[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY]

Ureolytic Activity of Urease at pH 8.9[1]

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The rate of hydrolysis of urea by urease was studied at pH 8.9_5 in absence of added buffers, by utilizing the buffering action of the products of the hydrolysis. The activity of urease was found to be very sensitive to the ionic strength of the solution. Qualitatively the effect is of the same type as previously observed at pH 7 and 7.5; it can be fitted by the Debye–Hückel equation for ionic activity coefficients. The magnitude of the effect, however, is very much greater, indicating a larger effective ionic charge at this high pH. At very low ionic strengths the activity of urease at pH 8.9_5 is only some 20% lower than at pH 7. The dependence of the rate on urea concentration also has been investigated. This dependence was shown to be described by the same kinetic parameters which, according to previous work, govern the rate in acid solutions. The significance of these findings for the general reaction mechanism is discussed.

Introduction

Extensive experimental data were presented from this Laboratory[2,3] on the activity of urease in weakly acid and neutral solutions. A kinetic mechanism was developed which describes the observations by a sequence of reactions obeying the Mass Action Law. Thus the reduction of enzymatic activity in acid solutions was interpreted as an inhibition by hydrogen ions. The reduction of enzymatic activity by electrolytes at pH 7 and 7.5 was left out of the mechanism because the magnitude of the effect and the pH range wherein it was observed were too small to attempt its detailed analysis. The effect was shown to be non-specific and to be representable by the Debye–Hückel equation for the ionic activity coefficients. Howell and Sumner[4] have shown that the activity of urease drops very rapidly above pH 7. For these experiments they used rather concentrated buffers. The observations, therefore, can be interpreted either as an inhibition by hydroxyl ions, similar to that exercised by hydrogen ions, or as an ionic effect. This uncertainty and the desire to test the proposed kinetic mechanism in basic solutions led to the present experiments.

The selection of buffers for experiments with urease in basic solutions presents considerable difficulties because both the phosphate and the borate buffers have been shown to be specific inhibitors.[2a,5] Those buffers, however, which were previously[2a] shown to be not inhibitory in acid solutions, have very poor buffering capacity in basic solutions. They must therefore be used at high concentrations for adequate pH control. This was the technique adopted by Sumner.[4] The present experiments were carried out over a very wide range of ionic concentrations at pH 8.95 ± 0.1, utilizing the buffering action of the products of the reaction, ammonium carbamate and/or ammonium carbonate,[6] in absence of other buffers. This is practicable because calculations based on the ionization constants of the electrolytes involved show that a few micromoles of ammonium carbonate per liter suffice to bring the pH of an unbuffered neutral solution to 8.9. On the other hand, even a saturated solution of ammonium carbonate was calculated to have a pH of only 9.2.

Experimental Details

The technique and the materials employed were the same as previously described,[2a] with the following modifications. The dilute enzyme solutions used in this work were prepared in a different manner. An aliquot of a freshly made solution of hydrogen sulfide (approximately 1.7 mM) was titrated with sodium hydroxide to a pH between 8.0 and 8.6 and an equal amount of alkali was then added to another aliquot of this solution. This aliquot, uncontaminated by the electrodes of the pH meter, was then chilled to 1° and stock urease solution[2a] added. The solution was allowed to warm to room temperature and was used after three hours of aging, by adding 1 cc. of it to 20 cc. of unbuffered urea solution, with or without neutral salts added. The following Table I shows that the enzyme causes a very rapid change of pH to 8.9, whereupon the pH remains constant. The data of Table I were obtained by withdrawing aliquots from the reaction mixture, to be sure that the electrodes of the pH meter had no effect on the enzyme. Figure 1 shows that the rate of ammonia production becomes constant after the first minute or two, that is, after the pH reaches a constant value of 8.95. The rate of hydrolysis at this pH was determined therefore by making rate runs for 2.5 minutes and for 10 minutes, taking the difference of ammonia yields and dividing by the time differential. Each set of runs made with the same dilute enzyme solution included also a few made at pH 7 under standard conditions.[2a] The data given

TABLE I

THE OBSERVED pH CHANGES DURING THE HYDROLYSIS OF UNBUFFERED UREA SOLUTIONS

Digestion time, min.	0	1	2	5	20	30	60
pH	7.0	8.8	8.95	8.97	8.98	9.00	8.95

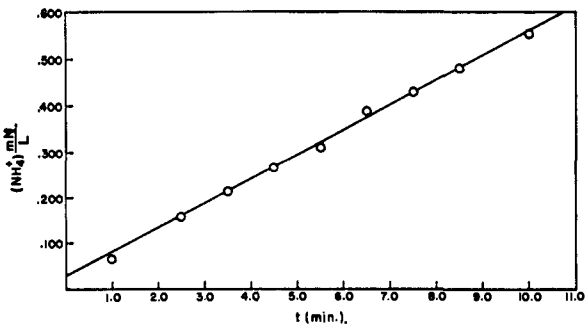

Fig. 1.—The observed rate of hydrolysis in unbuffered urea solutions: urea concentration 33.3 mM; average ionic strength = 0.3 mM.

(1) This work was made possible by a grant from the Rockefeller Foundation to Harvard University.

(2) (a) G. B. Kistiakowsky, P. C. Mangelsdorf, Jr., A. J. Rosenberg and W. H. R. Shaw, THIS JOURNAL, **74**, 5015 (1952); (b) G. B. Kistiakowsky and A. J. Rosenberg, *ibid.*, **74**, 5020 (1952).

(3) G. B. Kistiakowsky and W. H. R. Shaw, *ibid.*, **75**, 866 (1953).

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

(5) J. B. Sumner and G. F. Somers, "The Chemistry and Methods of Enzymes," Academic Press, Inc., New York, N. Y., 1947, p. 158.

(6) J. B. Sumner, D. B. Hand and R. G. Holloway, *J. Biol. Chem.*, **91**, 333 (1931).

in the following are the observed rates divided by the standard. All measurements were made at 25.0°. The enzyme was one of the samples prepared in this Laboratory.

Results

Figure 1 and other similar data omitted here show that the enzyme is not irreversibly denatured at *p*H 8.9. This confirms the observations of Howell and Sumner[4] and the experiments of Dr. A. Rosenberg[7] who noted that solutions of urease could be raised to a *p*H as high as 10, without altering the activity of the enzyme upon return to lower *p*H. One can feel confident, therefore, that measurements at *p*H 8.9 reveal the activity of the enzyme characteristic of this *p*H, rather than demonstrating the progress of an irreversible denaturation. The complete reproducibility of the present experiments, which agreed to the estimated precision of the measurements, about 4%, is further evidence that irreversible denaturation was absent.

The rate of urea hydrolysis shown in Fig. 1 was found to be 0.78 of that at *p*H 7 under our standard conditions. In contrast to this, the ratio found by Howell and Sumner[4] in 0.125 *M* citrate buffer was somewhat less than 0.1. Since one of the possible causes of this apparent discrepancy is the use of buffer solutions with a very high ionic strength (1.5 *M*) by the previous investigators, the effects of various ions on the activity of urease at *p*H 8.9 were systematically studied. The results, shown in Table II, demonstrate that electrolytes have indeed a profound effect on the activity of urease at this high *p*H. An extrapolation of the data (see below) to the ionic strength employed by Howell and Sumner indicates a ratio of rates at *p*H 8.9 and *p*H 7 equal to 0.07, in excellent agreement with their findings. Although the ionic effects at *p*H 8.9 are much larger, they are of the same nature as previously observed at *p*H 7 and 7.5. The specific nature of the ions involved appears to be immaterial and the rate is determined by the ionic strength of the solution. The reduction of enzymatic activity by electrolytes is independent of the concentration of the substrate, as shown by the data of Table II.

TABLE II

THE EFFECT OF ADDED ELECTROLYTES ON THE UREASE-CATALYZED HYDROLYSIS OF UREA AT *p*H 8.95 ± 0.1

Electrolyte	Ionic strength, m*M*	Av. urea concn., m*M*	Relative rate Obsd.	Cor.[a]	Calcd.[b]
NaCl	0.3	3.11	0.425	0.425	0.434
NaCl	9.8	3.14	.296	.295	.301
NaCl	24.0	3.17	.246	.244	.246
NaCl	47.8	3.18	.204	.202	.205
NaCl	119	3.21	.163	.160	.155
NaCl	238	3.23	.116	.114	.124
NaCl	0.33	99.85	.824		.805
NaCl	9.8	99.85	.540		.556
NaCl	24.0	99.85	.455		.457
Na_2SO_4	24.0	99.85	.470		.457
KNO_3	24.0	99.85	.44		.457
KBr	24.0	99.85	.44		.457
NaCl	47.8	99.85	.359		.380
NaCl	119.0	99.85	.291		.287
NaCl	238.0	99.85	.242		.230
Na_2SO_4	238.0	99.85	.230		.230
KNO_3	238.0	99.85	.236		.230
KBr	238.0	99.85	.237		.230

[a] Corrected to a urea concentration of 3.11 m*M*. [b] Calculated by equation (1) with parameters from Table IV.

(7) Unpublished data.

In the previous papers[2b,3] from this Laboratory, the effect of substrate concentration on the rate of hydrolysis was shown to be more complex than the simple Michaelis–Menten equation would predict. The deviations became apparent when rate measurements were extended to substrate concentrations below *ca.* 4 m*M*. To determine whether the same situation prevailed at *p*H 8.9, rate measurements were made at constant ionic strength over a wide range of urea concentrations. The results are shown in Table III. As in the lower *p*H range, a decided curvature is obtained when the inverse rate is plotted against inverse substrate concentration. On the other hand, the three-parameter equation employed previously[3] gives excellent fit with the data, as shown in column 3 of Table III. The parameters chosen for this calculation were: $V_m = 0.857$; $K = 1.91$ m*M*, and $K' = 4.20$ m*M*.

TABLE III

THE EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE AT *p*H 8.95 ± 0.1 AND 0.3 m*M* AVERAGE IONIC STRENGTH

Av. urea concn., m*M*	Relative rate Obsd.	Calcd.[a]
0.58	0.182	0.183
1.51	.326	.322
2.14	.372	.379
3.11	.425	.444
4.76	.482	.516
6.48	.59	.568
8.16	.590	.604
9.8	.655	.633
13.1	.671	.670
16.4	.684	.697
16.5	.702	.700
33.0	.766	.766
33.0	.782	.766
66.2	.815	.806
99.4	.824	.822

[a] Calculated from equation (2).

Discussion

The previously measured ionic strength effects at *p*H 7 and 7.5 were indistinguishable experimentally and were treated therefore as independent of *p*H. The magnitude of the observed effect at *p*H 8.9 is such as to show that ionic effects rise with *p*H. The data at all three *p*H have been replotted, therefore, in Fig. 2, after casting the Debye–Hückel equation for the ionic activity coefficients into the form

$$\frac{\sqrt{\mu}}{\log (V^0/V)} = \frac{1}{A} + \frac{B}{A}\sqrt{\mu} \qquad (1)$$

which emphasizes the more accurate data at higher ionic strengths. The ratio of the rate V (measured at an ionic strength μ) to the rate V^0 (calculated for zero ionic strength) is treated here as an activity coefficient. The constant A is that of the Debye–Hückel limiting law, while B introduces the well-known correction for a finite distance of closest approach. The vertical lines indicate experimental uncertainties, which appear to be so large at *p*H 7

and 7.5 because the ratio V/V^0 does not differ from unity by more than 30 to 40%. The large magnitude of the ionic effect at pH 8.9 reduces the uncertainty and therefore the good fit of the new data to a straight line is a strong evidence of the applicability of the Debye–Hückel equation. The parameters of the three lines are given in Table IV.

TABLE IV

THE PARAMETERS OF THE IONIC EFFECT

pH	7	7.5	8.95
V_m^0	1.48	1.45	0.95
A	0.4	0.5	2.5
B	1.4	1.2	2.2

The magnitude of the experimental uncertainty and the presence of three parameters make it possible to obtain satisfactory fit of experimental data over an appreciable range of each parameter, provided the others are properly chosen. In the graph given previously,[2a] the parameters A and B were taken to be the same at pH 7 and 7.5 and the parameter V^0 was then adjusted to obtain the best possible fit. In the present plot all three parameters were assumed to vary with pH and therefore the final values differ from those given in the earlier publication. The data of Table IV show that the effective ionic charge, given by the magnitude of the parameter A, increases rapidly with rising pH. The parameter B, proportional to ionic diameter exhibits the same trend. These trends are demonstrated by the comparison of the new data at pH 8.9 with those at pH 7.0 and 7.5. The variation within the range 7.0 to 7.5 is too small to be established with certainty by the experimental data. The enzymatic activity optimum, at very low ionic strengths, appears to lie between pH 7.0 and 7.5.

The rates calculated in column 3 of Table III were obtained from the previously[3] derived equation

$$V = \frac{k_3'[E_0][S]([S] + K')}{\alpha([S]^2 + 2[S]K') + KK'} \qquad (2)$$

of the "interacting site" mechanism, in which α corrects for the inhibition by hydrogen ions. Since the effect of ionic strength on enzymatic activity is independent of the substrate concentration, it must reside in the quantity $k_3'[E_0] = V_m$ of this equation. It is therefore appropriate to use V_m^0, that is V_m extrapolated to zero ionic strength, in the analysis of the pH dependence of the parameters of the equation. The specific form of the inhibition law fitting the data in acid solutions predicts no decrease of V_m^0 at high pH. To allow for the observed slight decrease from the value characteristic of pH 7, one may add to the proposed mechanism weak inhibiting reactions with hydroxyl ions similar to those previously postulated for hydrogen ions. This leaves the general form of equation (2) unaltered but changes α from a function which decreases with rising pH to one having a minimum at the pH of optimum enzymatic activity. The parameter K', as before, is independent of pH, whereas the pH dependence of V_m^0 and of K is identical. Table V shows that these requirements are met by the combined experimental

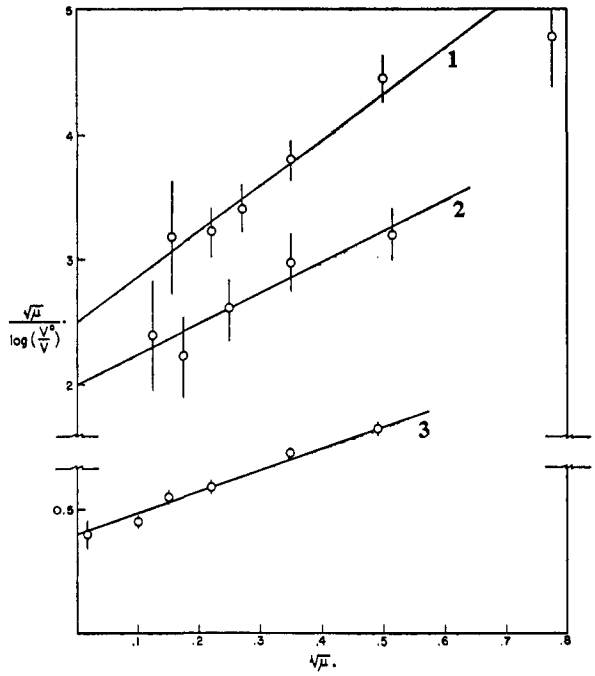

Fig. 2.—The effect of ionic strength on the rate of ureolysis at pH 7 (line 1), 7.5 (line 2) and 8.9 (line 3).

data exceedingly well. As regards the dependence of the rate on urea concentration, the proposed equation describes the data over an unusually wide range of substrate and hydrogen ion concentrations.

TABLE V

THE KINETIC PARAMETERS OF UREASE AT 25°

pH	K^1, mM	K, mM	V_m^0	K/V_m^0
5.43	4.3	1.72	0.76	2.26
6.00	4.4	1.97	1.00	1.97
6.50	4.5	2.56	1.29	1.98
7.00	4.2	2.59	1.48	1.75
7.48[a]	5.2	2.30	1.45	1.59
8.95	4.2	1.90	0.95	2.03

[a] Data on this pH are least reliable.

The ionic strength effect, however, remains unexplained. In essence, the experimental data mean that the concentration of hydroxyl ions *per se* has but slight effect on the enzymatic activity, but that it enhances the "inhibitory" action of the ionic atmosphere. If the direct action of hydroxyl ions on the enzyme is a reversible total inhibition of the active site, in the sense defined previously,[3] it might be subject to ionic effects. They should become more pronounced with increasing degree of inhibition, that is, with rising pH. Qualitatively this agrees with observations, but calculations cannot be made to represent numerically the observed trends of V_m^0 and of the ionic effect with pH.

The possibility has been considered that the inhibition by hydroxyl and hydrogen ions is of the "partial" type, in the sense that the products of the corresponding reactions retain some catalytic activity. The additional parameter thus made available does permit the elimination of the objectionable fractional exponent[3] in the inhibition by hydrogen ions. Some improvement in the simultaneous representation of the trends of V_m^0 and of

ionic effects in alkaline solutions is also obtained but is of slight significance because of the number of parameters involved. An independent proof of the partial nature of inhibition should be adduced before exploring this possibility further.

It is possible to represent the last step of the Michaelis-Menten mechanism by a sequence of reactions of the enzyme–substrate compound with hydrogen and hydroxyl ions, rather than with water. The electrolyte effect is then to be regarded as a kinetic, primary salt, effect. Such treatment, however, requires several parameters to describe the observed effect of pH on V_m^0 and does not facilitate the interpretation of the ionic strength effect.

The failure of simple mechanisms to account for the effect of the ionic strength on enzymatic activity emphasizes the possibility that it may be an indirect result of the interaction of the entire molecule of urease, as a zwitterion, with the surrounding ionic atmosphere. The isoelectric point of urease has been observed at pH 5.1.[8] Thus ionic effects become noticeable only when the enzyme carries a large net negative charge. If similar effects are found in the as yet unexplored range of pH below 5, their correlation with the zwitterion properties of the enzyme would gain much in plausibility.

(8) J. B. Sumner and D. B. Hand, THIS JOURNAL, **31**, 1255 (1929).

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

Oxidation of Aromatic Compounds in Aqueous Solution by Free Radicals Produced by Photo-excited Electron Transfer in Iron Complexes[1]

BY H. G. C. BATES[2] AND N. URI

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The kinetics of systems of the type $Fe^{3+}X^-$–aromatic substrate–ultraviolet light were investigated. The substrates chosen were benzoic acid and benzyl alcohol as monosubstituted benzene derivatives and *o*-, *m*- and *p*-toluic acids as disubstituted benzene derivatives. The kinetic scheme involves (1) light absorption, (2) primary dark back reaction, (3) dissociation of excited complex, (4) secondary dark back reaction, (5) free radical attack on the substrate, (6) oxidation of the free radical by the ferric ion pair and (7) combination of radicals at low ferric ion concentration. Benzyl alcohol was attacked in the side chain only and quantitatively oxidized to benzaldehyde. On the other hand, benzoic acid shows substitution in the benzene nucleus only. The respective ratios of *o*-, *m*- and *p*-hydroxybenzoic acid, were examined and under certain conditions these were found to be statistical (*i.e.*, 2:2:1). It appears that the benzene nucleus of *o*-toluic acid is not attacked (in contrast to the other toluic acids). Various ratios of the rate constants of the free radical reactions were evaluated quantitatively.

Introduction

In a recent paper concerned with the reactions of free radicals in aqueous solution Evans, Santappa and Uri[3] described in detail the system $Fe^{3+}X^-$–vinyl monomer–ultraviolet light (X^- being a monovalent anion such as OH^-, Cl^-, N_3^-). It was shown that the free radicals produced from the anion, X^-, by a photo-excited electron transfer reaction were capable of initiating polymerization. The work of Merz and Waters with Fenton's reagent[4] and particularly the very extensive examination by Stein and Weiss[5-8] of the reactions of aromatic compounds with OH radicals produced by this method and by the action of ionizing radiations, suggested a comparative study with the initiation system previously applied to the polymerization of vinyl compounds.[3,9-11]

A considerable advantage of this system over Fenton's reagent is that the production of free radicals is easily regulated by the variation of light intensity, and the problem of mixing conditions—important with Fenton's reagent—does not enter. The scope of this investigation was widened by the introduction, as substrates, of disubstituted benzene derivatives. The latter show interesting peculiarities which are not yet completely understood. Benzoic acid and benzyl alcohol were chosen as monosubstituted benzene derivatives and these two show remarkable differences in their behavior as substrates.

The problem of substitution in the benzene nucleus by organic free radicals was recently reviewed by Hey.[12] The whole field of aromatic substitution by free radicals is still largely undeveloped. This investigation does not fill the numerous gaps but merely supplies some more data, part of which seem to lend themselves to a satisfactory interpretation.

Experimental

Ferrous ion was determined colorimetrically as the *o*-phenanthroline complex in the presence of excess fluoride ion to avoid interference by ferric ion. The minimum excess of *o*-phenanthroline to ensure quantitative complex formation was calculated from the equilibrium measurements made by Lee, Kolthoff and Leussing.[13] The pH was regulated by addition of a biphthalate buffer. Ferric ion

(1) Presented at the 122nd Meeting of the American Chemical Society held in Atlantic City, N. J., September 14–19, 1952.

(2) Shell Petroleum Co., Ltd., (CIM), St. Helen's Court, London, E. C. 3, England.

(3) M. G. Evans, M. Santappa and N. Uri, *J. Polymer Sci.*, **7**, 243 (1951).

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(8) (a) G. Stein and J. Weiss, *ibid.*, 3245 (1949); (b) 3254; (c) 3256.

(9) M. G. Evans and N. Uri, *Nature*, **164**, 404 (1949).

(10) *Idem*, *J. Soc. Dyers and Colourists*, **65**, 709 (1949).

(11) H. G. C. Bates, M.Sc. Thesis, University of Manchester, 1949.

(12) D. H. Hey, Tilden Lecture, Chemical Society, London and Leeds, 1951.

(13) T. S. Lee, I. M. Kolthoff and D. L. Leussing, THIS JOURNAL, **70**, 2348 (1948).