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INHIBITION OF UREASE BY HYDROXYUREA*

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Abstract—Hydroxyurea was shown to be a potent inhibitor of urease; 50% inhibition occurred at a concentration of approximately 5×10^{-5} M. Preincubation of the enzyme with the inhibitor prior to addition of substrate yielded inhibition which progressed with time, whereas simultaneous addition of inhibitor and substrate to the enzyme reduced the degree of inhibition markedly. Reciprocal plots of velocity against substrate concentration, with and without inhibitor, showed the kinetics of inhibition to be predominantly noncompetitive after preincubation of enzyme with inhibitor. A small increase in apparent K_m in the presence of inhibitor indicated, however, a mixed inhibitory component. Inhibition was partially overcome by dialysis of enzyme–inhibitor mixtures. A tentative mechanism of inhibition was suggested, which involves hydrolysis of hydroxyurea by urease to form the actual inhibitory agent.

NUMEROUS aspects of the biochemical pharmacology of the unique antitumor agent, hydroxyurea, have been reported recently. These include a reduction of the DNA/RNA ratio in *Pseudomonas aeruginosa* with a concomitant alteration of the cytology of that organism;[1] an inhibition of incorporation of thymidine into Ehrlich ascites tumor cells,[2] HeLa cells,[3] and sand dollar (*Echinarachnius parma*) embryos;[4] a possible cleavage of acetyl CoA after conversion of hydroxyurea to hydroxylamine;[5] a reduction of the viscosity and sedimentation coefficient of isolated mammalian and phage DNA;[6] and an inhibition of conversion of cytidine monophosphate to deoxycytidine monophosphate in bone marrow.[7] An antimetabolite function of hydroxyurea in DNA synthesis has also been suggested.[8]

Davidson and Winter[9] developed a sensitive method for the determination of hydroxyurea in biological fluids. They hydrolyzed urea in serum with urease in Tris buffer at pH 7·4 and estimated the remaining hydroxyurea with the diacetyl monoxime reagent. They reported that hydroxyurea did not appear to inhibit urease, and that it actually served as substrate but with a rate of degradation only about 10^{-3} that of urea. In view of the report by Kobashi *et al.*[10] on the marked inhibition of urease by a number of mono-, di-, and tricarboxylic hydroxamic acids and nitrogen-containing acyl hydroxamic acids (hydroxyurea being the hydroxamic acid derivative of carbamic acid), the effect of hydroxyurea on urease has been investigated in this laboratory; a report follows.

EXPERIMENTAL

Jackbean urease, type IV, was obtained from Sigma Chemical Co. and had an activity of approximately, 2,500 modified Sumner units/g. Urea (Baker, reagent grade)

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was recrystallized from absolute ethanol and stored in a drying oven at 60°. Hydroxyurea was synthesized by the Hynes Chemical Research Corp., Durham, N. C. Elemental analysis showed: C, 15·49%; H, 5·24%; N, 37·04%. Calculated for $CH_4N_2O_2$: C, 15·79%; H, 5·29%; N, 36·84%.

Measurements of reaction rates were done with a Warburg respirometer at 30° in an air atmosphere. In all experiments the final reaction volume was 2·2 ml, and potassium citrate, pH 5·8, was present at a final concentration of 0·2 M. In most experiments 50 μg urease was used in each vessel. With the use of data on activity of this preparation, and the information on molecular weight and activity of crystalline urease as given by Seneca,[11] it was calculated that the final concentration of enzyme in vessels containing 50 μg was approximately 10^{-9} M.

To enhance precision of the determinations of reaction velocities, five readings of each manometer were recorded during the initial 10 min while the course of the reaction was linear with time. The slope of each plot was calculated by the method of least squares and was numerically equal to the velocity of the reaction in μliters CO_2 min^{-1}.

RESULTS

Initial kinetic studies of the enzyme showed a linear relationship of V (initial velocity) to E (enzyme concentration), demonstrating the absence of toxic impurities in the reagents, and of inhibitors or activators in the enzyme preparation. Similarly, a typical hyperbolic relationship between V and S (substrate concentration) was noted. The K_m (Michaelis constant) of the complex was calculated by least squares treatment

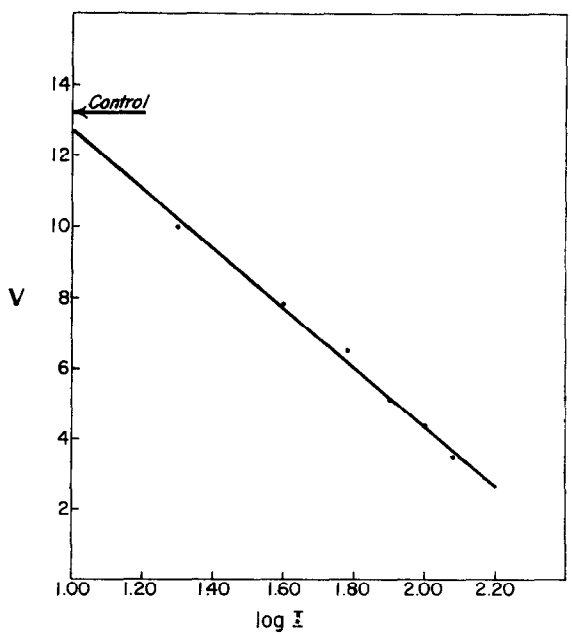


FIG. 1. Inhibition of urease by hydroxyurea. Initial velocity (V) = μl CO_2 min^{-1}; log inhibitor (I) = log μM hydroxyurea. Enzyme (E) = 50 μg and substrate (S) = 30 μmoles in 2·2 ml. E preincubated with I 20 min before addition of S.

of the reciprocal plots, and varied from $4{\cdot}0 \times 10^{-3}$ M to $5{\cdot}8 \times 10^{-3}$ M in several experiments.

The effect of hydroxyurea on urease is shown in Fig. 1. This figure shows the linear relationship of V to log I (inhibitor); the concentration giving 50% inhibition was calculated to be $5{\cdot}4 \times 10^{-5}$ M. These results were incompatible with those of Davidson and Winter,[9] who used an enzyme preparation virtually identical with that used in this work and found no inhibition of the enzyme by hydroxyurea; consequently, their protocol was compared with that used in the present work. Their analyses of hydroxyurea consisted first of adding the enzyme solution to serum containing urea and hydroxyurea to hydrolyze urea. The enzyme thus was brought into contact with both compounds simultaneously. In experiments such as are shown in Fig. 1, enzyme and inhibitor were added to the main compartments of the Warburg vessels prior to attachment to the manometers, and substrate was tipped in from the side arms after temperature equilibration. There was thus a 20-min period of contact of enzyme with inhibitor before addition of substrate. When the order of addition of the solutions to the vessels was changed, with enzyme being added from the side arms to a mixture of substrate and inhibitor in the main compartment, magnitude of the inhibition was markedly reduced. A typical experiment with inhibitor at 10^{-4} M showed that 66% inhibition resulted when substrate was added to enzyme + inhibitor, while only 7% inhibition occurred when enzyme was added to substrate + inhibitor.

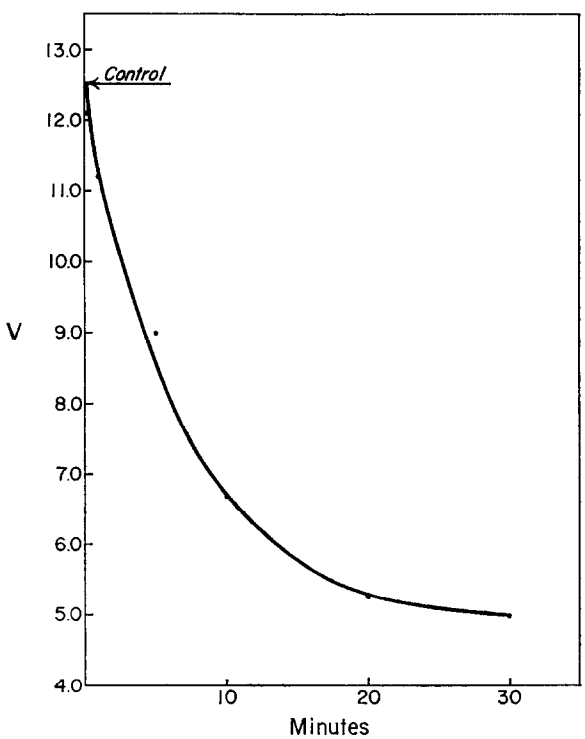


FIG. 2. Progress of inhibition of urease by hydroxyurea. $I = 5 \times 10^{-5}$ M; $E = 50$ μg and $S = 25$ μmoles in 2·2 ml. $V = \mu l\ CO_2\ min^{-1}$. Minutes indicate time of contact of E with I before addition of S.

To determine if onset of inhibition was immediate or delayed, a series of double side-arm vessels was set up so that inhibitor could be added to enzyme at precise times before addition of substrate. Thermal inactivation was not a factor in these experiments, since the protocol was arranged so that substrate was added to all vessels within a 2-min period. Figure 2 shows the progress of inhibition with time. This curve

TABLE 1. RESTORATION OF UREASE ACTIVITY BY DIALYSIS OF UREASE-HYDROXYUREA SOLUTIONS

	Experiment A		Experiment B	
	V (μl CO_2 min^{-1})	Per cent inhibition	V (μl CO_2 min^{-1})	Per cent inhibition
E, not dialyzed	16·5		15·5	
E, dialyzed	7·9		14·2	
$E + I$, not dialyzed	3·4	79	4·6	70
$E + I$, dialyzed	4·7	41	9·4	34

Enzyme concentration (E) = 100 μg/ml and inhibitor (I) = $2{\cdot}0 \times 10^{-4}$ M in dialysis sacks. Experiment A, dialyzed 20 hr against 5 changes of 100 volumes of glass-distilled water; experiment B, dialyzed 17 hr against 2 changes of 100 volumes of citrate buffer. Appropriate dialyzed and nondialyzed solutions of E alone were used as controls. Dialyzed and nondialyzed solutions maintained at 2° until assayed. Per cent inhibition is expressed as reduction in reaction velocity at 30° when 1·0 ml of each solution was tested with 30 μmoles substrate in the Warburg respirometer with a final reaction volume of 2·2 ml.

did not assume linearity when one of the co-ordinates was converted to the log scale; consequently, the kinetics of onset of inhibition apparently are not of first order.

In experiments to determine reversibility of the enzyme–inhibitor complex, enzyme and inhibitor were mixed and incubated at 30° for 30 min. After this, the solutions were dialyzed against 0·2 M potassium citrate, pH 5·8, or against glass-distilled water. Table 1 shows the results of two such experiments and reveals approximately 50 per cent restoration of activity by dialysis.

Reciprocal plots ($1/S$ against $1/V$) with and without I indicated a predominantly noncompetitive inhibition when E was preincubated with I for 20 min prior to addition of S. Results of one such determination are shown in Fig. 3. In none of the experiments did the control and inhibited plots intercept the $-1/S$ abscissa at precisely the same point, and the increase in apparent K_m ($=K_p$) induced by I ranged from 21·6 to 34·5 per cent in a number of experiments. V_{max} was consistently lowered by I in these experiments, the reduction ranging from 42·5% to 43·3%. Such an increase in apparent K_m in the presence of a decrease in V_{max} is suggestive of a mixed type of inhibition, even though the reciprocal plots appear principally noncompetitive. This is of course compatible with the experiments in which activity of E was partially, never completely, restored by dialysis.

A possible breakdown product of hydroxyurea, hydroxylamine, when added to enzyme 20 min before addition of substrate, resulted in only 1·3%, 2·6%, and 7·3% inhibition at concentrations of 10^{-5} M, 10^{-4} M, and 10^{-3} M respectively.

DISCUSSION

The foregoing data establish another pharmacological action of this interesting carcinostatic agent. These findings appear somewhat unique in that earlier reports of

actions of hydroxyurea have described almost exclusively effects on genetic mechanisms.[1–4, 6–8] The one previous exception is the report by Fishbein and Carbone[5] on the possible cleavage of acetyl CoA *in vivo* after hydrolysis of the drug to hydroxylamine.

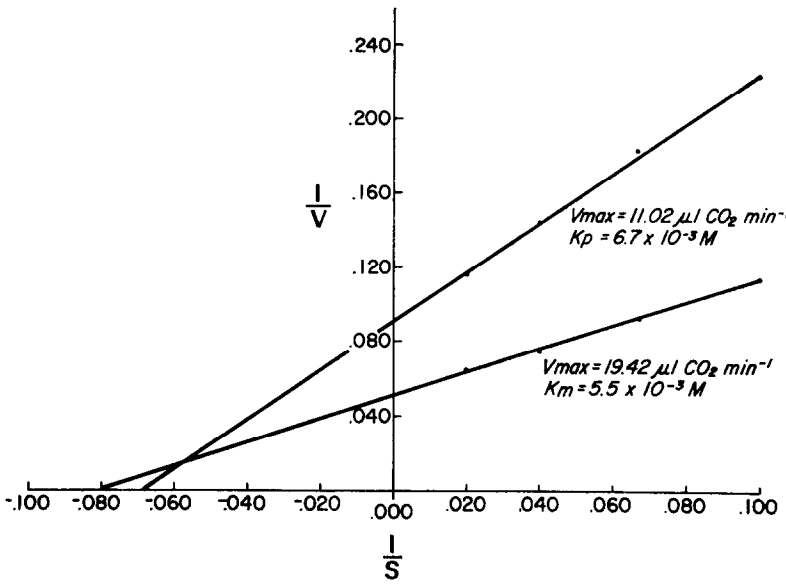
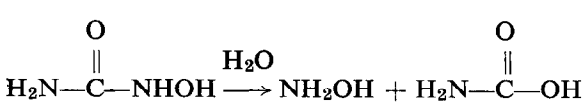


FIG. 3. Reciprocal plot of S against V, with and without I. $1/V = 1/\mu l\ CO_2\ min^{-1}$; $1/S = 1/\mu moles$ n 2·2 ml. $I = 5 \times 10^{-5}$ M. E preincubated with I 20 min before addition of S.

Discrepancies encountered in the earlier phases of this work between the current data and those of Davidson and Winter[9] appear to be resolved adequately. The marked protection afforded the enzyme by bringing it into contact with substrate and inhibitor simultaneously was the basis of those authors' report of negligible inhibition of the enzyme by the drug. Such a situation is strongly indicative of an inhibition mediated at the substrate binding site of the enzyme, a mechanism suggested by Kobashi *et al.*[10] for other hydroxamic acids.

The progression of inhibition with time most logically could be accounted for in one of two ways: (a) slow binding of the inhibitor to the enzyme, with inactivation being a function of the amount bound, or (b) conversion of a noninhibitory compound to an inhibitor. This latter possibility is extremely intriguing, since Davidson and Winter[9] found that hydroxyurea is altered by urease, albeit at a much lower rate than is urea. On theoretical grounds, two possible routes exist for enzymatic hydrolysis of hydroxyurea. The first to be considered

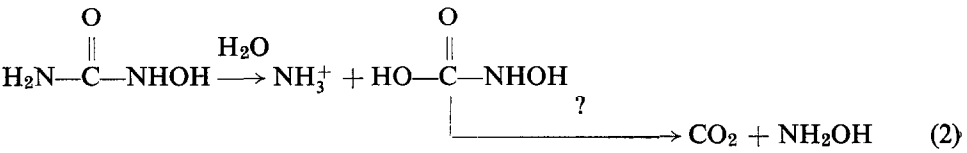


(1)

would yield initially carbamic acid and hydroxylamine. The carbamic acid would decompose rapidly to CO_2 and NH_3^+, and indeed has been shown by Wang and Tarr[12]

to be an intermediate in the hydrolysis of urea; this finding was confirmed by Gorin.[13] Hydroxylamine was not found to be significantly inhibitory to urease at concentrations equivalent to those of hydroxyurea used in this study.

An alternative pathway of hydrolysis would be



$$H_2N{-}\overset{\overset{O}{\|}}{C}{-}NHOH \xrightarrow{H_2O} NH_3^+ + HO{-}\overset{\overset{O}{\|}}{C}{-}NHOH \xrightarrow{?} CO_2 + NH_2OH \qquad (2)$$

which would yield initially NH_3^+ and N-hydroxycarbamic acid. This alternative appears particularly attractive, since the first step involves removal of an amino group; this is the first step in the hydrolysis of the natural substrate of the enzyme. The other compound, N-hydroxycarbamic acid, has never been described so far as can be determined. On the basis of its structural similarity to carbamic acid, it would be expected to be labile and undergo decomposition to CO_2 and hydroxylamine. During its finite existence, however, it may inactivate some fraction of the enzyme, or may be bound to it and thus stabilized while forming an inactive complex. An irreversible complex would be expected to yield predominantly noncompetitive inhibition kinetics, as was found to be the case. Such a hypothesis does not, however, account for the apparent mixed inhibitory component of the reciprocal plots, nor does it appear to be compatible with partial restoration of enzymatic activity of an enzyme–inhibitor solution by dialysis. A hypothesis that would seem to explain the mixed component could propose that hydroxyurea is a weak competitive inhibitor, while some metabolite of the drug is a noncompetitive inhibitor.

One final alternative should be mentioned: if hydroxyurea is hydrolyzed by either step suggested above, with hydroxylamine being almost surely a product in (1) and quite possibly a product in (2), this hydroxylamine formed in close proximity to the active site of the protein molecule may be inhibitory to the enzyme, whereas that exogenously added is not. Since no precedent currently exists for such a model in a cell-free system, the likelihood of the existence of this mechanism seems remote.

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