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# The Inhibition of Urease by Methylurea

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Methylurea inhibits jackbean urease. Experimental results describing the dependence of this inhibition on *p*H, substrate concentration and inhibitor concentration are reported. At *p*H 8.9 in unbuffered solutions and at *p*H 7.0 in maleate buffer, the inhibition is non-competitive. Between *p*H 7.0 and *p*H 6.0 a drastic change in character takes place and, in maleate buffers at the lower *p*H, methylurea functions as a competitive inhibitor. The inhibition index is found to depend on methylurea concentration raised to the second power. This result indicates participation of two moles of methylurea in equilibria involving the enzymic species. The similarity of these results to those reported earlier for the inhibition of urease by thiourea is noted. Interpretations based on various mechanisms are discussed.

## Introduction

Information in the literature concerned with the effect of methylurea on urease action has been limited to descriptive comments in papers devoted primarily to other topics. Takeuchi,[1] working with jackbean urease in phosphate buffer, observed a slight inhibition of urease by methylurea. Harmon and Niemann[2] also reported an inhibition that was not of the simple competitive type. They further concluded, however, that their data did not permit elucidation of the nature of the observed inhibitory action. In a study of the ureolytic activity of urease from *Corynebacterium renale*, Lister[3] noted inhibition by thiourea but not by methylurea. The qualitative nature of these observations and the lack of systematic data collected under a variety of experimental conditions made further study of the problem seem desirable.

## Experimental

Urease was extracted from jackbean meal by the method of Sumner as modified by Dounce.[4] In the final step of the preparation the precipitate was taken up in a cold 50% aqueous glycerol solution and stored in the refrigerator. Dilute solutions of this glycerol stock were prepared, allowed to stand overnight in the refrigerator, and then kept 2 hr. at reaction temperature in a water-bath prior to the kinetic runs. Since reproducible results were obtained without its use, hydrogen sulfide was not employed to stabilize the enzyme-containing solutions.

Kinetic runs were performed by mixing (in a water bath at 25.0°) 1 ml. of the urease solution with 25 ml. of the experimental solution. The reaction was allowed to proceed for a measured time and then stopped by addition of 3 ml. of 5.0 *N* HCl. The ammonium ion formed was determined by the ion exchange procedure previously described.[5]

For the runs at *p*H 8.9, urease produced its own buffer[6]; but, at *p*H 6.0 and *p*H 7.0, 0.1 *M* maleate buffers were employed. These buffers were prepared from Eastman White Label maleic anhydride.

In preparing the experimental solutions J. T. Baker analyzed C. P. urea was used without further purification, and methylurea was taken from a sample whose purity and preparation have been previously described.[7] All water was distilled and then passed through a Dowex-50 cation exchange column. The concentration of the solutions is, in general, expressed in millimoles per liter (m*M*/l.).

Glassware was first cleaned in a hot nitric-sulfuric acid bath and then rinsed several times with the purified water before use. General experimental techniques were quite similar to those employed in earlier work.[5,6]

## Results

Methylurea was found to inhibit urease at all *p*H values studied. Table I summarizes the data. The rate, $V$, at a particular urea concentration, S, in the presence of inhibiting methylurea at a concentration $I$ is reported. A rate ratio, $V_i/V_u$, of inhibited velocity to uninhibited velocity at each urea concentration is also given in column four of the table. Lineweaver–Burk plots are shown in Fig. 2.

In previous work[8] an inhibition index, $\phi$, was defined as

$$\phi = (V_u/V_i) - 1 \qquad (1)$$

A plot of this function (calculated from the rate ratios for *p*H 8.9 in Table I) against $I^2$ is presented in Fig. 1.

Although all data in Table I were collected at 25°, a few measurements were also made at 15°. These demonstrated that within our experimental error (av. deviation about 4%) the rate ratios at 15° did not differ from those found at the higher temperature.

## Discussion

Examination of the data collected at *p*H 8.9 (Table I, part A) reveals that the rate ratio, $V_i/V_u$, obtained at inhibitor concentrations of 15 and 30 m*M*/l. does not depend on substrate concentration. At an inhibitor concentration of 45 m*M*/l., there appears to be a slight trend in the ratios toward increasing inhibition with increasing urea concentration. This trend may be significant but most probably it simply reflects a larger experimental error involved in determining the decreased amount of ammonium ion produced at high inhibitor concentration. In any event the rate ratio, at fixed inhibitor concentration, seems to be (aside from the slight trend noted above) essentially independent of substrate concentration. This conclusion also applied to the data at *p*H 7.0. At *p*H 6.0, however, a vastly different situation is encountered. The rate ratio increases rapidly with increasing substrate concentration and only a slight inhibition by methylurea can be detected at a urea concentration of 333 m*M*/l. Thus a profound change in the nature of the inhibition has taken place during the one unit *p*H change from 7.0 to 6.0. This change is dramatically

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

(3) A. J. Lister, *J. Gen. Microbiol.*, **14**, 478 (1956).

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

(6) G. B. Kistiakowsky and W. H. R. Shaw, *ibid.*, **75**, 2751 (1953).

(7) W. H. R. Shaw and B. Grushkin, *ibid.*, **82**, 1022 (1960).

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

TABLE I

THE EFFECT OF *p*H AND UREA CONCENTRATION ON THE INHIBITION OF UREASE BY METHYLUREA[a,b]

| S, m*M*/l. | *I*, m*M*/l. | $V \times 10^3$, (m*M*/l.)/min. | $V^i/V_u$, obsd. | calcd.[c] |
|---|---|---|---|---|
| | A. *p*H 8.9, unbuffered[d] | | | |
| 3.33 | 00.0 | 2.64 | 1.00 | |
| 3.33 | 15.0 | 1.37 | 0.518 | 0.492 |
| 3.33 | 30.0 | 0.505 | .190 | .195 |
| 3.33 | 45.0 | 0.243 | .092 | .097 |
| 7.00 | 00.0 | 3.44 | 1.00 | |
| 7.00 | 6.00 | | 0.830 | .857 |
| 7.00 | 9.00 | | .710 | .729 |
| 7.00 | 12.0 | | .617 | .600 |
| 7.00 | 15.0 | 1.77 | .515 | .492 |
| 7.00 | 15.0 | | .516 | .492 |
| 7.00 | 21.0 | | .349 | .330 |
| 7.00 | 24.0 | | .283 | .274 |
| 7.00 | 30.0 | 0.67 | .195 | .195 |
| 7.00 | 30.0 | | .196 | .195 |
| 7.00 | 45.0 | 0.310 | .090 | .097 |
| 7.00 | 45.0 | | .088 | .097 |
| 33.3 | 00.0 | 4.40 | 1.00 | |
| 33.3 | 15.0 | 2.28 | 0.520 | .492 |
| 33.3 | 30.0 | 0.865 | .196 | .195 |
| 33.3 | 45.0 | 0.385 | .088 | .097 |
| 333 | 00.0 | 4.81 | | |
| 333 | 15.0 | 2.48 | .516 | .492 |
| 333 | 30.0 | 0.937 | .195 | .195 |
| 333 | 45.0 | 0.404 | .084 | .097 |
| | B. *p*H 7.0, 0.1 *M* maleate buffer | | | |
| 3.33 | 00.0 | 3.44 | 1.00 | |
| 3.33 | 15.0 | 1.56 | 0.454 | .443 |
| 3.33 | 30.0 | 0.577 | 0.167 | .165 |
| 7.00 | 00.0 | 4.55 | 1.00 | |
| 7.00 | 15.0 | 2.00 | 0.440 | .443 |
| 7.00 | 30.0 | 0.756 | 0.166 | .165 |
| 33.3 | 00.0 | 5.77 | 1.00 | |
| 33.3 | 15.0 | 2.57 | 0.445 | .443 |
| 33.3 | 30.0 | 0.938 | 0.162 | .165 |
| 333 | 00.0 | 6.25 | 1.00 | |
| 333 | 15.0 | 2.83 | 0.453 | .443 |
| 333 | 30.0 | 0.962 | 0.154 | .165 |
| | C. *p*H 6.0, 0.1 *M* maleate buffer | | | |
| 3.33 | 00.0 | 1.59 | 1.00 | |
| 3.33 | 15.0 | 0.721 | 0.454 | 0.440 |
| 3.33 | 30.0 | 0.240 | 0.151 | .164 |
| 7.00 | 00.0 | 2.22 | 1.00 | |
| 7.00 | 15.0 | 1.25 | 0.563 | .555 |
| 7.00 | 30.0 | 0.50 | 0.225 | .238 |
| 33.3 | 00.0 | 2.63 | 1.00 | |
| 33.3 | 15.0 | 2.16 | 0.810 | .820 |
| 33.3 | 30.0 | 1.38 | 0.524 | .534 |
| 333 | 00.0 | 2.80 | 1.00 | |
| 333 | 15.0 | 2.76 | 0.958 | .975 |
| 333 | 30.0 | 2.74 | 0.950 | .914 |

[a] All measurements were made at 25°. [b] The symbols are: *S*, urea concn.; *I*, methylurea concn.; *V*, reaction rate; $V_u$, uninhibited rate; $V_i$, inhibited rate. [c] Calculated from equations 1 and 5 using parameters from Table II. [d] The ammonium carbamate produced by urease buffers the solution to this *p*H.

reflected in the Lineweaver–Burk plots[9,10] of the data shown in Fig. 2.

(9) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

(10) These plots do not show the deviation from linearity noted earlier[11] since measurements have not been made over a wide enough substrate concentration range.

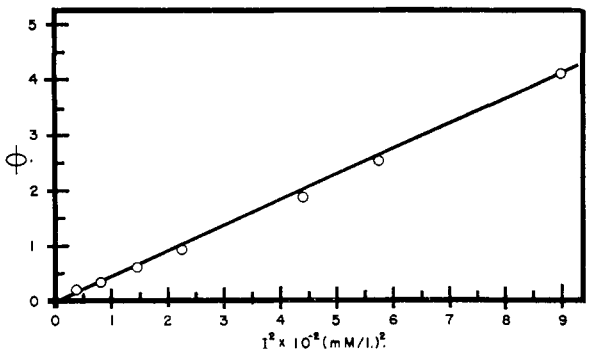


Fig. 1.—The inhibition index, $\phi$, as a function of methylurea concentration squared, $I^2$. Measurements were made in unbuffered 7.00 m*M*/l. urea solutions (*p*H 8.9) at 25°.

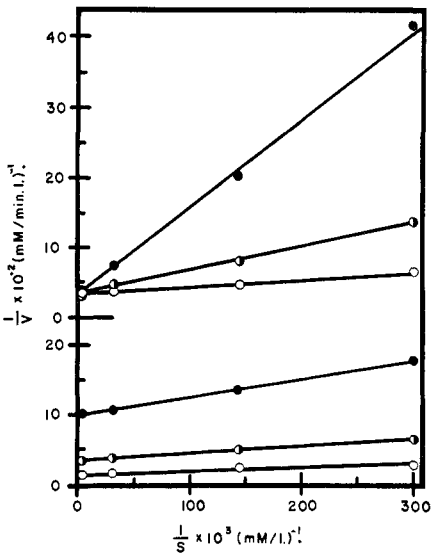


Fig. 2.—Lineweaver–Burk plots for various methylurea concentrations: ○, no methylurea; ◑, 15 m*M*/l.; ●, 30 m*M*/l. Inverse rate, $1/V$, is plotted against inverse substrate concentration, 1/S, at *p*H 6 (upper family of lines) and *p*H 7 (lower family of lines).

The inhibition index derived from the inhibited Michaelis–Menten mechanism

$$E + S \underset{K_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{K_3} E + P;\ K_m = \frac{k_2 + k_3}{k_1} \quad (2)$$

$$E + xI \rightleftarrows EI_x;\ K_1 = \frac{[EX_x]}{[E][I]^x} \quad (3)$$

$$ES + yI \rightleftarrows ESI_y;\ K_2 = \frac{[ESI_y]}{[ES][I]^y} \quad (4)$$

can be written[8] as

$$\phi = \frac{K_1 K_m I^x + K_2 S I^y}{S + K_m} \quad (5)$$

Inhibition indices at a fixed substrate concentration of 7.00 m*M*/l. were calculated (eq. 1) from the *p*H 8.9 rate ratio data in Table I. Plots of $\phi$ *vs.* various functions of I were then constructed until a straight line relationship was obtained. For inhibitor concentrations ranging from 6.00 to 45.0 m*M*/l., $\phi$ was found to be directly pro-

portional to $I^2$ (Fig. 1). This finding makes it possible to conclude (eq. 5) that $x = y = 2$.

As noted in the first paragraph of this section the rate ratio at a fixed inhibitor concentration appears to be essentially independent of substrate concentration at *p*H 8.9 and *p*H 7.0. Since the inhibition index is related to the rate ratio by eq. 1, $\phi$ is also independent of S. This can only be true (eq. 5) if $K_1 = K_2 = K$. In terms of the mechanism this implies that the free enzyme and enzyme-substrate have the same affinity (eq. 3 and 4) for methylurea. Thus at *p*H 8.9 and *p*H 7.0, eq. 5 can be written simply as

$$\phi = KI^2 \qquad (6)$$

Values for $K$ determined from the data are recorded in Table II, and rate ratios calculated from these parameters using eq. 1 and 6 are reported in column five of Table I.

TABLE II

THE PARAMETERS DESCRIBING THE INHIBITION[a,b]

| *p*H | $K \times 10^3$ (m$M$/l.)$^{-2}$ |
|---|---|
| 6.0 | 12[c] |
| 7.0 | 5.6 |
| 8.9 | 4.6 |

[a] The values reported are equilibrium constants derived from treatment of the data in Table I according to the inhibited Michaelis–Menten mechanism. [b] All measurements were made at 25°. [c] At this *p*H a Michaelis constant of 2.95 m$M$/l. was assumed. At *p*H 7.0 and *p*H 8.9 the inhibition is independent of the urea concentration.

At *p*H 6.0 the non-competitive ($K_1 = K_2 = K$) inhibition observed at high *p*H is replaced by a competitive ($K_2 = 0$) one as indicated by the common intercept of the lines in Fig. 2. The inhibition index for this type of inhibition (eq. 5) will depend on S and decrease with increasing substrate concentration. The apparent value of $K_m$ for the substrate concentration range employed in this investigation was calculated from the slope to intercept ratio of the uninhibited Lineweaver–Burk plot at *p*H 6.0. This ratio was in agreement with the $S_{1/2}$ parameter of 2.95 m$M$/l. found in other work.[11] This value was, therefore, used to determine the equilibrium constant ($K_1$) reported for *p*H 6.0 in Table II. Rate ratios calculated from these parameters are compared with experimentally determined ones in part C of Table I.

It is important to note that the simple Michaelis–Menten mechanism fails to explain rate data over the wide range of substrate concentration studied in other investigations.[6,11,12] Different mechanisms have been devised that give a much better representation of the data. These are based on the idea of two interacting[6,8,11] or two independent[12] sites. At *p*H 7.0 and *p*H 6.0 application of the interacting site mechanism[8] leads to the conclusion that the species $E_2S$ and $E_2S_2$ have equal affinity for methylurea. At these same *p*H values the independent site mechanism makes it necessary to assume that the affinities of I for E, I for ES, I for E′ and I for ES′ are all equal. At *p*H 6.0 the inhibition is no longer substrate independent. Interpretations in terms of the more sophisticated mechanisms are consequently more complex at this *p*H. A generalized treatment based on the interacting site mechanism applied to thiourea inhibition has been presented.[8] Completely analogous equations and conclusions apply to the present case. Independent site treatment yields involved expressions with several adjustable parameters. It seemed best, therefore, to use the simple Michaelis–Menten mechanism to interpret the limited data at *p*H 6.0.

The effect of methylurea on ureolytic activity is similar in many respects to the effect of thiourea studied earlier.[8] The changing character of the inhibition with *p*H and the dependence on $I^2$ are characteristic features of both inhibitions. Methylurea, however, is a much stronger inhibitor than thiourea. At *p*H 6.0 and a substrate concentration of 7 m$M$/l., for example, it requires approximately a sixty fold higher concentration of thiourea than methylurea to reduce the rate by a factor of two.

Exploratory runs with other urea derivatives were also made; and a rough, tentative inhibitory sequence was established. In order of decreasing inhibitory effectiveness the sequence is methylurea $\gg$ *sym*-dimethylurea $>$ *unsym*-dimethylurea $>$ phenylurea. Harmon and Niemann[2] have also reported that methylurea $>$ *t*-butylurea $>$ *n*-butylurea.

Apparently profound changes in the enzyme take place between *p*H 6.0 and *p*H 7.0. The salt effect[5,12] is negligible at 6.0 but becomes pronounced at 7.0; and, as noted above, the thiourea and methylurea inhibitions drastically alter their character in this same one unit *p*H interval. Explanation of these effects must probably be made in terms of structural changes in the enzyme protein, but a clearer understanding of the specific nature of the phenomena involved must await the outcome of additional research.

**Acknowledgment.**—It is a pleasure to express our gratitude to the Administration of The University of Texas for the special assignment given to W. H. R. S. that provided time for research and the preparation of this paper.

(11) G. B. Kistiakowsky and A. J. Rosenberg, *J. Am. Chem. Soc.*, **74**, 5020 (1952).

(12) G. B. Kistiakowsky and W. E. Thompson, *ibid.*, **78**, 4821 (1956).