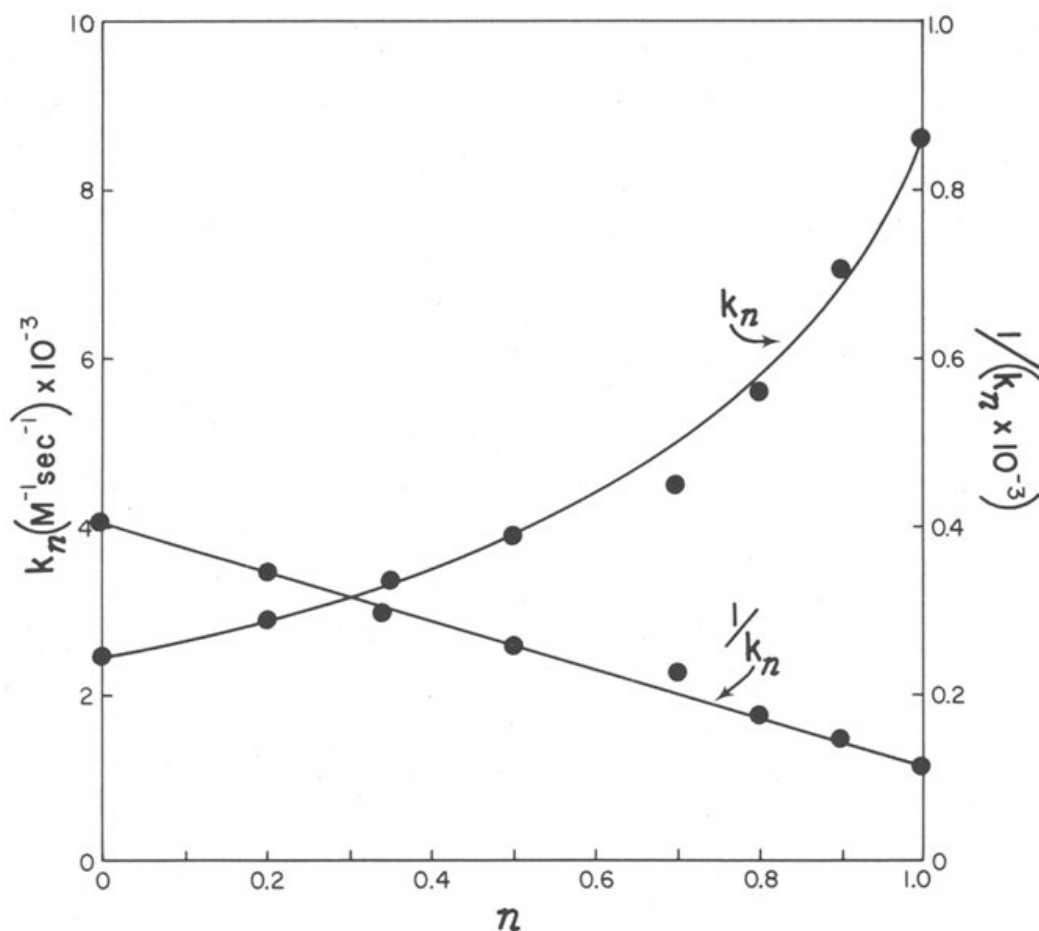


Fig. 12. Plot of k_n and $1/k_n$ vs. n for formyltetrahydrofolate synthetase subunit association. Data from Table VII. (Reproduced with permission from J. A. K. Harmony *et al.*⁽²⁾)

When the equilibrium constant K for Eq. (134) is plotted vs. n a curve going up to the right and bulging down results. One can imagine a process analogous to that just proposed to account for the rate data: Each of the two cations which bind the tetramer do so by occupying simultaneously two cationic binding sites (one on each monomer). The binding is accomplished by the expulsion from each site of one of the protons of $\phi^R \simeq 0.3$ proposed above. The equilibrium solvent isotope effect would thus arise from the conversion of four reactant protons of $\phi^R \simeq 0.3$ to four product protons of $\phi^P \simeq 1.0$:

$$K_n = K_0 \frac{(1 - n + 1.0n)^4}{(1 - n + n\phi^R)^4} \quad (138)$$

$$K_n^{-1} = K_0^{-1} (1 - n + n\phi^R)^4 \quad (139)$$

A plot of $(K_n^{-1})^{1/4}$ vs. n is linear, generating a value for ϕ^R of 0.36, which agrees well with the 0.33 obtained from the kinetic data, and giving a value of $K_{D_2O}/K_{H_2O} \simeq 60$.

At this point there is no proof that the subunit-association SIE's arise as suggested above. Nevertheless, this example shows how such results can be analyzed and interpreted and that very large SIE's are easily accounted for by reasonable chemical mechanisms.

It is to be hoped that the preceding six examples and the chapter as a whole will prove both informative and useful in the determination of biochemical transition-state structures. To date relatively few instances of the application of the proton inventory-solvent isotope technique exist, yet their variety and the results obtained indicate the power of the method. It will indeed be interesting to see further biologically interesting mechanisms emerge as a result of its use.

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