the solubility of HBr to the extent here observed.

It is of interest to note that with addition of HBr, the conductance of the solution increases. For a ratio 0.05 for combined HBr to $AlBr_3$, the extreme conductance increase for the higher concentration of salt is 16% and for the lower concentration 20%.

Hydrogen bromide was condensed on aluminum bromide and thereafter was evaporated at −78°. The amount of hydrogen bromide that was evaporated was measured and the pressure was measured as a function of the $HBr/AlBr_3$ ratio. The pressure varied continuously until no HBr was left behind; no breakpoint could be observed. Under the conditions of this experiment a compound was not formed.

V. Summary

1. The conductance of aluminum bromide in nitrobenzene on addition of dimethyl ether has been measured at 25°. In pure nitrobenzene the conductance decreases continuously and markedly up to a molar ratio of unity; thereafter the conductance increases sharply and approximately linearly with increasing molar ratio of ether to salt. With an impure solvent, the minimum comes at a molar ratio slightly less than unity.

2. With ammonia, the conductance passes through a not very pronounced maximum and then decreases to a value differing little from that of the pure salt at a molar ratio of unity. Thereafter, the conductance increases linearly with increasing molar ratio.

3. On addition of trimethylamine, the conductance decreases continuously along a rather complex curve to a minimum value at a molar ratio of unity. Thereafter the conductance increases linearly with increasing ratio of amine to salt.

4. On addition of hydrogen bromide, the conductance increases slightly with increasing hydrogen bromide concentration.

5. The solubility of dimethyl ether and of hydrogen bromide in nitrobenzene have been determined at 25°. From these data, it is possible to determine the concentration of the free additions in solution.

6. In pure nitrobenzene, dimethyl ether is completely combined with aluminum bromide up to a molar ratio of unity. In less pure solvent, there is a measurable concentration of ether beginning with the first additions at molar ratios much below unity.

7. Assuming an equilibrium between aluminum bromide monoetherate, dietherate and ether, an equilibrium constant may be obtained that is constant as a function of the free ether concentration. However, this constant, while holding for certain aluminum bromide concentrations, differs from that at other concentrations.

8. The maximum in the conductance curves of aluminum bromide on addition of ammonia would seem to indicate the existence of a dimeric compound.

9. On condensing hydrogen bromide on aluminum bromide, and thereafter evaporating the hydrogen bromide at −78°, no evidence was found of the formation of a compound between the two components.

PROVIDENCE, R. I. RECEIVED MARCH 12, 1949

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE CATHOLIC UNIVERSITY OF AMERICA]

The Molecular Kinetics of the Urea–Urease System. I. The Kinetic Laws

BY K. J. LAIDLER AND J. P. HOARE

Introduction

The urease-catalyzed hydrolysis of urea, which follows the stoichiometric equation

$$CO(NH_2)_2 + 2H_2O = 2NH_3 + CO_2$$

has a number of unusual kinetic features. One of these relates to the influence of the urea concentration upon the rate of reaction: as the concentration is increased from zero the rate first increases linearly, and later shows a falling-off from linearity, reaching a maximum, and then decreasing.[1] A second characteristic is that the energy of activation is sensitive to the oxidation–reduction potential of the reaction system, and under certain circumstances depends upon the temperature.[2] The present work was undertaken with the primary object of investigating the concentration effect, and of obtaining some information as to the molecular kinetics and energetics of the reaction. The results have also shed some light on the variation of energy of activation with the temperature and on the nature of the intermediate complex which is formed; this will be presented in a later paper (Part II).

Experimental

The Enzyme Preparation.—Urease was prepared from finely ground jack-bean meal and crystallized according to Sumner's method.[3] The enzyme was stored in concentrated solution at 2° under which conditions its activity showed no significant change over a period of ten days. In all of the work the water employed was laboratory distilled water which had been redistilled using an all-glass apparatus.

The urease solution used in the kinetic runs was prepared by diluting 5 cc. of the concentrated solution to 100

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

[2] I. W. Sizer, *J. Gen. Physiol.*, **22**, 719 (1939); *J. Biol. Chem.*, **132**, 209 (1940); Sizer and A. A. Tytell, *ibid.*, **138**, 631 (1941).

[3] J. B. Sumner, *ibid.*, **69**, 435 (1926).

cc. The activity of the urease solution used in the work was constantly determined by the following procedure.[2] The amount of ammonia nitrogen produced in five minutes at 20° by 5 cc. of the solution mixed with 5 cc. of 3% urea solution in a 0.5 *M* phosphate buffer of *p*H 6.2 was determined by the method used in the runs (see below). The amount of active urease present was then calculated on the basis of Sumner's result that under these conditions 1 g. of pure urease produces 133,000 mg. of ammonia nitrogen. Most of the runs were carried out using a urease solution that produced about 0.2 mg. of ammonia nitrogen in five minutes, this corresponding to a weight of 1.5×10^{-3} mg. of pure urease. The molarity of the urease in the reaction system, calculated assuming a molecular weight of 483,000,[4] was 3.1×10^{-10}.

Kinetic Procedure.—Reaction rates were determined by mixing 5 cc. of the diluted urease solution with 5 cc. of a urea–buffer solution prepared by dissolving the required amount of urea in a 1 *M* phosphate buffer. The reaction was stopped after various periods of time by rapid addition of 2 cc. of *N* hydrochloric acid. The mixture was then Nesslerized in a 100-cc. flask and the amount of ammonia nitrogen produced was determined colorimetrically using a calibrated Aminco Neutral Wedge Photometer.

Experimental Data.—The results obtained in a typical run are given in Table I, which shows the number of moles per liter, *c*, of urea remaining at time *t*, and in Fig. 1, which shows a plot of these results. Rates were determined by measuring slopes, some values corresponding to the same run being included in Table II.

TABLE I

THE CONCENTRATION OF UREA REMAINING AT TIME *t*, *T* 30°, *p*H 6.6

sec.	$c \times 10^2$, (mole liter^{-1})
0	1.49
600	1.21
1200	0.980
1800	.722
2100	.648
2400	.595
3000	.528

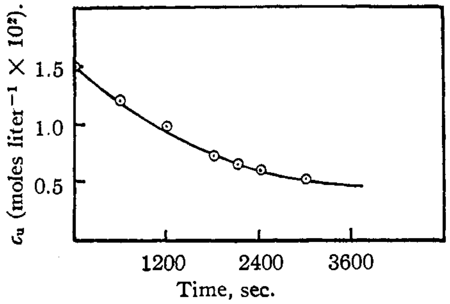

Fig. 1.—Plot showing concentration of urea, c_u, as a function of time; *T*, 30°; *p*H, 6.6.

TABLE II

$c \times 10^2$, mole liter^{-1}	$(-dc/dt) \times 10^8$, mole liter^{-1} sec.$^{-1}$
1.49	5.36
1.21	4.52
0.857	3.45
.714	2.80
.643	2.26

The best value of the initial rate was obtained by plotting log $(-dc/dt)$ against log *c* and obtaining the value of log $(-dc/dt)$ which corresponds to the initial value c_0 of *c* (*i. e.*, 1.49×10^{-2} mole per liter in the example given).

Results and Discussion

The "Order" of the Reaction.—Previous investigations have all been concerned with the initial rate of the reaction, the measurements being made within the first 2% of the total reaction.[1,2] The present work has indicated this to be the most satisfactory procedure, but some information has been obtained as to the variation of the concentrations of the reactant and products with the time.

The matter was investigated by analyzing the results employing a graphical version of van't Hoff's differential method. If the rate, *v*, of a reaction is related to the concentration *c* of reactant by the general equation

$$v = -dc/dt = kc^n$$

where *k* is the specific rate constant, the slope of a plot of log *v* against log *c* gives the value of *n*. If the reaction is of a simple type a straight line is obtained, and a single order, equal to the slope of the line, can be assigned to the process. This procedure can be applied to a single run, in which case the initial rate can be obtained by extrapolation back to the initial conditions. It can also be applied to initial rates obtained at a series of initial concentrations.

The results of applying both procedures to the present data are included in Fig. 2. Each of the dotted curves represents a run at a given initial concentration, and therefore corresponds to the change of concentration with time. The full curve shows the variation of initial rate with

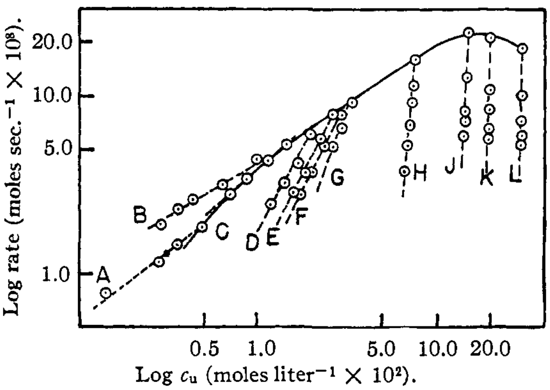

Fig. 2.—Plot of log (rate) *vs.* log c_u; the broken lines are for concentration changes in a single run, the full curve the initial rates and concentrations. The apparent order is given by the slope. The broken curves relate to the following initial concentrations of urea, all in moles per liter × 10²: A = 0.498, B = 0.997, C = 1.49, D = 1.99, E = 2.49, F = 2.99, G = 3.49, H = 7.48, J = 14.96, K = 19.94, L = 29.91.

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[4] J. B. Sumner, N. Gralen and I. B. Eriksson-Quensel, *ibid.*, **125**, 37 (1938).

initial concentration, the initial rates having been obtained from the dotted curves by extrapolation.

The variation of initial rate with concentration corresponds to the results obtained in previous investigations.[1] At low concentrations the plot of log v against log c_0 has a slope of unity, indicating that the rate varies linearly with the initial concentration. The slope falls to zero, indicating an order of zero, and finally becomes negative; the apparent order of the reaction is then negative.

A significant feature of Fig. 2 lies in the discrepancy between the order with respect to time (*i. e.*, the slopes of the dotted curves) and that with respect to concentration (the slopes of the full curve). In particular it is to be noted that in the neighborhood of the maximum, where the order with respect to concentration is close to zero, the slopes of the dotted curves are very high, indicating a rapid falling off of the rate as reaction proceeds. Such differences between the two orders of a reaction have been discussed by Letort,[5] who points out that due regard to them leads to important conclusions as to reaction mechanisms. A higher order with respect to time than is found with respect to concentration indicates that products are interfering with the course of reaction.

In the present instance it was found that ammonium ions interfere with the reaction. This was established by carrying out runs in the presence of added amounts of ammonium ions, added in the form of ammonium acetate which does not appreciably affect the *p*H of the solution. The rate-curves shown in Fig. 3 illustrate the marked inhibition brought about by the addition of 1.25 times as many ammonium ions as are produced in the complete hydrolysis of the urea present. Further work on the influence of inhibitors on the reaction will be carried out in the near future. It has not been found possible to arrive at a simple kinetic law which took account of the inhibition observed.

In view of this result it was felt that the most satisfactory data could be obtained by measuring initial rates, and this procedure was therefore employed in all subsequent work.

The Intermediate Complex.—In order to explain the fact that the rates of enzyme-catalyzed reactions vary by less than the first power of the substrate concentration and generally reach a limiting rate at high concentrations, Michaelis and Menten proposed a kinetic scheme involving the formation of an intermediate complex between enzyme and substrate.[6] The existence of such complexes has been confirmed in a number of investigations of various kinds.[7] The situation is closely analogous to a simple surface-catalyzed reaction for which the kinetics become of zero order when the surface is saturated.

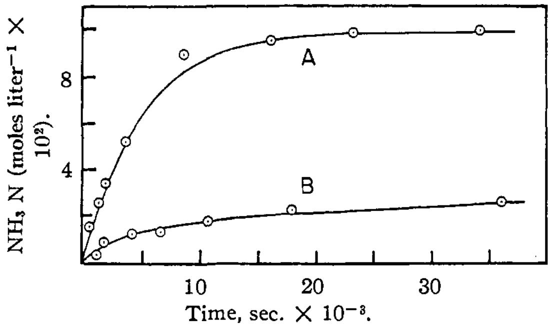

Fig. 3.—Curves showing the inhibiting effect of ammonium ions on the reaction; the lower curve B is for a run in which ammonium ions had been added initially, the upper curve A for no addition.

That an extension of the Michaelis–Menten treatment is necessary for the case of the urea hydrolysis is indicated by the decreasing rate at high urea concentrations. This may be explained in terms of a model in which a urea molecule and a water molecule must, for reaction to occur, become adsorbed on neighboring sites on the urease molecule: at high concentrations the urea becomes adsorbed on both sites and the reaction is therefore inhibited. Let the sites for urea and water be denoted as S_1 and S_2, respectively, and let θ_1 be the fraction of urease molecules having S_1 covered by urea. If the fraction of urease molecules having S_2 covered by urea is θ_2, the fraction which simultaneously have S_1 covered by urea and S_2 covered by water is $\theta_1(1 - \theta_2)$. The concentration of such complexes, which can undergo reaction, is $c_e\theta_1(1 - \theta_2)$, where c_e is the total molar concentration of urease. If the adsorption processes obey the Langmuir adsorption isotherm the fractions θ_1 and θ_2 are defined by the equations

$$\theta_1/(1 - \theta_1) = K_1c_u$$
$$\theta_2/(1 - \theta_2) = K_2c_u$$

where K_1 and K_2 are the equilibrium constants for adsorption on the two sites, and c_u is the total concentration of urea. Rearrangement of these equations gives

$$\theta_1 = K_1c_u\,/(1 + K_1c_u)$$

and

$$1 - \theta_2 = 1/(1 + K_2c_u)$$

The concentration of molecules having a urea molecule adsorbed on S_1 and a water molecule on S_2 is therefore

$$c_x = \frac{K_1c_uc_e}{(1 + K_1c_u)(1 + K_2c_u)}$$

If k_0 is the specific rate of reaction of the complex, the over-all rate of reaction is given by

$$\begin{aligned} v &= k_0c_x \\ &= \frac{k_0K_1c_uc_e}{(1 + K_1c_u)(1 + K_2c_u)} \end{aligned}$$

This equation is clearly of the right general form. At sufficiently low concentrations $K_1c_u < 1$ and $K_2c_u < 1$, so that the rate is

$$v \simeq k_1K_1c_uc_e$$

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(5) M. Letort, Thesis, Paris, 1937.

(6) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913); *cf.* G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

(7) *Cf.* K. G. Stern, *J. Biol. Chem.*, **114**, 473 (1936); B. Chance, *ibid.*, **151**, 553 (1943).

i. e., proportional to the first power of both the urea and the urease concentrations. At high concentrations $K_1c_u > 1$ and $K_2c_u > 1$, and the rate becomes

$$v \simeq k_0c_e/K_2c_u$$

the rate is then inversely proportional to the urea concentration. At intermediate concentrations the rate passes through a maximum, and it may readily be shown that if $K_1 = K_2 = K$, $c_u(\text{max}) = 1/K$.

The best agreement between the theory and the experimental curve has been obtained by taking the values $k_0K_1c_e = 5.73 \times 10^{-10}$ sec.$^{-1}$ and $K_1 = K_2 = 7.56$ liter mole^{-1}; in Fig. 4 the points are experimental and the curve theoretical. There is seen to be some discrepancy in the region immediately before the maximum, and the same type of deviation has been found at several *p*H's and temperatures. Various explanations may be advanced for the slight disagreement between theory and experiment: perhaps the most important factor is the failure of the Langmuir isotherm owing to the strong electrostatic forces involved.

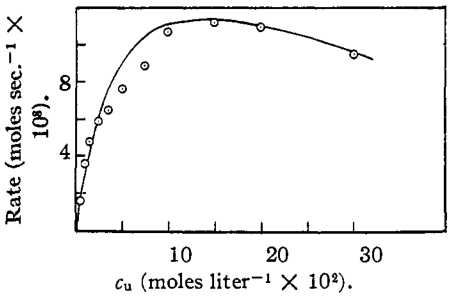

Fig. 4.—The points are experimental initial rates plotted as a function of initial urea concentrations; the curve represents the best fit obtainable with the rate law $v = k_0K_1c_uc_e/(1 + K_1c_u)(1 + K_2c_u)$.

In a further publication (Part II) the temperature dependence of the rate will be described, and values for heats and entropies corresponding to k_0 and K will be derived.

Alternative Reaction Mechanisms.—The model proposed above offers what appears to be a plausible interpretation of the experimental results, particularly of the decrease in rate at high urea concentrations, but the possibility of alternative mechanisms should be borne in mind: the deviation of the experimental points in Fig. 4 from the theoretical line might suggest that an entirely different mechanism should apply. We have considered various other models, but can find no improvement in the agreement; one of these, somewhat similar to the one proposed, is the formation of a urease–urea complex followed by the reaction of this with water, the activity of which decreases with increase of urea concentration. Much more complicated models could doubtless be devised to give better agreement, but in the absence of additional evidence there would appear to be no advantage in introducing further complexities.

It may be noted that the kinetic law proposed here can be deduced without assuming that sites 1 and 2 are neighboring sites; it is also conceivable that the adsorption of urea on a site which is distant from site 1 would bring about a decrease in the rate by bringing about a change in structure of the enzyme equivalent to a reversible deactivation. Irreversible enzyme deactivations are in fact sometimes found at urea concentrations higher than those used in the present experiments (*e. g.*, at 3 *M* with monamine oxidase), so that a reversible effect would not be surprising in the system under investigation.

Experiments using mixed solvents are planned with the object of proving or disproving the proposed mechanism; however, it is anticipated that an unequivocal interpretation of the results will be difficult because (1) the change in dielectric constant will itself introduce changes in rate which will be hard to treat theoretically, and (2) most of the plausible models predict the same type of effect when the solvent is varied.

Summary

1. The rate of hydrolysis of urea catalyzed by urease has been measured over a range of initial concentrations of urea, the temperature being 30° and the *p*H 6.2.

2. The order with respect to time is greater than the order with respect to concentration, indicating that the reaction is inhibited by products: this effect has been traced to the ammonium ion.

3. In agreement with earlier work the initial rate is found at first to increase linearly with concentration, to reach a maximum and subsequently fall. This effect is attributed to the existence of two neighboring active sites on the urease molecule: for reaction to occur a urea molecule must be adsorbed on one and a water molecule on the other. This concept is developed quantitatively in terms of Langmuir adsorption isotherms, and the theoretical rate equation compared with the experimental. Alternative reaction models are discussed briefly.

WASHINGTON, D. C. RECEIVED JANUARY 28, 1949