made 1 $M$ in sodium hydroxide. To these were added a 300-fold, 150-fold and 75-fold excess of hydrogen peroxide, respectively, over the amount required to reduce a 3.40 × $10^{-4}$ $M$ $RuO_4^-$ solution to $RuO_4^{=}$. The absorption spectrum of the $RuO_4^{=}$ formed by reduction was measured on the Cary Spectrophotometer in the first two cases and at selected wave lengths on the Beckman in the third case. The absorption spectra were the same within experimental error when corrected for differences in dilution. Hydrogen peroxide in alkaline solution has an absorption band which becomes significant at 370 mμ at the concentrations used in these experiments. This is below the wave length measured (Table III). The values given in Table III are believed to be accurate to within 4%. The spectrum of $RuO_4^{=}$ shown in Fig. 1 was obtained with solutions containing no peroxide.

To verify that the spectrum of only a single oxidation state was being measured, powdered zinc was added to one solution and the spectrum followed for several days. A black precipitate formed and the absorption of the solution decreased, but the shape of the curve remained the same.

Marshall and Rickard[13] and Silverman and Levy[2] have reported the molar extinction coefficient of $RuO_4^{=}$ at 465 mμ to be 1742 ± 12, and 1735, respectively. Their spectra show a distinct maximum at 375 mμ, indicating the presence of some $RuO_4^-$ in their $RuO_4^{=}$ solutions. This may account for deviations from Beer's law and the lower molar extinction coefficients found by the former authors.

It has been reported[13] that ruthenate solutions could not be kept without change in composition and precipitation of hydrated ruthenium dioxide, even in 2 $M$ potassium hydroxide solutions. We have had orange solutions of potassium ruthenate in approximately 1 $M$ sodium hydroxide which have shown no signs of precipitation in more than one year.[14] Here, as with the +7 and +8 oxidation states, the reduction of ruthenium appears to be very sensitive to minute traces of impurities.

(13) E. D. Marshall and R. R. Rickard, *Anal. Chem.*, **22**, 795 (1950).

(14) See also ref. (2), p. 5.

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[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY]

# The Effects of Electrolytes on Urease Activity[1]

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A sensitive quantitative analytical procedure for the determination of ammonia has been developed. The ammonium ion is separated from interfering substances by ion-exchange, and its concentration is determined photometrically after nesslerization. The effect of enzyme concentration, *p*H and added electrolytes on the catalytic activity of urease has been studied. Measurements were made in the presence of various added salts utilizing a number of buffer systems. Over a range of more than $10^3$, the activity of the enzyme is strictly proportional to its concentration. Below *p*H 7, over the thousand-fold concentration range studied, sodium maleate buffers and other added salts are without effect on the activity of urease. At *p*H 7 and 7.5 the enzymic activity decreases slightly with increasing electrolyte concentration. This effect is not specific for a large number of ions. Its interpretation as a salt effect is consistent with the data. Specific inhibition of the enzyme was caused by several other salts. It is suggested that the real inhibitory substances in phosphate buffers may be complexes formed by the alkali cations with the various buffer species. This explains the different behavior of the enzyme in sodium and potassium phosphate buffers as observed by others.

It is well known that the activity of urease is sensitive to changes in *p*H and that several electrolytes are inhibitory. No conclusive evidence exists[2] that urease requires activators for its functioning, as do certain other enzymes.

Harmon and Niemann[3] have shown that phosphate buffers inhibit urease competitively. In a more recent publication Fasman and Niemann[4] concluded that phosphate ions activate this enzyme, and that the inhibition is due to sodium and potassium ions. Their concept of a complex pattern of simultaneous activation and inhibition by the several components of a buffer, including the alkali ions, makes a quantitative treatment of urease kinetics most difficult if not impossible. A kinetic study, undertaken in this Laboratory, made it necessary to determine whether or not the components of the buffers used affect the activity of urease in the complex manner suggested by Fasman and Niemann.

We are not concerned here with the behavior of urease in the total absence of ions, nor with the effects on it of traces of ions. The objective of these experiments is to demonstrate whether or not there exists a "plateau" of ureolytic activity in electrolyte solutions. If the specific activity is virtually unaffected by large changes in concentration and kind of ions present, as well as by changes in the concentration of the enzyme itself, then the effects of *p*H, substrate and inhibitor concentrations, temperature, etc., should be interpretable by the standard kinetic procedures.

## Experimental Details

The procedure consisted of rapidly mixing in a thermostat at 25° a small volume (0.5 cc.) of buffered urease and a buffered solution of urea (25 cc.), adding strong acid after a known time interval, and determining the ammonia formed. The thermostat was controlled to 0.01° and extremely fast mixing was obtained with the aid of glass-enclosed magnetic stirrers, so that reaction times as short as 60 sec. were reproducibly obtained. The analytical procedure (developed by A. J. R.) consisted of the following steps: (1) neutralization of the acidified reaction mixture; (2) absorption of the ammonium ion on Dowex 50 resin contained in an ion-exchange column; (3) washing the resin with ammonia-free water; (4) elution of the ammonium ion with 0.02 $N$ sodium hydroxide; (5) Nesslerization of the resultant solution; and (6) determination of the optical density of the Nesslerized samples with a Lumetron photoelectric colorimeter after a 15-min. waiting period. The absolute amount of ammonium ion was obtained by comparison with a calibration chart derived from the Nesslerization of known quantities of ammonium ion. Thus the method is similar to that

(1) This work was made possible by grants from the American Cancer Society and from the Rockefeller Foundation, to both of which the authors wish to express their sincere gratitude.

(2) J. B. Sumner and K. Myrback, "The Enzymes," Academic Press, Inc., New York, N. Y., 1951.

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

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

developed by Folin.[5] Experiments showed that the ion exchange columns adsorbed, and then released, ammonium ion quantitatively, provided that the following conditions were met: (1) the concentration of alkali ions in the starting solution was below 0.7 $N$; (2) the commercially obtained resin was pretreated by cycling it three times from the hydrogen to the sodium form; and (3) the contact time for adsorption and elution was three minutes or longer. Experiments showed that the analytical results were not affected by the presence of urea, hydrogen sulfide, protein, dioxane, glycerol, ethylene glycol, nor any of the anions considered below. The standard deviation of a single determination in this series was 1.7%, whereas the s.d. of a direct Nesslerization and optical density measurement was 1.5%. These figures apply to quantities of ammonia in the range from $10^{-6}$ to $10^{-5}$ mole in 50 cc., the sensitivity of the method being about $2 \times 10^{-8}$ mole with the 1-cm. cell used in the photometer. It might be noted that in this concentration range Beer's law applies quite accurately to Nessler solutions.

Several preparations of urease were used in the course of this work. Sample 1 was obtained from Synzyme Laboratories as a suspension in 50% glycerol, with a specific activity of 65,000 S.U. Other samples were prepared locally, essentially by the method of Dounce.[6] Specific activity varied in these samples from less than 10,000 S.U. upward, two and three precipitations being used. The enzyme samples were stored in 50% glycerol at 2° and their activity was well maintained. Enzyme sample No. 2 lost only 10% of its specific activity over 4 months; the more active sample No. 7 changed its activity by a factor of 2 in 3 months. Prior to use the enzyme was diluted in appropriate buffer made 0.004 $M$ in hydrogen sulfide. The dilute enzyme solutions were aged several hours before use. Tests showed that no changes in enzyme activity occurred on further aging, except for a slow deactivation. All glassware was cleaned in a mixture of hot sulfuric and nitric acids and rinsed repeatedly in glass-distilled water which had been passed through a mixed bed of Dowex-50 and IRA 400 to ensure freedom from ammonium and heavy metal ions. This water was used in making all solutions.

The electrolytes used in this work were Merck Reagent and Mallinckrodt Analytical Reagent grade employed without further purification. Maleate buffers were prepared from Eastman White Label maleic anhydride; identical results were obtained after several recrystallizations of commercial maleic acid. Buffers were made by titrating weighed amounts of the corresponding acids or anhydrides with alkali hydroxides. Final adjustments for experiments in Table II, parts B and C were made by adding traces of sulfuric acid. $p$H was measured with the Beckman $p$H meter, model G. Urea was Baker Analyzed, C.P. grade. Tests showed that aging of buffer and urea solutions prior to use had no effect on enzyme activity. Comparisons of urea and of buffer salts from different batches gave identical results, within the precision of the analytical method.

Each series of runs included several made under standard conditions (0.1 $M$ sodium maleate buffer at $p$H 7.0 with 33.3 m$M$ urea at 25.0°) to avoid uncertainties arising from the dilution of the stock enzyme solutions, from their slow deterioration with time of storage, and from different activities of stock solutions of the several preparations of the enzyme used. The relative rates given below are ratios of observed rates to those under standard conditions. Each value is the average of two or three measurements. The changes in absolute rates from day to day were slight, as indicated by the previously given slow rate of deactivation of the stock solutions. Each set of measurements also included blanks (necessitated by the presence of traces of ammonia in urea solutions). They were run under identical conditions except that the acid used to quench the reaction was added before the enzyme. Ammonium ion concentration in the blank was deducted from that in the actual run.

## The Results

The rate of urea hydrolysis was found to be constant in time. Six runs with 33 m$M$ urea at $p$H 7 in 0.1 $N$ sodium maleate, and from 90 to 420 sec. in duration had a standard deviation of 1.7% for a single measurement; twelve runs with 1000 m$M$ urea and ranging in duration from 60 to 360 sec. had a standard deviation of 2.3%. There was thus no evidence of deterioration of the enzyme nor of the inhibition by ammonia noted by Hoare and Laidler[7] at higher concentrations. Hence the quantity of ammonia liberated could be used as the measure of the rate of urea hydrolysis. The activity of the enzyme was found to be proportional to its concentration in an experiment in which rates of ammonia production at $p$H 7 (7.5 m$M$ in urea, with 0.1 $M$ sodium maleate buffer) were compared at enzyme concentrations which were in the ratio 1:25:100. The rates were found to be in the ratio of 1:25.1:108. The highest of these enzyme concentrations is approximately that used by Fasman and Niemann, the lowest is of the same order of magnitude as used in the other experiments here reported, in which $10^{-6}$ to $10^{-5}$ mole of ammonia was usually obtained in 3 min. in 25 cc. of solution. The ratio of enzyme concentrations used in this and in Fasman and Niemann's research was obtained by comparing the intercepts of the Lineweaver and Burk plots of their data and those given in Table IV.

Fasman and Niemann's conclusion that alkali ions inhibit urease was based largely on their finding that urease activity differs by as much as a factor of two in sodium and potassium phosphate buffers. If the conclusion were correct, other buffers might be expected to exhibit the same effect. The following Table I shows the results of experiments in which sodium and potassium salts of maleic, malonic and citric acids were used as buffers. The rates in these six buffers are seen to be virtually identical at every substrate concentration. The residual variations, not exceeding 5.5% may be attributed to the comparatively high concentrations of the buffers. This is confirmed by Table II, Part A, showing systematic measurements of urease activity at $p$H 7.0 as a function of maleate buffer concentration and of added salts. The activity is seen to be a function of ionic strength alone at lower ionic concentrations.[8] At higher

Table I

Effects of Three Anions and Two Cations on Ureolytic Activity at $p$H 7. Anion Concentration 0.1 $M$

| Anion | Cation | Average urea concentration | Relative rate |
|---|---|---|---|
| Maleate | Na | 33.2 | 1.00 |
| Maleate | K | 33.2 | 1.03 |
| Citrate | Na | 33.2 | 1.00 |
| Citrate | K | 33.2 | 1.02 |
| Maleate | Na | 2.2 | 0.473 |
| Maleate | K | 2.2 | .499 |
| Citrate | Na | 2.2 | .498 |
| Citrate | K | 2.2 | .477 |
| Maleate | Na | 1.063 | .308 |
| Maleate | K | 1.063 | .326 |
| Citrate | Na | 1.063 | .321 |
| Citrate | K | 1.063 | .314 |
| Malonate | Na | 1.075 | .298 |
| Malonate | K | 1.075 | .304 |
| Maleate | Na | 1.075 | .316 |

(5) O. Folin and R. D. Bell, *J. Biol. Chem.*, **29**, 329 (1917).

(6) A. L. Dounce, *ibid.*, **140**, 307 (1941).

(7) J. P. Hoare and K. J. Laidler, This Journal, **72**, 2487 (1950).

(8) For concentrations below the ionic strength of 0.25 $M$ this conclusion is reached with a 90% level of confidence.

concentrations slight individual electrolyte effects become evident. The dependence on ionic strength is the same at all urea concentrations, including some which are omitted from the table for the sake of brevity.

A comparison of the rates in sodium and potassium maleates was also made at an enzyme concentration 100 times higher than those used for Tables I and II. The ratio of rates in 0.1 *M* buffers with 7.5 m*M* urea was 0.99 at *p*H 7.

TABLE II

RELATIVE RATES OF HYDROLYSIS IN THE PRESENCE OF ELECTROLYTES AT SEVERAL *p*H VALUES

| Enzyme No. | Maleic acid, *M* | Cation, *M* | Added anion | Ionic strength, *M* | Urea concentration, m*M* 0.646 | 1.30 | 3.27 | 33.3 | 66.7 | 83.3 | 167 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (A) *p*H 7.0 | | | | | | | | | | | |
| 1 | 0.01 | 0.017 Na | .... | 0.024 | 0.266 | 0.406 | 0.677 | | | 1.21 | 1.24 |
| 2 | .01 | .017 Na | .... | | | | .638 | | | | 1.26 |
| 2 | | .017 K | .... | | | | .632 | | | | 1.21 |
| 1 | .02 | .034 Na | .... | .048 | .237 | .387 | .626 | | | | |
| 2 | .01 | .050 Na | $SO_4$ | .072 | | | .601 | | | | |
| 1 | .03 | .052 Na | .... | | | .375 | | | | 1.20 | 1.19 |
| 2 | .03 | .052 Na | .... | | | | .600 | | | | |
| 2 | .03 | .052 K | .... | | | | .602 | | | | 1.15 |
| 1 | .05 | .086 Na | .... | .122 | .227 | .360 | .599 | 1.05 | | 1.14 | 1.14 |
| 2 | .05 | .086 Na | .... | | .227 | .359 | .595 | | | | |
| 3 | .05 | .086 Na | .... | | .224 | .360 | .595 | | | | |
| 2 | .05 | .086 K | .... | | | | .590 | | | | 1.12 |
| 2 | .01 | .163 Na | $SO_4$ | .245 | | | .573 | | | | |
| 2 | .01 | .240 Na | Cl | | | | .561 | | | | |
| 1 | .1 | .172 Na | .... | | .211 | .344 | | 1.00 | | | |
| 2 | .1 | .172 Na | .... | | | | .576 | | | | 1.11 |
| 2 | .1 | .172 K | .... | | | | .563 | | | | 1.12 |
| 2 | .01 | .41 Na | $SO_4$ | .613 | | | .551 | | | | |
| 2 | .01 | .60 Na | Cl | | | | .475 | | | | |
| 2 | .01 | .60 K | Cl | | | | .425 | | | | |
| 2 | .25 | .43 Na | .... | | | | .512 | | | | .95 |
| 2 | .25 | .43 K | .... | | | | .504 | | | | .90 |
| (B) *p*H 6.50 | | | | | | | | | | | |
| 2 | .009 | .014 Na | $SO_4$ | .017 | | | .627 | | | | |
| 2 | .026 | .041 Na | $SO_4$ | .050 | | | .631 | | | | |
| 2 | .043 | .068 Na | $SO_4$ | .083 | | | .637 | | | | |
| 2 | .050 | .073 Na | .... | .095 | | | .643 | | | | |
| 2 | .087 | .136 Na | $SO_4$ | .165 | | | .608 | | | | |
| 2 | .195 | .326 Na | $SO_4$ | .415 | | | .543 | | | | |
| (C) *p*H 6.00 | | | | | | | | | | | |
| 2 | .009 | .014 Na | $SO_4$ | .017 | | | .520 | | | | |
| 2 | .017 | .027 Na | $SO_4$ | .033 | | | .519 | | | | |
| 2 | .05 | .060 Na | .... | .070 | | | | .94 | | | |
| 2 | .051 | .081 Na | $SO_4$ | .098 | | | .524 | | | | |
| 2 | .05 | .112 Na | Cl | .122 | | | | .95 | | | |
| 2 | .05 | .112 Na | $NO_3$ | | | | | .95 | | | |
| 2 | .05 | .091 Na | $SO_4$ | | | | | .95 | | | |
| 2 | .87 | .104 Na | .... | | | | | .92 | | | |
| 2 | .087 | .135 Na | $SO_4$ | .167 | | | .527 | | | | |
| 2 | .173 | .269 Na | $SO_4$ | .331 | | | .502 | | | | |
| 2 | .017 | .413 Na | $SO_4$ | .613 | | | .489 | | | | |
| (D) *p*H 5.43 | | | | | | | | | | | |
| 5 | 0.10 | .011 Na | .... | .013 | | | | | .68 | | |
| 5 | .025 | .027 Na | .... | .031 | | | | | .68 | | |
| 5 | .050 | .053 Na | .... | .063 | | | | | .68 | | |
| 2 | .050 | .053 Na | .... | | | | | | .69 | | |
| 5 | .125 | .133 Na | .... | 1.28 | | | | | .69 | | |
| (E) *p*H 7.48 | | | $PO_4$ | | | | | | | | |
| 3 | ... | .012 Na | 0.0075 | 0.016 | | | | | | 1.17 | |
| 3 | ... | .024 Na | .0150 | .031 | | | | | | 1.10 | |
| 3 | ... | .056 Na | .030 | .062 | | | | | | 1.06 | |
| 3 | ... | .10 Na | .060 | .124 | | | | | | 1.00 | |
| 3 | ... | .19 Na | .12 | .25 | | | | | | 0.91 | |
| 3 | .10 | .19 Na | .... | .28 | | | | | | 0.91 | |

Relative rates at different ionic strengths and *p*H are presented in Table II. Parts B, C and D show that the effect of electrolytes at *p*H lower than 7 is too slight to be observed, except at the highest concentrations tried.

To avoid the disturbing effect of a changing *p*H on urease activity, the above experiments were so designed that the *p*H did not vary by more than 0.05 unit in the course of each run. Therefore more dilute buffers could not be used. A different kinetic procedure, however, permitted the extension of the measurements to greater dilutions. The method (developed by P. C. M., Jr., for other purposes) consisted in following the changes in the electrolytic conductivity caused by the hydrolysis of urea. The Jones Conductivity Bridge and associated electronic equipment were used for these measurements. The thermostated urea–urease solutions were contained in a special stirred conductivity cell of 80-cc. volume with blank platinum electrodes, and were protected from atmospheric carbon dioxide by a layer of mineral oil. Conductivity changes were calibrated by adding known amounts of a solution of crystalline ammonium carbamate. Table III compares the results thus obtained with those of simultaneous runs made by the standard procedure. Aliquots from the same stock solution of enzyme were used. The specific activity is seen to be the same even though the enzyme concentrations differed by more than a factor of ten and buffer concentrations by a factor of $10^3$.

TABLE III

RATE OF HYDROLYSIS IN VERY DILUTE BUFFERS AT *p*H 6

| Urea concn., m*M*/l. | Sodium maleate, moles/l. | Urease added, cc. stock soln.[a] | Observed rate of urea hydrolysis, moles/min. | Rate in moles/min. cc. of enzyme stock |
|---|---|---|---|---|
| 1.07 | $5.0 \times 10^{-2}$ | $2.3 \times 10^{-3}$ | $4.65 \times 10^{-7}$ | $2.02 \times 10^{-4}$ |
| 1.16 | $6.7 \times 10^{-5}$ | $1.28 \times 10^{-4}$ | $2.75 \times 10^{-8}$ | $2.15 \times 10^{-4}$ |
| 2.19 | $5.0 \times 10^{-2}$ | $2.3 \times 10^{-3}$ | $6.68 \times 10^{-7}$ | $2.90 \times 10^{-4}$ |
| 2.31 | $6.7 \times 10^{-5}$ | $1.28 \times 10^{-4}$ | $3.80 \times 10^{-8}$ | $2.79 \times 10^{-4}$ |

[a] Calculated from dilution factor.

Because of the use of ion exchange resin, only alkali cations could be present at high concentrations in the reaction mixtures. The experiments at *p*H higher than 7.0 were thus handicapped by the lack of suitable buffers. Table II, Part E, shows very nearly the same dependence of ureolytic activity on phosphate buffer concentration at *p*H 7.48 as was observed for several other electrolytes at *p*H 7.0. The rate, at the highest concentration, was the same as in the maleate buffer. Lower concentrations of maleate could not be used because of poor buffer capacity. It thus appears that at *p*H 7.48 and the high urea concentration used, the phosphate buffer has no detectable inhibitory action and that the character of electrolyte effects at this *p*H is the same as at *p*H 7.

A few experiments indicated that in water–dioxane mixtures (25% dioxane) the electrolyte effects were more pronounced. Thus at *p*H 6.7 the change in sodium malonate buffer from 0.01 to 0.04 *M* decreased the rate by 7%, whereas at *p*H 7.0 the decrease was 12%.

The activity of urease in the presence of certain other anions was different from that in the several buffers discussed so far. As Table IV shows, the activity in all such cases was lower, thus pointing clearly to inhibition rather than activation. The inhibition by sodium phosphate at *p*H 7.0 appears to be of the competitive type, as was concluded by Harmon and Niemann for potassium phosphate. This inhibition increases rapidly with decreasing *p*H, in complete accord with the results of Howell and Sumner.[9]

TABLE IV

INHIBITION OF UREOLYTIC ACTIVITY BY CERTAIN ANIONS

| Anion | Concn., *M* | Cation | Concn., *M* | Urea concn., m*M* | Relative rate |
|---|---|---|---|---|---|
| | | *p*H 6.00, Enzyme No. 2 | | | |
| Maleate | 0.087 | Na | 0.104 | 3.27 | 0.527 |
| Malonate | .052 | Na | .088 | 3.27 | .534 |
| Succinate | .050 | Na | .087 | 3.27 | .389 |
| Benzoate | .139 | Na | .120 | 3.27 | .092 |
| Phthalate | .048 | Na | .085 | 3.27 | .031 |
| Phosphate | .109 | Na | .117 | 3.27 | .026 |
| | | *p*H 7.00, Enzyme No. 7 | | | |
| Maleate | 0.10 | Na | | 33.2 | 1.00 |
| Maleate | .05 | Na | | 33.2 | 1.03 |
| Phosphate | .0667 | Na | | 33.2 | 0.81 |
| Phosphate | .0667 | Na | | 14.8 | .60 |
| Phosphate | .0667 | Na | | 2.2 | .184 |

## Discussion

The specific activity of urease appears to be a well defined quantity. For the several enzyme preparations used in these investigations, the specific activity was found to be virtually independent of the concentration of the enzyme. Below *p*H 7, it was also uninfluenced by the presence of electrolytes in widely varying concentration. Identical rates were observed in the presence of any of several anions and two cations, making hypothetical inhibitions or activations of the enzyme by these ions highly improbable. Certain other ions have been shown to act as inhibitors but none were found to enhance the activity. This conclusion is not invalidated by the circumstance that traces of citrate buffer, hydrogen sulfide, glycerol and impurities from the jackbean meal were carried into the urea solutions with the enzyme. The enzyme has been shown to retain a constant specific activity over a dilution range of several thousand, although the amounts of impurities were correspondingly varied. The experiments leave open the possibility that in a complete absence of some or all ions the behavior of the enzyme would be entirely different. What they do establish is the existence of a very level and broad "plateau" of ureolytic activity which justifies the concept of a well-defined "specific activity" of urease.

At *p*H 7 and higher the electrolytes appear to cause a measurable reduction of ureolytic activity. The character of the effect is such as to suggest electrostatic interactions of the Debye–Hückel type. The general Debye–Hückel equation can be cast into the form

$$\frac{1}{\log (A_0/A)} = \frac{1}{C\sqrt{\mu}} + B$$

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

Here $A_0$ and $A$ are, respectively, the activities at zero ionic strength and at an ionic strength $\mu$; $B$ and $C$ are constants. The smallness of the electrolyte effects magnifies the effect of accidental errors of measurement and therefore the data at $p$H 7 were averaged for each ionic strength over all urea concentrations. The results are plotted in Fig. 1. The ratio of activities at zero ionic strength and at $\mu$ = 0.122 has been arbitrarily chosen as 1.28 to give the best fit. A very good fit is indeed possible, the averages of experimental points falling close to a straight line. The data at $p$H 7.5 fall on the same line. The slope of this line, 1.97, is a value associated in the Debye–Hückel theory with a single univalent ion in aqueous solutions at 25°. The intercept leads to a value of 1.5 for the ionic diameter constant $B$, well within the range observed for common ions.[10]

In view of experimental uncertainties, the fit of experimental points to a line of this slope may be accidental. The conservation of charge requires the entry of at least two ionic activity coefficients into any Brönsted rate equation or into any equilibrium expression. It should be noted, however, that the $p$H control with the glass electrode keeps the hydrogen and hydroxyl ion activities roughly constant. Their activity coefficients, therefore, could not appear in the resultant theoretical equations. The results suggest therefore the participation of one of these ions in the reaction mechanism.

The rather abrupt disappearance of the salt effect between $p$H 7 and 6 is an interesting clue to the kinetic mechanism of the ureolytic activity. Some other properties of polyelectrolytes show abrupt changes in the salt effect with $p$H. Thus the binding of certain ions by human serum albumen is greatly enhanced at basic $p$H.[11] The solubility of several proteins changes differently with ionic strength on the basic and acid sides of the isoelectric point.[12]

The results of the present experiments demonstrate that several anions, including those of phosphate buffers, are inhibitors of urease. The increase of the inhibition with decreasing $p$H, already observed by Sumner, indicates that the inhibitors must be some less ionized species than the $PO_4H^{=}$ ion. If anions are the inhibitors, then the different rates observed by Fasman and Niemann in sodium and potassium phosphates call for an explanation. This may lie in the impurities of the salts used or in the complexing tendency of phosphate ions. The formation of adducts involving sodium and potassium ions has been demonstrated for partially ionized monoesters of phosphoric acid.[13,14] A difference in the behavior of sodium and potassium orthophosphates on pyrolysis is very well known and the existence of salts of the composition $MH_5$-

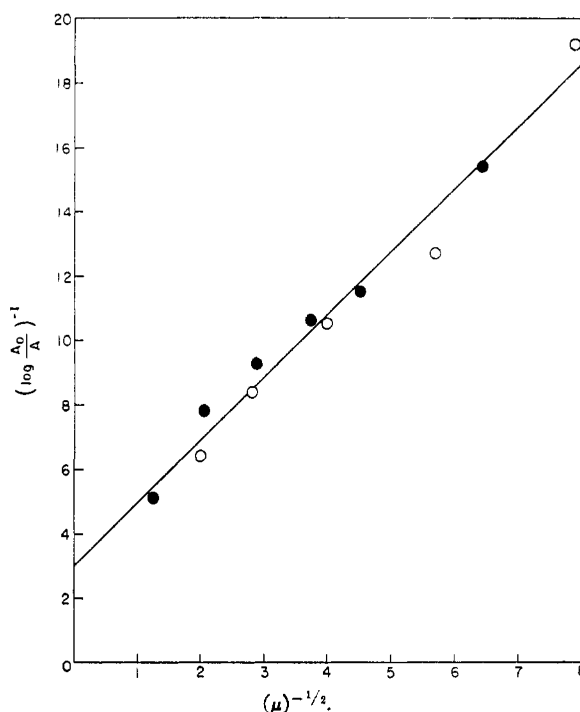


Fig. 1.—The effect of ionic strength upon the rate of hydrolysis of urea: solid circles, data at $p$H 7 averaged over all urea concentrations and electrolytes; open circles, data at $p$H 7.5 in sodium phosphate buffer.

$P_2O_8$ has been demonstrated.[15] Professor G. Scatchard informs us that the osmotic coefficients of sodium and potassium orthophosphates at $p$H 7 are substantially different at molal concentration,[16] clearly indicating differential complexing with the two cations. Warburg observed that a complex magnesium fluorophosphate was the inhibitor of enolase.[17] This evidence and the non-linear dependence of inhibition on phosphate buffer concentration observed by Fasman and Niemann support the hypothesis of inhibition by compounds of cations with partially ionized anions of phosphate buffers.

Fasman and Niemann have noted that their results are inconsistent with those of Harmon and Niemann. They suggest that the different enzyme concentrations used in the two investigations may be responsible. There appears to be some internal irreproducibility in Fasman and Niemann's experiments, as shown by a comparison of their Figs. 2 and 6. In view of the presently demonstrated independence of specific activity from enzyme concentration, another explanation appears to be more probable. Three of the more impure enzyme samples used by us (No. 2, 4 and 5) indicated slight competitive inhibition by maleate and malonate buffers when freshly prepared. The effect vanished on storage of the stock solutions. Thus the rate of hydrolysis in 1.3 m$M$ urea with 0.05 $M$ sodium maleate at $p$H 7 rose by 8% in the first five days after preparation and by an additional 12% in the following few weeks, while the rate in 167 m$M$ urea remained constant. For several

(10) H. S. Harned and D. B. Owen, "The Physical Chemistry of Electrolyte Solutions," Reinhold Publishing Corp., New York, N. Y., 1950.

(11) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, THIS JOURNAL, **74**, 202 (1952).

(12) See E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943.

(13) T. Wagner-Jauregg and A. Wildermuth, *Ber.*, **77**, 481 (1944).

(14) O. M. Friedman and A. M. Seligman, THIS JOURNAL, **73**, 5292 (1951).

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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.

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# The Kinetics of Urea Hydrolysis by Urease[1]

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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

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