months thereafter this enzyme sample gave reproducible results, some of which were shown among the present data. In fact, all the seven enzyme preparations used in these and related experiments gave completely consistent results after aging.

These observations suggested that one might be dealing with a mixture of two ureolytically active proteins, one of which was comparatively unstable under conditions of storage. If so, their sensitivity to rapid irreversible denaturation might be expected to be different. Experiments were made in which the enzyme was denatured thermally until only *ca.* 10% of original activity remained. This was done by heating an aged enzyme sample, buffered at *p*H 7, to 96° for six minutes. Then a portion of an un-aged enzyme was denatured by adding it to a strong solution of hydrochloric acid. Measurable reaction was observed at *p*H 2.2 at 25°, the order of the reaction being about 12 in hydrogen ion concentration. The kinetic parameters of urea hydrolysis were then determined, using the active residues from the denaturation treatments. They were found to be identical with those of the untreated enzymes. The hypothesis of several distinct active proteins in the original samples is thus very improbable. It does not seem profitable in view of this to speculate on the nature of the aging effect. It may be significant, however, that Fasman used freshly prepared enzyme solutions throughout his experiments. It is conceivable that the same factor which caused a slight initial irreproducibility of the present experiments in maleate buffers at very low urea concentrations also affected some of the Fasman and Niemann results in more concentrated urea and phosphate buffers.

CAMBRIDGE, MASS.

[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY]

The Kinetics of Urea Hydrolysis by Urease[1]

BY G. B. KISTIAKOWSKY AND ARTHUR J. ROSENBERG

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The rate of hydrolysis has been measured over about 5000-fold urea concentration range at several *p*H. It was found in agreement with earlier work that the rate first increases, reaches a maximum and then slowly falls off with rising urea concentration. The latter effect is best interpreted as an inhibition by urea. The Michaelis–Menten mechanism of enzymatic catalysis does not fit the data accurately at lower concentrations. An excellent fit is obtained by three-parameter equations which can be derived from the assumption that two types of Michaelis–Menten sites are present or that pairs of identical sites mutually influence their kinetic parameters. One of the parameters of these equations is found to be invariant to changes in *p*H and to addition of dioxane. The other parameters all show very similar dependence on these variables. The relation of the present data to those of earlier workers and their kinetic significance are discussed.

The kinetics of the enzymatic hydrolysis of urea have been the subject of several investigations, most of which were carried out in phosphate buffers. The demonstration of the complex nature of competitive inhibition[2] of urease by components of phosphate buffers has invalidated most of the quantitative conclusions about reaction mechanism drawn from experiments using this buffer. It appears, however, that the kinetics are consistent with the Michaelis–Menten mechanism,[3] except that at very high urea concentrations the rate falls off instead of approaching a limiting value.[4] The Michaelis constant is of the order of 2–3 m*M*,[5] the activity of urease passes through a maximum between *p*H 6.7 and 7, and the temperature coefficient at high urea concentration corresponds to an activation energy of 8000 cal.[6,7]

In the preceding paper[8] it was shown that the effects of alkali maleates and certain other electrolytes are slight and non-specific, being in the nature of the salt effect in ionic reactions. A study of the kinetics in such buffers might well reveal the reaction mechanism, which is of considerable interest because of the extreme specificity of urease.

Experimental Details

The experiments were carried out by the technique recently described.[8] Since the present experiments deal largely with the effect of urea on the rate of hydrolysis, extensive tests were made to prove the independence of the analytical results from the urea concentration. Some of these tests have already been outlined.[8] Moreover, it was found that the analytical results remained unchanged when urea was added to the reaction mixture after acidification, but prior to the passing of the solution through the ion exchange resin bed. Hence it is believed that the analytical procedure introduced no errors greater than those evidenced by random variations. Their magnitude is shown by the following data: thirty-one runs at *p*H 7 in 0.05 *M* sodium maleate buffer using 0.65, 1.30 and 16.7 m*M* urea, which were spread over a period of more than six months and involved three enzyme preparations and different batches of all the chemicals, gave a standard deviation of a single measurement equal to 3.7%. If three runs, in which the rate was lower by about 12%, are excluded from this calculation, the standard deviation reduces to 1.9%. Occasional rates considerably lower than normal occurred throughout this research, with an average frequency of less than 10%. Unintentional deactivation of the enzyme or its inhibition by accidentally introduced heavy metal ions appear to be the most probable explanations. To protect the results from these sources of errors, all measurements were made at least in duplicate. Altogether use was made of four preparations of urease, differing in activity from more than 60,000 S.U. per gram to considerably less than 10,000. No difference between the results obtained with these

(1) This work was made possible by a grant from the American Cancer Society to which the authors wish to express their sincere gratitude.

(2) G. D. Fasman and C. Niemann, THIS JOURNAL, **73**, 1646 (1951).

(3) J. B. Sumner and S. F. Howell, *J. Biol. Chem.*, **104**, 619 (1934).

(4) (a) J. B. Sumner and G. F. Somers, "The Chemistry and Methods of Enzymes," Academic Press, Inc., New York, N. Y., 1947; (b) K. J. Laidler and J. P. Hoare, THIS JOURNAL, **71**, 2699 (1949).

(5) K. M. Harmon and C. Niemann, *J. Biol. Chem.*, **177**, 601 (1949).

(6) I. W. Sizer, *J. Gen. Physiol.*, **22**, 719 (1939).

(7) G. B. Kistiakowsky and R. Lumry, THIS JOURNAL, **71**, 2006 (1949).

(8) G. B. Kistiakowsky, Paul C. Mangelsdorf, Jr., Arthur J. Rosenberg and W. H. R. Shaw, *ibid.*, **74**, 5015 (1952).

samples could be detected. Aliquots from the same dilute solution of the enzyme were used over a period of several hours. The reproducibility of the results within each set of runs indicated the stability of such dilute solutions over the limited time intervals involved. In the following the data are given as ratios of observed rates to those obtained with an aliquot of the same dilute enzyme solution under standard conditions, namely, 33.3 mM urea at pH 7 in 0.1 M sodium maleate buffer at 25°. The reproducibility of these relative rates, when compared over a period of months and obtained with different preparations of the enzyme and with different batches of the other chemicals, was as good as the reproducibility of absolute rates within each set of runs. This, we believe, rules out the possibility that the results presented below are distorted through inhibition by trace impurities.

The Results and Discussion

The use of a sensitive and accurate analytical technique,[8] unaffected by the concentration of urea or of buffer salts, made it possible to extend the observations over a very wide range of urea concentrations. The hydrolysis of urea during each run was so slight that it had to be allowed for only at the lowest urea concentrations. Under these conditions there was no observable inhibition by the products,[9] inasmuch as the rate did not change with the progress of hydrolysis.[8]

Numerous results obtained under a variety of experimental conditions are presented in condensed form in Table I. Table II shows the data at pH 7 and 25° in more detail. These data reproduce the general features reported by others: rise of rate with increasing urea concentration and then a gradual fall.

TABLE I

THE DEPENDENCE OF THE RELATIVE RATE OF HYDROLYSIS ON UREA CONCENTRATION

The concentrations of urea are in millimoles/liter and are the mean values during the run. All rate values are averages of two or more measurements, the standard deviation of individual measurements being about 2%.

Solvent	H_2O	H_2O	H_2O	H_2O	H_2O–$C_4H_8O_2$	H_2O
Temp., °C.	25	25	25	25	25	9.9
pH	5.43	6.00	6.50	7.48	6.70	7.00
Ionic strength, M	0.10	0.10	0.10	0.10	0.07	0.12
Urea concn.						
0.315		0.129	0.136			
.348					0.083	
.576					.128	
.640	0.177	.220	.243	0.218		.119
1.29	.270	.329	.381	.342	.206	.186
3.27	.397	.523	.640	.537	.345	.298
5.30	.475					
6.61						.372
8.30	.536		.886	.750	.479	
10.0						.413
16.7	.615	.814	1.03	.853	.542	.457
33.3	.647	.940	1.14	.971	.573	.493
53.3				.995		
58.3	.665					
66.7	.690				.585	
83.3	.721	.970		1.02		.528
125	.700			1.05		
167	.723			1.07		.532
333	.725			1.05		.510
500	.689			1.00		.495
667				0.962		
750	.685					
833						.448
1000				.897		
1200	.610					
1333				.800		.395
1600	.56					
1830				.685		
2000	.50					

TABLE II

THE DEPENDENCE OF THE RATE OF HYDROLYSIS ON UREA CONCENTRATION AT 25°, pH 7.00, AND 0.10 M IONIC STRENGTH

Urea concn., mM	Relative rate	Rate cor. by eq. (2)	Calcd. by eq. (3)	Calcd. by eq. (5)
0.320	0.130		0.137	0.126
0.646	.226		.232	.223
1.29	.362		.366	.359
3.27	.600		.598	.596
8.30	.846		.830	.825
16.7	.975		.966	.965
33.3	1.03		1.06	1.07
58.3	1.15		1.11	1.12
83.3	1.16	1.17	1.13	1.14
125	1.18	1.20	1.15	1.15
167	1.15	1.17	1.16	1.17
250	1.15	1.19	1.17	1.18
333	1.13	1.19	↓	↓
416	1.14	1.22	↓	↓
500	1.11	1.21	↓	↓
583	1.08	1.19	↓	↓
667	1.07	1.21	(1.20)	(1.20)
833	1.00	1.17		
1000	0.99	1.22		
1333	0.91	1.23		

As already pointed out by Laidler and Hoare,[4b] the fall-off of rates at high urea concentrations is too pronounced to be explained by a decrease in the activity of water, but can be interpreted as an inhibition by urea. A very good fit to the present data is obtained by an empirical equation

$$1/V_m = 1/V_{obsd} - K(S)^{1.4} \qquad (1)$$

where S stands for the concentration of urea, V for the rate of hydrolysis, and V_m for the limiting rate at high urea concentrations in absence of the fall-off. An equally good fit is obtained by an equation derived from the inhibition mechanism

$$E + nS \rightleftharpoons ES_n;\ K_1^i$$
$$E + mS \rightleftharpoons ES_m;\ K_2^i$$

where K_1^i and K_2^i are inhibition equilibrium constants

$$\frac{1}{V_m} = \frac{1}{V} - K_1^i(S)^{n-1} - K_2^i(S)^{m-1} \qquad (2)$$

To fit this equation to experimental data it is necessary to determine the value of V_m. This can be done very readily by successive approximations, since V_m differs from the observed maximal rate by not more than a few per cent. The results are shown in Fig. 1, the large scatter of points being due to the coördinate system chosen and the small extent of inhibition. The trend of the experimental points with urea concentration is representable by a straight line with a finite intercept. It follows that

(9) J. P. Hoare and K. J. Laidler, THIS JOURNAL, **72**, 2487 (1950).

two inhibitory reactions must be postulated, one involving two molecules of urea combining with the active site and the other three. The magnitude of the cubic term is in doubt because of the uncertainty as to: (a) whether the concentration or the activity[10] of urea should be used in the kinetic equation; (b) how the concentration of water is involved; and (c) the effect of the dielectric constant of the medium on the activity of urease. Moreover, it is possible that the fall-off at the highest urea concentration is related to the well known denaturing effect of urea on proteins. Within the range of urea concentrations here involved, the dielectric constant varies from 81 to 86.[11] A substantial dependence of ureolytic activity on dielectric constant is suggested by the observations in mixed solvents. At *p*H 6.7 and an ionic strength of 0.12 *M* the rate was found to be reduced by a factor of two in the presence of 25% by volume (3 mole per cent.) of dioxane. In a similar mixture with ethylene glycol the rate was reduced by a factor of 1.6. The dielectric constant of the former mixture is 55,[12] that of the latter is presumably somewhat higher. The enzyme showed no irreversible denaturation in these mixed solvents. The dependence of the rate on *p*H in 0.05 *M* sodium maleate was similar to that observed in aqueous solutions, the maximum being at *p*H 6.7.

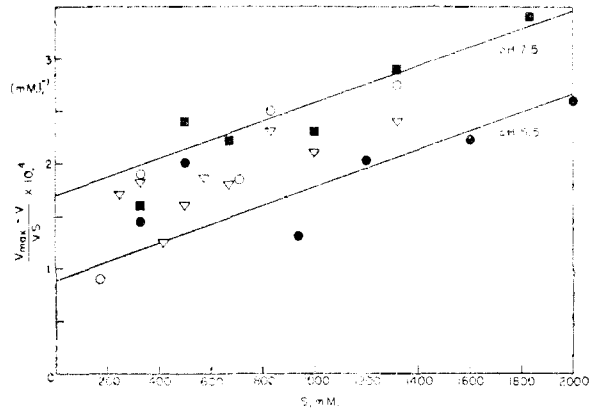

Fig. 1.—Inhibition of the rate of hydrolysis by high concentrations of urea: solid circles, *p*H 5.5; triangles, *p*H 7; open circles, *p*H 7 at 10°; solid squares, *p*H 7.5.

The plot of Fig. 1 permits a reliable calculation of the maximal rate, V_m, in absence of inhibition at very high urea concentrations. Inhibition is seen to become insignificant when urea concentration drops to *ca.* 100 m*M*. Therefore, the rates at lower concentrations can be accurately corrected for this inhibition. Such corrected rates are shown in column 3 of Table II and are suitable for a test of the Michaelis–Menten mechanism. The conclusion reached from such a test is that under no experimental conditions tried does it fit completely the data. They can be described quite well (Table II, column 4) by an empirical equation

$$V = \frac{k(E_0)(S)^n}{K + (S)^n} \qquad (3)$$

(10) G. Scatchard, W. J. Homer and S. E. Wood, THIS JOURNAL, **60**, 3061 (1938).

(11) R. Fürth, *Ann. phys. Chem.*, **70**, 69 (1923).

(12) H. Hartmann, *Z. physik. Chem.*, **191A**, 157 (1942).

wherein the fractional exponent n varies according to conditions of *p*H and temperature. This is demonstrated by Fig. 2, wherein the quantity $\log [S(V_m - V)/V]$ has been plotted against $\log S$. According to the Michaelis–Menten mechanism the above quantity should be independent of urea concentration. If the more general equation (3) applies, the data should lie on a straight line, whose slope is equal to $1 - n$. The experimental data are presented in the form of vertical lines, whose length is equal to twice the standard deviation of each measurement. The lines are longer at one end of the graph because of the coördinate system chosen. It is seen that all seven conditions studied give deviations from the Michaelis–Menten mechanism in the same direction. The exponents of equation (3) vary from 0.8 to 0.9. An error of a few per cent. in the values of V_m would cause the experimental points to fall on a curve, but would not make the quantity $S(V_m - V)/V$ independent of urea concentration. The independence of the relation between the rate and urea concentration from the concentration of the buffers[8] eliminates the latter as the source of the trends in Fig. 2. Accidental inhibition by impurities can be ruled out because of: (a) the reproducibility of the results over the entire period of this investigation, and (b) the several tests discussed in the experimental sections.[8]

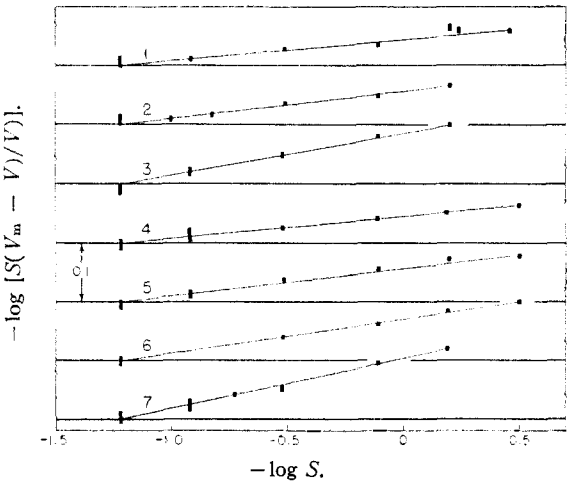

Fig. 2.—A test of the Michaelis–Menten equation. Substrate concentration in millimoles/l. The vertical distance between solid lines is 0.1 log unit. Line 1 refers to data at *p*H 6.7, 25° in presence of 25% dioxane; line 2, *p*H 7.0 at 10°, in water; line 3, *p*H 7.5 at 25°; line 4, *p*H 7.0 at 25°; line 5, *p*H 6.5 at 25°; line 6, *p*H 6.0 at 25°; and line 7, *p*H 5.4 at 25°.

It has not been possible to derive equation (3) on the basis of a kinetic mechanism involving mass action law for individual reactions. The following considerations, however, lead to useful information about the mechanism of urea hydrolysis. It was shown[8] that the hydrolysis is strictly linear in the concentration of the enzyme. Hence the general rate expression derivable from a mechanism involving parallel and consecutive elementary steps obeying the mass action law must take the form of the quotient of two algebraic polynominals in urea

concentration. The numerator must start with the linear term and the denominator with a zero order term, to express the general observed features of the rate expression embodied in equation (3). The retention of the linear terms leads, of course, to the Michaelis–Menten equation. The next more complex expression involves quadratic terms in urea concentration. Among several mechanisms which lead to expressions of this type are two which are closely related to the Michaelis–Menten scheme. They involve: (a) two types of active sites on the enzyme, differing in their Michaelis–Menten rate parameters, and (b) 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. This latter hypothesis is analogous to the currently favored treatment[13] of the equilibrium between oxygen, hemoglobin and oxyhemoglobin. Applied to urease, the two mechanisms are

$$\text{E} + \text{S} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{ES} \xrightarrow{k_3} \text{E} + \text{P}$$

(a)

$$\text{E}' + \text{S} \underset{k_2'}{\overset{k_1'}{\rightleftarrows}} \text{E}'\text{S} \xrightarrow{k_3'} \text{E}' + \text{P}$$

$$\text{E}_2 + \text{S} \underset{k_2}{\overset{2k_1}{\rightleftarrows}} \text{E}_2\text{S} \xrightarrow{k_3} \text{E}_2 + \text{P}$$

(b)

$$\text{E}_2\text{S} + \text{S} \underset{2k_2'}{\overset{k_1'}{\rightleftarrows}} \text{E}_2\text{S}_2 \xrightarrow{2k_3'} \text{E}_2\text{S} + \text{P}$$

The usual steady-state treatment leads to the equations

$$V = \frac{1}{2}\frac{[k_3(E_0)K_A' + k_3'(E_0')K_A](S) + [k_3(E_0) + k_3'(E_0')](S)^2}{(S)^2 + (S)[K_A + K_A'] + K_AK_A'} \quad (4a)$$

$$V = \frac{k_3(E_0)(S)[K_B' + (k_3'/k_3)(S)]}{(S)^2 + 2(S)K_B' + K_BK_B'} \quad (4b)$$

(E_0) stands here for the total concentration of active sites, free and combined with urea (S). The constants K_A, etc., are Michaelis constants defined by $K = (k_2 + k_3)/k_1$. The equations are mathematically identical, the difference being in the physical interpretation of the four parameters of each equation. Their form is such that it has not been possible to devise a satisfactory procedure for fitting them to the experimental data. To do so it would be necessary to extend the measurements to much lower urea concentrations. However, the good fit of equation (3) to experimental data around the point $V/V_m = 1/2$ means that $k_3E_0 = k_3'E'$ in (4a) or $k_3 = k_3$ in (4b). This has been confirmed by a trial and error fitting of equation (4), which gave acceptable results centering around the same selection of the above parameters. Thus equations (4) reduce to the form

$$V = \frac{k_3(E_0)(S)\left[\frac{K_A + K_A'}{2} + (S)\right]}{(S)^2 + (S)[K_A + K_A'] + K_AK_A'} \quad (5a)$$

$$V = \frac{k_3'(E_0)(S)\,[K_B' + (S)]}{(S)^2 + 2(S)K_B' + K_BK_B'} \quad (5b)$$

(13) See J. Wyman, Jr., *Advances in Protein Chem.*, **4**, 407 (1948).

The fitting of these three-parameter equations has been carried out by a method entirely analogous to that employed in the analysis of the hemoglobin–oxyhemoglobin equilibria.[4a] It is based on the circumstance that when the parameters of equation (3) are so chosen that best fit is obtained around the point $V/V_m = 1/2$, the parameters of equations (5) become uniquely related to them. The results of such an analysis are shown in column 5 of Table II. The deviations between the calculation and experiment are no larger than random experimental errors. To cover the entire experimental range it becomes necessary to add inhibition by urea to mechanisms (a) or (b) which results in higher terms in (S) of the denominator of equation (5). Therefore at least four parameters are needed to describe the data over the whole range of about 5,000-fold variation in urea concentration, which is not altogether satisfactory. It was felt that if the parameters involved showed widely different dependence on *p*H and temperature and were randomly affected by the addition of dioxane, the suggested mechanisms would not be tenable. Fortunately, this is not the case, as the following Table III shows. It is a summary of all kinetic parameters, derived by the application of equations (2), (3), (4a) and (4b) to the data of Tables I and II. Prior to considering their dependence on the above mentioned variables, some comments on the limits of errors and their relation to previous work are in order.

As Fig. 1 shows, the accuracy of the determination of the quadratic inhibition constant of equation (2) is poor, but the trend to higher inhibition with rising *p*H is definite. The experimental data are inadequate to decide on the trends in the cubic inhibition constant. Temperature has no sigficant effect on either of the inhibition constants. The indicated limits of error in the exponent n and in $S'^{1/2}$, that urea concentration at which the rate has decreased to $1/2V_m$, are approximately twice the standard deviation. The same applies to the uncertainties indicated for the values of the relative V_m. This maximal rate has been shown[8] not to depend on ionic strength below *p*H 7 when the ionic strength was less than 0.2 *M*. At higher *p*H a dependence was observed and therefore V_m has also been given for very low ionic strength, the extrapolation being made with the aid of Fig. 1 of the preceding paper.[8] A consequence of this ionic effect is that the *p*H of optimal activity, which has been assumed to be at approximately 6.7 according to the work of Sumner,[4a] is actually between 7 and 7.5 in very dilute ionic solutions.

Equation (5b) was chosen to be fitted to experimental data and therefore the uncertainties, equal to approximately twice the standard deviation, are given only for its kinetic parameters. The parameters of equation (5a) were derived from those of equation (5b) by an obvious algebraic procedure and the uncertainties are of comparable relative magnitude.

The observed trend of V_m with *p*H agrees semiquantitatively with that reported by Sumner,[4a] but the decrease he observed in citrate buffer at lower *p*H was somewhat steeper than shown by

TABLE III

THE KINETIC PARAMETERS OF UREA HYDROLYSIS BY UREASE

Solvent	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O–$C_4H_8O_2$
Temp., °C.	25	25	25	25	25	9.9	25
*p*H	5.43	6.0	6.5	7.0	7.48	7.0	6.7
K_1 (mM)	0.9×10^{-3}			1.2×10^{-4}	1.6×10^{-4}	1.4×10^{-4}	
K_2^1 (mM)	$.9 \times 10^{-3}$			0.9×10^{-7}	1×10^{-7}	0.9×10^{-7}	
n, eq. (3)	.77 ± 0.03	0.80 ± 0.025	0.86 ± 0.02	0.88 ± 0.025	0.80 ± 0.03	0.90 ± 0.025	0.86 ± 0.02
$S_{1/2}$, mM, eq (3)	2.70 ± .2	2.95 ± .15	3.40 ± .10	3.30 ± .15	3.45 ± .15	2.65 ± .15	3.1 ± .10
V_{max}; $\mu = 0.12\ M$	0.76 ± .02	1.00 ± .02	1.29 ± .02	1.19 ± .02	1.10 ± .02	0.54 ± .01	0.66; $\mu = 0.07$
V_{max}; $\mu \rightarrow 0$	.76	1.00	1.29	1.52 ± .04	1.45 ± .04		
K_A, mM	1.00	1.13	1.49	1.60	1.32	1.40	1.42
K_A', mM	7.63	7.77	7.53	6.80	9.04	5.96	6.78
K_B, mM	1.72 ± 0.13	1.97 ± 0.10	2.56 ± 0.10	2.59 ± 0.13	2.30 ± 0.2	2.24 ± 0.16	2.34 ± 0.20
K_B', mM	4.30 ± 0.33	4.45 ± 0.26	4.51 ± 0.20	4.20 ± 0.25	5.18 ± 0.4	3.18 ± 0.32	4.10 ± 0.35

Table III. His data indicated that optimum *p*H was slightly raised by the lowering of urea concentration,[4a] whereas the meaning of the data of Table III is precisely the opposite. Sumner's measurements, however, were made at only two urea concentrations and one of them was in the range where substantial inhibition (Fig. 1) is observed; the discrepancy appears to be within the combined experimental error.

Laidler and Hoare[4] found inhibition at high urea concentrations to be linear in urea concentration, whereas the present results indicate a more complex relationship. Their experiments were carried out in a phosphate buffer and the inhibition[5] by the buffer altered the relation between their inhibition constant and the true Michaelis constant. The present data show that the inhibition constant is comparatively very small. Whether the inhibition is due to a competition between urea and water, as Laidler and Hoare proposed, or to other causes, will be considered in a subsequent paper.

The $S_{1/2}$ observed presently agrees very well with that deduced by Harmon and Niemann[5] through extrapolation to zero concentration of phosphate buffers at *p*H 7.

None of the earlier experiments with urease extended to low enough concentrations of urea to detect deviations from the Michaelis–Menten mechanism. The present experiments show the existence of such deviations, although they do not lead to a unique identification of their cause. The identity of kinetic parameters obtained by the use of several preparations of the enzyme differing widely in purity and their invariance to extensive heat and acid denaturation, reported previously,[8] eliminate the possibility that protein or other impurities were the cause of the complex nature of the kinetics. If the objective of a kinetic analysis is the interpretation of experimental data with the least number of adjustable parameters, then equations (5a) and (5b) would appear to be the first choice. In the case of hemoglobin, because the affinity of the second site for oxygen is enhanced by the reaction of the first site, only the interaction hypothesis explains the experimental dissociation curves. In the case of urease the interaction is of opposite sign and therefore the hypothesis of different sites cannot be excluded.

It appears that the deviations from the Michaelis–Menten mechanism are not limited to urease. Thus Kauzmann, Chase and Brigham[14] interpreted their data on the catalysis of luciferin oxidation by luciferase in terms of this mechanism. Actually seven sets of their data out of eight show very systematic deviations from the straight lines of the Lineweaver–Burk plots. The fit of equation (3) to the experimental points is very good and exponents n so calculated are of the order of 0.8. This is, of course, no proof that equation (5b) describes their data but is at least suggestive.

The empirical rate expressions for these two enzymes may also be interpreted by more radical departures from the Michaelis–Menten mechanism. Both enzymes catalyze reactions between two substrates, water and urea in one case, luciferin and the oxidizing agent in the other. The Michaelis–Menten mechanism is an assymetric one, in that one of the reactants (*e.g.*, water) is assumed to be involved in only one step of the over-all reaction sequence. This notion may be discarded and the enzyme may be assumed capable of reversibly forming compounds with either of the two reactants. These compounds may then combine reversibly with the other reactant. The double adduct is assumed to undergo the final reaction, yielding products and enzyme. The calculations of Mr. P. C. Mangelsdorf, Jr., in this Laboratory show that such a mechanism can formally explain our observations. The number of *ad hoc* assumptions involved, however, is disconcertingly large.

A very interesting correlation between the numerical magnitude of the several kinetic parameters and *p*H emerges from Table III. The smaller of the two K's, both in (5a) and (5b), are definitely dependent on *p*H, whereas the larger ones (K') are invariant to such changes and are even not effected by the addition of dioxane. Moreover, the changes of the smaller K with *p*H and the corresponding changes of the quadratic inhibition constant are very nearly proportional to changes of V_m. It thus appears that a unique mechanism could be made responsible for the changes of all these parameters with *p*H. Thus a complete analysis of urease kinetics by means of a limited number of adjustable parameters and the mass action law may be feasible. An attempt in this direction will be presented in a subsequent paper from this Laboratory.

From the data of Table III the activation energy of V_m is found to be 8850 ± 200 cal., which is in very satisfactory agreement with some of the previous work.[6,7] Sumner[4a] has determined the turnover number of nearly pure urease under controlled conditions and enough information is avail-

(14) W. J. Kauzmann, A. M. Chase and E. H. Brigham, *Arch. Biochem.*, **24**, 281 (1949).

able from his own work and the data of previous papers to convert his specific activity to that in maleate buffer at *p*H 7 and 25°. He has given[3] the molecular weight of urease as 480,000 and evidence has been presented[15] that such a molecule contains four active sites. Thus the expression for k_3' per active site is found to be $k_3' = 7 \times 10^9 \exp(-8850/RT)$ sec.$^{-1}$. This is written as a unimolecular rate constant since the kinetic function of water is not known. The numerical magnitude of the pre-exponential factor is somewhat unusual, being much smaller than the median value for reactions involving small molecules.

From the data on the temperature dependence of the Michaelis constants in Table III the following expressions are readily obtained

$\ln K_B = (-6.5 \pm 5)/R - (1600 \pm 1500)/RT$ and
$\ln K_B' = (-0.5 \pm 5)/R - (3100 \pm 1500)/RT$

There has been considerable discussion in the literature concerning the kinetic meaning of the Michaelis constant. On the first glance the invariance of K' to changes in *p*H and solvent, as compared with substantial changes in V_m, suggests that $k_2' > k_3'$ and therefore that K' has the meaning of a thermodynamic equilibrium constant k_2'/k_1'. By the same argument, then, $K = k_3/k_1$. This is, of course, not impossible but another interpretation appears more likely. The values of V_m here reported are relative to the rates measured under a standard set of conditions. Hence $V_m = k_3'(E_0)/(k_3'(E_0))_{standard}$; it involves not only the dependence of k_3' but also that of (E_0) on such variables as *p*H, temperature, etc. It seems rather arbitrary to assume that the latter dependence is nil. Acid–base ionization equilibria[16] may result in a fraction of the catalytic sites being inactive. These equilibria may be the entire cause of the *p*H dependence of V_m, in which case the previous argument as to the nature of K' is invalid and the comments on the temperature dependence of V_m may have to be revised. It is clear that the interpretation of the nature of the Michaelis constant must await the elucidation of the complete mechanism of urea hydrolysis by urease.

(15) J. F. Ambrose, G. B. Kistiakowsky and A. G. Kridl, THIS JOURNAL, **73**, 1232 (1951).

(16) L. Michaelis, *Biochem. Z.*, **33**, 182 (1911).

CAMBRIDGE, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF COLORADO]

Mechanisms of Elimination Reactions. VII. The Alkaline Dehydrohalogenation of Chloro- and Bromo-Maleate and Fumarate[1]

BY STANLEY J. CRISTOL AND ARTHUR BEGOON[2]

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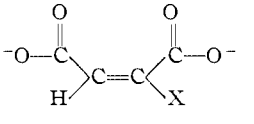
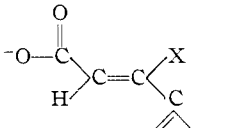
The kinetics of the alkaline dehydrohalogenation in water and in aqueous ethanol of the halofumarate ions and halomaleate ions have been studied. The determinations of the reaction order and of the effect of ionic strength upon rate constant have been interpreted to indicate that the reaction is between the bivalent ion of the salt and hydroxide ion. The extent of the superiority of *trans* over *cis* elimination has been measured, and this has been considered in terms of coulombic repulsions and in terms of mechanistic differences between *cis* and *trans* elimination.

In many bimolecular elimination reactions, a stereochemical preference is observed, *trans* substituents being more readily removed than corresponding *cis* substituents.[3] This paper is a continuation of a program relating to the factors contributing to relative reactivities in *cis–trans* systems. Two factors have been suggested as being of particular importance, one[4] involving *cis* repulsions and one[3,5] being based upon a postulated concerted one-stage mechanism for the *trans* process and a multiple-stage mechanism for the *cis* process.

As further tests of the relative importance of these factors, it seemed worthwhile to study the alkaline dehydrohalogenation of halomaleates (I) and halofumarates (II). Here, assuming that the attack is by hydroxide ion upon the hydrogen atom of the bivalent ion, it is seen that electrostatic repulsion will be greater between the negative hydroxide ion and the carboxylate group (which



I



II

bears a whole negative charge) than between the hydroxide ion and the negative end of a carbon–halogen dipole. Thus if electrostatic repulsions were the determining factor in *trans vs. cis* elimination, the formation of sodium acetylenedicarboxylate from sodium halomaleate might be expected to be more rapid than from sodium halofumarate.

Michael[6] has reported that chlorofumaric and bromofumaric acids lose hydrogen halide more rapidly than the corresponding halomaleic acids upon treatment with excess aqueous alkali. In view of the fact that the argument given above is based upon reaction of the dicarboxylate ion, rather than upon either of the acid salts or the free acid with hydroxide ion, it seemed desirable to study the reaction more extensively than did Michael. Accordingly we have determined the

(1) Previous paper in series: S. J. Cristol, N. L. Hause, A. J. Quant, H. W. Miller, K. R. Eilar and J. S. Meek, THIS JOURNAL, **74**, 3333 (1952).

(2) Deceased December 15, 1951.

(3) Appropriate references have been given earlier (S. J. Cristol, N. L. Hause and J. S. Meek, THIS JOURNAL, **73**, 674 (1951)).

(4) W. Hückel, W. Tappe and G. Legutke, *Ann.*, **543**, 191 (1940).

(5) S. J. Cristol, THIS JOURNAL, **69**, 338 (1947).

(6) A. Michael, *J. prakt. Chem.*, **52**, 289 (1895).