

^{13}C KINETIC ISOTOPE EFFECTS IN THE UREASE-CATALYZED HYDROLYSIS OF UREA

I. TEMPERATURE DEPENDENCE

K. R. LYNN AND PETER E. YANKWICH

Noyes Laboratory of Chemistry, University of Illinois, Urbana, Ill. (U.S.A.)

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SUMMARY

The ^{13}C kinetic isotope effects in the urease-catalyzed hydrolysis of urea have been measured with several preparations of the enzyme in two buffers (maleic acid-maleate, and Tris-sulfuric acid) in which the kinetics have been studied previously. The results indicate that the mechanism of the hydrolysis possesses complexities not reflected in the gross rate phenomena which do influence the isotope fractionation. The isotopic analogues of the several mechanisms found adequate to explain the kinetics of the reaction are examined in detail. It is concluded that complexity at the molecular level, probably involving temperature-dependent interconversion of two or more types of active sites (directly, or indirectly through enzyme conformational changes), is responsible for the isotope-effect results obtained.

INTRODUCTION

Kinetic isotope fractionation effects afford a method for the elucidation of the changes in bonding, about a position of isotopy in a molecule, which occur during the passage of that molecule from an initial reagent state to the transition state (or activated complex configuration). The method has been applied with success to the investigation of the mechanistic details of a number of chemical reactions; BIGEISEN AND WOLFSBERG¹ have published recently a comprehensive review of such applications.

A number of brief studies of isotope fractionation in enzyme-catalyzed reactions have been reported in the recent literature. SELTZER, HAMILTON AND WESTHEIMER² investigated the ^{13}C isotope effect in the decarboxylation of [$1\text{-}^{13}\text{C}$]oxalacetic acid catalyzed by the enzyme isolated from *Micrococcus lysodeikticus* and observed a very small effect (approx. 0.2%). RABINOWITZ *et al.*³, reported a large ^{14}C isotope effect in the reaction of formic acid and formic dehydrogenase. Appreciable carbon isotope effects (*i.e.* greater than 0.2%) have been found in several studies of the urease-catalyzed hydrolysis of urea. SCHMITT, MYERSON AND DANIELS⁴ examined the carbon isotope fractionation during hydrolyses of [^{13}C]urea and [^{14}C]urea in phosphate buffers at pH 5.0. Only one preparation of enzyme was employed in this work, and phosphate

Abbreviations: TIF, temperature-independent factor; TDF, temperature-dependent factor.

buffers have been reported^{5,6} to inhibit the activity of urease. More recently, RABINOWITZ *et al.*^{3,7} measured the varying specific activity of $^{14}\text{CO}_2$ produced during the hydrolysis of ^{14}C urea by urease in 3 *M* acetate buffer at pH 5.0 and 37°; they found that the minimum in specific activity of $^{14}\text{CO}_2$ occurred at about 10% reaction and was equivalent to an approximately 10% isotope effect. A ^{14}C kinetic isotope effect of this magnitude would be considered large even in a purely thermal reaction; further, the kinetics of the urease-catalyzed hydrolysis have not been studied extensively in acetate buffer.

Two buffer systems are known in which there is no apparent inhibition of urease by the buffer itself, and in which the kinetics of the hydrolysis of urea have been studied. These systems are maleic acid-maleate, which was employed by KISTIAKOWSKY *et al.*⁸, and Tris-sulfuric acid, which was used by WALL AND LAIDLER⁹. KISTIAKOWSKY AND ROSENBERG⁹ interpreted their kinetics results in terms of a pair of two-site mechanisms, though the deviations from simple Michaelis-Menten kinetics are modest; WALL AND LAIDLER found that the hydrolysis conformed to simple Michaelis-Menten kinetics, but their investigation was not as comprehensive as that of KISTIAKOWSKY *et al.*

This article reports the results obtained in the first part of a broad investigation of the ^{13}C kinetic isotope effect in the urease-catalyzed hydrolysis of urea containing ^{12}C and ^{13}C at their natural abundance levels. The atomic displacements which occur in the actual rate-determining step of a chemical reaction contribute a temperature-independent factor to the observed ratio of the isotopic specific rate constants¹⁰⁻¹². This TIF is accessible only through a study of the temperature dependence of the isotope effect; it is to such an investigation that this research is devoted. The steps in the reaction which precede that which is rate-determining, as well as the genuine vibrational motions occurring during the rate-determining process, contribute a TDF to the ratio of the isotopic specific rate constants¹³; TDF is a primary derived quantity in an investigation of isotope effect temperature dependence, but, because of our interest in the molecular details of dynamic processes, TIF is the ultimate object.

EXPERIMENTAL

Reagents

Urease was prepared from jackbean meal (Nutritional Biochemicals Corp.) by the method of SUMNER¹⁴ and DOUNCE¹⁵. From one to four crystallizations of the enzyme were carried out with different preparations; but, as has been reported by other workers¹⁶⁻¹⁸, the ease with which urease is denatured makes repeated crystallization unprofitable as a method of purification. The several preparations of urease were stored as solutions in 50% aqueous glycerol (Fisher "Certified" reagent) in stoppered vessels at pH 7.0; a refrigerated bath was employed to maintain a storage temperature of 3-5°. From these reservoirs, small aliquots were withdrawn as required and diluted with the appropriate buffer. Solutions so prepared were then allowed to "age"^{6,18}, at the temperature at which the reaction was to be carried out, for not less than two hours before being used.

The urea (Baker "Analytical Reagent") was used without further purification. Maleic acid-maleate buffers were prepared from maleic anhydride (Eastman "White Label") and sodium hydroxide (Mallinckrodt "A.C.S. Grade"). The buffers of Tris

and sulfuric acid were prepared by titrations of solutions of the amine (Commercial Solvents Corp.; recrystallized) with standard acid ("Acculute", Anachemia Chemicals, Ltd.). The citric acid-citrate buffers employed in the crystallization of urease¹⁵ were obtained by mixing solutions of citric acid and sodium citrate (both Baker and Adamson "A.C.S. Grade"). All buffer solutions were calibrated against standards (Leeds and Northrup Co.) using a Beckman pH Meter (Model G).

The water used throughout this work was de-ionized by passage through a mixed bed of ion exchange resins (Rohm and Haas, Amberlite I.R.A.-400; Dow Chemical Co., Dowex-50). Before use all apparatus was cleaned with either an acid⁶ or detergent bath, rinsed liberally with de-ionized water, and then rinsed several times with the buffer solution to be employed.

Procedure for kinetics measurements

The stability and reproducible behavior of each preparation of urease employed in the isotope effects measurements was established by observation of the kinetics of the hydrolysis. The procedures employed in this phase of the investigation were those of KISTIAKOWSKY *et al.*⁶; k_{exp} values appearing later in this report are pseudo-first order rate constants. Unless specifically noted, isotope effect measurements were made only with preparations of urease which gave a constant value of k_{exp} over whatever extent of reaction was examined and one which was invariant within experimental error for a period sufficient to permit completion of the isotope fractionation studies. The stock solutions of enzyme were diluted to concentrations of the order of a few SUMNER Units¹⁹/ml, a range used in the previous kinetics studies cited. Estimation of the protein content of the enzyme preparations was made by conventional procedures²⁰.

Procedure for isotope effect measurements

Into flasks (200 ml) carrying two side-arms and a "break-seal" tube connected through a capillary were measured aliquots of the buffer solution (85 ml) and of a standard solution of urea (0.5 *M*) in that buffer (10 ml). Five milliliters of a solution of the enzyme in the same buffer were placed in one side-arm, and in the other an identical volume of 5 *M* sulfuric acid. The reaction flasks were evacuated to approx. 20 mm Hg on a vacuum manifold, sealed from the atmosphere, and removed from the manifold. The vessels were then placed in a constant ($\pm 0.1^\circ$) temperature bath and 2 h allowed for attainment of thermal equilibrium and "aging" of their contents. Reaction, begun by tilting the side-arm containing the enzyme solution, was allowed to proceed for a pre-determined time interval (usually somewhat less than that required for the hydrolysis to reach 3% of completion²¹), and then "quenched" by denaturation of the enzyme with the acid in the second side-arm. The extent to which reaction had proceeded was checked by determination of the concentration of ammonium ion in the mixture using the method employed in the conventional kinetics measurements. Product carbon dioxide was distilled from the now strongly acidic medium to the "break-seal" tube, which was then sealed; the gas was purified and dried by distilling several times, on a vacuum manifold, between traps at -78° and -196° through columns packed with magnesium perchlorate. Carbon isotope analyses were carried out with a Consolidated-Nier Isotope-Ratio Mass Spectrometer;

the analytical procedures and methods for correcting the raw output data have been described in earlier publications from this laboratory^{22, 23}.

Conditions employed

The isotope fractionations in the hydrolyses catalyzed by a number of preparations of urease were measured at several temperatures between 1.8° and 43.2°; another group of preparations was studied only at 11.4°. All experiments were carried out at pH 6.5; the urea concentration in all reacting solutions was 0.050 *M*. During reaction, solutions in maleate-buffers had a buffer concentration of 0.032 *M* and ionic strength of 0.066 *M*; in the experiments with Tris-sulphuric acid buffers, the buffer concentration was 0.130 *M* and the ionic strength 0.127 *M*.

RESULTS

Symbols

Individual enzyme preparations are designated by Roman numerals; Arabic subscripts to these numerals indicate the number of crystallizations (by the procedure of DOUNCE¹⁵) to which the extracted material was subjected before the preparation of the final "reservoir" solutions. The enzyme concentration employed in any series of experiments with a given preparation of urease was constant, the same volume of reservoir solution being used in each run. In the Tables, maleate-buffers are indicated by the symbol M and Tris-sulphuric acid buffers by T; the appended Arabic numerals indicate the order of preparation of the buffer stock solutions.

Calculation of results

The isotopic pair of reactions under consideration may be represented as follows:



When the reaction is allowed to proceed to the extent of 5% or less of completion²¹,

$$(R_u/R_p) = (k_{12}/k_{13}) \quad (3)$$

where R_u is the corrected ratio of ($^{13}\text{CO}_2/^{12}\text{CO}_2$) observed for carbon dioxide obtained by combustion (in a conventional Pregl apparatus) of a sample of substrate urea, R_p is the analogous ratio of the product carbon dioxide, and k_{12} and k_{13} are the isotopic experimental pseudo-first order specific rate constants. For the experiments reported here, $R_u \times 10^6 = 10800 \pm 2$. This result is the average obtained for four combustions of urea. (Uniformly in this paper, appended errors are average deviations from the mean.)

Tabulations of experimental results

Experimental rate constants, k_{exp} , and isotopic rate constant ratios, (k_{12}/k_{13}), observed for 8 enzyme preparations at various temperatures are collected in Tables I and II; in Tables IA, IB, and IC, the isotope effects are shown for each experiment, while in Table II only the averaged results are given.

The sensitivity of the catalytic activity of urease to the presence of heavy metal ions is well known, even 10^{-11} *M* silver ion causing reversible inactivation of the enzyme²⁰. Colorimetric tests with diphenylthiocarbazone showed that the maximum

TABLE I
EFFECTS OF TEMPERATURE ON RATE AND ISOTOPE FRACTIONATION

Temperature	Buffer	k_{exp} ($\text{sec}^{-1} \times 10^5$)	$R_p \times 10^6$	(k_{12}/k_{13})	Average (k_{12}/k_{13})
A. Preparation VIII ₁					
2.3	M11	0.22	10 691	1.0102	1.0102 \pm 0.0001
			10 694	1.0099	
			10 690	1.0103	
11.4	M11	0.31	10 675	1.0117	1.0121 \pm 0.0003
			10 666	1.0126	
			10 672	1.0120	
			10 670	1.0122	
19.0	M10	0.55	10 689	1.0104	1.0099 \pm 0.0008
			10 701	1.0093	
			10 684	1.0109	
			10 706	1.0088	
31.0	M10	1.00	10 751	1.0046	1.0051 \pm 0.0004
			10 746	1.0050	
			10 738	1.0058	
			10 748	1.0048	
B. Preparation IX ₂					
3.5	M12	0.076	10 681	1.0111	1.0106 \pm 0.0003
			10 694	1.0099	
			10 686	1.0107	
11.4	M12	0.13	10 715	1.0079	1.0079 \pm 0.0003
			10 720	1.0075	
			10 716	1.0078	
			10 710	1.0084	
21.8	M12	0.25*	10 761	1.0036	1.0041 \pm 0.0004
			10 753	1.0044	
			10 751	1.0046	
			10 761	1.0036	
C. Preparation XII ₂					
1.8	M16	0.30	10 675	1.0117	1.0119 \pm 0.0002
			10 671	1.0121	
	T ₂	0.29	10 603	1.0186	
11.4	M16	0.51	10 670	1.0122	1.0123 \pm 0.0001
			10 668	1.0124	
	T ₂	0.46	10 608	1.0181	1.0183 \pm 0.0002
			10 604	1.0185	
20.5	M17	1.00**	10 674	1.0118	1.0117 \pm 0.0001
			10 676	1.0116	
	T ₂	1.00**	10 636	1.0154	1.0156 \pm 0.0002
			10 633	1.0157	
31.0	M16	1.7	10 672	1.0120	1.0120 \pm 0.0001
	M17	1.7	10 673	1.0119	
	T ₂	1.7	10 619	1.0170	
			10 620	1.0168	

* Rate measurements indicated inactivation occurring within time required for isotope fractionation experiments.

** Estimated from absorption measurements after fractionation experiments.

TABLE II
EFFECTS OF TEMPERATURE ON RATE AND ISOTOPE FRACTIONATION

Temperature	Buffer	$k_{\text{exp}} (\text{sec}^{-1} \times 10^4)$	No. of Experiments	Average (k_{13}/k_{12})
<i>Preparation I₁</i>				
11.4	M1	0.004	11	1.0347 ± 0.0022
31.0	M1	0.017	12	1.0338 ± 0.0017
43.2	M1	0.040	10	1.0327 ± 0.0020
<i>Preparation XIII₂</i>				
2.6	M28	4.8	4	1.0108 ± 0.0004
	T3	5.0	3	1.0116 ± 0.0001
11.4	M27	8.6	4	1.0094 ± 0.0004
	T2	8.6	4	1.0106 ± 0.0003
20.5	M27	17.0*	2	1.0104 ± 0.0004
<i>Preparation XIV₂</i>				
2.6	T3	0.070	2	1.0154 ± 0.0001
11.4	T3	0.12	2	1.0144 ± 0.0002
	T4	0.12	4	1.0145 ± 0.0002
31.0	T4	0.25*	3	1.0112 ± 0.0002
<i>Preparation XV₄</i>				
2.6	M19	0.013	4	1.0238 ± 0.0020
11.4	M18	0.023	4	1.0167 ± 0.0006
31.0	M19	0.10*	4	1.0218 ± 0.0013
<i>Preparation XVI₃</i>				
2.6	M19, M20	0.0094	4	1.0107 ± 0.0017
31.0	M19	0.065	4	1.0232 ± 0.0008

* Rate measurements indicated inactivation occurring within time required for isotope fractionation experiments.

concentration of heavy metal ions in the water used during these experiments was less than $10^{-8} M$. However, to establish that heavy metal ions were without effect on our results, hydrolyses were carried out at 11.4° with several enzyme preparations in the presence and absence of $10^{-4} M$ EDTA (Hack Chemical Co.), a powerful sequestering agent. The results of these experiments are collected in Table III.

The specific activity of a preparation of urease may be defined as the rate of reaction in a standard hydrolysis mixture per unit weight of protein present. KISTIAKOWSKY *et al.*^{9,20} have reported that the specific activity is essentially constant for all preparations of urease which have attained a stable and reproducible degree of catalytic activity. Comparative data bearing on this point, and isotope effect data, were obtained for several preparations at 11.4° ; these results are shown in Table IV.

The Arrhenius activation energy, E_A , computed for each preparation used in the study of temperature effects, is shown in Table V. The precision of the individual k_{exp} values averaged about $\pm 3\%$; if this were the only source of imprecision in the E_A values, they would be reliable to ± 0.4 kcal/mole.

TABLE III
TESTS FOR EFFECTS OF POSSIBLE HEAVY METAL ION CONTAMINATION*

Buffer**	$k_{exp} (sec^{-1} \times 10^5)$	No. of Experiments	Average (k_{12}/k_{13})
<i>Preparation XIV₃</i>			
M21	0.06	6	1.0185 ± 0.0003
M21E	0.06	4	1.0187 ± 0.0004
<i>Preparation XVIII₂</i>			
M26	0.77	2	1.0162 ± 0.0007
M26E	0.77	2	1.0178 ± 0.0000
<i>Preparation XIX₁***</i>			
M25	2.2	5	1.0148 ± 0.0014
M25E	2.2	4	1.0134 ± 0.0015
<i>Preparation XX₂***</i>			
M26	3.2	4	1.0178 ± 0.0002
M26E	3.2	3	1.0174 ± 0.0009
<i>Preparation XXVI₁</i>			
T9	4.6	3	1.0208 ± 0.0010
T9E	4.6	3	1.0189 ± 0.0030

* All experiments at 11.4°.

** E indicates buffer containing 10^{-4} M EDTA.

*** Preparations XIX₁ and XX₂ were from the same crude extract of meal.

TABLE IV
RELATION OF ENZYME SPECIFIC ACTIVITY TO OBSERVED RATE AND ISOTOPE FRACTIONATION*

Buffer	$k_{exp} (sec^{-1} \times 10^5)$	Specific activity**	No. of experiments	Average (k_{12}/k_{13})
<i>Preparation XXI₁***</i>				
M27	37.0	9.0	4	1.0209 ± 0.0007
T5	37.6	9.0	2	1.0143 ± 0.0006
<i>Preparation XXII₂***</i>				
M27	37.0	9.0	4	1.0165 ± 0.0027
T5	37.6	9.0	2	1.0060 ± 0.0015
<i>Preparation XXIII₁</i>				
T7	1.04	0.035	2	1.0054 ± 0.0004
<i>Preparation XXV₁</i>				
T7	1.04	0.017	4	1.0061 ± 0.0007

* All experiments at 11.4°.

** Specific activity given in units of $sec^{-1} (mg \text{ of protein})^{-1} \times 10^3$.

*** Preparations XXI₁ and XXII₂ were from the same crude extract of meal.

TABLE V
ARRHENIUS ACTIVATION ENERGIES
M, maleate; T, Tris-sulphuric acid buffer.

Preparation	Buffer	E_A , (kcal/mole)
I ₁	M	12.2
VIII ₁	M	10.0
IX ₂	M	10.1*
XII ₂	M	10.4
XII ₂	T	10.6
XIII ₂	M	11.2
XIII ₂	T	10.0*
XIV ₃	T	9.8*
XV ₄	M	11.8
XVI ₂	M	11.3

* From experiments at two temperatures only.

DISCUSSION

Kinetics results

The final concentration of urea in all reacting solutions, 0.050 *M*, is sufficient to yield essentially the maximum rate of hydrolysis, but not so high as to produce substrate inhibition. The values of E_A refer, therefore, to k_3 , the specific rate constant for the formation of products by breakdown of the enzyme-substrate complex, and the values of k_{exp} being proportional to k_3 . The figures for E_A recorded in Table V appear to be significantly different from those reported for the same buffers by other investigators. The average E_A for the experiments in maleate-buffers is 11.0 ± 0.6 kcal/mole, while KISTIAKOWSKY AND ROSENBERG⁹ found 8.85 ± 0.2 kcal/mole; the average value in Tris-sulphuric acid buffers is 10.1 ± 0.3 kcal/mole, while WALL AND LAIDLER⁸ recorded figures at two acidities from which we estimate $E_A = 8.6$ kcal/mole at pH 6.5. Our value for E_A in Tris-sulphuric acid buffers is not as reliable as the small error appended seems to indicate, and the difference between our average values in the two buffer systems employed is small. The average value of E_A derived from our experiments in maleate-buffers is based on data collected at a pH one-half unit lower than the figure given by KISTIAKOWSKY AND ROSENBERG; and, even after allowance for this fact, we believe the difference between the two is real.

As our discussion of the isotope effects and of the lack of correlation between isotope fractionation and rate results will indicate, it is quite possible that the results tabulated above were secured with urease preparations inhibited or otherwise contaminated in a manner which could not have been reflected in the results of any kinetics test which was performed. On the other hand, our higher E_A values may be due simply to the fact that a wider range of temperature was employed in the experiments reported here than in those of the earlier investigators. It is worthy of note that there does not appear to be an effect of buffer type on k_{exp} (*vide*: Preparation XII₂, Table IC; Preparation XIII₂, Table II; Preparations XXI₁ and XXII₂, Table IV).

Our higher value for the Arrhenius activation energy may reflect the occurrence of partially non-competitive inhibition, or the existence of a temperature-dependent equilibrium between two or more configurations of the active site on the enzyme

(though the activation energies associated with activity of the different types of site should not differ by more than a few kcal/mole). Inhomogeneity of urease at the molecular level has been proposed^{18, 20} and indications of it sought^{6, 8, 9, 16-18, 24}, and the evidence is increasingly positive. It is entirely probable that many of the physical characteristics of urease would be only subtly altered by reactive site interconversion, and the chemical detectability of such an equilibrium situation would be low if the activation energy differences among the site types were as small as indicated above. Isotope fractionation, however, is a very sensitive probe.

Isotope effect results

The average error of values of (k_{12}/k_{13}) in Tables I and II is ± 0.0009 , which corresponds to $\pm 4.7\%$ of the average isotope effect (*i.e.* of the average deviation from unity of the isotopic ratio of specific rate constants); an error of this size is comparable with those reported from similar studies of very well-behaved thermal reactions. SCHMITT, MYERSON AND DANIELS⁴ reported a 1.0% ¹³C isotope effect, while the result given by RABINOWITZ *et al.*⁷, is equivalent to about a 5% effect; none of our results is as high as the latter, and their average is almost double the former, the range (all temperatures) being 0.41-3.47%.

A number of conclusions can be drawn from the data in the Tables without more detailed analysis. First, the magnitudes of the isotope effects are reasonable for a process involving bond rupture^{1, 13, 25, 26} and an "equilibrium" before the rate-determining step; second, there is no correlation (for the several preparations at any one temperature of reference) between k_{exp} and (k_{12}/k_{13}) ; third, neither k_{exp} nor (k_{12}/k_{13}) shows influence of possible heavy metal ion contamination of the reacting mixtures (Table III); fourth, there appear to be inconsistent effects of buffer type (but no effect of using different preparations of the same buffer) on the isotope fractionations observed for different samples of enzyme (maleate results lower than Tris-sulphuric acid, Preparation XII₂, Table IC; about the same, Preparation XIII₂, Table II; higher, Preparations XXI₁ and XXII₂, Table IV), but there is no similar effect on k_{exp} ; fifth, there is no correlation between (k_{12}/k_{13}) and enzyme specific activity (Table IV, especially the results for Tris-sulphuric acid buffers); sixth, while one would expect (k_{12}/k_{13}) to decrease uniformly with increasing temperature, the observed temperature dependence varies in magnitude and sense from preparation to preparation (normal: Preparation IX₂, Table IB, and Preparation I₁, Table II; reverse: Preparation XVI₃, Table II; zero: Preparation XII₂-M, Table IC, and Preparations XIII₂ and XIV₂, Table II; composite: Preparation VIII₁, Table IA, Preparation XII₂-T, Table IC, and Preparation XV₄, Table II; some of these assignments are altered if isotope effect data are rejected which are suspect because of the kinetics observations, but the general chaos is not reduced).

Normal isotope effects in enzyme reactions

Before discussing the isotope fractionation data in detail, we shall derive expressions which permit one to make an educated guess as to the isotope effects which one would expect to observe in the study of an uncomplicated enzymic reaction. The simple Michaelis-Menten mechanism is represented as follows:



where E represents a molecule of enzyme, S a substrate molecule, ES the enzyme-substrate complex, and P the products. The Briggs-Haldane treatment leads to

$$V_0 = \frac{k_3(E_0)(S_0)}{K + (S_0)} \quad (5)$$

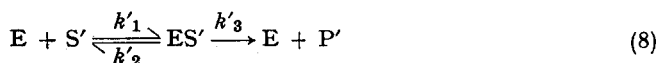
where V is the velocity, the subscripts 0 denote initial values, and

$$K = \frac{k_2 + k_3}{k_1} \quad (6)$$

At the levels of (S_0) employed in this research, Eqn. 5 yields

$$k_{\text{exp}} = k_3 \quad (7)$$

The competition of an isotopic substrate molecule, S' , for reaction with the enzyme requires the addition to the mechanism of the following steps:



Application to Eqns. 4 and 8 of the steady-state approximation yields²⁷⁻²⁹

$$\frac{V_0}{V'_0} = \frac{k_3 K'(S_0)}{k'_3 K(S'_0)} \quad (9)$$

which, upon rearrangement, becomes

$$\frac{R_u}{R_p} = \frac{V_0}{V'_0} \cdot \frac{(S'_0)}{(S_0)} = \frac{k_3 K'}{k'_3 K} = \frac{(k_3/k'_3)}{(K/K')} = \frac{k_{12}}{k_{13}} \quad (10)$$

It is important to note that, unlike k_{exp} , at no concentration is the experimentally accessible quantity (k_{12}/k_{13}) independent of the substratum constants K and K' .

The specific rate constant ratio (k_3/k'_3) refers to the process in this hydrolysis in which, presumably, the C-N bond is ruptured. If this step required an activation energy characteristic of an uncatalyzed thermal reaction (30-35 kcal/mole), the TIF in (k_3/k'_3) would be about 1.015, and the TDF would be approx. 1.039 at the lowest temperature studied in the experiments and 1.034 at the highest; thus we would expect $(k_3/k'_3) = 1.055$ near 0° and = 1.050 near 45°. BUIST AND BENDER³⁰ recently have summarized the evidence for a strong direct correlation between activation energy and the magnitude of the kinetic isotope effect³⁰⁻³⁴, and the result of the enzymic catalysis on the hydrolysis of urea should be to reduce both TIF and TDF (and, therefore, the expected temperature dependence) in (k_3/k'_3) . It seems reasonable that (k_3/k'_3) for the enzyme reaction should be of the order of 1.030-1.040 and change by perhaps 0.003-0.004 over the range of temperatures employed in this research.

Estimation of reasonable values for (K/K') is more difficult, primarily because one must first have an estimate of the relative magnitudes of k_2 and k_3 . LAIDLER³⁵ has interpreted the non-competitive pH dependence of the rate of urease-catalyzed urea hydrolysis³⁶ as evidence that k_3 is insignificant in comparison with k_2 and that K has, therefore, the form of an equilibrium constant. While we shall adopt this view as our own, we prefer to base it on evidence brought to our attention by NIEMANN AND HEIN³⁷: that with chymotrypsin and several other enzymes, determination of the various parameters of Eqn. 5 has shown that changes of several orders of magnitude are produced in k_3 by variation of the structure of the substrate without being

accompanied by significant changes in K . The specific rate constants k_1 and k_2 refer to the forward and reverse of the same process; their TIF's are the same³⁸. If our view that k_3 is small with respect to k_2 is not correct, there would be associated with K a small temperature-independence—probably smaller than that in (k_3/k'_3) for there is no evidence that k_3 for urease-urea is large with respect to k_2 and the TIF in k_1 has the opposite sense to that in k_3 in contributing to the character of K .

The form of (K/K') , when K and K' are regarded as equilibrium constants, is that of the equilibrium constant for an isotopic exchange reaction. The value of such a (K/K') can be calculated, in principle, by methods which have been described by UREY³⁹ and BIGEISEN AND MAYER⁴⁰; however, the necessary molecular data are not available in the present case. It is necessary to estimate the value; this problem has been considered in some detail by a number of workers^{1, 13, 22, 23, 26, 41, 42}. When K is of the order of 0.04 M , (K/K') is found experimentally to be approx. 1.010 (see refs. 26, 42). The earlier kinetics studies on ureolysis by urease^{8, 9} indicate $K = 0.002$ – $0.005 M$, and we estimate that (K/K') should be approx. 1.010–1.015 and change by perhaps 0.001–0.002 over the range of temperature in the present experiments.

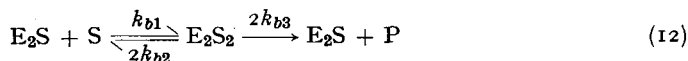
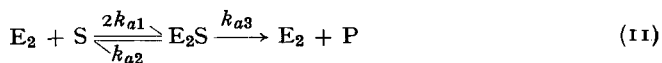
The predictions for (k_3/k'_3) and (K/K') can be combined to yield the following extremes for (k_{12}/k_{13}) : a mean of 1.030 with a change of 0.003 over the temperature range of the experiments; a mean of 1.015 with a change of 0.001 with temperature. Where normal temperature dependence was observed, these estimates contain the experimental observations, within reasonable limits, except for the cases where the temperature dependence was unusually high and led to very low isotope effects at the higher temperatures (Preparation VIII₁, Table IA, and Preparation IX₂, Table IB). The problem of abnormally large temperature dependence of normal sense has been encountered in the investigation of isotope fractionation in some purely thermal reactions in solution^{22, 23, 26, 41, 42} and is not yet understood.

These few very small isotope effects might be due to enzyme inactivation occurring at the higher temperatures, but neither the plots of $\log k_{\text{exp}}$ versus $(1/T)$ or of $\log (k_{12}/k_{13})$ versus $(1/T)$ for those sets of runs show sufficient deviation from linearity to support such a conclusion. Four sets of runs are identified as having been carried out under conditions such that the kinetics measurements showed the possibility of enzyme inactivation. It is to be noted that of these four sets, two (Preparation IX₂, Table IB, and Preparation XIV₂, Table II) show a lower isotope effect than expected, one (Preparation XV₄, Table II) a higher, and one (maleate-results, Preparation XIII₂, Table II) exhibits no difference from the results obtained at lower temperatures.

Eqn. 10 should be applied strictly only to the results obtained in Tris-sulphuric acid buffers, for it is in that system that the work of WALL AND LAIDLER⁸ indicates that the hydrolysis follows the simple Michaelis-Menten mechanism. Consideration of these experiments reveals that all of the isotope effect results lie in the expected magnitude range. Further, all of the isotope fractionations in Tris-sulphuric acid buffers exhibit temperature dependence normal in sense and nearly normal in magnitude. Since our value for the experimental activation energy in Tris-sulphuric acid buffers is only about 1 kcal/mole higher than that reported by WALL AND LAIDLER, one might well conclude that the isotope effects in that buffer are explained adequately by the simple treatment outlined above.

KISTIAKOWSKY AND ROSENBERG⁹ proposed two double-site mechanisms to

explain their kinetics results for the hydrolysis in maleate buffers. Their second, or "B", mechanism assumes that there are on each molecule of the enzyme "pairs of identical Michaelis-Menten active sites with interaction, such that the kinetic parameters of a site are altered when the neighbor site becomes combined with urea". Where *a* represents a site of first combination and *b* a site of second combination, the mechanism is:



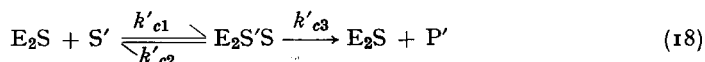
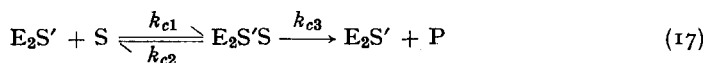
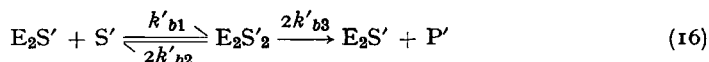
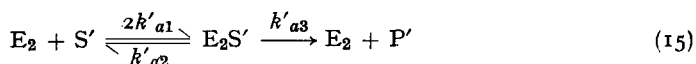
The steady-state treatment leads to:

$$V_0 = \frac{(E_0)(S_0)[K_b k_{a3} + (S_0)k_{b3}]}{(S_0)^2 + 2(S_0)K_b + K_a K_b} \quad (13)$$

At high substrate concentrations, Eqn. 13 yields

$$k_{\text{exp}} = k_{b3} \quad (14)$$

The availability of a competing isotopic substrate molecule, *S'*, requires the inclusion of the following steps in the mechanism:



A number of equalities can be employed to simplify the mathematics involved in the steady-state treatment of the mechanism represented by Eqns. 11, 12, 15-18. While transmission of kinetic effects between the sites is assumed, transmission of isotope effects between the sites would be of second order importance; hence: $k_{b3} = k_{c3}$, $k'_{b3} = k'_{c3}$, $K_b = K_c$, and $K'_b = K'_c$. The four substances E_2 , E_2S , $E_2S'S$, and E_2S' form a stoichiometric cycle, and there is a thermodynamic requirement that $K_c K'_a = K'_c K_a$. When these equalities are used, the following expression is obtained for high substrate concentrations:

$$\frac{R_u}{R_p} = \frac{k_{c3} K'_a}{k'_{c3} K_a} = \frac{(k_{c3}/k'_{c3})}{(K_a/K'_a)} = \frac{k_{12}}{k_{13}} \quad (19)$$

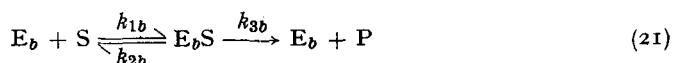
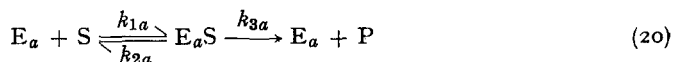
which is exactly the same form as Eqn. 10. The similarity of Eqns. 7 and 14, and 10 and 19, means that at high substrate concentrations neither conventional kinetics nor isotope fractionation experiments can distinguish between enzyme behavior characterized as simple Michaelis-Menten on the one hand or interacting pairs of sites on the other.

Abnormal isotope effects in enzyme reactions

To account for the observation of reverse, zero, and, possibly, composite temperature dependence of these isotope effects, it is necessary to postulate mechanisms more

complicated than those discussed above. The likely schemes would seem to involve the accessibility in the enzyme preparations of more than one kind of active site, but without direct interaction of the different kinds. One can, of course, explain any findings by taking a sufficiently large number of parameters; to avoid this situation we confine the following development to cases involving only two kinds of active site.

The first, or "A", mechanism of KISTIAKOWSKY AND ROSENBERG⁹ assumes "two types of active site on the enzyme, differing in their Michaelis-Menten parameters". It is clear from the formulation that either there are two distinct types of enzyme in a preparation, each with its own kind of enzymic site, or that each enzyme molecule contains both types of site, that only one kind can be occupied at a time, and that the relative reactivity of the two types is constant. Where the different site types are distinguished by the subscripts *a* and *b*, the mechanism is:



Application of the steady-state treatment leads to:

$$V_0 = \frac{1}{2} \cdot \frac{[k_{3a}(E_{a0}) + k_{3b}(E_{b0})] (S_0)^2 + [k_{3a}(E_{a0})K_a + k_{3b}(E_{b0})K_b] (S_0)}{(S_0)^2 + [K_a + K_b] (S_0) + K_aK_b} \quad (22)$$

At high substrate concentrations, Eqn. 22 yields

$$k_{\text{exp}} = \frac{k_{3a}(E_{a0}) + k_{3b}(E_{b0})}{(E_{a0}) + (E_{b0})} \quad (23)$$

from which we obtain the following relation for the experimental activation energy:

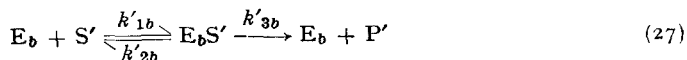
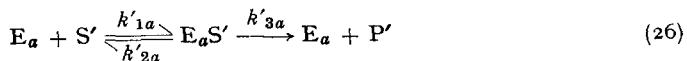
$$E_A = \frac{E_{Aa}k_{3a}X_{a0} + E_{Ab}k_{3b}X_{b0}}{k_{3a}X_{a0} + k_{3b}X_{b0}} \quad (24)$$

where E_{Aa} and E_{Ab} are the activation energies for operation of the two kinds of catalytic sites and X_{a0} and X_{b0} are their initial mole fractions in the preparation. KISTIAKOWSKY AND ROSENBERG's results led them to the conclusion that

$$k_{3a}(E_{a0}) = k_{3b}(E_{b0}), \text{ or } k_{3a}X_{a0} = k_{3b}X_{b0} \quad (25)$$

They did not consider the possibility of interconversion of the two types of sites, nor is it clear from their exposition whether or not the condition laid down by Eqn. 25 is presumed to hold at all temperatures; if it does, X_{a0} and X_{b0} being taken as temperature-invariant, then $E_{Aa} = E_{Ab}$, a seeming inconsistency with the assumption that the two sites have different rate parameters. It is likely that the two activation energies differ at most by a few kcal/mole; under such circumstances, Eqn. 24 leads to the prediction that over a range of temperature such as employed in the experiments reported here the activation energy would change by only a few tenths of one kcal/mole, an un-noticeable amount⁴⁴.

Isotopic competition is added to the mechanism through the following steps:



The steady-state treatment of Eqns. 20, 21, 26, and 27 yields the following expression applicable to high substrate concentrations:

$$\frac{R_u}{R_p} = \frac{k_{3a}X_{a0} + k_{3b}X_{b0}}{\frac{k'_{3a}K_aX_{a0}}{K'_a} + \frac{k'_{3b}K_bX_{b0}}{K'_b}} = \frac{k_{12}}{k_{13}} \quad (28)$$

The form of Eqn. 28 is such that the temperature dependence of (k_{12}/k_{13}) which would be observed depends upon the relative values of $k_{3a}X_{a0}$ and $k_{3b}X_{b0}$; if one of these factors is much larger than the other, (k_{12}/k_{13}) will have all the properties of the appropriate single-site version of Eqn. 10. Given comparable values of $k_{3a}X_{a0}$ and $k_{3b}X_{b0}$, (k_{12}/k_{13}) will have some weighted average behavior; it is the possibility of such weighting, particularly if it is variable, that makes this model attractive for the explanation of anomalous temperature dependences.

The applicability of Eqn. 28 to the experimental data tabulated above can best be tested, and demonstrated, by means of specific calculations. It is necessary to assign values to the various isotope effects at a pair of temperatures. As a matter of convenience, the selected input parameters in Table VI are given for 11.4° and

TABLE VI
PARAMETERS FOR ISOTOPE EFFECT CALCULATIONS

Ratio of isotopic constants (sub-case)	Temperature	
	11.4°	43.2°
k_{3a}/k'_{3a}	1.030	1.027
k_{3b}/k'_{3b}	1.040	1.036
K_a/K'_a (LS)	1.020	1.016
K_a/K'_a (LL)	1.012	1.010
K_b/K'_b (LS)	1.012	1.010
K_b/K'_b (LL)	1.020	1.016

43.2°. At pH 6.5, one of the K values was found experimentally to be about five times the other⁹, both were a few thousandths molar, and (these measurements being made at pH 7.0) their ratio was relatively insensitive to temperature change. We conclude from these facts that (K_a/K'_a) and (K_b/K'_b) are somewhat different. These ratios of constants and (k_{3a}/k'_{3a}) and (k_{3b}/k'_{3b}) are assigned magnitudes and temperature dependences within the ranges estimated above. There is no way of deciding from the little pertinent data available²⁶ whether the larger kinetic isotope effect should be associated with the larger or the smaller of the substratum isotope effects; such a decision is debased by the argument above concerning the relative magnitudes of k_2 and k_3 . Accordingly, the calculations are made for both possibilities, identified as sub-cases LL, and LS, respectively. While there are some guideposts to the selection of reasonable values for the isotope effect parameters, there is a degree of arbitrariness in all the choices; in particular, the figures tabulated were picked so that in one certain extreme the net isotope effect would have a small abnormal temperature dependence—one of such size that it would have been hidden in experimental errors of the average magnitude recorded above.

Consider first a situation in which there is no interconversion of the two types of sites. We assume that k_{3a} and k_{3b} have similar magnitudes at the two temperatures,

their differences being associated with a small difference in activation energy, 2.0 kcal/mole. The results of calculations for this model are shown in Table VII. For all cases the temperature dependence is small; (k_{12}/k_{13}) is relatively insensitive to the proportions of the two sites for the LL combination of isotope effect parameters, but quite sensitive for the LS combination. The variation of (k_{12}/k_{13}) with temperature is near the limit of detectability except for the LS situation at $X_{a0} = 0.75$, where it has a sense the reverse of normal. No case yields a temperature dependence of large magnitude. It is, of course, possible to confer normal temperature dependence on (k_{12}/k_{13}) so calculated by assigning almost identical values to the a and b sets of input parameters, thus reducing Eqn. 28 to the form of Eqn. 10; such assignments, however, deny the basic postulate of the model which requires that the two sites

TABLE VII
CALCULATED ISOTOPE EFFECTS: TWO TYPES OF ACTIVE SITE WITHOUT INTERCONVERSION

X_{a0}	Sub-case	(k_{12}/k_{13}) calculated for	
		11.4°	43.2°
0.25	LL	1.0192	1.0191
	LS	1.0231	1.0229
0.50	LL	1.0187	1.0185
	LS	1.0187	1.0195
0.75	LL	1.0182	1.0178
	LS	1.0142	1.0156

have different Michaelis-Menten parameters. The same result is achieved when very different values are assigned to k_{3a} and k_{3b} , or, if they are equal at some one temperature, their activation energies are assumed to be very different; the effect of such characterizations is to reduce the model to the simple Michaelis-Menten form. The most uncertain element in the estimated isotopic specific rate constant ratios in Table VI is TIF, which was taken as approx. 1.012. A slightly lower value for TIF would bring the general predictions of this model into correspondence with the smaller isotope effects observed with Preparation XII₂-M, Table IC, and Preparation XIII₂, Table II.

Next we consider a variant of the KISTIAKOWSKY AND ROSENBERG "A" mechanism, subject to the limitation imposed by Eqn. 25; but it will be assumed that the activation energies E_{Aa} and E_{Ab} differ by 2.0 kcal/mole. The limitation and the assumption taken together imply the equilibrium

$$E_a \rightleftharpoons E_b; \Delta H = \pm 2.0 \text{ kcal/mole} \quad (29)$$

The values of k_{3a} and k_{3b} are chosen to maximize the effect of the site interconversion, (X_{a0}/X_{b0}) having reciprocal values at the two temperatures, and the larger of (k_{3a}/k'_{3a}) and (k_{3b}/k'_{3b}) is associated with the site species assigned the higher activation energy. In addition to the groupings of ratios of isotopic constants denoted by the symbols LL and LS, one must consider in this version of the model the situations in which the enthalpy change in Eqn. 29 is taken with positive (P) or negative (N) sign. It is not necessary to tabulate the results for the four cases PLL, PLS, NLL, and NLS, because they yield the same results: $(k_{12}/k_{13}) = 1.0187$ at 11.4° and $= 1.0183$ at 43.2°.

As in the variant of the "A" mechanism considered immediately above, this version accounts well for those sets of (k_{12}/k_{13}) results which show little change with temperature; application to normal temperature dependence requires assumption of virtual identity of the kinetic properties of the two types of enzymic site.

The condition imposed by Eqn. 25 is apparently too restrictive, and other temperature-dependent site-interconversion mechanisms can be devised which are not subject to that limitation; in effect, such mechanisms involve an enthalpy of conversion which is independent of the activation energies associated with the different site types. However, if the process of interconversion involves changes in the general or local conformation of the enzyme, or modest structural changes in the sites themselves, the associated enthalpy change cannot be too large. In characterizing such a model for the situation in urease it has been assumed that $E_{Ab} - E_{Aa} = 2.0$ kcal/mole, that $\Delta H = \pm 12.4$ kcal/mole, and matters have been adjusted so that $(X_{a0}/X_{b0}) = 3$ at one temperature and $= 1/3$ at the other, and k_{3a} has been taken equal to k_{3b} at the lower temperature. As above, these choices are made to secure the maximum influence on (k_{12}/k_{13}) of the interconversion of sites. The results of the calculations for this model are shown in Table VIII. It is apparent from consideration of these

TABLE VIII
CALCULATED ISOTOPE EFFECTS: TWO TYPES OF ACTIVE SITE WITH INTERCONVERSION

Case	(k_{12}/k_{13}) calculated for	
	11.4°	43.2°
PLL	1.0182	1.0191
PLS	1.0142	1.0229
NLL	1.0192	1.0178
NLS	1.0231	1.0156

figures that this model is capable of reproducing all of the observed varieties of temperature dependence of (k_{12}/k_{13}) except those which we designated composite. Close correspondence between the calculated results and the various sets of experimental observations may require adjustment of the several thermal and isotopic input parameters, but none of these adjustments is major. There does not appear to be any way of deciding at the present time whether the LL and LS pairings of the kinetic and substratum isotope effects are equally reasonable. This is a matter of some importance since it is the LS combination of constant ratios which yields the higher temperature dependence of (k_{12}/k_{13}) , whether its sense be normal or reverse.

Effects of inhibition

It was suggested in the first part of this DISCUSSION that one explanation for the observation of experimental activation energies slightly higher than those reported previously was the occurrence of partially non-competitive inhibition in the hydrolyses. The isotope effects furnish some information bearing on this possibility.

We have examined the several major classes of non-product, non-substrate inhibition which might apply in the enzymic hydrolysis of urea. The single new element involved in the analysis of the isotope effect consequences of inhibition arises in the

value to be assumed for the ratio of the isotopic inhibition constants K_I and K'_I , which are the dissociation constants, respectively, for the reactions



and



These reactions involve the binding to isotopic enzyme-substrate complexes of a single kind of inhibitor species, I. Even if the binding of the inhibiting substance occurs close to the active site, deviation of (K_I/K'_I) from unity would require that isotope effects be transmitted from the site of substratum to the inhibitor site. As stated in the argument concerning the isotopic case of KISTIAKOWSKY AND ROSENBERG's "B" mechanism, such transmission effects should be of second order or lesser importance; hence, $K_I = K'_I$. The result of this equality is to reduce all but one of the expressions for (k_{12}/k_{13}) where inhibition is included in the mechanism to the simple Michaelis-Menten form of Eqn. 10.

Where the inhibition is partially non-competitive, however, (k_3/k'_3) must be replaced by a ratio of effective rate constants defined as follows:

$$\frac{k_{\text{eff}}}{k'_{\text{eff}}} = \frac{k_3 + k_{3I}(\text{I})/K_I}{k'_3 + k'_{3I}(\text{I})/K_I} \quad (32)$$

where k_{3I} and k'_{3I} apply to the decomposition of EIS and EIS', and (I) is the inhibitor concentration. The effect of temperature on this ratio of effective rate constants depends upon the activation energy difference between k_3 and k_{3I} , the isotope effects associated with (k_3/k'_3) and (k_{3I}/k'_{3I}) , and the enthalpy of dissociation of the EIS complexes. Consistent with the reasoning detailed in earlier paragraphs, if I is truly an inhibitor the activation energy E_{AI} will be somewhat larger than E_A and (k_{3I}/k'_{3I}) will exceed (k_3/k'_3) . The temperature dependence of $(k_{\text{eff}}/k'_{\text{eff}})$ will thus always be normal in sense and intermediate in magnitude between that expected for (k_3/k'_3) and (k_{3I}/k'_{3I}) . Partially non-competitive inhibition cannot, therefore, be employed to explain isotope effect temperature dependences normal in sense but either abnormally large or small in size, reverse in sense, or zero

CONCLUSIONS

Kinetic and isotope effect data for 17 enzyme preparations are shown in Tables I-IV; for 16 of the preparations results are available for 11.4°. If the hydrolysis of urea catalyzed by urease were an uncomplicated reaction, the same value of (k_{12}/k_{13}) would have been obtained with each of the 16 preparations at 11.4°; such is not the case. The scattered values of k_{exp} for these same sets of experiments are not unexpected, because the gross enzyme concentration is implicit in k_{exp} ; however, concentration effects as such should not appear in (k_{12}/k_{13}) , nor have they ever been found in the analogous ratios obtained from investigations on thermal reactions of considerable variety. It is surprising that we found no appreciable effect of buffer type on the values of k_{exp} , because the values reported for k_3 in Tris-sulphuric acid and maleate-buffer systems by earlier workers^{8,9} stand in the ratio 10-30, depending upon the conditions of pH and temperature for which one chooses to make the comparison. The most likely interpretation of this fact is that the enzyme concentrations computed in the earlier studies were inconsistent with each other.

The results recorded for four preparations studied in both buffer systems (Preparation XII₂, Table IC; Preparation XIII₂, Table II; and Preparations XXI₁ and XXII₂, Table IV) show no effect of the buffer on k_{exp} , but the isotope effects are found to have all possible relations to each other: maleate higher than Tris-sulphuric acid, maleate lower than Tris-sulphuric acid, and maleate the same as Tris-sulphuric acid; further, in each particular buffer, the isotope effects associated with the several enzyme preparations are found to be high, low, and normal in magnitude, a fact which shows that the scattering of the isotope effects is not an artifact of the buffer chosen (and it must be recalled that the original investigators of the enzyme kinetics selected these buffers, for their general "good behavior" and specific lack of inhibitory character).

It is our opinion that the isotope effect results indicate a complexity of character of urease preparations which is not reflected strongly in the gross kinetic properties of such preparations. It seems likely that this complexity consists in there being in urease several molecular species, perhaps indistinct in the large, but different in the small. The extreme isotope effect temperature dependences seem to require inter-convertibility of the site types, but whether such processes involve conformational changes limited to the locale of the enzymic site or of a more general nature is not a fact derivable from the present experiments. It is reasonable that the distribution of urease species depend upon the nature of the buffer and the detail of each individual preparation and crystallization¹⁶⁻¹⁸, the latter because of possible interaction between urease and varying proportions of inert protein, if for no other reason. There is support for this view of urease in the results of recent experimental studies^{45, 46}, and it is similar in some ways to the "induced-fit" theory of KOSHLAND⁴⁷⁻⁴⁹.

It was pointed out in the INTRODUCTION that a primary object of isotope fractionation studies was TIF, the temperature-independent factor in a ratio of isotopic specific rate constants. It is apparent that the TIF (k_{12}/k_{13}) for a system such as urease-urea is a composite quantity. The information which TIF is expected to contain is the nature of the motion in the reaction coordinate leading to the products of the rate-determining step in a chemical process. Only by isolation of individual reaction paths in an enzymic reaction can meaningful TIF values be obtained. It would appear that the urease-urea system was not an admirable choice in this regard.

Investigations of the effects on both rate and isotope fractionation of variations (such as pH, ionic strength, etc.) in the reaction media may assist in the further elucidation of the nature of the enzyme catalyst. Such studies are in progress in this laboratory.

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REFERENCES

- ¹ J. BIGELEISEN AND M. WOLFSBERG, *Advances in Chemical Physics*, 1 (1958) 30.
- ² S. SELTZER, G. A. HAMILTON AND F. H. WESTHEIMER, *J. Am. Chem. Soc.*, 81 (1959) 4018.
- ³ J. L. RABINOWITZ, J. S. LEFAIR, H. D. STRAUSS AND H. C. ALLEN, JR., *Biochim. Biophys. Acta*, 27 (1958) 544.
- ⁴ J. A. SCHMITT, A. L. MYERSON AND F. DANIELS, *J. Phys. Chem.*, 56 (1952) 917.
- ⁵ G. D. FASMAN AND C. NIEMANN, *J. Am. Chem. Soc.*, 73 (1951) 1646.
- ⁶ G. B. KISTIAKOWSKY, P. C. MANGELSDORF, A. J. ROSENBERG AND W. H. R. SUTOW, *J. Am. Chem. Soc.*, 74 (1952) 5015.
- ⁷ J. L. RABINOWITZ, T. SALL, J. N. BIERLY, JR. AND O. OLEKSYSHYN, *Arch. Biochem. Biophys.*, 63 (1956) 437.
- ⁸ M. C. WALL AND K. J. LAIDLER, *Arch. Biochem. Biophys.*, 43 (1953) 299, 307, 312.
- ⁹ G. B. KISTIAKOWSKY AND A. J. ROSENBERG, *J. Am. Chem. Soc.*, 74 (1952) 5020.
- ¹⁰ J. BIGELEISEN, *J. Chem. Phys.*, 17 (1949) 675.
- ¹¹ J. BIGELEISEN AND M. WOLFSBERG, *J. Chem. Phys.*, 21 (1953) 1927; 22 (1954) 1264.
- ¹² H. S. JOHNSTON, W. A. BONNER AND D. J. WILSON, *J. Chem. Phys.*, 26 (1957) 1002.
- ¹³ J. BIGELEISEN, *J. Phys. Chem.*, 56 (1952) 823.
- ¹⁴ J. B. SUMNER, *J. Biol. Chem.*, 69 (1926) 435.
- ¹⁵ A. L. DOUNCE, *J. Biol. Chem.*, 140 (1941) 307.
- ¹⁶ J. B. SUMNER AND I.-B. ERIKSSON-QUENSEL, *J. Biol. Chem.*, 125 (1938) 37.
- ¹⁷ A. D. McLAREN, E. SHEPPARD AND J. WAGMAN, *Nature*, 162 (1948) 370.
- ¹⁸ A. J. SOPHIANOPOULOS, *Dissertation*, Purdue University, 1959.
- ¹⁹ J. B. SUMNER AND V. A. GRAHAM, *Proc. Soc. Exptl. Biol. Med.*, 22 (1925) 504.
- ²⁰ J. F. AMBROSE, G. B. KISTIAKOWSKY AND A. G. KRIDL, *J. Am. Chem. Soc.*, 73 (1951) 1232.
- ²¹ J. Y.-P. TONG AND P. E. YANKWICH, *J. Phys. Chem.*, 61 (1957) 540.
- ²² P. E. YANKWICH AND R. L. BELFORD, *J. Am. Chem. Soc.*, 75 (1953) 4178.
- ²³ P. E. YANKWICH AND R. L. BELFORD, *J. Am. Chem. Soc.*, 76 (1954) 3067.
- ²⁴ A. E. FRANKLIN AND J. H. QUASTEL, *Science*, 110 (1949) 447.
- ²⁵ P. E. YANKWICH AND A. E. VEAZIE, *J. Am. Chem. Soc.*, 80 (1958) 1835.
- ²⁶ P. E. YANKWICH AND R. M. IKEDA, *J. Am. Chem. Soc.*, 82 (1960) 1891.
- ²⁷ J. B. S. HALDANE, *Enzymes*, Longmans, Green and Co., London, 1930, p. 82.
- ²⁸ M. B. THORN, *Nature*, 164 (1949) 24.
- ²⁹ R. J. FOSTER AND C. NIEMANN, *J. Am. Chem. Soc.*, 73 (1951) 1552.
- ³⁰ G. J. BUIST AND M. L. BENDER, *J. Am. Chem. Soc.*, 80 (1958) 4308.
- ³¹ G. A. ROPP AND V. F. RAAEN, *J. Chem. Phys.*, 22 (1954) 1223.
- ³² E. M. MAGEE AND F. DANIELS, *J. Am. Chem. Soc.*, 79 (1957) 829.
- ³³ G. A. RUSSELL, *J. Am. Chem. Soc.*, 79 (1957) 3871.
- ³⁴ G. A. ROPP, C. J. DANBY AND D. A. DOMINEY, *J. Am. Chem. Soc.*, 79 (1957) 4944.
- ³⁵ K. J. LAIDLER, *The Chemical Kinetics of Enzyme Action*, Oxford, 1958, p. 192.
- ³⁶ K. MYRBÄCK, *Acta Chem. Scand.*, 1 (1947) 142.
- ³⁷ C. NIEMANN AND G. E. HEIN, private communication.
- ³⁸ J. BIGELEISEN, *Can. J. Chem.*, 30 (1952) 443.
- ³⁹ H. C. UREY, *J. Chem. Soc.*, (1947) 562.
- ⁴⁰ J. BIGELEISEN AND M. G. MAYER, *J. Chem. Phys.*, 15 (1947) 261.
- ⁴¹ J. BIGELEISEN, *J. Chem. Phys.*, 23 (1955) 2264.
- ⁴² P. E. YANKWICH AND R. M. IKEDA, *J. Am. Chem. Soc.*, 81 (1959) 5054.
- ⁴³ P. E. YANKWICH, R. L. BELFORD AND G. FRAENKEL, *J. Am. Chem. Soc.*, 75 (1953) 832.
- ⁴⁴ G. B. KISTIAKOWSKY AND R. LUMRY, *J. Am. Chem. Soc.*, 71 (1949) 2006.
- ⁴⁵ E. L. KUFF, G. H. HOGEBOM AND M. J. STRIEBICH, *J. Biol. Chem.*, 212 (1955) 439.
- ⁴⁶ J. M. CREETH AND L. W. NICHOL, *Biochem. J.*, 77 (1960) 230.
- ⁴⁷ D. E. KOSHLAND, *Proc. Natl. Acad. Sci. U.S.A.*, 44 (1958) 98.
- ⁴⁸ J. A. THOMA AND D. E. KOSHLAND, *J. Am. Chem. Soc.*, 82 (1960) 3329.
- ⁴⁹ D. E. KOSHLAND, *J. Cellular Comp. Physiol., Suppl.* 1, 54 (1959).