Urease Catalysis

II. INHIBITION OF THE ENZYME BY HYDROXYUREA, HYDROXYLAMINE, AND ACETOXYDROXAMIC ACID

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In the preceding paper (1), hydroxyurea was shown to be a substrate for urease and to be associated with the development of a marked inhibition of the enzyme during the course of the reaction. The lack of significant inhibition during the hydrolysis of urea at equal concentrations suggested that the hydroxylamine formed during hydroxyurea hydrolysis was the responsible agent. The inhibitory effects of both compounds are here extended to urea hydrolysis and found to differ significantly. Hydroxylamine has previously been shown to inhibit some but not all sulfhydryl-dependent enzymes, and it had been presumed that this effect resulted from nonspecific oxidation of -SH groups to the disulfide form (2). The studies reported below indicate, however, that this is not the mechanism by which hydroxylamine inhibits urease. The inhibitory effects of hydroxyurea also led to the exploration of inhibition by acetohydroxamic acid, which is not hydrolyzed by urease (1). This compound was found to be a potent and irreversible inhibitor of the enzyme.

EXPERIMENTAL PROCEDURE

The reagents and analytic methods used are detailed in the preceding paper (1). The designation of urease preparations and their corresponding activity in modified Sumner units (assayed at 30°) is repeated here for convenience: I, 3.4 units per mg; II, 7.8 units per mg; III, 48 units per mg. In addition, sulfhydryl determinations were done by the method of Ellman (3), and modified only by use of the buffer system described below. The 5,5'-dithiobis-(2-nitrobenzoic acid) was purchased from the Aldrich Chemical Company.

Some additional points are pertinent with regard to the assay method for urea and hydroxyurea (4). Because of its extreme sensitivity, care must be exercised in the handling of tubes and cuvettes. The 5 μg or so of urea on the fingertip produce significant readings in the 2-ml volume of the Technicon Auto-Analyzer cup. Interspersing frequent water blanks provides a check on the absence of contamination. Sulfhydryl compounds, although not measured themselves, do inhibit color formation by hydroxylurea. That this does not represent reduction of hydroxylamine is shown by its occurrence also with urea assay. By hydroxyurea. That this does not represent reduction of oxidized. The initial inhibition of color development with urea was about 4% and 80%, respectively, for 1 mM concentrations of cysteine and mercaptoethanol. With hydroxyurea, the corresponding values were 13% and 46%. Substrate assays were therefore delayed when these mercaptans were present (always at 5-fold lower final concentrations). Control assays without mercaptans were also run to assure that no interference was present at the time of assay.

Tris-maleate buffers, prepared by mixing equimolar solutions of Tris base and maleic acid, were used throughout. Each of the constituents has been shown to lack inhibitory effects on urease (5, 6). In combination they provide sufficient buffering capacity from pH 5 to 9 for the low levels of substrate employed. To avoid the inhibitory effects of the metal cations (7), no NaOH or KOH was used in buffer preparations. The buffers were prepared fresh every few days from refrigerated stock solutions or from the solid compounds. The pH-activity curves of urease hydrolysis of urea and hydroxyurea in this buffer show maxima at pH 7.0 with less than 10% fall in rate from pH 6.5 to 7.5. Unless specified, all incubations were run in 0.1 M buffer, pH 7.0, at 25 ± 2°. For kinetic analysis of hydroxylamine inhibition, the buffers were nitrogen-saturated for 3 hours before use.

Enzyme incubations and determination of initial velocities were performed as described in the preceding paper (1). When possible, inhibitor rate constants were determined by comparison of initial substrate decay rates in enzyme solutions preincubated for varying time intervals with and without inhibitor. Where this approach was not satisfactory (i.e. t less than 10 minutes), the following procedure was used. Reactions were initiated by the addition of urease to substrate-containing media with and without inhibitor. Each of these was assayed at 1-minute intervals. The measured points were connected by smooth curves, by employing French curves and the squint test (8). Instantaneous rates were determined from the inhibition curve at selected times by the use of a tangent meter. Comparative rates from the control curve were measured at points where corresponding substrate concentrations were obtained (rather than at the same time intercepts). This provides a fractional inhibition corrected for changes in the control rate due to product inhibition and substrate decline.

In all enzyme-inhibitor preincubation experiments, the addition of substrate (to start the reaction) involved less than 8% dilution of the incubation media. By the use of 1-minute interval time schedules, each experiment was completed within 4 hours of inception.

RESULTS AND DISCUSSION

The biphasic nature of hydroxyurea hydrolysis by urease was shown in the preceding paper to represent a rapid development...
of enzyme inhibition that appears to reach a maximum level short of complete inhibition (1). From the data, it was estimated that the hydrolysis of 0.055 μmole of hydroxyurea per unit of urease would produce maximal inhibition.

Because of the 120-fold difference in hydrolytic rates, the inhibitory effects of hydroxyurea were extended to urea hydrolysis by way of dilution experiments. After incubation of 2.5 mM hydroxyurea with 12.2 units per ml of urease II for 2, 10, and 30 minutes, aliquots of the solution were diluted 100-fold in buffer and assayed with 2.5 mM urea. Maximum urease inhibition would be anticipated after about 5-minute incubation with hydroxyurea. The results are presented in Figs. 1 and 2. The final concentrations of enzyme and the two substrates following dilution provide active urea hydrolysis in the control experiments, that is unaffected by the low level of hydroxyurea present. After 2-minute preincubation with hydroxyurea, however, Fig. 1 demonstrates about 80% inhibition of urea hydrolysis. After the longer preincubation periods, the inhibition is greater than 90%. The initial lag phase of complete inhibition would seem to be due to a rapidly dissociable enzyme-inhibitor complex. Constant slopes are observed thereafter in the specimens preincubated for 10 to 30 minutes. This indicates that the residual enzyme inhibition is irreversible and has reached a maximum by 10 minutes of preincubation with hydroxyurea. In Fig. 2 urea assay is delayed for varying times after dilution of the hydroxyurea urease solution. After preincubating urease with hydroxyurea for 30 minutes, there is almost complete inhibition of urea hydrolysis initially. During the assay the rate of hydrolysis increases to about 5% of the control rate and remains at this level thereafter. When urea addition is delayed for 60 minutes after the dilution, the rate of hydrolysis is constant at this same level throughout the 40-minute assay period. Thus, over periods as long as 100 minutes after dilution, there appears to be a fixed level of inhibition. The findings are similar following a 10-minute preincubation of urease with hydroxyurea.

For a reversible enzyme-inhibitor complex, a 100-fold dilution of inhibitor should result in almost complete dissociation of the complex and hence in a very low level of inhibition. In this case, however, the residual inhibition of urea hydrolysis is approximately as great as that of hydroxyurea hydrolysis before the dilution.

There are at least two components, therefore, involved in hydroxyurea inhibition of urea hydrolysis by urease. The major fraction of inhibition is irreversible, although this does not specify whether a tightly bound complex or a chemical reaction with the enzyme is responsible. The inhibition is nevertheless partial in that urea hydrolysis proceeds at about 5% of its control rate. This inhibition is entirely comparable to that observed during hydroxyurea hydrolysis. Superimposed upon this is a relatively reversible inhibitory component presumably due to a dissociable enzyme-inhibitor complex. This component is not manifest during hydroxyurea hydrolysis.

The great difference in the hydrolysis rates of urea and hydroxyurea permits a ratio analysis of the affinity of urease for the two substrates. At low enzyme concentrations, hydroxyurea is not hydrolyzed to a measurable extent and may be viewed purely as a competitive inhibitor of urea hydrolysis. Employing equimolar mixtures of the two substrates, the maximal rate will be lower than that for urea alone. The degree of depression of $V_{max}$ will depend on the relative affinities of the two substrates for the active center of urease. The formulation employed is that of Webb (9).

$$K_I = \frac{V_{mix} - V_i}{V_s - V_{mix}}$$

i.e. with HU to denote hydroxyurea,

$$\frac{K_HU}{K_{area}} = \frac{V_{area} - V_{HU}}{V_{area} - V_{HU+area}}$$

The data obtained in a representative experiment are presented in Fig. 3. The direct measurement of the Michaelis constants for both substrates (1) indicates that the $K_m$ for hydroxyurea is 2.5 to 8 times lower than that for urea. The evidence presently
available (10) also suggests that the \( K_m \) for urea is a true dissociation constant, i.e. \( K_m = K_s \). If this is also true for hydroxyurea, then its equimolar presence would dictate, by the above formulation, a 3.5- to 9-fold decrease in the maximal rate of urea hydrolysis. Reciprocal plots of initial velocities in analogous but shorter experiments demonstrated a decline in maximal rate of 3.75-fold. This is within the expected range, and may, therefore, be taken as added evidence that the two substrates are degraded at the same active site. Put another way, the agreement between the Michaelis constant for hydroxyurea as a substrate (\( K_m = 1.25 \) to \( 1.6 \) mm) and its dissociation constant as a competitive inhibitor of urea (\( K_I = 1.45 \) to \( 3.6 \) mm) suggests that the premise involved, i.e. \( K_m = K_s \) for both substrates competing at the same site, is valid for this system (11).

A more impressive feature of Fig. 3, however, is the development of marked urease inhibition when both substrates are present. In the absence of hydroxyurea, the exponential pattern of urea hydrolysis discloses no evidence for product inhibition. In the presence of increasing concentrations of hydroxyurea, the degradation of urea becomes progressively inhibited. Thus a family of inhibition curves is obtained analogous to that seen with hydroxyurea hydrolysis (1). In sum, the presence of hydroxyurea results in similar patterns of urease inhibition with regard to the hydrolysis of either substrate.

The effects of hydroxyurea on urease catalysis in these experiments are those of irreversible inhibition progressing with substrate decay. While this could be due to gradual enzyme inactivation by the undegraded hydroxyurea, it is more suggestive of product inhibition. Absence of the phenomenon during urea hydrolysis would favor hydroxylamine as the product responsible. Considering its large reduction-oxidation potential (12) in comparison with those of the common sulfhydryl compounds (13), hydroxylamine might reasonably be expected to produce irreversible inhibition of sulfhydryl-dependent enzymes by oxidizing these reactive groups. The reversal of enzyme inhibition by mercaptans would then be taken as evidence that oxidation did not pass the disulfide stage (14). Direct oxidation has been stated to be the cause of hydroxylamine inhibition observed in two out of five sulfhydryl-containing enzymes tested (2). Similarly, the inhibition of urease by 1 \( \mu \)M hydroxylamine was considered proof that the inhibition produced by hydroxyurea (also at 1 \( \mu \)M) was due to oxidation of the enzyme (15).

The study of urease inhibition by hydroxylamine was undertaken with these points in mind. In preliminary experiments, hydroxylamine inhibition of hydroxyurea hydrolysis was found to be increased upon preincubation of the enzyme with the inhibitor. Thus, 2.5 \( \mu \)M hydroxylamine produced 10% inhibition without preincubation, and 27% inhibition after a 45-minute preincubation period. The inhibition by hydroxylamine, however, was much less than that which develops during hydroxyurea hydrolysis, which makes an accurate analysis difficult. Urea hydrolysis, which proved much more sensitive, was therefore used to study the kinetics of hydroxylamine inhibition of urease.

The \( pH \) curve for hydroxylamine inhibition of urease-urea hydrolysis was determined over the range \( pH \) 5 to 9. Maximum inhibition occurred at \( pH \) 7 to 7.5, conveniently the same as the \( pH \) maximum for substrate activity. Kinetic analyses were, therefore, confined to this region. Inhibition by hydroxylamine was consistently reversed by addition of equimolar amounts of cysteine or mercaptoethanol. As far as could be determined experimentally (Fig. 4), reversal was immediate in appearance, and was not facilitated by preincubation of urease with the mercaptan. Although the data to this point were compatible with irreversible oxidative inactivation of urease by hydroxylamine, further study of the reaction failed to confirm this mechanism.

On preincubation of hydroxylamine with urease for periods up to 2 hours prior to urea assay, constant fractional inhibitions were attained after 5- to 10-minute preincubation. The degree of inhibition was proportional to the concentration of hydroxylamine employed \( \left( 10^{-5} \right) \) to \( 10^{-4} \) \( M \). Since the inhibitor was always in excess of the enzyme concentration \( \left( 10^{-4} \right) \), hydroxylamine inhibition must be a reversible reaction. The preincubation effect, therefore, reflects a low rate constant for the formation of enzyme-inhibitor complex.

Confirmation was provided by a comparative dilution experiment (Table 1). This is offered as a simple method for simultaneously assessing the rate of formation, reversibility, and residual activity of an enzyme-inhibitor complex. Following preincubation of the inhibitor with a level of enzyme well in excess of that sufficient for substrate assay, comparable dilutions are made in two sets of buffers. One of these contains sufficient inhibitor to maintain its preincubation level unchanged. In this latter set of dilutions, the equilibrium, \( I \left( E/\left(E-I\right) \right) \), will not change with time, since the ratio of free and complexed enzyme is not altered by dilution. The same result would be expected for an irreversible inhibitor, since the rate of development of further inhibition will be decreased by the factor of dilution, in this case 50-fold. An irreversible inhibitor, moreover, should produce the same effect in the other set of dilutions, which contain
omitting hydroxylamine. The enzyme dilution in each case was for a reversible inhibitor with the complexed enzyme totally inhibition upon dilution may be compared with that calculated containing,

Expected (VEIS = 0)

FIG. 4. Reversal of hydroxylamine inhibition of urease I upon addition of mercaptans. All tubes contained 0.136 unit per ml of urease. The lowest curve indicates the control rate of hydrolysis in the presence (O) and absence (●) of the same concentration of HCl as that liberated by hydroxylamine hydrochloride. The upper three curves represent the rates of hydrolysis after preincubation of the enzyme with 1 mM hydroxylamine for 3 (●), 6 (●), and 9 (△) minutes prior to assay. Addition of urea to start the reaction and of mercaptans at the times noted by the arrows, involved respective volume increases of 5% and 1%.

TABLE I
Comparative dilution experiment assessing reversibility, rate of development, and completeness of hydroxylamine inhibition of urease I

<table>
<thead>
<tr>
<th>Time diluted before assay</th>
<th>Inhibition at different inhibitor dilutions</th>
<th>0</th>
<th>1:50</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>60</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>62</td>
<td>22.5</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>67.5</td>
<td>17</td>
</tr>
<tr>
<td>Expected (VEIS = 0)</td>
<td></td>
<td>60-70</td>
<td>3-5</td>
</tr>
</tbody>
</table>

no added inhibitor. In the case of hydroxylamine and urease, a sharp fall in inhibition occurs when the inhibitor is also diluted, indicating a reversible reaction. The further decline of inhibition upon delaying the assay is evidence that equilibrium is not attained instantaneously. Finally, the observed decline in inhibition upon dilution may be compared with that calculated for a reversible inhibitor with the complexed enzyme totally inactive, or obtained from the graphic treatment by Strauss and Goldstein (16). In the present case, the large discrepancy suggests that the inhibition is partial, i.e., the urease-hydroxylamine complex retains some catalytic activity.

A direct search for sulfhydryl oxidation was made with the hydroxylamine assay previously described (1). In contrast to the immediate disappearance of hydroxylamine upon addition of ferric salts, no perceptible loss occurred after 2-hour incubation of 1 mM hydroxylamine with 20 mM cysteine at pH 4.5 and 8.1, or 20 mM mercaptoethanol at pH 6.4 and 8.5, or of 10 mM hydroxylamine with 100 mM mercaptoethanol at pH 7. Extension of these negative findings to the sulfhydryl groups of urease III was accomplished by the use of 5,5'-dithiobis-(2-nitrobenzoic acid) (3). With 1 μM urease III and 50-fold this level of the reagent the number of sulfhydryl groups per mole of enzyme was 10.5 and 11 in two experiments. This is close to the value of 11.5 reported by Helleman, Chinard, and Deitz (14) with porphyrin, p-iodosobenzoate, and iodoacetamide. Like these other compounds, 5,5'-dithiobis-(2-nitrobenzoic acid) may be sterically prevented from reacting with a second complement of sulfhydryl groups titrated by p-chloromercuribenzoate. In any case, there was no decrease in titrable —SH groups after preincubation of the enzyme for 1 hour with 2.5 mM hydroxylamine (or acetyldioxynamic acid). The possibility was considered that hydroxylamine might oxidize a single pair of —SH groups in some particular spatial arrangement, or sterically "hidden" from assay. If this occurred, one might expect urease to "catalyze" the oxidation of hydroxylamine by low molecular weight sulfhydryl compounds, through alternate oxidation and reduction of the enzyme's favorably oriented pair of —SH groups. However, the incubation of 10-8 M urease III with 25 μM cysteine and 2.5 mM hydroxylamine resulted in no loss of cysteine after 1 hour.

It seems reasonable to conclude, then, that sulfhydryl oxidation by hydroxylamine is too slow to be of significance in its role as a urease inhibitor in these experiments. Indeed, a surprisingly slow reaction rate with even potent reducing agents is a well-known feature of hydroxylamine (12). Urease inhibition by this agent was consequently analyzed according to the usual kinetics for reversible inhibitors. Although an estimate of the rate constant is presented later in this report, it sufficed for equilibrium measurements to preincubate enzyme and inhibitor for 30 minutes prior to substrate assay.

The experimental data obtained was analyzed by double reciprocal plots for substrate (Fig. 5) and inhibitor (Fig. 6), both of which demonstrated noncompetitive inhibition. Further categorization was then effected according to the formulation developed by Friedenwald and Maengwyn-Davies (17) and expanded by Webb (18). This construction provides for partial competitive inhibition, where a measures the decreased affinity for substrate in the E-I complex and would be infinity for the usual complete inhibitor. For noncompetitive inhibition a = 1, and β represents the fractional rate of product formation from E-I-S as compared to E-S, and would be 0 for a complete inhibitor. Hydroxylamine inhibition of urease was incomplete in type. This was evidenced in the reciprocal substrate plots

1 The assumption is that the fractional inhibition, i, represents the fraction of enzyme complexed with inhibitor, E-I/(E + E-I); at equilibrium, \( K = I (E/E-I) \) so that with subscripts to denote initial and final levels (with respect to dilutions): \( I = (1 - i)/i_0 = I_0 (1 - i)/I_0 \), from which a corresponding \( i_0 \) may be calculated for any observed \( i_0 \).
by a decline in the calculated values for $K_I$ with decreasing concentrations of hydroxylamine, and in the reciprocal inhibitor plots by an ordinate intercept greater than 1. The latter plot permits a direct determination of the inhibitor constants, since the intercept $= 1/1 - \beta$ and the slope $= K_I/1 - \beta$. To obtain these constants from the more widely used reciprocal substrate plot, the values for the apparent dissociation constant ($K_I'$) must be replotted against the corresponding inhibitor concentrations $I$ according to the equation: $K_I' = K_I/(1 - \beta) + [\beta/(1 - \beta)]I$, which will give a straight line with slope $= \beta/1 - \beta$ and an intercept $= K_I/1 - \beta$. Both procedures were used here and the values averaged for $K_I$ and $\beta$. Four experimental runs gave the following average values for the urease-hydroxylamine inhibitor constants: $\beta = 0.22$ and $K_I = 0.21$ mm. The inhibition kinetics by this schema were reasonably reproducible, although more complex treatments, involving interacting substrate sites, have been devised for urease kinetics (10).

The reversible inhibition of urease by hydroxylamine with a maximum level of 80% cannot fully explain the irreversible inhibition with a maximum of 95% observed with hydroxyurea. Furthermore, sulfhydryl compounds, which were shown to reverse hydroxylamine inhibition, had no effect on the inhibition by hydroxyurea of urease catalysis.

The possibility that a stable intermediate or product of a side reaction is responsible for the inhibition was eliminated by the following crossover experiment. During a 5-hour incubation of 10.2 units per ml of urease I with 3 mm hydroxyurea, aliquots assayed at 10 minutes, 4 hours, and 5 hours showed the amount of substrate degraded to be 0.45, 1.26, and 1.45 mm, respectively. At the end of 5 hours, an aliquot was diluted 100-fold along with an equal amount of untreated urease, and the combination was tested for urea-cleaving activity. Since the reaction with hydroxyurea results in a constant 95% inhibition of urease within 10 minutes, the prolonged incubation has provided at least 3 times the necessary amount of whatever product is responsible. This would certainly be adequate to produce a "crossover" inhibition of the untreated urease. Yet no inhibition was observed in the mixture relative to untreated urease alone, even when urea acetyl was delayed for periods up to 1 hour after dilution.

There remains the possibility that the substrate itself is an irreversible inhibitor. That is, hydroxyurea may act at some point remote from the active site to inactivate the enzyme. The development of inhibition would then depend upon the competing rates for hydroxylamine inhibition of, and hydroxyurea degradation by, urease. For this reason, acetohydroxamic acid, which is structurally similar to hydroxyurea but is not hydrolyzed by urease (1), was tested. This compound was found to be a complete, irreversible inhibitor of urease.

Kobashi, Hase, and Uehara (20) have recently demonstrated urease inhibition by a wide variety of hydroxamic acids. Although their data is expressed in terms of dissociation constants, which are more appropriate for reversible inhibitors, they do remark that the inhibition was progressive during incubation. It is also known that acetamide, which differs from acetohydroxamic acid only with regard to the $\text{CONH}_2$ grouping, does not inhibit urease (19). The $\text{CONH}_2$ grouping thus appears to be the critical factor for urease inhibition. Since this grouping is also present in hydroxyurea, the hypothesis that this compound plays a dual role as substrate and inhibitor seems reasonable by analogy.
inhibition than can be accounted for by the same concentration of hydroxylamine. On the other hand, hydroxylamine inhibition is much more rapid in onset and quickly reaches a fixed level indicative of the equilibrium state for that particular concentration of inhibitor. Acetohydroxamic acid inhibition progresses to completion, and is first order with respect to enzymatic activity, as shown in Fig. 8 by an exponential plot (21). A similar treatment of hydroxylamine inhibition (from a separate experiment) is also shown in Fig. 8. In this instance, the rate law measures the approach to a reversible equilibrium state which will vary with the inhibitor concentration.

From the half-times in Fig. 8, the derived bimolecular rate constants for the two inhibitors are summarized in Table II, along with the pertinent equations. For acetohydroxamic acid, the agreement over a 10-fold difference in concentrations is good; and the average value, \( k = 1060 \text{ M}^{-1} \text{ min}^{-1} \) at 25°C, may be compared to cholinesterase inhibition by organophosphorus compounds, having \( k = 10^4 \) to \( 10^5 \text{ M}^{-1} \text{ min}^{-1} \) at 37°C (21). The equally rapid development of inhibition in the presence of substrate suggests that acetohydroxamic acid is a noncompetitive inhibitor in contrast to the organophosphorus compounds, where substrate prevents the development of inhibition.

Although the values for hydroxylamine also show good agreement, the rate constant for this inhibitor can be regarded only as an estimate. The rate of inhibition will be limited in range for a reversible inhibitor by its dissociation constant (22). In the case of hydroxylamine, it is thus not possible to select concentrations which will yield inhibitions (about 50%) and rates \( (i > 10 \text{ minutes}) \) conducive to accurate determination. In addition, the additive contribution of the enzyme-inhibitor complex to the total rate will cause any partial inhibitor to deviate from the exponential rate law progressively as equilibrium is approached. For these reasons the rate constant for hydroxylamine inhibition is intended only to indicate an order of magnitude similar to that of acetohydroxamic acid.

In contrast to Takeuchi (15), we must conclude that the inhibition of urease by hydroxylamine and hydroxyurea is related to complex formation rather than enzyme oxidation. In addition to the direct evidence against sulfhydryl oxidation, the inhibitory activity of these compounds (acetohydroxamic acid > hydroxyurea > hydroxylamine) is inverse to their oxidizing power. Although more widely known as an oxidizing agent, hydroxylamine has been shown to complex with NAD (23). The hydroxylamine oxidation potentials for hydroxamic acids, studies in this laboratory have demonstrated that the oxidation of hemoglobin by hydroxylamine is immeasurably rapid, even at low hydroxylamine concentrations, while 50% oxidation by large concentrations (0.1 M) of hydroxyurea and acetohydroxamic acid takes 19 hours and 55 hours, respectively (at 25°C).

![Fig. 7. Temporal development of urease inhibition in the presence of acetohydroxamic acid (AHA), hydroxyurea (HU), and hydroxylamine (HA). The time scale denotes the intervals for which urease, 0.272 unit per ml, was preincubated with inhibitor, prior to assay with 4 mm urea. The fractional inhibitions \( (i) \) are expressed relative to the control rates of enzyme preincubated with buffer for the same time intervals.](image)

![Fig. 8. Exponential plot of the rates of inhibition of urease by acetohydroxamic acid (AHA) and hydroxylamine (HA). The final fractional inhibition attained is denoted by \( i_f \). Inhibitor concentrations present are: O, 18 \( \mu \text{M} \) acetohydroxamic acid; \( \bullet \), 180 \( \mu \text{M} \) acetohydroxamic acid; \( \Delta \), 16 \( \mu \text{M} \) hydroxylamine; and \( \Delta \), 1 mm hydroxylamine.](image)
Acetohydroxamic acids are characterized by their color complexes with divalent metal ions, and direct evidence has been obtained for their complex formation with urease (29).

Assuming a common enzyme site for all three inhibitors, one may postulate a structural mechanism which would explain the observed findings. This depends upon the thermodynamic evidence that the urease molecule "opens up" during hydrolysis (5), and presumes that the restriction of this unfolding by ternary complex formation with two enzyme sites prevents or decreases enzymatic catalysis. The hydroxamic acid group (—CONHOH) would be essential for tight binding and irreversibility, while their complex formation with urease (20).

Hydroxyurea is currently under study in the chemotherapy of cancer, with potential application primarily as an antileukemic agent (24). It has been suggested by ourselves (25) and others (20) that the drug may act as a depot for hydroxylamine (and subsequently, hyponitrite), which upon release, would prevent cell proliferation by condensation or cleavage reactions with DNA (26, 27), or by cleavage of acetyl-CoA (25). The findings reported here provide one route by which this might occur, although according to present evidence on urease distribution (28, 29), the reaction would be limited to gastrointestinal bacteria.

The noncompetitive inhibition pattern of hydroxylamine, a product of hydroxyurea hydrolysis, is analogous to that of NH₂⁺ and urea has provided additional evidence that both substrates are degraded at the same site, and that the Michaelis constants of both probably represent true dissociation equilibria.

**Summary**

Urease inhibition occurring during the hydrolysis of hydroxyurea has been shown by dilution studies to be irreversible. Hydroxylamine, on the other hand, was found to be a reversible, partial, noncompetitive inhibitor of the enzyme, the kinetic constants with respect to urea hydrolysis being: K_i = 0.2 mm, β = 0.2 (the fractional reaction rate of inhibitor-complexed enzyme as compared to free enzyme), and the rate constant k = 10⁶ M⁻¹ min⁻¹ at 25°. Although not critically studied, hydroxylamine inhibition of hydroxyurea hydrolysis was much less impressive. Acetohydroxamic acid was demonstrated to be an irreversible, complete and probably noncompetitive inhibitor of urease with k = 10⁷ M⁻¹ min⁻¹ at 25°.

Inhibition by hydroxylamine was instantly reversed by cysteine and mercaptoethanol, which had no effect on hydroxyurea or acetohydroxamic acid inhibition. In addition, hydroxylamine was observed to exert no oxidative effect on the sulphydryl groups of urease and of mercaptans in direct studies. By structural analogy with acetohydroxamic acid, it is argued that hydroxyurea probably acts simultaneously as substrate and irreversible inhibitor of urease.

The kinetics of urease hydrolysis of combinations of hydroxyurea and urea has provided additional evidence that both substrates are degraded at the same site, and that the Michaelis constants of both probably represent true dissociation equilibria.

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