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Kinetics of the Urease-catalyzed Hydrolysis of Urea at *p*H 4.3

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The rate of hydrolysis of urea by urease at *p*H 4.3 is subject to complex effects by neutral salts. These are interpreted as a superposition of a specific competitive inhibition by several anions and of a general acceleration determined by the ionic strength of the solution. After correction for these effects, the rate shows a dependence on substrate concentration which does not follow the Michaelis–Menten equation. The data do fit a three-parameter rate equation which is derived by assuming the existence of two active sites with Michaelis constants: $K_M = 0.42$ mM and $K'_M = 8.4$ mM. The dependence of enzymatic activity on *p*H, over the range from 4.3 to 8.9, does not agree with the assumption that it is due only to an equilibrium between the active neutral and inactive acidic and basic forms of the enzyme. The hydrolysis of urea by urease at *p*H 6 and 7 was found not to be inhibited by ammonium ions in sodium maleate buffers. The isotopic exchange between $N^{15}H_3$ and urea in solution at *p*H 7 is slightly accelerated by urease but the rate of this reaction is slower by approximately a factor of one hundred than is the rate of hydrolysis under these conditions. The mechanism of enzymatic urea hydrolysis is discussed and it is concluded that, because $k_3 << k_2$, the Michaelis constants of urease are of the form $K_M = k_2/k_1$, *i.e.*, are equilibrium constants for the first step of the reaction.

Introduction

Earlier work in this Laboratory on the enzymatic hydrolysis of urea has revealed a rather complicated kinetic behavior[1-3] over a *p*H range from 5.4 to 8.9. Its most notable features are the dependence of the rate on substrate concentration in a manner which even in dilute urea solutions does not follow the simple Michaelis–Menten mechanism, and the influence of the ionic strength of the solution on rate parameters. The latter effect is negligibly slight in the *p*H range from 5.4 to 6.5 but rises rapidly with *p*H in alkaline solutions. The isoelectric point of urease is at *p*H 5.1[4] and it seemed desirable therefore to extend kinetic studies to more acid solutions in which the net charge on urease molecules is opposite to that in the *p*H range already investigated. Howell and Sumner[5] showed that in acid solutions urease has different activity in acetate and in citrate buffers, but otherwise the information is very meager.

Hoare and Laidler[6] reported strong inhibition of urea hydrolysis by ammonium ions. As the following will show, kinetic evidence may be interpreted as indicating that the Michaelis constant (K_M) of urease is the equilibrium constant of the reaction ES = E + S. It was suggested to us by Professor F. Lynen that the rapid step in the enzymatic hydrolysis therefore may actually be: $EH + (NH_2)_2CO = E(NH_2)CO + NH_3$, where EH represents the free enzyme. Experiments are described below which explore the relative importance of this mechanism.

(1) G. B. Kistiakowsky, P. C. Mangelsdorf, A. J. Rosenberg and W. H. R. Shaw, THIS JOURNAL, **74**, 5015 (1952).
(2) G. B. Kistiakowsky and A. J. Rosenberg, *ibid.*, **74**, 5020 (1952).
(3) G. B. Kistiakowsky and W. H. R. Shaw, *ibid.*, **75**, 2751 (1953).
(4) J. B. Sumner and D. B. Hand, *ibid.*, **51**, 1255 (1929).
(5) S. F. Howell and J. B. Sumner, *J. Biol. Chem.*, **104**, 619 (1934).
(6) J. P. Hoare and K. J. Laidler, THIS JOURNAL, **72**, 2487 (1950).

Experimental Details.—The apparatus and techniques were those employed and described previously,[1,2] except as noted below.

The ion-exchange columns employed to isolate ammonium ions for analysis were charged with Dowex 50W-X8 resin.

The enzyme used in all experiments, except as indicated, was obtained from the Sigma Chemical Company, St. Louis, Missouri, as Urease Powder, Type II. This material, however, developed cloudiness in solution on acidification to *p*H 4.3 and therefore it was subjected to purification. The method followed was that of Sumner and Dounce as modified previously in this Laboratory.[1] The final precipitate was dissolved in 50% aqueous glycerol and stored at 0°. Enzyme solutions prepared in this manner usually contained an activity estimated at about four Sumner units per milliliter. For reaction rate measurements, subsidiary stock solutions were made by diluting the above ten- to twenty-fold with ammonia-free water which was made 2% saturated in H_2S. They were kept in absence of buffers at room temperature. One ml. of dilute enzyme solution was used in each reaction mixture, which had a volume of 21 or 26 ml.

All rate measurements were made at 25 ± 0.01°, using techniques previously described.

Experimental Results. The Choice of a *p*H and a Buffer.—The choice of *p*H 4.3 for kinetic studies was a compromise between the desire to work as far on the acid side of the isoelectric point of urease as possible, and experimental difficulties caused by denaturation of the enzyme, which becomes quite fast at *p*H 4. The rate of enzymatic hydrolysis was measured at *p*H 4.3 in four 0.1 *M* buffers: sodium acetate, maleate, malonate and citrate, and was found to be in the ratio: 1.0:0.75: 0.5:0.2. Accordingly, sodium acetate buffer was selected for subsequent experiments. As will be seen later, however, even this buffer competitively inhibits the enzyme.

The Ratio of Enzymatic Activities at *p*H 4.3 and *p*H 7.0.—In previous work in this Laboratory the rates were measured relative to that in an arbitrarily chosen standard, for which was used a 33.3 m*M* solution of urea, buffered at *p*H 7.0 with 0.1 *M* sodium maleate. A subsidiary reference solution buffered at *p*H 4.3 was advantageous for the present work and the first experiments made were concerned with the determination of the ratio *R* between the rates of hydrolysis in a 33.3 m*M* solution of urea buffered at *p*H 4.3 with 0.04 *M* acetate buffer and the standard solution defined above. This apparently simple task led to entirely unexpected findings. Their gist is that the ratio *R* is not a constant but is a rather complex function of the past history of the enzyme solution used for the determination. Of the extensive experimentation devoted to this problem only the most significant aspects will be here presented. Figure 1

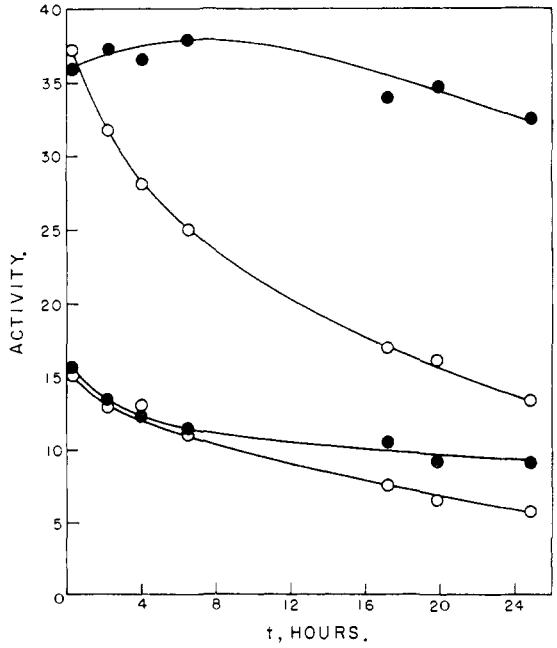

Fig. 1.—Effect of storage at 25° on the activity of dilute enzyme solutions. Solid circles: solution buffered at *p*H 7, tested at *p*H 7 (upper curve) and at *p*H 4.3 (lower curve). Open circles: unbuffered enzyme solution, tested at *p*H 7 (upper curve) and at *p*H 4.3 (lower curve).

shows changes in the activity of two dilute enzyme solutions on standing at room temperature. Solution 1 was buffered at *p*H 7.1 with 0.01 *M* sodium maleate; solution II was unbuffered and had a *p*H of 5.3. Aliquots from each enzyme solution were added to (a) the standard urea solution at *p*H 7.0 and (b) a urea solution buffered at *p*H 4.3 with 0.1 *M* acetate. Figure 1 shows that the unbuffered enzyme solution gradually loses its activity. The percentage loss is the same whether the test is made at *p*H 7 or 4.3, and therefore the ratio *R* remains substantially constant, at 0.43. In contrast, the enzyme solution buffered at *p*H 7 shows different trends of activity with aging when tested at *p*H 7 and at 4.3. The ratio *R* decreases from 0.43 for the fresh sample to 0.28 after 24 hours. Figure 1 shows a slight initial rise of activity of the buffered enzyme solution when tested at *p*H 7. This was observed only occasionally, while the downward trend of *R* with aging was a completely consistent effect. Experiments similar to those shown in Fig. 1 were extended to several weeks. The ratio *R* continued to drop but appeared to trend to an asymptotic value in the neighborhood of 0.20. The rate of decrease was not reproducible, the same change requiring only a few hours in some experiments but days in others. The temperature of storage influenced the rate of decrease. Thus in comparable experiments the value $R = 0.25$ was attained after 25 days of storage at room temperature but only after 112 days at 0°.

The substitution of sodium acetate for maleate when buffering the enzyme solution at *p*H 7 gave the same trends of *R*. On the other hand, enzyme solutions buffered at *p*H 5.4 and 4.4 (with 0.0125 *M* maleate buffer) gave constant *R* ratios, and in both cases they were equal to that obtained with unbuffered enzyme solution (*p*H 5.3). Thus, the effect which is observed when the enzyme is buffered at *p*H 7 does not occur when it is buffered at *p*H 5.4 and 4.4 and, furthermore, it does not appear to depend on the buffering electrolyte.

It was thought that perhaps the reason for the observed effect is that the enzyme stored at *p*H 7 becomes progressively more sensitive to the sudden change in *p*H it suffers when it is added to a urea solution at *p*H 4.3. An enzyme solution buffered at *p*H 7 with 0.028 *M* sodium acetate was prepared. During the period of a week, aliquots of it were suddenly brought to *p*H 4.3 by addition of dilute acetic acid. After holding the enzyme at this *p*H for 135 seconds, a solution of maleate buffer and NaOH was added to bring the *p*H back to 7. The enzymatic activities of this solution, as well as of the original stock solution, were then measured. The acid treatment reduced the activity measured at *p*H 7 by a constant factor of 0.800 ± 0.006 during the entire period; at the same time the ratio *R* dropped from 0.40 to 0.28. The proposed explanation is therefore untenable. However, a sudden change in *p*H does have a slight deleterious effect on the activity of urease. The half-life of urease stored at *p*H 4.3 is 2 to 4 hours, and therefore an exposure to this *p*H for 135 seconds should cause only an insignificant reduction in activity.

The length of time required for significant trends in the ratio *R* suggested bacterial contamination as a possible cause. A new stock solution of urease was prepared, to an aliquot of which a few drops of

toluene were immediately added. The treated and the untreated portions of the stock solution were then used to make dilute enzyme solutions buffered at pH 7, toluene being added again to the treated solution. Both these solutions were found to denature at the same rate, retaining 56 and 58% of the initial activity after standing for 27 days at room temperature. These data were obtained with urea solutions buffered at pH 4.3. The R ratios also decreased identically, from 0.25 to 0.20 for the treated and from 0.26 to 0.20 for the untreated solution.

The initial R ratios in these experiments were substantially lower than those observed with the first enzyme preparation. Some of the new enzyme was therefore made into the unbuffered dilute solution and some into one buffered at pH 4.7. Both gave R ratios of only 0.29. A new stock solution of enzyme was prepared using glassware which was freshly washed in the acid bath. A dilute enzyme solution buffered at pH 4.8 gave R = 0.36. A urease preparation sold by E. R. Squibb Company (Control No. 74369)' was tried then. A dilute solution buffered at pH 5.0 gave R = 0.42. This value was obtained using aliquots of the same buffer solutions which were used when the R value of 0.29 (see above) was found, indicating that impurities in the solutions were not responsible for the difference.

Clearly even the initial R ratios are subject to variations from one enzyme preparation to another. The cause of this may be a step in the preparation of the stock solutions, in which urease is extracted at room temperature and pH 7.5, almost the exact conditions which cause decreasing R ratios.

Experiments were made with a freshly made and an aged enzyme solution buffered at pH 7, using urea at 2.95 and at 33.3 mM concentration and pH 4.3. Fresh enzyme gave an apparent Michaelis constant equal to 2.5 mM. After 15 days of storage the value observed was 3.9 mM. This indicates that a competitive inhibitor is being introduced into the enzyme solutions in storage. Two dilute enzyme solutions were then made, one using fresh ammonia-free water, the other using a pH 7 buffer which was stored in a volumetric flask for 7 days. The R ratios observed for these solutions immediately after preparation were 0.39 and 0.35, respectively. The difference between the ratios is in the direction expected if an inhibitor was introduced into the aged buffer.

Electrolyte effects were studied at two urea concentrations[7] in the presence of 0.04 M acetate buffer, except for the experiments on the effect of the buffer salts themselves. The results are shown in Table I. In the more concentrated urea solution all the electrolytes tried, except potassium phosphate, accelerate the rate. In solutions of the same ionic strength, the acceleration is the same, except with potassium perchlorate and sodium acetate. In the more dilute urea solution only sodium chloride accelerates the rate to the same extent as it does at the higher urea concentration. The other electrolytes either accelerate less or retard the rate. These results may be interpreted by the existence of two simultaneous electrolyte effects. One is a general acceleration of the rate by all electrolytes, due to changing ionic strength of the solutions. The other is a specific and competitive inhibition of the enzyme by most of the electrolytes tested. The buffer electrolyte falls into this general pattern.

(7) A few experiments were also made with 400 mM urea solutions. The results were qualitatively in agreement with those at lower urea concentrations.

Dependence on substrate concentration is complicated experimentally by the influence of the buffer on the activity of the enzyme, and it was therefore necessary to extrapolate the results to zero concentration of buffer. As will be shown below, if the buffer electrolyte both accelerates the rate because of changes in the ionic strength of the solution and at the same time competitively inhibits the enzyme, there should be a linear relationship between the concentration of the buffer and the quantity $10^{A\sqrt{\mu}}/V$, where A is the proportionality constant derived from the observed electrolyte effect, treated as due to ionic strength

TABLE I

EFFECT OF ADDED ELECTROLYTES ON THE RATE OF UREA HYDROLYSIS

Solutions buffered at pH 4.3 with 0.04 M sodium acetate, except in experiments on the buffer salts.

Salt	Concn. of salt (M)	Total ionic strength, μ	Relative rate
A. Solutions 33.3 mM in urea			
HAc + NaAc	0.021	0.00598	0.93
	.040	.0115	1.00
	.050	.0143	1.03
	.100	.0286	1.13
	.400	.121	1.25
NaCl	.030	.042	1.32
KNO_3	.030	.043	1.34
Na_2SO_4	.010	.042	1.37
NaCl	.060	.074	1.52
KNO_3	.060	.074	1.52
KI	.060	.073	1.52
Na_2SO_4	.020	.073	1.44
$KClO_4$	.060	.072	1.11
KH_2PO_4	.010	.0214	0.045
B. Solutions initially 3.00 mM (av. 2.94 mM) in urea			
HAc + NaAc	0.021	0.00598	0.97
	.040	.0115	1.00
	.050	.0143	1.00
	.100	.0286	1.02
	.200	.0624	0.89
NaCl	.015	.0273	1.15
KNO_3	.0151	.0272	1.08
Na_2SO_4	.00505	.0270	0.95
NaCl	.030	.0432	1.32
KNO_3	.0303	.0427	1.12
Na_2SO_4	.0101	.0427	0.92
NaCl	.060	.0732	1.51
KCl	.060	.0728	1.36
KNO_3	.0606	.7039	1.10
NaI	.060	.0728	1.07
KI	.060	.0728	1.04
Na_2SO_4	.0202	.0738	0.87
$KClO_4$	.060	.0735	0.77
KH_2PO_4	.015	.0275	No reaction
NaH_2PO_4	.015	.0275	detected

(see below), μ is the ionic strength and V is the observed rate. In Fig. 2 some of the data are plotted in this manner, and it is seen that straight lines are indeed obtained at urea concentrations of 100 mM and less. There was evidence that deviations occur at larger urea concentrations (400 and 1000 mM), probably because of added complications due to inhibition by urea.[2] The intercepts of the straight lines with the ordinate axis are then the rates at different urea concentrations, extrapolated to zero buffer concentration (and hence also to zero ionic strength). The extrapolated values, including some not shown in Fig. 2, are given in Table II.

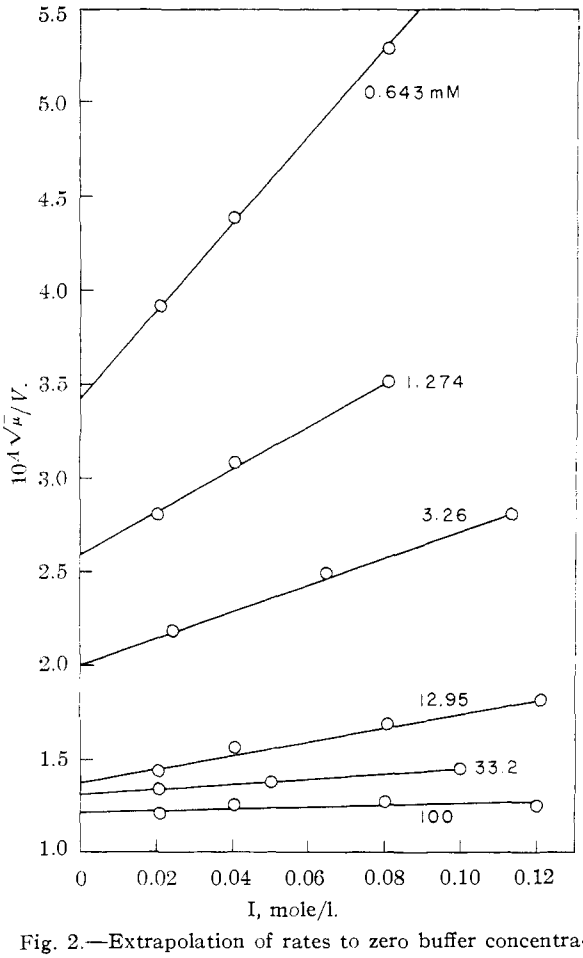

Fig. 2.—Extrapolation of rates to zero buffer concentration, using eq. 3. Figures in graph show concentrations of urea in mM/l. Abscissa shows concentration of buffer in M/l.

Test for Inhibition by Ammonium Ions and the Isotopic Exchange between Urea and Ammonium Ions.—As noted in the Introduction, the reversible reaction of urea with urease could be a metathetic reaction of the type EH + $(NH_2)_2CO$ = $E(NH_2)$-CO + NH_3 rather than an association reaction: EH + $(NH_2)_2CO$ = $EH(NH_2)_2CO$. If the former is the case, the inhibition by ammonium ion described by Hoare and Laidler[6] finds a ready explanation. The existence of such a reaction can be demonstrated by carrying out enzymatic urea hydrolysis in the presence of labeled ammonia and testing the residual urea for isotopic enrichment. To determine favorable conditions for such experiments a study of the reported inhibition by ammonium ions was first undertaken. For this purpose sodium maleate buffers at pH 6.0 and 7.0 were used, and the rate of hydrolysis was measured at each pH in two solutions, one containing added ammonium nitrate, and the other not. The results are shown in Table III. The initial concentrations of ammonium ion chosen were such that the rates in the two solutions should have been in the ratio of 1:5, or less, according to the interpretation of Hoare and Laidler. Table III shows, however, that the rates are identical, within the experimental error, which was unusually large in these experiments. The inhibition by ammonium ions is clearly absent, in maleate buffers at least.

TABLE II

RATE OF UREA HYDROLYSIS AT pH 4.3 EXTRAPOLATED TO ZERO BUFFER CONCENTRATION

Av. urea concn. (mM)	Relative rate V^0	Av. urea concn. (mM)	Relative rate V^0
0.643	0.292	3.26	0.500
0.658	.289	6.56	.588
1.274	.386	6.57	.589
1.281	.380	12.95	.733
1.929	.458	33.2	.765
3.24	.525	100	.823

TABLE III

TESTS FOR INHIBITION BY AMMONIUM IONS IN SODIUM MALEATE BUFFERS

Initial (NH_4NO_3) = 0			Initial (NH_4NO_3) = 30.5 mM		
Time, min.	μM NH_4^+ produced	μM/min.	Time, min.	μM NH_4^+ produced	μM/min.
A. Test at pH 7; initial urea concn. 22.6 mM; volume of reaction mixture 31 ml.					
0	0		0	0	
31.5	130	4.13	23.0	81	3.52
67.5	243	3.60	53.0	200	3.77
97.0	290	2.99	83.5	266	3.19
Initial (NH_4NO_3) = 0			Initial (NH_4NO_3) = 15 mM		
B. Test at pH 6; initial urea concn. 15.0 mM; volume of reaction mixture 21 ml.					
0	0		0	0	
13.5	40.5	3.00	11.0	24	2.18
24.0	84	3.50	21.7	68	3.13
34.5	131	3.80	32.5	113	3.47
46.5	185	3.98	44.0	190	4.32
61.0	237	3.90	57.5	254	4.42
74.0	294	3.98	71.0	307	4.32

Notwithstanding this negative result, the experiments on the exchange between urea and ammonium ions in the presence of the enzyme were carried out. The experimental procedure was as follows. To a solution 0.01 M in urea, 0.05 M in NH_4NO_3 containing 62.5% $N^{15}H_3$, buffered at pH 6.9 with 0.1 M sodium maleate, was added unbuffered enzyme solution. After 70 minutes the reaction was stopped by adding hydrochloric acid as usual and the reaction mixture, after neutralization, was passed slowly through an ion-exchange column. The effluent was collected. A small aliquot of it was tested for ammonium ions by means of Nessler reagent, and none was found. Enough glacial acetic acid was added to make the solution

35% in acid, and the urea was precipitated as dixanthylurea by a 4% solution of xanthydrol in methyl alcohol. The precipitate was dried and from its weight it was determined that 68% of the initially present urea had been hydrolyzed. The dixanthylurea was converted to nitrogen and other products by the Dumas method and the isotopic composition of the nitrogen determined with a Consolidated Isotope-Ratio mass spectrometer, Model 21-201.

Another reaction mixture, identical to the first except that it contained no enzyme, was allowed to stand the same length of time and was then analyzed to determine whether non-enzymatic exchange takes place. Finally, some urea used in these experiments was precipitated by xanthydrol and the product analyzed as above. The results shown in Table IV indicate that isotopic exchange is accelerated by the presence of enzyme only to a very slight extent, since complete exchange would have produced nitrogen approximately 45% N^{15}.

Errors.—The random errors involved in the rate measurements described in the preceding sections were determined by: (a) passing solutions containing identical amounts of ammonium sulfate through nine ion exchange columns and analyzing for ammonium ion and (b) by carrying out nine identical enzymatic hydrolyses and analyzing for ammonium ion. In both cases the standard

TABLE IV

MASS SPECTROMETRIC ANALYSES OF NITROGEN DERIVED FROM UREA

Source of N_2	$\frac{\text{Mass } 29}{\text{Mass } 28}$ ± Std. dev. of mean	No. of determinations
Enzymatic	0.0196 ± 0.00025	3
Non-enzymatic	.0102	1
Reference	.00766 ± 0.000014	6

deviation of individual measurements was 2%, showing that the analytical procedure was the limiting factor. Rate measurements were usually carried out in triplicate and hence the standard deviation of the mean was about 1%. To this must be added errors resulting from comparison of the rate with that in the standard solution, from incomplete identity of buffer solutions used in experiments widely separated in time, etc. An overall standard deviation of *ca.* 3% appears to be a fair estimate of combined errors.

Interpretation of the Results. The ratio of enzymatic activities at pH 4.3 and 7.0 is subject to considerable variations. The experiments have not completely identified the cause but have made it rather probable that a slow introduction of a competitive inhibitor into the enzyme solutions is causing them. The data presented in Table I show that several anions inhibit the enzyme strongly at pH 4.3. Extraction from glass of some of its anionic constituents by neutral enzyme solutions appears to be the most likely cause of the observed variations in the ratio R. Its true value, therefore, is no less than the highest observed, which is $R = 0.46$, and while it has not been determined with high precision, the uncertainty has only an unimportant effect on the other results described in this paper.

The electrolyte effects are complex but are rationally interpreted as the superposition of a general accelerating action and some specific competitive inhibitions. The data of Table I strongly suggest that the latter are due to anions, and a comparison with the effect of electrolytes at higher pH[1-3] shows it to be limited to low pH. Presumably, the competitive inhibition is due to the combination of anions with some of the positively charged groups of the enzyme molecule. As the positive charges disappear on raising the pH, so do the inhibitory effects of the anions. For this reason the maleate buffers are non-inhibitory in the pH range studied previously,[2] and even the very strong inhibition by phosphate ions vanishes at pH 7.5.[2] A more thorough investigation of the effects of pH on inhibition by anions should permit the identification of the protein groups at which binding takes place and also establish with certainty which state of polyvalent anions (*e.g.*, $H_2PO_4^-$, $HPO_4^=$, etc.) is most effective in causing inhibition. It is interesting to note that Scatchard and Black,[8] in their study of the effect of various uni-univalent electrolytes on the isoionic point of human serum albumin, found that the effect increases in the order NaCl, $NaNO_3$, NaI and $NaClO_4$. Changing the cation has only a much smaller effect. The competitive inhibitory properties of these salts (or their potassium analogs) were now found to increase in the same order (see Table I). This suggests that the specific enzymatic effects of the ions may be due to differences in their inherent tendency to bind to proteins.

That the data obtained fit quantitatively the proposed mechanism of competitive inhibition will be shown below after analyzing the accelerating effect of electrolytes. This we assign to the action of the ionic strength in the solution on the activity coefficient of a charged species. Figure 3 shows the logarithmic plot of the rates against the square root of the ionic strength. Experimental points for 33.3 mM urea are seen indeed to fall on a straight line, as demanded by the Debye–Hückel limiting law. The slight fall-off at the highest ionic strength is in the direction predicted when the two-parameter[3] Debye–Hückel equation is used in obtaining an expression for the rate V^μ as a function of ionic strength μ

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

The parameter A was found to have the value of 1.3 and is thus intermediate in magnitude between the values observed at pH 7.5 and 8.9.[3] However, the interesting point is that the sign of A has been reversed on going from basic to acidic solutions. The significance of this finding will be considered later in conjunction with the analysis of pH effects.

The determination of A permits a quantitative treatment of the effect of the buffer on the reaction rate. The combination of the ionic strength effect and of competitive inhibition applied to the simple Michaelis–Menten mechanism leads to rather unsatisfactory results in that either the inhibition constant or the Michaelis constant shows a strong trend with the concentration of the substrate.

(8) G. Scatchard and E. S. Black, *J. Phys. Chem.*, **53**, 88 (1949).

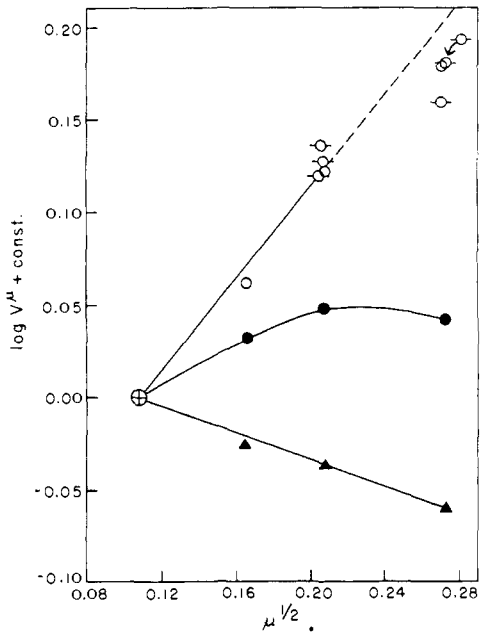

Fig. 3.—Logarithmic plots of relative rates V^{μ} against the square root of ionic strength: barred open circles, several neutral salts in 33.3 mM urea concentration; open circles, NaCl in 2.94 mM urea; solid circles, KNO_3 in 2.94 mM urea; solid triangles, Na_2SO_4 in 2.94 mM urea solutions; crossed circle, buffered solution without added electrolytes.

More self-consistent is the combination of the above effects with the hypothesis of two independent active sites, a mechanism which will be shown below to be applicable in the absence of buffer. It leads to the rate equation

$$V^{\mu} = 10^{A\sqrt{\mu}} \left[\frac{k_3^{\circ}(E_0)(S)}{(S) + K_M(1 + K_I(I))} + \frac{k_3^{\circ\prime}(E_0')(S)}{(S) + K_M'(1 + K_I'(I))}\right] \quad (2)$$

where $k_3^{\circ}(E_0)$ and $k_3^{\circ\prime}(E_0')$ are the respective V_{max}° of the two sites in solutions of zero ionic strength. The simplest form of the inhibition reaction: E + I = EI; K_I = (EI)/(E)(I) has been used because it leads to an equation adequately describing the experimental data. We shall assume $k_3^{\circ}(E_0) = k_3^{\circ\prime}(E_0')$ because it has been shown previously[2] that a satisfactory representation of urease kinetics is possible with this reduction of adjustable parameters. Denoting by V° the rate at zero ionic strength and zero inhibitor concentration, neglecting a term in $(I)^2$ in the numerator and a term in (I) in the denominator, which become important at buffer concentrations of 0.1 M and higher, equation 2 can be expanded into

$$\frac{10^{A\sqrt{\mu}}}{V^{\mu}} = \frac{1}{V^{\circ}} + (I)\frac{K_M K_M'(K_I + K_I') + (K_M K_I + K_M' K_I')(S)}{k_3^{\circ}(E_0)(S)\,[2(S) + K_M + K_M']} + \cdots \quad (3)$$

This equation shows that a straight line relationship should exist between $10^{A\sqrt{\mu}}/V^{\mu}$ and the concentration of the inhibitor. That this is indeed the case is shown by Fig. 2 for different concentrations of urea and buffer.

Calculations have been made for three assumed ratios of the inhibition constants in equation 2: (1) $K_I = K_I'$; (2) $K_I/K_I' = K_M/K_M'$ and (3) $K_I/K_I' = K_M'/K_M$, of which the third relation appeared most likely because the inhibition constants are defined for the reaction E + I = EI whereas the Michaelis constants (when regarded as equilibrium constants) are written for the reverse process: ES = E + S. Substituting into eq. 3 the later-to-be-established values of the Michaelis constants, K_M = 0.42 mM and K_M' = 8.4 mM, the inhibition constants are obtained from a comparison of the experimental ratio (intercept)/(slope) with the corresponding expression derived from eq. 2 and 3. The results are shown in Table V. The compounding of errors which is involved in this calculation results in a substantial scatter of results. It is seen, however, that the assumption of identical inhibition constants results in only very slight systematic trends of K_I over a urea concentration range from 0.6 to 100 mM. Measurements at 400 and 1000 mM urea concentration give inhibition constants entirely out of line with the values shown. Equation 3, however, is inapplicable in this concentration range because it fails to take into account the inhibition by urea.[2]

TABLE V

INHIBITION CONSTANTS OF SODIUM ACETATE BUFFER SALTS AT *p*H 4.3

Assumptions: (I): $K_I = K_I'$; (II): $K_I = K_I' K_M/K_M'$; (III): $K_I = K_I' K_M'/K_M$

(Urea), mM/l.	Exptl. (Fig. 2) intercept/slope	K_I (M^{-1}) I	II	III
0.64	0.147	5.1	0.36	15
0.66	.144	5.3	.37	16
1.28	.146	6.1	.39	23
1.28	.212	4.2	.27	16
2.94	.300	3.9	.23	21
3.26	.276	4.4	.25	24
3.26	.421	2.9	.16	16
12.9	.363	6.5	.35	53
33.3	.970	4.9	.26	46
33.3	.741	6.3	.34	60
100.0	2.13	5.8	.31	58

The rate as a function of substrate concentration, after the extrapolation to zero buffer concentration, is shown in Table II. The experimental points show a strong curvature in a Lineweaver–Burk plot. The data were analyzed by the method of Hill[9] for the hypothesis of two independent sites with equal values of $k_3^{\circ}(E_0)$. The two Michaelis constants resulting from this computation are K_M' = 8.4 and K_M = 0.42 mM. Figure 4 shows the experimental data and the curve drawn with these parameters. The agreement with the hypothesis of two sites is well within the experimental errors. As in previous work on urease, the data leave open the choice between the hypothesis of two independent sites with different Michaelis constants and of two identical sites with interaction. The latter was emphasized in previous work but the following will show that a slight advantage accrues from the use of the independent site hypothesis.

The *p*H and Electrolyte Effects.—Converting the previously determined rate parameters[3] at different *p*H to those corresponding to the inde-

(9) A. V. Hill, *Proc. Physiol. Soc.* [iv], *J. Physiol.*, **40** (1910).

pendent site hypothesis, and adding the parameters now evaluated, results in the data shown in Table VI.[10]

The widely accepted explanation of an optimum pH value for V°_{max} is that proposed by Michaelis and Davidsohn[11] and Michaelis and Pechstein.[12] Their ideas may be embodied in the reaction mechanism

$$\begin{array}{ccccc} E^- + H^+ & & ES^- + H^+ & & \\ \uparrow\downarrow K_a & & \uparrow\downarrow K_{as} & & \\ S + EH & \underset{k_1}{\overset{k_2}{\rightleftharpoons}} & EHS & \xrightarrow{k_3} & EH + P \\ +H^+ \downarrow\uparrow K_b & & +H^+ \downarrow\uparrow K_{bs} & & \\ EH_2^+ & & EH_2S^+ & & \end{array} \quad (4)$$

which attributes changes in V°_{max} solely to changes in the concentration of the active form of the enzyme–substrate compound, EHS. It follows that

TABLE VI

KINETIC PARAMETERS OF UREASE AT SEVERAL pH AND 25°

pH	V°_{max} Obsd.	V°_{max} Calcd.	K_M, mM	K'_M, mM	A (eq. 1)	K_M/V°_M	K'_M/V°_{max}
4.3	0.40	0.11	0.42	8.4	1.3	1.05	21
5.4	0.76	(0.76)	1.00	7.6	0	1.32	10
6.0	1.00	1.22	1.13	7.8	0	1.13	7.8
6.5	1.29	1.41	1.49	7.5	0	1.15	5.8
7.0	1.48	(1.48)	1.60	6.8	−0.4	1.08	4.6
7.5	1.45	1.48	1.32	9.0	−0.5	0.91	6.2
7.5			(1.60)	(7.5)		(1.10)	(5.1)
8.9	0.95	(0.95)	1.09	7.3	−2.5	1.15	7.7

$$V^{\circ}_{max} = \frac{k_3(E_0)}{1 + \frac{K_{as}}{(H^+)} + \frac{(H^+)}{K_{bs}}} \quad (5)$$

where (E_0) is the (constant) total concentration of the enzyme.

The experimental values of V°_{max} at pH 5.4, 7.0 and 8.9 were used to evaluate the three parameters in equation 5, yielding $k_3(E_0) = 1.53$, $K_{as} = 7.9 \times 10^{-10}$, $K_{bs} = 3.9 \times 10^{-6}$. The values of V°_{max} shown in column 3 of Table VI were calculated with these parameters. It is clear that equation 5 does not represent quantitatively experimental data in acid solutions. On the basic side of optimum pH, insufficient data are available to draw any conclusions.

The postulate that the species EH_2S^+ is also capable of yielding the products of hydrolysis, but with a smaller rate constant than k_3, would improve somewhat the agreement of calculated and observed V°_{max}, as almost any additional adjustable parameter would. The agreement, however, cannot be made perfect because the observed changes in V°_{max} spread over too wide a pH range to be fitted by a single ionization constant. It may be that the increased positive charge which the enzyme molecule acquires, as the pH of its environment is lowered, causes an increase in the acid dissociation constants,[13] including those of groups which have control over the enzymatic activity. This effect would explain the observed deviations from eq. 5, providing a change in the ionization constants of about a factor of ten was occurring over the pH range investigated.

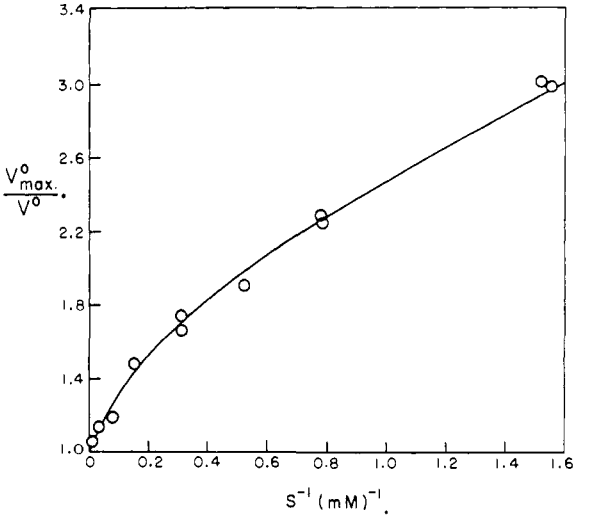

Fig. 4.—A Lineweaver–Burk plot of relative rates at zero ionic strength. Solid line calculated from the three-parameter rate equation.

The mechanism given in (4) leads to expressions of the following type involving the Michaelis constants of the two sites defined by eq. 2

$$\frac{K_M}{V^{\circ}_{max}} = \frac{k_2 + k_3}{k_1 k_3 (E_0)}\left[1 + \frac{K_a}{(H^+)} + \frac{(H^+)}{K_b}\right] \quad (6)$$

Table VI shows that for the unprimed site the ratio K_M/V°_{max} is constant over the entire pH range investigated.[14] On the other hand, the ratio K'_M/V°_{max} is seen to pass through a minimum, as it should if K_a and K_b have values not greatly different from the hydrogen ion concentrations under consideration. On the basis of mechanism (4) one must thus conclude that the unprimed site is not subject to ionization when it is not combined with the substrate, whereas the primed site does ionize under these conditions. It is interesting to note that the experimental K'_M/V°_{max} fails to fit eq. 6 in precisely the same manner as V°_{max} fails to fit eq. 5. Thus ionization constants which themselves are functions of pH would explain equally well both observations.

It might seem that the observed electrolyte effects are best treated as a "secondary salt effect" on the equilibria defined by (4) but this proposition is inconsistent with the experimental results. If the charges associated with the catalytic activity of the enzyme are treated as residing on small ions, the equilibria of the enzyme–substrate compound defined by (4) call for a secondary salt effect quite different from that observed. Namely, the increase of ionic strength on both sides of the optimum pH would decrease the apparent activity of

(10) Only limited experimental data were obtained to calculate K_M and K'_M, at pH 7.5. The parameter directly determined from the experimental data by the Hill method is the product $K_M K'_M$. If one accepts its experimental value, 11.9 (mM)^2, but assumes that the separation into the two factors[2] was slightly in error, one can obtain a value of $K_M/V^{\circ}_{max} = 1.10$, thus entirely consistent with the values at other pH, by the choice of $K'_M = 7.5$, $K_M = 1.6$ mM. The resulting value of K'_M is also more consistent with the rest of the data. The recalculated values are bracketed in Table VI.

(11) L. Michaelis and H. Davidsohn, *Biochem. Z.*, **35**, 386 (1911).

(12) L. Michaelis and H. Pechstein, *ibid.*, **59**, 77 (1914).

(13) *Cf.* P. Doty and G. Ehrlich, *Ann. Rev. Phys. Chem.*, **3**, 81 (1952).

(14) This expresses the experimental finding that at very low urea concentrations and zero ionic strength the observed rate of hydrolysis is completely independent of pH.

the enzyme by lowering the activity coefficient of the ionized (inactive) species. This difficulty may be overcome by a fairly trivial change in the Michaelis–Pechstein mechanism, *viz.*, the assignment of a charge to the intermediate (active) form. Thus, for instance, if the equilibria are

$$EH_2S \overset{K_{bs}}{\rightleftharpoons} EHS^- + H^+ \overset{K_{as}}{\rightleftharpoons} ES^- + 2H^+ \quad (7)$$

the concentration of EHS^- will increase with increasing ionic strength on the acid side and will show the opposite behavior on the basic side. But other difficulties remain, among which the following should be mentioned. (1) The observed electrolyte effects are quite asymmetric with respect to the optimum *p*H. From the optimum near *p*H 7 they remain insignificant down to at least *p*H 5.4, even though, according to Table VI and eq. 5, the ionization equilibrium substantially reduces the concentration of the active form before *p*H 5.4 is reached. On the other side, the basic electrolyte effect becomes marked at *p*H 7, *i.e.*, right at the optimum. (2) An interpretation of the electrolyte effect as the secondary salt effect requires, in general, that both Michaelis constants be also subject to electrolyte effects, because according to (4) they are given by

$$K_M = \frac{k_2 + k_3}{k_1} \frac{1 + \frac{a_{H^+} f_{EH}}{K_b' f_{EH_2}} + \frac{K_a' f_{EH}}{a_{H^+} f_E}}{1 + \frac{a_{H^+} f_{EHS}}{K_{bs}' f_{EH_2S}} + \frac{K_{as}' f_{EHS}}{a_{H^+} f_{ES}}} \quad (8)$$

where a_{H^+} is the activity of hydrogen ion and the *f*'s are the activity coefficients of the various species. (The equilibrium constants in eq. 8 are written with a prime, to distinguish them from those of eq. 5 and 6 which, for simplification, are written as involving concentrations and not activities.) From eq. 8 it is seen that if the Michaelis constants exhibit a *p*H dependence, they should also show an ionic strength dependence. Experimentally, K_M is strongly *p*H dependent and K_M' less so; however, neither shows an ionic strength effect. This constitutes irrefutable evidence against the hypothesis considered.

An alternative interpretation is that ionic strength affects the rate parameter k_3 and is therefore in the nature of a primary salt effect. To be subject to such effects in dilute electrolyte solutions, a reaction must involve two charged species. If the combined active site is assumed to have the properties of an amphoteric electrolyte, it might acquire positive charge at *p*H 4.3 and negative charges at *p*H 7 or higher. The observed salt effect would then be qualitatively explained by assuming that the enzyme–substrate compounds react with a hydrogen ion rather than with a neutral water molecule. But if so, then V_{max} should show a very strong dependence on *p*H, since the hydrogen ion concentration varies by a factor of 40,000 over the range investigated. The observed mild dependence of V_{max} on *p*H in absence of electrolytes is definitely not consistent with this conclusion. And what dependence there is, becomes awkward to explain because the enzyme–substrate compound is assumed to be active regardless of its state of ionization and hence eq. 5 cannot be appealed to. Thus one is led to reject this simple hypothesis. Since the relegation of electrolyte effects to changes in the concentration of active enzyme has also led to logical inconsistencies, the detailed mechanism of electrolyte effects remains unresolved. It is probable that to arrive at the correct explanation, the polyelectrolyte nature of the enzyme protein cannot be as lightly passed over as was done above; moreover, the rate-determining step may be an intramolecular reaction between (charged) parts of the enzyme molecule, rather than an intermolecular one as assumed above.

In any case, it is significant that the electrolyte effect is identically the same regardless of the concentration of urea. Hence V_{max} and K_M/V_{max} (for both sites) respond in identical fashion to changes in ionic strength. Effects of ionizations defined by eq. 5 and 6 fail to explain this. Hence the rate constants must themselves be functions of ionic strength. The alternatives are either that all three rate constants, k_1, k_2 and k_3, depend identically on ionic strength, or that k_3 alone is dependent. The former alternative is unlikely because k_1 is the rate constant of a reaction in which an *uncharged* molecule of urea combines with the enzyme. An analogous reaction, that of the hydration of carbon dioxide by carbonic anhydrase, has been studied in this Laboratory by Dr. R. P. Davis,[15] who was able to show in a much more certain fashion than is possible with urease, that the rate constants k_1 and k_2 of carbonic anhydrase are independent of ionic strength while k_3 depends on it. If the same holds for the urea–urease reaction, then it follows that for both active sites $k_3 << k_2$ and the Michaelis constants are essentially the equilibrium constants: $K_M = k_2/k_1$.

Isotopic Exchange.—This form of the Michaelis constant has an important bearing on the significance of the isotopic nitrogen exchange experiments described in this paper. If ammonia is hydrolyzed from urea molecules in the course of the first (fast) reaction step, then a virtually complete isotopic exchange should have occurred in the experiments in which a high concentration of ammonia was present. This was shown not to be the case and moreover it was found that ammonia does not inhibit the reaction.[16] Hence urea combines with the enzyme without loss of ammonia. The latter must be split off only in the last and slower reaction step, which results also in the formation of carbamic acid, according to Wang and Tarr.[17]

The present exchange experiments indicate that a slight acceleration of isotopic exchange between urea and aqueous ammonia is caused by the enzyme. Compared to the rate of urea hydrolysis, this reaction is slower by two orders of magnitude. Nonetheless it cannot be attributed to enzymatic synthesis of urea from the added ammonia and the carbon dioxide which is formed upon hydrolysis of carbamic acid. The equilibrium between urea, car-

(15) Unpublished data.

(16) Assuming that the inhibition observed by Hoare and Laidler is not due to experimental errors, the inhibiting species in their experiments must have been ammonium phosphate anions. Compare ref. 1 for a similar interpretation of different rates observed in sodium and in potassium phosphate buffers.

(17) J. H. Wang and D. A. Tarr, THIS JOURNAL, **77**, 6205 (1955).

bon dioxide and ammonia in dilute aqueous solutions is so far on the dissociation side[18] that enzymatic synthesis of urea in the present experiments accounts for only $10^{-5}\%$ exchange, instead of the 1% actually observed. The mechanism by which N^{15} is incorporated into urea is therefore uncertain and it may only be suggested that it is due to re-

(18) G. N. Lewis and G. H. Burrows, THIS JOURNAL, **34**, 1515 (1912).

synthesis of urea from carbamic acid and ammonia, before the former decomposes into ammonia and carbon dioxide.

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CAMBRIDGE, MASS.

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

The Oxygen Exchange between Oxy-anions and Water; Bromate and Iodate Ions[1]

BY T. C. HOERING, R. C. BUTLER[2] AND H. O. MCDONALD

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The kinetics of the isotopic exchange reaction between bromate ion and water has been studied. In the region of 0.02 to 0.80 *M* bromate ion, the reaction follows the rate law ($R = k(H)^{+2}(BrO_3^-)$ where $k = 6.60 \times 10^{-3}$ liter^2 moles^{-2} second^{-1} at 30.00° and ionic strength 0.900. The activation energy is 14,230 cal./mole. The course of the reaction is not affected by light, added Br_2 or increased surface area. The dependence on the water concentration was not measured. The reaction proceeds faster in D_2O with k_D/k_H equal to 1.72. The corresponding exchange between iodate ion and water is complete in the time of separation of one minute. The exchange was studied under a number of different conditions. Different methods of separating the reactants were used. The rate of the bromate–water exchange is compared with that of the reduction of bromate ion by halide ions.

Introduction

This paper is a report of quantitative measurements on the rate of exchange of isotopic oxygen between bromate ion and water. It also describes attempts to measure the kinetics of the iodate–water exchange.

The exchange of isotopic oxygen between bromate ion and water has been studied qualitatively by several workers[3] who have reported the exchange to be slow. The exchange of many oxy-anions and water occurs in acid solutions. Bromic acid is a strong acid and is a good oxidizing agent in acid solutions. Under the conditions of these experiments, bromic acid is stable toward decomposition. The exchange between iodic acid and water has been studied qualitatively[3] and a fast exchange has been reported. Iodic acid is only partially dissociated, is a good oxidizing agent and is more stable toward decomposition than bromic acid. The rates of oxidation by iodic acid are generally more rapid than by bromic acid.

Experimental

Materials.—Oxygen-eighteen tagged potassium bromate and potassium iodate were prepared by the electrolytic oxidation of potassium bromide and potassium iodide in O^{18}-tagged water.[4] All chemicals were reagent grade. The water used as solvent was redistilled from alkaline permanganate.

Procedures.—The bromate water exchange was studied in solutions maintained at constant ionic strength 0.900 with added sodium nitrate. The exchange of oxygen between nitrate ion and the solvent is very slow under the conditions of these experiments.[5] The proper amounts of nitric acid, sodium nitrate and O^{18}-tagged bromate were placed in a volumetric flask and diluted to the mark with water of normal isotope content. The hydrogen ion concentration of each run was determined by the titration of 0.250-ml. samples of the reaction mixture. The reactants were separated by the precipitation of silver bromate. These precipitates were converted to O_2 for isotopic analysis by thermal decomposition.

The iodate exchange was studied by dissolving O^{18}-tagged potassium iodate in solutions of HNO_3 or KOH of the desired *p*H. The iodate fraction was separated by the precipitation of silver iodate in acid solutions or barium iodate in alkaline solutions. These precipitates were decomposed to O_2 for isotopic analysis. In some experiments, samples of water were vacuum distilled from the reaction mixture. The water was allowed to reach isotopic equilibrium with CO_2 and the CO_2 was used for isotopic analysis.[6]

The isotopic analyses were made on an isotope ratio mass spectrometer designed for the measurement of small differences in isotope content.[7] Measurements of 34/32 ratios were made on oxygen. The carbon dioxide samples which were equilibrated with the water from the iodate exchange had relatively small differences in their O^{18} content. These differences were measured by observing the change in the ratio of mass 46 to mass 44 when rapidly shifting back and forth between a standard CO_2 sample and the unknown sample.

Calculations.—The bromate exchange study requires a measurement of the change in O^{18} content of the bromate fraction as a function of time. The half-time for the exchange was found by plotting the log of one minus the fraction exchanged *versus* time.[8] The rate of the reaction which leads to isotopic exchange was then calculated by[8]

$$R = \frac{3(BrO_3^-) \times (H_2O)}{3(BrO_3^-) + (H_2O)} \times \frac{0.693}{t_{1/2}} \qquad (1)$$

where R is the rate in gram-atom oxygen/liter-second when the quantities in parentheses are the formal concentrations of the exchanging species and $t_{1/2}$ the half-time for exchange in seconds.

If a single reaction path is available for the reaction which leads to isotopic exchange, the rate law at a given temperature may be given by

$$R = k(H^+)^m(BrO_3)^n(H_2O)^p\Gamma \qquad (2)$$

(1) This work supported by funds supplied by the U. S. Atomic Energy Commission and by the National Science Foundation.

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