[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY]

On the Mechanism of the Inhibition of Urease[1]

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The inhibition of urease by three urea analogs, sulfamide, acetamide and thiourea, was investigated. The first compound has no observable effect on the enzyme. The second inhibits it very slightly. Only the inhibition by thiourea was strong enough for a quantitative study. The results show this reaction to be instantaneous and reversible. Both the competitive and the uncompetitive inhibitions are observed. They are quadratic in the concentration of thiourea and depend on *p*H. The kinetic treatment of complex enzyme inhibitions is shown to be greatly facilitated by the application of Cramer's method for the solution of simultaneous linear equations and the use of inhibition indices. This technique is applied to the previously studied kinetics of the "uninhibited" ureolysis and a complete mechanism is derived which properly represents the dependence of the rate on urea concentration, varied between 0.3 mM and 2000 mM and on *p*H in the range from 5.5 to 7.0. The inhibition by thiourea is included in this mechanism. Finally, Cramer's method is shown to be useful in a reinterpretation of certain observations on the behavior of urease in phosphate buffers, in presence of high concentrations of urea and in sodium sulfite solutions.

The inhibition of enzymes has been the object of extensive experimental work; much of it has been concerned with the inhibition by structural analogs of the substrates.[2] Urease was shown to be inhibited by many compounds, including several urea-like substances. Some of these inhibitions where shown to be reversible, but most of the experiments were of qualitative character.[3] The quantitative significance of others was distorted by the use of phosphate buffers which were subsequently shown to be inhibitors themselves.[4,5]

The experiments described below deal with the quantitative effect on urease of three urea-like compounds: sulfamide, acetamide and thiourea. Acetamide was reported not to be an inhibitor.[3] So was thiourea[6] but weak inhibition might have escaped detection. Indeed, Sizer and Tytell[7] observed that thiourea affected the activity of urease. The interesting diamide of sulfuric acid, sulfamide, has not until now been tested with urease. Its very close analogy to urea[8] suggested its test as an inhibitor and, perchance, as a substrate.

The mathematical treatment of the reversible inhibition of enzymes was developed some time ago.[9,10] It is based on the Michaelis–Menten kinetics of the uninhibited catalysis and is limited to comparatively simple inhibition mechanisms. The experiments of an earlier paper[11] demonstrated significant deviations of the kinetics of the uninhibited ureolysis from the Michaelis–Menten mechanism; the data presented below reveal a somewhat more complex pattern of inhibition than is dealt with in earlier theoretical discussions. Therefore, a mathematical procedure had to be found which permits a more generalized treatment of the reversible inhibition of enzymes. With the help of this procedure, kinetic equations were derived from a complete reaction mechanism which describe satisfactorily the effects of several variables on the catalytic activity of urease.

Experimental Details

The experimental procedure was similar to that recently described.[5] One ml. of a thermostated solution of urease was added with stirring to 20 ml. of a thermostated solution containing urea, buffer salts and inhibitor. The enzyme was inactivated after a measured time interval by adding 2 N hydrochloric acid; the solution was then analyzed for ammonia produced. The analytical procedure employed was also similar to that previously described, except that the columns contained Permutit, recommended by Folin[12] for the determination of ammonia, instead of Dowex 50 ion exchange resin. Both techniques yield satisfactory results, but the use of Dowex 50 resin permits a quantitative determination of smaller quantities of ammonia. On the other hand, the absorption of ammonia in a Permutit can be carried out directly with the acid solutions obtained on destroying the enzyme. In the present experiments the urea concentration was kept at 7 mM or higher and hence high sensitivity was not required. The columns contained 3.6 g. of white granular Permutit.[12a] Prior to use, they were washed with 20 cc. of 2% NaOH solution followed by 150 cc. of ammonia-free water, then by 60 cc. of 2% acetic acid and finally by 220 cc. of ammonia-free water. Twenty-four ion exchange columns were set up and an experiment usually involved that many analyses. They included inhibited rate runs, rate runs made with the same urea and buffer salt concentrations in absence of inhibitor and blanks. Aliquot portions of the same dilute urease solutions were used for all. In blank runs strong acid was added before the enzyme. After absorption of the ammonium ion in Permutit at a flow rate of a drop per second and washing with 300 ml. of water, the columns were eluted with 20 ml. of 2% NaOH. The eluent was diluted to 250 ml. and Nesslerized. A 10-ml. aliquot was used in the 1-cm. cell of the Lumetron photometer. About 10^{-7} mole of ammonia could be detected with certainty. The runs were usually so arranged that from 1×10^{-6} to 4×10^{-6} mole of ammonia were produced.

Glass-distilled water was used, after passing through a bed of Dowex 50 and IRA 400 to avoid accidental inhibition of the enzyme. The glassware was cleaned in a nitric–sulfuric acid-bath, washed, and kept protected from dust until ready to use. J. T. Baker analyzed urea was used without further purification. Thiourea (Eimer and Amend, Tested Purity Reagent) was recrystallized from ethanol and then from water; m.p. 80°. Sulfamide was synthesized by the method of Degering and Gross[13] with about 40% yield. Analysis gave: S, 33.7 (33.3); N, 29.0 (29.2); H, 4.33 (4.17); m.p. 94°. The experiments were carried out in maleate buffers.[5] The concentration of the buffer in all experiments was 0.005

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

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

(3) T. Takeuchi, *J. Biochem. (Japan)*, **17**, 47 (1933).

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

(5) G. B. Kistiakowsky, A. J. Rosenberg, P. C. Mangelsdorf, Jr., and W. H. R. Shaw, THIS JOURNAL, **74**, 5015 (1952).

(6) O. Ambros and H. Münch, *Z. physiol. Chem.*, **187**, 252 (1930).

(7) I. W. Sizer and A. A. Tytell, *J. Biol. Chem.*, **138**, 635 (1941).

(8) L. F. Audrieth, M. Sveda, H. H. Sisler and M. J. Butler, *Chem. Revs.*, **26**, 49 (1940).

(9) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(10) F. H. Johnson, H. Eyring and W. Kearns, *Arch. Biochem.*, **3**, 1 (1943).

(11) G. B. Kistiakowsky and A. J. Rosenberg, THIS JOURNAL, **74**, 5020 (1952).

(12) (a) O. Folin and R. D. Bell, *J. Biol. Chem.*, **29**, 329 (1917); (b) J. C. Whitehorn, *ibid.*, **56**, 751 (1923).

(13) E. F. Degering and G. C. Gross, *Ind. Eng. Chem.*, **35**, 751 (1943).

M. Syn-Zyme Laboratories urease, with a specific activity of 60,000 S.U., was used for approximately one-third of the experiments here reported. Subsequently urease was prepared by the method of Sumner as modified by Dounce.[5] The final precipitate was taken up in chilled 50% glycerol. The specific activity of this preparation was about 10,000 S.U., remaining substantially constant for the duration of all experiments. To obtain stable dilute solutions of urease, it was necessary to dilute the stock solution with chilled water made 0.004 *M* in hydrogen sulfide and buffered to the *p*H of the run. These enzyme solutions were allowed to stand 3 hours before use.

The standard deviation of individual rate measurements was about 2 to 3%. Thus 12 tests of the analytical procedure using aliquots of an ammonia solution, had a s.d. of 2%. Eight hydrolyses carried out under identical conditions had a s.d. of 1.8%. The effect of several substances used in these experiments on the analytical results was negligible. Upon determining the quantity of ammonia formed by the enzyme in the presence of a potential inhibitor, the same amount of ammonia was added to a solution of the inhibitor, buffer salts and urea. This solution was then analyzed for ammonia. In all cases discussed below the two analyses agreed to within the precision of the analytical procedure.

Results

Sulfamide is not hydrolyzed by urease. The tests were conducted at a concentration of 0.25 *M* in sulfamide with solutions buffered at *p*H 6, 7 and 3, as well as in an unbuffered solution. If any reaction took place, less than 0.03% was hydrolyzed. No detectable inhibition by sulfamide was observed at a concentration of 0.5 *M*, *p*H 6 and *p*H 7, with 7.5 m*M* urea at 25° in 0.05 *M* maleate buffer.

Acetamide was tested for inhibition at *p*H 7 and 25° with 7.5 m*M* urea. A reduction of the rate of hydrolysis by 14 ± 4% was observed with 0.5 *M* acetamide. The effect was too weak for a quantitative study.

Thiourea was found to be an inhibitor. The reversibility of inhibition was demonstrated making use of the observation that at *p*H 7 thiourea showed no detectable inhibition below 0.05 *M* but reduced the rate by about 35% at 0.5 *M* concentration. Aliquot portions of an enzyme solution were added to two flasks, one of which was then made 0.5 *M* in thiourea, while the other was diluted to the mark with water. One-ml. portions of the two enzyme solutions were added to 20 ml. of 9 m*M* urea after 10, 20, and 30 minutes standing. The ratio of rates with the enzyme not treated by thiourea to those with treated enzyme was 0.98 ± 0.04. Thus even 30 minutes standing with 0.5 *M* thiourea has no effect on subsequent activity of the enzyme in sufficiently dilute thiourea solution.

The rate of ammonia production in the presence of thiourea was found to be independent of the total reaction time. The final concentration of ammonium ion in these experiments, which lasted from 1 to 5 minutes, was not more than 1×10^{-3} *M*. Thus the inhibition by thiourea appears to be an instantaneous and reversible reaction. The plots of inverse rate against inverse urea concentration are straight lines at a given thiourea concentration. This is shown in Fig. 1 for *p*H 7 and *p*H 6 at 25°.

As Fig. 1 demonstrates, *p*H alters the form of the inhibition law. At *p*H 6 only the slope of the lines is influenced by the presence of thiourea; at *p*H 7 the intercept is changed also. It is seen, moreover, that the extent of inhibition is not linear in thiourea at constant urea concentration.

A summary of all measurements is given in Table I. The data are presented in the form of inhibition indices,[10] each figure being the average of at least two separate determinations. The results at *p*H 6.7 are intermediate in character between those at *p*H 7 and 6. The results at 15° are very similar to those at 25°, the effect of temperature on the inhibition being evidently slight.

Discussion

The reversible inhibition of urease by thiourea is more complex than is predicted by the equations for a simple competitive or an uncompetitive inhibition. This follows because both the slopes and the intercepts of the Lineweaver–Burk plots[9] of Fig. 1 are altered by thiourea. Moreover, the

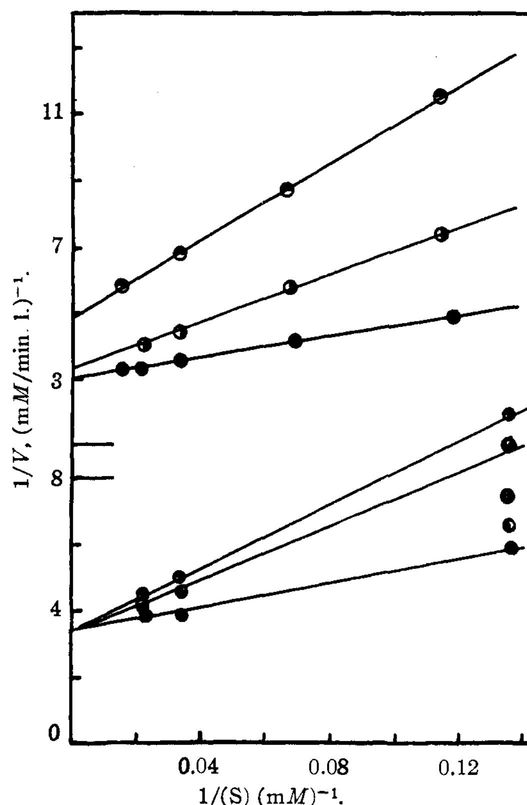


Fig. 1.—Inhibition by thiourea, a plot of inverse rate against inverse urea concentration: upper part, *p*H 7.0; lower part, *p*H 6.0, at 25°: ●, no thiourea; ◐, 0.3 *M*; ◑, 0.5 *M*; ◒, 0.7 *M*; ◓, 0.8 *M*.

straight lines of Fig. 1 are not an assurance that the analysis of thiourea inhibition may be based on the Michaelis–Menten mechanism of the uninhibited reaction. It is true that the deviations from this mechanism make themselves felt only outside that range of urea concentrations which was here investigated.[11] However, a rigorous kinetic treatment should make allowances for them, until proof is found that they are without effect on the inhibition measurements. Finally, the *p*H affects both the uninhibited reaction and the form of the inhibition law. The reaction mechanisms which attempt to allow for these various observations contain perforce many intermediate reaction steps. The working out of the rate equations becomes unwieldy and the results lack clarity. This difficulty has been overcome by the use of Cramer's method[14] for the solution of simultaneous linear equations. Its application permits a rapid survey and systematization of the kinetic consequences of various reaction mechanisms. Thus acceptable mechanisms can be readily identified and the rate equations cast into more instructive form.

To apply Cramer's method, several basic assumptions must be made about the reaction mechanisms. Some are universally used, as the mass action law expressions for the individual reaction steps and the steady state assumption for the intermediates. It will be assumed additionally that all the inter-

(14) *Cf.* H. Margenau and G. M. Murphy, "The Mathematics of Physics and Chemistry," D. Van Nostrand Co., Inc., New York, N. Y., 1951, p. 299.

mediates involved in the reaction mechanisms contain the enzyme molecule. Thus the initiation of "free" reaction chains in solution is excluded. Also, all reaction steps will be taken as linear in the concentrations of the intermediates. This is probably justified in the case of urease because it was shown[5] that the specific activity of this enzyme is independent of its concentration. However, the final justification for all of these assumptions comes only from the success of the treatment.

Cramer's Method.—If σ_i denotes a particular intermediate species (including the free enzyme) and E_0 the total enzyme concentration, the conservation equation is

$$E_0 = \sum_{i=1}^{n}[\sigma_i] \qquad (1)$$

The steady-state equation for the intermediate σ_j may be written as

$$[\dot{\sigma_j}] = 0 = \sum_{i=1}^{n-1} f_i[\sigma_i] \qquad (2)$$

Here $f_i[\sigma_i]$ is the total rate of formation of the intermediate σ_j from an intermediate σ_i. It may involve several parallel reactions. The summation includes the term $f_j[\sigma_j]$, which represents the total rate of decomposition of the intermediate σ_j. This again may consist of several parallel reactions. The coefficients f are therefore sums of products of the rate constants and concentrations of the substances which enter the reaction mechanism.

Equation (2) is written for each intermediate, except for the free enzyme. According to Cramer's rule, the concentration of the σ_j species is then

$$[\sigma_j] = \Delta_j/\Delta \qquad (3)$$

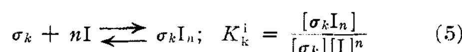
where Δ is the determinant of the coefficients on the right of eq. (1) and (2), while Δ_j is the same determinant but with the column containing the coefficients of σ_j having been replaced by E_0, 0, 0, 0....

The over-all rate of the reaction is represented by

$$V = \sum k_l[\sigma_l] = \sum k_l\Delta_l/\Delta \qquad (4)$$

where $k_l[\sigma_l]$ represents the rate at which the active intermediate σ_l breaks down into the final products.

The resultant equations are substantially simplified when added substances act as "total" inhibitors. That is, they react with the free enzyme or some other intermediate to form adducts which do not lead to the final products, except through the reversal of the addition reaction. In contrast, the product of the reaction of a "partial" inhibitor with some intermediate would break down more slowly into the final products than the "uninhibited" intermediate. Of course, the implication of eq. (2) in regard to total inhibitors is that the products of their reaction are in equilibrium with the uninhibited species. Thus, for a total inhibitor I reacting with the k-th intermediate



$$\sigma_k + n\mathrm{I} \rightleftharpoons \sigma_k\mathrm{I}_n;\quad K_k^i = \frac{[\sigma_k\mathrm{I}_n]}{[\sigma_k][\mathrm{I}]^n} \qquad (5)$$

where K_k^i is the inhibition equilibrium constant. Then for m mutually non-interacting inhibitors, the determinant Δ separates into a sum of subdeterminants

$$\Delta = \Delta_0 + \Delta_1 + \Delta_2 \ldots \Delta_m \qquad (6)$$

where Δ_0 contains no terms referring to inhibition. Each of the remaining m determinants contains only terms referring to a single total inhibitor. The sub-determinant Δ_0 is obtained by writing the conservation and the steady-state equations for the reaction mechanism in absence of the total inhibitors. The sub-determinant Δ_1 is derived from Δ_0 by replacing each of the coefficients unity of the conservation equation by $K_k^1[\mathrm{I}]^{n_1}$ which defines the inhibition equilibrium between the first inhibitor and the appropriate uninhibited species σ_k. Δ_2 is similarly derived for the second inhibitor, etc.[15]

The advantage of these expressions is realized when use is made of the inhibition index ϕ to characterize the extent of inhibition.[10] For the m-th inhibitor ϕ is defined as

$$\phi_m = (V_u - V_i)/V_i \qquad (7)$$

where V_i is the rate in the presence of the m-th inhibitor and V_u is the rate in its absence. In terms of the sub-determinants of eq. (6)

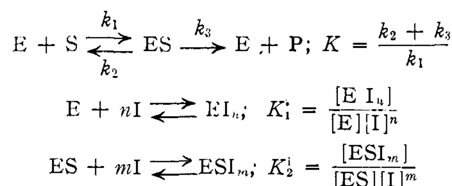
$$\phi_m = \Delta_m/(\Delta - \Delta_m) \qquad (8)$$

Thus for mutually non-interacting inhibitors the experimentally observed inhibition index ϕ_{tot} is simply the sum of the inhibition indices one would obtain from each inhibitor acting alone

$$\phi_{tot} = \sum_{i=i}^{m}\phi_i \qquad (9)$$

This is not true for the conventional expression, the fractional inhibition $(1/(1 + 1/\phi))$.

These general statements will be now applied to a simple example, the inhibited Michaelis–Menten mechanism, defined by the reactions



$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_3} \mathrm{E} + \mathrm{P};\quad K = \frac{k_2 + k_3}{k_1}$$

$$\mathrm{E} + n\mathrm{I} \rightleftharpoons \mathrm{EI}_n;\quad K_1^i = \frac{[\mathrm{E\,I}_n]}{[\mathrm{E}][\mathrm{I}]^n}$$

$$\mathrm{ES} + m\mathrm{I} \rightleftharpoons \mathrm{ESI}_m;\quad K_2^i = \frac{[\mathrm{ESI}_m]}{[\mathrm{ES}][\mathrm{I}]^m}$$

The equations for the concentrations of the intermediates are

$$E_0 = ES + E$$
$$\dot{ES} = 0 = -K\,ES + S.E$$

The determinants are

$$\Delta_0 = \begin{vmatrix} 1 & 1 \\ -K & S \end{vmatrix};\quad \Delta_{ES} = \begin{vmatrix} E_0 & 1 \\ 0 & S \end{vmatrix};\quad \Delta_1 = \begin{vmatrix} K_2^iI^m & K_1^iI^n \\ -K & S \end{vmatrix}$$

and therefore the inhibited rate is

$$V_i = \frac{k_3\Delta_{ES}}{\Delta_0 + \Delta_1} = \frac{k_3E_0S}{S + K + K_1^iI^nK + K_2^iI^mS}$$

The expression for the inhibition index

$$\phi = \frac{\Delta_1}{\Delta_0} = \frac{K_1^iI^nK + K_2^iI^mS}{S + K} = \phi^{E} + \phi^{ES} \qquad (10)$$

shows that the latter may be represented as a sum of terms. Each represents the contribution to ϕ

(15) Further details of these techniques and a discussion of more general types of inhibition and activation than are considered here may be found in the Ph.D. Thesis of W. H. R. Shaw, Harvard University, 1951.

from the reaction of the inhibitor with one particular intermediate. This separation of ϕ into additive terms is not limited to the Michaelis–Menten mechanism or the presence of one inhibitor only. It follows from the expansion of the determinant Δ_1 in terms of the sum of first row elements multiplied by their cofactors and so, quite generally, the inhibition index is the ratio of the sum of the concentrations of inhibited intermediates to the sum of the concentrations of uninhibited intermediates.

TABLE I

OBSERVED AND CALCULATED THIOUREA INHIBITION INDICES

Urea concn. (moles/l.) $\times 10^3$	Thiourea concn. (moles/l.)	$\phi_{obsd.}$[a]	$\phi_{calcd.}$[b]
	A. pH 7.0, 25°		
7	0.05	0.00	0.006
7	.10	.05 ± 0.05	.02
7	.20	.10 ± .04	.10
7	.30	.24 ± .05	.22
7	.40	.44 ± .06	.38
7	.50	.60 ± .06	.60
7	.70	1.10 ± .09	1.17
7	.80	1.42 ± .1	1.53
7	1.0	2.7 ± .2	2.5
8.6	0.5	0.52 ± .06	0.55
14.5	.5	.38 ± .05	.45
29.6	.5	.30 ± .05	.35
8.6	.8	1.42 ± .1	1.41
14.6	.8	1.10 ± .09	1.14
29.5	.8	0.91 ± .07	0.89
59.4	.8	0.72 ± .07	0.74
	B. pH 6.7, 25°		
7.1	0.3	0.24 ± 0.05	0.19
7.1	.5	0.57 ± .06	0.52
7.1	.8	1.30 ± .1	1.3
14.5	.8	0.98 ± .09	0.92
29.4	.8	.63 ± .06	.64
44.4	.8	.53 ± .06	.52
	C. pH 6.0, 25°		
7.34	0.3	0.09 ± 0.03	0.09
7.34	.5	.25 ± .04	.25
7.34	.7	.50 ± .05	.50
7.34	.8	.66 ± .05	.65
29.4	.6	.11 ± .04	.14
29.4	.7	.16 ± .04	.19
29.4	.8	.21 ± .05	.25
44.4	.6	.03 ± .03	.09
44.4	.7	.09 ± .04	.12
44.4	.8	.14 ± .04	.15
	D. pH 7.0, 15°		
7.25	0.3	0.33 ± 0.05	0.22
7.25	.5	0.66 ± .06	0.61
7.25	.8	1.57 ± .10	1.55
14.7	.8	1.2 ± .09	1.2
29.6	.8	0.98 ± .07	0.95
44.6	.8	0.88 ± .07	0.85
	E. pH 6.0, 15°		
7.2	0.3	0.00	0.06
7.2	.5	.11 ± 0.04	.16
7.2	.8	.43 ± .05	.41
14.6	.8	.24 ± .05	.25
29.6	.8	.09 ± .04	.14
44.55	.8	.03 ± .03	.09

[a] Uncertainties indicated are standard deviations. [b] Calculated from eq. 10.

Application to Thiourea.—Equation (10) gives good agreement with the present observations if it is assumed that two moles of thiourea inhibit an active site, that is, if both n and m are taken equal to two. The values of the inhibition constants are conveniently obtained from the intercept and the slope of a plot of $\phi(S + K)/I^2$ against S at a given pH and temperature. In these calculations, for the sake of simplicity, the Michaelis constant K was assumed to be independent of pH and was taken equal to 4.0 mM at both temperatures. This value of K gives an adequate fit of the present data on the uninhibited reaction; it is consistent with the earlier data[11] if the comparison is limited to the same range of substrate concentrations. Table I shows the degree of agreement of the observed inhibition indices with the calculated ones. Table II presents a summary of the inhibition constants. It is seen that while the equilibrium $E + 2I = EI_2$ is only slightly affected by changes of pH, the equilibrium constant of the reaction $ES + 2I = ESI_2$ is approximately proportional to the hydroxyl ion concentration. Quastel[16] found a similar pH dependence of the reversible inhibition of saccharase by some basic dyes. Denoting the fractional inhibition by I, he described his results by the equation

$$pH - \log (I/(1 - I)) = \text{Constant}$$

If this equation is rearranged to give the inhibition index, one obtains

$$\phi = \text{Const.}\,(OH^-)$$

TABLE II

THE PARAMETERS OF THE INHIBITION BY THIOUREA

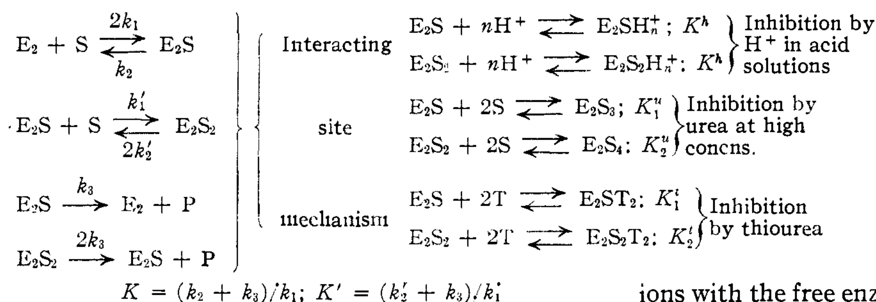
pH	t, °C.	K_1^t (mole/l.$^{-2}$)[a] eq. 10	K_1^t (mole/l.$^{-2}$)[a] eq. 16[b]	K_2^t (mole/l.$^{-2}$)[a] eq. 10	K_2^t (mole/l.$^{-2}$)[a] eq. 16
7.0	25	5.0	4.5	0.9	0.9
6.7	25	5.0	4.6	0.4	0.4
6.0	25	2.9	3.6	~0	~0
7.0	15	5.0	4.9	1.0	
6.0	15	1.8	2.9	~0	~0

[a] The errors in these highly derived quantities can be estimated only crudely. For K_1^t they are of the order of 15% and are still larger for K_2^t. [b] Constants for 15° calculated assuming the same pH dependence as at 25°.

Applications to the Interacting Site Model.—In an earlier paper[11] the rate data on the uninhibited reaction at lower urea concentrations were fitted to a three-parameter rate equation which was derived from the "interacting site" reaction mechanism. The decrease of rates at high urea concentrations was attributed to inhibition by urea and was treated separately.

With the aid of Cramer's method, it is feasible to present a unified treatment of these observations and to include the dependence of the kinetic parameters on pH and the inhibition by thiourea. The experimental data are described by the following set of reactions which does not include processes at basic pH

(16) J. H. Quastel and E. D. Yates, *Enzymologia*, **1**, 60 (1936).



$K = (k_2 + k_3)/k_1$; $K' = (k'_2 + k_3)/k'_1$

The six inhibition reactions will be regarded as those of non-interacting total inhibitors, kinetically the simplest case. Then the expression for the rate of hydrolysis, according to the rules enumerated in the preceding sections, is

$$V = \frac{k_3\Delta_{E_2S} + 2k_3\Delta_{E_2S_2}}{\Delta_0 + \Delta_h + \Delta_u + \Delta_t} \tag{11}$$

The two terms in the numerator refer to the two intermediates which yield the products. The first term in the denominator is the determinant of the uninhibited interacting site mechanism. The following terms deal successively with the inhibition by hydrogen ions, by urea, and by thiourea.

If thiourea is not present and the concentration of urea is so low that the determinant Δ_u is insignificant in comparison with the other two terms, the explicit rate equation is

$$V = \frac{k_3E_0S(S + K')}{\alpha(S^2 + 2SK') + KK'} \tag{12}$$

in which α is defined by

$$\alpha = 1 + K^h(\mathrm{H}^+)^n \tag{13}$$

Equation (12) is identical with eq. (5b) of the previous paper[10] except for the presence of the term α which allows for the pH effect on the rate. A comparison of the two equations shows that V_m, or k_3E_0, of eq. (5b) is k_3E_0/α of eq. (12). Similarly, K_B of (5b) is K/α of eq. (12). But K'_B of eq. (5b) is K' of equation (12). Thus eq. (12) predicts that the ratio of the experimental V_m to K_B should be independent of pH. It also requires that the experimental K'_B be independent of pH. Since these are precisely the conclusions which were drawn from experimental data, eq. (12) does describe the effect of pH on the kinetic parameters of the uninhibited reaction. The quantitative effect of pH on V_m can also be reproduced, choosing the following expression for α

$$\alpha = 1 + 8.07 \times 10^2\,(\mathrm{H}^+)^{1/2} \tag{13a}$$

A comparison of calculated and observed V_m is shown in Table III. While the significance of an inhibiting reaction involving one-half of a hydrogen ion is only formal, equally good agreement can be obtained by using integer exponents with two or more terms in eq. (13a). However, the experimental data on the uninhibited reaction show rather definitely that the effect of pH on the rate decreases at low urea concentrations. Therefore, within the framework of the proposed mechanism, an inhibiting reaction of hydrogen ions with the free enzyme (E_2) must be insignificant.

TABLE III

CALCULATED[a] AND OBSERVED LIMITING RATES AT VARIOUS pH AND 25°

pH		5.4	6.0	6.5	7.0
V_m	Obsd.	0.76	1.00	1.29	1.52[b]
	Calcd.	0.72	1.04	1.30	1.51

[a] Calculated from eqs. 12 and 13a. [b] Extrapolated to low ionic strength.

The urea inhibition index is defined by the expression

$$\phi_u = \frac{V_m - V}{V} = \frac{2K'K^u_1S^3 + K^u_2S^4}{\alpha(S^2 + 2K'S) + KK'} \tag{14}$$

Here, V_m is the rate predicted for high urea concentrations from the interacting site mechanism without inhibition and V is the rate derived from the complete mechanism, including inhibition by urea. If the inhibition, being weak, is observable only when $S \gg K'$, eq. (14) simplifies to

$$\phi_u = \frac{V_m - V}{V} = \frac{2K'K^u_1S + K^u_2S^2}{\alpha} \tag{15}$$

Except for the meaning of the proportionality constants, this approximate form is identical with the two-term equation which was fitted[11] to experimental data. Thus the stepwise procedure of the previous paper[11] is justified, in particular the use of V_m, defined by (15), in the analysis of the kinetics at lower urea concentrations.

Changes of pH should have the same effect on the inhibition constants according to (15) as they have on V_m. The experimental values of K^u_1, Table III of the previous paper,[11] show indeed approximately the trend required by eq. (13a) and (15). The experimental uncertainties in the second inhibition constant K^u_2 are too large to establish a definite trend with pH. One can only conclude that it is not large and hence not in direct conflict with the theoretical predictions.

The thiourea inhibition index is defined by

$$\phi_t = \frac{\Delta_t}{\Delta_0 + \Delta_h} \cong \frac{2K'K^t_1T^2S + K^t_2T^2S^2}{\alpha(S^2 + 2K'S) + KK'} \cong \frac{2K'K^t_1T^2 + K^t_2T^2S}{\alpha(S + 2K')} \tag{16}$$

The second approximation is valid in a narrow range of urea concentrations where neither the inhibition by urea nor the deviations from the Michaelis–Menten mechanism are significant. This is the range covered in the present investigation. In this range, the dependence of the inhibition index on urea and thiourea concentrations is the same whether one uses eq. (16) or eq. (10), derived from the Michaelis–Menten mechanism. Thus, the present measurements are equally consistent with both treatments. Equation (16) requires the inhibition constants to change with pH in the same manner as V_m of the uninhibited reaction. Calculating the inhibition constants by eq. (16) and the values of K' taken from the previous paper,[11] one finds indeed a satisfactory constancy of the first inhibition constant K^t_1, as shown in column 4 of Table II. The second inhibition constant, K^t_2 however, shows a strong dependence on pH

It would seem that the proper stoichiometry of this inhibition reaction would include the gain of a negative charge by the intermediate $E_2S_2T_2$.

Application to the Phosphate Buffers.—The data of Howell and Sumner[17] indicate that in phosphate buffers the inhibition by urea nearly vanishes at low pH. Since these buffers have been shown to be strong inhibitors of urease, the problem may be treated as a case of three non-interacting total inhibitors: urea, hydrogen ion and some components of phosphate buffers. The expression for the corresponding inhibition index becomes then

$$\phi_u = \frac{\Delta_u}{\Delta_0 + \Delta_h + \Delta_p} \quad (17)$$

Here Δ_p is the sub-determinant containing all terms referring to reactions with the components of phosphate buffers. Its exact form is not known but its numerical magnitude must increase with decreasing pH when the phosphate concentration is held constant, because the extent of inhibition then increases. Thus the dependence of ϕ_u on pH is not determined by the slight changes in the term Δ_h, as it is in the non-inhibiting maleate buffers. Instead, the denominator of eq. (17) increases rapidly in magnitude because of Δ_p. Hence the fall off of rates at high substrate concentrations may be expected to vanish at low pH and high phosphate concentrations, as observed.

Application to Sulfite Inhibition.—The inhibition of urease by components of sodium sulfite solutions was studied in phosphate buffers and the conclusion was drawn that the inhibiting specie is the bisulfite ion.[18] This conclusion is subject to doubt because no allowance was made for the simultaneous inhibition by phosphate. The expression for the sulfite inhibition index is

$$\phi_s = \frac{\Delta_s}{\Delta_0 + \Delta_h + \Delta_p} \quad (18)$$

where Δ_s contains all terms referring to the inhibition by sulfite. The other sub-determinants have the meaning defined previously. Because of the rapid rise of the numerical magnitude of Δ_p with decreasing pH, the magnitude of ϕ_s must decrease, unless Δ_s increases. The experiments showed the inhibition index to increase with decreasing pH; the most likely explanation is that the concentration of the inhibiting sulfite species increases with decreasing pH at a constant molality of sulfite and phosphate solutions. The bisulfite ion does behave in this fashion. Thus the main conclusion of the earlier paper,[18] that the bisulfite ion is the inhibitor, is probably correct. However, the quantitative calculations given there need revision to allow for the inhibition by phosphate buffers.

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

(18) J. F. Ambrose, G. B. Kistiakowsky and A. G. Kridl, THIS JOURNAL, **72**, 317 (1950).

Conclusion

The use of Cramer's method and the inhibition index has made it possible to treat rigorously rather complex mechanisms applicable to the kinetics of the urea hydrolysis by urease. Good agreement with experimental data is thus obtained over a very wide range of urea concentrations and a fairly wide pH range by an equation which involves only rate or inhibition constants and the concentrations of the reactant and the inhibitors. Of the available experimental findings only one has been left out of consideration. It is the effect of ionic strength on the activity of the enzyme in basic solutions.[5] But the pH range in which it was observed (pH 7.0 and 7.5) is too narrow to evaluate from experiments its trend with pH. Hence, experimentally the behavior of urease in basic solutions is still poorly defined and its analysis by the procedure used here is premature.

The plausibility of the proposed mechanism is supported by the comparative simplicity of the Mass Action Law expressions which are used for the individual reaction steps. Nonetheless, we have not been able to develop a self-consistent model of the active site in urease which explains in structural terms all the postulated reactions. This suggests that the interacting site model and the inhibition reactions which follow from it represent some over-simplification of the reaction mechanism. As noted previously,[11] other mechanisms of the uninhibited reaction may be constructed which reduce to a three-parameter equation fitting the experimental data. A change in the mechanism of the uninhibited reaction must be accompanied by changes in the stoichiometry of the inhibiting reactions to fit the experimental data. It is thus possible that some still unexplored mechanisms will represent the whole array of experimental data by means of more plausible inhibition reactions. On the other hand, the possibility exists that some of the basic assumptions underlying the present treatment are inapplicable. For instance, the use of ideal solution laws, implicit in the Mass Action Law expressions, may be in doubt. More experimental data should settle these questions. Until then the construction of a chemical structural model of the active site can be but speculation. Even the structural requirements for urea analogs to act as inhibitors are puzzling. The presence of two primary amido groups and of the carbonyl groups are neither necessary nor sufficient, because sulfamide is not an inhibitor but thiourea and acetamide are.

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