Competitive Inhibition of Enzyme Activity by Urea*

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It is widely accepted that urea and guanidine act as protein denaturants by breaking intramolecular hydrogen bonds (1). Loss of catalytic activity in the presence of urea is thought to occur by elimination of bonds contributing to the tertiary structure of enzyme molecules. Subsequent restoration of structural and catalytic properties by removal of the denaturant, "reversible denaturation," is usually interpreted as evidence of the reformation of the ruptured hydrogen bonds.

Although there exists a large literature describing denaturation of enzymes by urea, there have been few kinetic analyses of the reversible inhibition of enzyme activity at relatively low levels of urea. Although ribonuclease activity appears to be unaffected in 8 M urea (2), it has been shown that the ribonuclease molecule is actually extensively unfolded in such solutions, but the process is reversed by its substrate, ribonucleic acid (or other polyanions), so that there is no apparent influence on the enzymatic activity of the molecule (3). Pepsin (4) and carboxypeptidase (5) have been shown to retain their activity at relatively high urea concentrations. Reversible inactivation of trypsin (6), lysozyme (7), invertase (8), Cypridina luciferase (9), and phosphoglucomutase (10) by urea has been reported, whereas chymotrypsin (6) and aldolase (11) have been seen only to undergo irreversible inactivation in urea solution. Hill, Schwartz, and Smith (12) have studied the effect of relatively low concentrations of urea on the activity of papain; their data indicate that, with c-benzoyl-L-argininamide as substrate, urea competitively inhibited papain.

The present studies have demonstrated that in the presence of low concentrations of urea, milk xanthine oxidase exhibits an immediate inhibition which is readily reversed by dilution. Kinetic analysis revealed that urea serves as a formally competitive inhibitor of this enzyme.

Twenty-one diverse enzymes have been similarly tested for their susceptibility to inhibition by urea and the nature of the inhibition characterized in each case. The results have revealed interesting differences in patterns of urea inhibition and are considered to bear on the mechanism of formation of the enzyme-substrate complex in each case. The competitive inhibition of enzymes by urea has been studied under different conditions in an effort further to understand the significance of the inhibition.

EXPERIMENTAL PROCEDURE

Xanthine oxidase, lactic dehydrogenase, histidase, horseradish peroxidase, alkaline phosphatase, acid phosphatase, tyrosinase, liver alcohol dehydrogenase, yeast alcohol dehydrogenase, uricase, and carboxypeptidase were obtained from Worthington Biochemical Corporation. The other enzymes were prepared as described below.

Xanthine Oxidase—This enzyme was purified as previously described (13). Assays were conducted in 0.05 M potassium phosphate, pH 7.8, containing 0.005% Versene-Fe³⁺, at 25° unless otherwise specified. Enzyme activity usually assayed according to Kalckar (14) with xanthine as substrate by observing the increase in absorbancy at 295 mμ. When salicylaldehyde was the substrate, salicylic acid production was estimated from the increase in absorbancy at 290 mμ.

Liver Aldehyde Oxidase—A crude preparation from rabbit liver was obtained by the procedure of Hurwitz (15). Subsequent adsorption and elution from calcium phosphate and alumina CY gels permitted 100-fold purification. The preparation was assayed by estimation of pyridine formation, measured at 300 mμ, with N-methyl nicotinamide as the substrate, and by estimation of salicylic acid formation, followed at 290 mμ, when salicylaldehyde was used as the substrate. The temperature and buffer were the same as those used with xanthine oxidase.

Urease—Urease was assayed by following the diminution in absorbancy at 290 mμ as uric acid is oxidized by the enzyme; conditions of assay were identical with those for xanthine oxidase.

Lactic Dehydrogenase—Twice-recrystallized rabbit muscle enzyme was assayed in terms of DPNH oxidation by following absorbancy at 340 mμ in cuvettes with a light path of 10 cm. Initial DPNH concentration was 3 × 10⁻⁵ M whereas the concentration of pyruvate was varied. All assays were performed in phosphate buffer, pH 6.8, at 25°.

r-Amino Acid Oxidase—This enzyme was prepared from moccasin venom by the method of Singer and Kearney (16) and was assayed spectrophotometrically by measuring the change in absorbancy at 290 mμ as the enzyme catalyzed the oxidation of L-leucine in 0.13 M Tris-HCl buffer, pH 7.2, at 25°.

Histidase—An aqueous extract of lyophilized cells of Pseudomonas fluorescens was used as the enzyme source. Assay of enzyme activity was performed by the method of Tabor and Mehler (17).

Tyrosinase—The assay method was based on the increase in absorbancy at 280 mμ when the enzyme acts on L-tyrosine. Assays were conducted in 0.17 M potassium phosphate buffer, pH 6.5, at 25°. The reaction mixture was oxygenated for 3 minutes before addition of the enzyme.

Liver alcohol dehydrogenase was assayed by the method of Theorell and Bonnichsen (18), with 1 × 10⁻³ M DPN and varying concentrations of ethanol or cyclohexanol (19).
**Yeast alcohol dehydrogenase** was assayed according to Racker (20) with $5 \times 10^{-3}$ M DPN and varying levels of ethanol.

**Peroxidase** was assayed spectrophotometrically by measuring the increase in absorbancy at 460 nm when o-dianisidine was used as the substrate in 0.01 M phosphate buffer, pH 6.0.

**Alkaline Phosphatase**—Intestinal alkaline phosphatase was assayed spectrophotometrically at 298 nm with o-carboxyphenyl phosphate as substrate (21) in 0.13 M glycine buffer, pH 9.1, containing $3.3 \times 10^{-4}$ M MgCl$_2$.

**Acid Phosphatase**—The principle and method of assay were similar to those for alkaline phosphatase. However, MgCl$_2$ was omitted from the medium and 0.05 M acetate buffer, pH 5.0, was used in place of glycine.

**Hexokinase**, obtained from the Sigma Chemical Company, was assayed by the method of Crane and Sols (22) with glucose as the variable substrate.

**Rhodanese** was prepared from fresh beef liver by ammonium sulfate fractionation of a homogenate which had been subjected to basic lead acetate treatment (23). The procedure of Sörbo (24) was used for assay.

**Sulfite-cytochrome c reductase** (25) was prepared from dog liver by the method developed in this laboratory. It was assayed spectrophotometrically by following the change in absorbancy at 550 nm when cytochrome c is reduced by the enzyme in presence of sulfite, in 0.05 M potassium phosphate buffer, pH 7.8.

**Uridine diphosphate glucose dehydrogenase**, obtained from Dr. E. A. Davidson of this laboratory, was assayed by measuring the increase in absorbancy when DPN is reduced in the presence of uridine diphosphate glucose, in 0.1 M glycine buffer, pH 9.6.

**Cytochrome Oxidase**—Two preparations, one made according to Smith and Stotz (26) and the other a purified soluble preparation (27) generously donated by Dr. D. E. Green, were used. Activity assays were performed with p-phenylenediamine as reductant for cytochrome c (28) in 0.1 M phosphate buffer, pH 6.0.

**Carboxypeptidase** was assayed with carbobenzoxyglycyl-n-phenylalalanine as substrate in 0.025 M Tris buffer, pH 7.65, containing 0.1 M NaCl. The change in absorbancy at 231 nm was measured spectrophotometrically (29).

**Hexose 1,6-diphosphatase**, prepared from beef liver, was obtained from Dr. W. L. Byrne of this laboratory, and was assayed by estimation of the inorganic phosphate liberated from fructose 1,6-diphosphate in 0.05 M Tris-histidine buffer, pH 6.5, at 37°C, by the Fiske-SubbaRow method (30).

**Urea**—The initial studies with xanthine oxidase were conducted with reagent grade urea (Mallinckrodt) which had been recrystallized from a solution containing Versene Fe-3. However, since this precaution proved to be unnecessary, the practice was discontinued and subsequent studies were conducted with unrecrystallized reagent grade urea. All solutions were prepared fresh daily.

**RESULTS**

The reversible denaturation of enzymes by urea is exemplified by the effect of urea on xanthine oxidase, shown in Fig. 1. Inclusion of urea in an assay medium containing $5 \times 10^{-5}$ M xanthine resulted in an inhibition, the magnitude of which was a linear function of urea concentration, up to about 5.0 M urea. When brief exposure of the enzyme to urea was followed by abrupt diminution of the urea concentration by 30-fold dilution before assay, little or no inhibition of enzyme activity was observed. Thus, whereas 5.0 M urea present in the assay mixture caused 86% inhibition of xanthine oxidase, exposure of the enzyme to this level of urea for 5 minutes at 25°C followed by dilution and assay resulted in no detectable inhibition. Prolonged incubation of the enzyme with concentrations of urea in excess of 5 M resulted in irreversible inhibition, the extent of which was a function of the exposure time. Thus, the data in Fig. 1 indicate a rapid, reversible inhibition of xanthine oxidase by urea, followed by a much slower, irreversible denaturation. It seemed possible that the dilution-reversible inhibition could specifically be due to an effect of urea at the active site of the enzyme. Accordingly, the effect of varying the concentration of xanthine on the extent of urea inhibition was investigated. The results are presented in Fig. 2. Lineweaver-Burk plots (31) of the data indicate that urea is formally competitive with respect to xanthine. The $K_i$ for urea, determined by the method of Dixon (32) was found to be 0.2 M.
The effect of urea on the activity of a number of diverse enzymes was then investigated. These studies indicated that urea can serve as an inhibitor of many enzymes and that the type of inhibition is dependent on the enzyme. In general, inhibition is of the competitive type with respect to organic substrates but is not competitive with those enzymes which catalyze reactions involving inorganic substrates. Table I lists the enzymes studied for which urea is a competitive inhibitor; $K_i$ is shown for each case. It is noteworthy that urea appears to be most effective as an inhibitor of enzymes acting on larger substrates such as xanthine, uridine diphosphate glucose, and the pyruvate- DPNH system.

Two of the enzymes studied, milk xanthine oxidase and hepatic aldehyde oxidase, can each catalyze the oxidation of two widely differing types of substrates. As shown in Table I, in each instance the $K_i$ for urea is independent of the type of substrate. Thus, $K_i$ for urea is 0.2 mM when xanthine oxidase is acting on either xanthine or salicylaldoxide whereas the $K_m$ values for the two substrates differ by two orders of magnitude. Similarly, $K_i$ was 0.8 mM for rabbit liver aldehyde oxidase when oxidizing either salicylaldoxide or $N^\text{3}$-methylhistioimidam.

As mentioned earlier, the inhibition by urea of several other enzymes was not formally competitive. Fig. 3 shows a representative experiment dealing with the effect of urea on yeast inorganic pyrophosphatase. A list of enzymes for which urea inhibition is not competitive is presented in Table II.

It is significant that the enzymes for which urea inhibition is not competitive, viz. inorganic pyrophosphatase, sulfito-cytochrome $c$ reductase, catalase, and rhodanese, all have inorganic compounds as substrates. Further, the $K_i$ for urea, in the instances where it could be determined, is very high. The rather low susceptibility of catalase to urea inhibition is indicative of a very large $K_i$ for this enzyme. The only exceptions to the observed demarcation between enzymes acting on organic versus inorganic substrates were the alcohol dehydrogenases from liver and yeast. These were noncompetitively inhibited by urea, in such a manner as to lower maximal velocity without affecting the apparent $K_m$. The $K_i$ for urea was relatively low in these cases, in contrast to the other enzymes in this list. The liver enzyme was inhibited essentially to the same extent, whether it was oxidizing ethanol or cyclohexanol.

In view of the similarity of action of urea and guanidinium salts as hydrogen-bonding agents, it was of interest to determine whether guanidine can also serve as a competitive inhibitor of enzymes. Fig. 4 demonstrates competitive inhibition of xanthine oxidase by guanidine at concentrations far lower than those required for inhibition by urea. The Dixon plots (32) indicate a $K_i$ of 0.006 mM for guanidine. The effect of guanidine was tested on two other enzymes, histidase and tyrosinase, for both of which urea is a competitive inhibitor. Guanidine inhibition was again of the competitive type in these instances, and, as seen in Table III, $K_i$ for guanidine was, in each case, much less than that for urea. These data are in conformity with the known superiority of guanidine as a protein denaturant. Formamide, which is known to denature proteins (2), also inhibits xanthine oxidase competitively with an efficiency slightly less than that of urea; $K_i$ for formamide is about 0.35 mM.

Several of the enzymes tested were not inhibited by urea. Hexokinase, fructose 1,6-diphosphatase, alkaline phosphatase, peroxidase, and cytochrome oxidase retained full activity in the presence of 2 mM urea. Indeed, the activities of cytochrome oxidase and of alkaline phosphatase were enhanced somewhat by this concentration of urea.

### Table I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine oxidase</td>
<td>Xanthine</td>
<td>$3 \times 10^{-5}$</td>
<td>0.2</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Salicylaldoxide</td>
<td>$8 \times 10^{-7}$</td>
<td>0.2</td>
</tr>
<tr>
<td>Uridine diphosphate</td>
<td>glucose</td>
<td>$1.1 \times 10^{-4}$</td>
<td>0.3</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>Pyruvate</td>
<td>$4 \times 10^{-5}$</td>
<td>0.6</td>
</tr>
<tr>
<td>Histidase</td>
<td>$L^\text{-Histidine}$</td>
<td>$5.1 \times 10^{-3}$</td>
<td>0.7</td>
</tr>
<tr>
<td>Aldehyde oxidase</td>
<td>$N^\text{3}$-Methylhistioimidam</td>
<td>$3 \times 10^{-4}$</td>
<td>0.8</td>
</tr>
<tr>
<td>Aldehyde oxidase</td>
<td>Salicylaldoxide</td>
<td>$1.5 \times 10^{-4}$</td>
<td>0.8</td>
</tr>
<tr>
<td>L-Amino acid oxidase</td>
<td>$L^\text{-Leucine}$</td>
<td>$1 \times 10^{-3}$</td>
<td>1.4</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>$o^\text{-Carboxyphenyl}$</td>
<td>$1.1 \times 10^{-4}$</td>
<td>1.5</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>L-Tyrosine</td>
<td>$1.7 \times 10^{-4}$</td>
<td>1.7</td>
</tr>
<tr>
<td>Uricase</td>
<td>Uric acid</td>
<td>$3.4 \times 10^{-5}$</td>
<td>2.2</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>Carbobenzoxy glycin</td>
<td>$5 \times 10^{-3}$</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**Fig. 3.** Inhibition of inorganic pyrophosphatase by urea. Velocity is calculated as $\mu$moles of orthophosphate formed in 15 minutes in 0.02 mM veronal-acetate buffer, pH 7.2, at 37°C.
TABLE II
Enzymes not competitively inhibited by urea

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Type of inhibition</th>
<th>$K_m$ (M)</th>
<th>$K_i$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase (liver)</td>
<td>Ethanol</td>
<td>Uncompetitive</td>
<td>$8.7 \times 10^{-4}$</td>
<td>0.35</td>
</tr>
<tr>
<td>Alcohol dehydrogenase (liver)</td>
<td>Cyclohexanol</td>
<td>Uncompetitive</td>
<td>$4 \times 10^{-4}$</td>
<td>0.48</td>
</tr>
<tr>
<td>Alcohol dehydrogenase (yeast)</td>
<td>Ethanol</td>
<td>Uncompetitive</td>
<td>$3.3 \times 10^{-4}$</td>
<td>0.9</td>
</tr>
<tr>
<td>Pyrophosphatase</td>
<td>Pyrophosphate</td>
<td>Uncompetitive</td>
<td>$1.6 \times 10^{-4}$</td>
<td>4.3</td>
</tr>
<tr>
<td>Sulphite-cytochrome c reductase</td>
<td>Sulfite</td>
<td>Uncompetitive</td>
<td>$2.8 \times 10^{-4}$</td>
<td>7.0</td>
</tr>
<tr>
<td>Catalase</td>
<td>Hydrogen peroxide</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Rhodanese</td>
<td>Thiosulphate</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Rhodanese</td>
<td>Cyanide</td>
<td>None</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>Glucose, ATP</td>
<td>None</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>Ferrocytochrome c</td>
<td>None</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Hexose diphosphatase</td>
<td>Fructose 1,6-diphosphate</td>
<td>None</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>o-Carboxyphenylphosphate</td>
<td>None</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Hydrogen peroxide, o-dianisidine</td>
<td>None</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

Effect of Deuterium Oxide on Urea Inhibition of Xanthine Oxidase—The inhibition of xanthine oxidase by urea was studied in a medium of 99.5% deuterium oxide containing 0.05 M phosphate buffer, pH 7.8. Gutfreund and Sturtevant (33) have observed that, in a 50% D,O medium, xanthine oxidase activity was depressed but that there was no effect on the $K_m$. Similar results were obtained in 99.5% of D$_2$O in the present study. Further, no change was observed in the $K_i$ for urea inhibition, or in the competitive nature of this inhibition.

Effect of Anions on Urea Inhibition of Xanthine Oxidase—The inhibition of xanthine oxidase by urea has been studied in the presence of several anions. KF, KBr, and KCl, at 1 M concentration, had no effect on enzyme activity nor did they significantly alter the $K_m$ for xanthine. Sulfate was similarly ineffective. Acetate, at 1 M, inhibited xanthine oxidase activity by nearly 50% in a noncompetitive manner. Thiocyanate, at the same level, inhibited the enzyme almost completely. The effect of the addition of each of these anions on the urea inhibition of xanthine oxidase was then studied. The results are presented in Table V. Sulfate has no effect on the competitive inhibition of the enzyme by urea. Fluoride, acetate, chloride, bromide, and thiocyanate, in increasing order of effectiveness, sensitize the enzyme to urea inhibition. The inhibition ceases to be purely competitive in the presence of several of these anions. Thiocyanate, at 0.1 M, was nearly as effective as 1.0 M bromide.

Effect of Sodium Dodecyl Sulfate—The anionic detergent sodium dodecyl sulfate is a protein denaturant (34) and has been shown to inhibit several enzymes (35, 36). A kinetic study of

TABLE III
Comparative inhibitory effects of urea and guanidine

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$ for urea</th>
<th>$K_i$ for guanidine</th>
<th>$K_i$ urea</th>
<th>$K_i$ guanidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine oxidase</td>
<td>0.2</td>
<td>0.006</td>
<td>33.3</td>
<td>7.7</td>
</tr>
<tr>
<td>Histidase</td>
<td>0.7</td>
<td>0.009</td>
<td>77.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>0.75</td>
<td>0.25</td>
<td>7</td>
<td>7.7</td>
</tr>
</tbody>
</table>

$p$ Values at $15^\circ$.

TABLE IV
Effect of temperature on $K_i$ for urea inhibition

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (M)</th>
<th>$K_i$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine oxidase</td>
<td>Xanthine</td>
<td>$0.7 \times 10^{-4}$</td>
<td>$0.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Aldehyde oxidase</td>
<td>N-Methyl nicotineamide</td>
<td>$3.2 \times 10^{-4}$</td>
<td>$5.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>L-Amino acid oxidase</td>
<td>L-Leucine</td>
<td>$1.25 \times 10^{-3}$</td>
<td>$0.8 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
TABLE V

Effect of anions on inhibition of xanthine oxidase by urea

The anions were added as the appropriate potassium salts at a concentration of 1.0 M except for thiocyanate which was present at a level of 0.1 M. Urea was present in all experiments at 1.0 M. The data shown are the percentage inhibitions of the activity in control vessels containing neither urea nor the indicated anions.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Inhibition of activity</th>
<th>Type of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xanthine concentration × 10^5 M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>1.35</td>
</tr>
<tr>
<td>None</td>
<td>61.5</td>
<td>46.5</td>
</tr>
<tr>
<td>Sulfate</td>
<td>74.4</td>
<td>53.4</td>
</tr>
<tr>
<td>Fluoride</td>
<td>57.5</td>
<td>40</td>
</tr>
<tr>
<td>Acetate</td>
<td>71.9</td>
<td>55.6</td>
</tr>
<tr>
<td>Chloride</td>
<td>71.8</td>
<td>63.5</td>
</tr>
<tr>
<td>Bromide</td>
<td>66.0</td>
<td>54.0</td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>66.0</td>
<td>54.0</td>
</tr>
</tbody>
</table>

The inhibition of xanthine oxidase by this detergent showed that the inhibition is noncompetitive (Fig. 5). However, the inhibition of xanthine oxidase by urea is still competitive in the presence of sodium dodecyl sulfate, the $K_i$ for urea being 0.1 M as compared with 0.2 M in the absence of the detergent. The inhibition of tyrosinase by the detergent is also noncompetitive. Alkaline phosphatase was not inhibited even at comparatively high levels of dodecyl sulfate.

**DISCUSSION**

The present demonstration of the competitive inhibition by urea of several enzymes of diverse activities indicates that this is likely to be a general phenomenon extending to many enzymes not investigated in this study. Papain is so inhibited (12), and Inagaki (37) has recently presented kinetic data showing competitive inhibition of glutamic acid dehydrogenase by urea. Several enzymes, including glyceraldehyde 3-phosphate dehydrogenase (38), penicillinase (39), and ribonuclease (2), have been found to be protected by their substrates against inactivation by urea, and it seems likely that a competitive relationship between urea and the respective substrates exists in these cases. The competitive nature of the inhibition of many enzymes by low concentrations of urea suggests that inhibition results from interference with the formation of the appropriate enzyme-substrate complex. Guanidine and formamide are similarly inhibitory. All three compounds are known to be strong hydrogen-bonding agents. It appears, therefore, prima facie, that these compounds compete with substrates by reversible, hydrogen-bonding attachment at the active sites of enzymes. The combination of urea with protein molecules is nonspecific and probably occurs over a large area, with numerous molecules of urea combining with each molecule of enzyme. But the inhibition of enzyme activity would seem to reflect the attachment of urea specifically at the active site, in view of the relatively low concentrations of urea required for inhibition, and of the competitive nature of the inhibition. Analysis of the competitive inhibition of several enzymes by urea in the present study, by the method of Johnson, Eyring, and Williams (40), indicates that 1 or 2 molecules of urea per molecule of enzyme are involved in the formation of the enzyme-urea complex which leads to the observed inhibition. Chase and Krotkov (8) and Osborne and Chase (9) have presented similar figures for invertase and luciferase whereas Hill et al. (12) have calculated that, in the case of papain, 4 molecules of urea are bound to each molecule of enzyme. These figures indicate a rather specific effect of urea within a small region of the enzyme molecule, presumably the active site.

Klotz and Luborsky (41) have postulated that the combination of an enzyme with its substrate involves the cooperative formation of an iceslike structure of water molecules between the enzyme and the substrate, by analogy to their interpretation of the manner in which various organic ions are bound to bovine serum albumin. Urea and related protein denaturants, which prevent dye-binding to proteins, are presumed to occasion enzyme inactivation by dissociating such cooperative iceslike structures through hydrogen bond formation with the water molecules of the lattice. The present results, although not lending support to the "ice-lattice" theory, do not definitively contradict it.

However, the possibility should be considered that, at the binding sites of enzymes in the free state in aqueous medium, there are functional groups which are solvated with hydrogen-bonded water. Formation of enzyme-substrate complexes may require displacement of this water by the substrate (42) which, in turn, forms hydrogen bonds at the active site. The competitive action of urea might then be ascribed to the displacement of water molecules by urea in a similar manner.

It is not inherent in this concept that every substrate is hydrogen-bonded to the enzyme in the enzyme-substrate complex. Particularly when relatively large substrates are involved, it is conceivable that a portion of the substrate molecule overlays an area of the protein to which water is bonded. Displacement of such water by urea would also prevent formation of the enzyme-substrate complex simply because of the large volume of the urea molecule compared to that of water. This would be partic-
ularly significant if formation of the complex is accomplished by an "induced fit" as proposed by Koshland (43). The difference between the strength of the hydrogen bond and that of the deuterium bond is very small (44). If urea inhibition is accomplished by displacement of hydrogen-bonded water, there should then be no significant difference in its ability to "compete" with H$_2$O or D$_2$O. The nonvariance of $K_i$ for xanthine (34) and $K_i$ for urea inhibition when determined in a 99.5% D$_2$O medium is in accord with this hypothesis.

**Urea Inhibition of Metallo-enzymes**—From the data here presented, it appears that, in general, urea is relatively ineffective as an inhibitor of those metallo-enzymes whose metal component is involved in the formation of the enzyme-substrate complex. Thus, hexokinase and alkaline phosphatase which show an absolute dependence upon the presence of Mg$^{++}$, and the ferriporphyrin proteins, peroxidase and cytochrome oxidase, are completely unaffected by urea even at 2 M concentration. Uricase, a copper-protein (45), carboxypeptidase, a zinc protein (46), catalase, a hemoprotein, and inorganic pyrophosphatase which is dependent upon Mg$^{++}$ in the medium are inhibited only at very high concentrations of urea. Much evidence suggests that the metal of many metal-requiring or metal-containing enzymes serves as a bridge between enzyme and substrate in the formation of the enzyme-substrate complex (47). It is extremely unlikely that urea can prevent chelation or coordination of the enzyme or substrate by a metal. Similarly, it is unlikely that urea could competitively inhibit the action of enzymes on inorganic substrates such as pyrophosphate, sulfitc or peroxide since, in these instances, electrostatic forces rather than hydrogen-bonding or van der Waals' forces must be involved in formation of the enzyme-substrate complex.

Xanthine oxidase contains both iron and molybdenum, yet the present studies have shown that this enzyme exhibits the lowest $K_i$ for urea among the enzymes studied. This finding suggests that neither metal is involved in the binding of purines or aldehydes to xanthine oxidase. Further, since the $K_i$ for urea inhibition is identical to both types of substrates, it is evident that the same binding site is involved in both oxidations.

**Influence of Anions on Urea Inhibition**—The synergistic effects of dodecyl sulfate and of various anions on urea inhibition are not presently understood. In the presence of dodecyl sulfate, itself a noncompetitive inhibitor of xanthine oxidase, the $K_i$ for urea is lowered from 0.2 M to 0.1 M and the inhibition remains competitive. Alkaline phosphatase, which is unaffected by urea, is also insensitive to dodecyl sulfate. It is of interest, in this context, that cytochrome oxidase, which is not inhibited by urea, has also been shown to be fully active in the presence of the detergent. The order of effectiveness of the anions, thiocyanate > bromide > chloride > acetate > fluoride > sulfate, in accentuating urea inhibition of xanthine oxidase is identical with that found in studies of protein denaturation by urea (48, 49) and bears a striking similarity to the Helminthol lyotropic series.

A clear distinction is warranted between the immediate, dilution-reversible effect of urea on proteins, observed in these studies as a competitive inhibition of enzymic activity, and the essentially irreversible denaturation resulting from prolonged exposure to high concentrations of urea. The former appears to reflect reversible hydrogen bonding of urea with loci on the protein surface ordinarily bonded to water, whereas the latter may be presumed to result from the disruption of the tertiary structure of the protein as urea molecules displace intramolecular hydrogen-bonded groups.

**SUMMARY**

Xanthine oxidase has been found to be competitively inhibited by low levels of urea. Analysis of urea inhibition of a series of diverse enzymes indicates that urea is, in general, a competitive inhibitor of enzymes which act upon organic substrates. Guanidine is much more effective than urea as a competitive inhibitor of the same group of enzymes. Relatively higher concentrations of urea are required to effect inhibition of enzymes which act upon inorganic substrates and such inhibitions are either "non-competitive" or "uncompetitive."

In general, enzymes which require metal ions as cofactors or which contain a metal as an integral part of the enzyme molecule were found to be resistant to inhibition by urea.

$K_i$ for urea inhibition decreases with decreasing temperature. Urea inhibition remains competitive in 99.5% D$_2$O, in 50% glycerol, and in the presence of inhibitory levels of dodecyl sulfate. A number of anions enhance the inhibition of xanthine oxidase by urea; in the more marked instances the inhibition is no longer competitive.

These findings are discussed and a possible mechanism for the competitive inhibition of enzymes by urea is presented.

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