N-Substituted N'-Hydroxythioureas[1]

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A series of N-substituted N'-hydroxyureas and N'-hydroxythioureas were prepared, and their inhibitory properties have been examined in several microbial systems. The hydroxythiourea analogs proved to be toxic to growth of *Lactobacillus arabinosus*, *Leuconostoc dextranicum*, and *Streptococcus faecalis* at about 2 μg/ml; however, the growth inhibition was reversed by adding supplements of cysteine and other sulfhydryl compounds not of natural origin. The toxicity of these derivatives may be due to protein denaturation since in an isolated enzyme system (yeast alcohol dehydrogenase) the enzyme is progressively deactivated by increasing concentrations of N-substituted N'-hydroxythioureas, and the toxicity is negated by the presence of a variety of SH compounds.

In contrast to the rather extensive studies which have been carried out on the biological properties of hydroxyurea as well as other urea derivatives, it was surprising to observe that little data had been reported concerning the physiological properties of hydroxythiourea derivatives. Thiourea *per se* has been utilized by plant physiologists in a variety of biological systems to affect germination and growth,[3] and certain thiourea derivatives have been widely examined as chemotherapeutic agents.[4,5]

Hydroxyurea apparently inhibits the synthesis of deoxyribonucleic acid in *Escherichia coli*,[6,7] ascites tumor cells,[8] and rat liver.[9] It has also proven to be effective against a variety of leukemias and thus has been chosen for clinical evaluation.[10] In addition, N-methyl-N'-hydroxyurea has been shown to be an impressive antimicrobial agent which is active against pathogenic organisms.[11]

The present study is concerned with the inhibitory properties of some N-substituted N'-hydroxythioureas in various microbial systems, as well as a selected enzyme system, and with the reversal of these toxicities by sulfhydryl compounds. For comparative purposes, the corresponding N-substituted N'-hydroxyureas were also prepared and examined in concurrent assays.

Experimental Section

Microbiological Assay Procedures.—For the lactic acid bacteria a previously described amino acid medium[12] was employed with the addition of calcium pantothenate at 0.2 μg/ml, and with additional modifications as noted: for *Streptococcus faecalis* 8043, 20 μg/ml of L-glutamine was added aseptically without heating to each assay tube; for *Leuconostoc dextranicum* 8086, 0.02 μg/ml of pantethine was added, and the phosphate concentration was increased fourfold. All of the lactic bacteria assays were incubated at 30°. *Lactobacillus arabinosus* 17-5 and *S. faecalis* were read at 15–18 hr; whereas, *L. dextranicum* usually required 24 hr for optimum growth. Assays with *E. coli* 9723 were carried out in a previously described inorganic salts–glucose medium[13] and incubated at 37° for 15–18 hr.

The amount of growth was determined using a Bausch and Lomb Model 20 spectrophotometer set at 600 mμ. The interpretation of microbial data in these studies was based on essentially complete inhibition of growth.[14] Test solutions of the N-substituted N'-hydroxythioureas were made up just prior to use because of the instability of the derivatives.

Studies with Yeast Alcohol Dehydrogenase.—Yeast alcohol dehydrogenase was purchased from Worthington Biochemical Corp. as twice-crystallized, lyophilized material, and used directly in the following enzymic assay procedures. A stock solution was prepared at 1 mg/ml in 0.1 *M* phosphate buffer at pH 7.5 and stored in the refrigerator at about 5°. Dilutions of this stock solution were made for the indicated experimental assays by adding 0.1 ml of the stock solution to 9.9 ml of 0.01 *M* phosphate buffer, pH 7.5, containing 0.1% gelatin. This solution contains 10 μg/ml of enzyme and retains constant activity for at least an 8-hr period. The general assay procedure was patterned after that of Vallee, *et al.*,[15,16] and the spectrophotometric readings were determined on a Beckman Model DB spectrophotometer at 340 mμ with a recorder attached. Nicotinamide–adenine dinucleotide (NAD) was also purchased from Worthington Biochemicals.

The basic assay solution contained 1000 μmol of EtOH, 0.125 μmol of NAD, and 2 μg of enzyme adjusted to 3-ml volume with 0.032 *M* pyrophosphate buffer, pH 8.8. The N-substituted N'-hydroxythiourea was made up fresh daily in 0.1 *M* phosphate buffer, pH 7.5 Various concentration levels of the inhibitors were then preincubated with the diluted enzyme solution for 0-, 15-, 30-, 45-, and 60-min periods. The substrate and coenzyme were then added and the relative reaction rates determined by measuring the change in optical density between 30 and 45 sec after mixing and placing in the spectrophotometer. Normally, a 2-min time spectrum was utilized to establish the relative linearity of the reaction under these treatment conditions. The control cuvet was treated in the same manner except that the enzyme was omitted.

Synthesis of N-Substituted N'-Hydroxyureas and -Thioureas.—All of these compounds were synthesized through a procedure similar to that of Kjellin.[17] The appropriate isocyanate or isothiocyanate in C_6H_6 or Et_2O was treated with a slight molar excess of $HONH_2$[18] to yield ultimately products which were recrystallized from EtOAc–C_6H_{14} (Table I).

Results and Discussion

As indicated in Table I, a variety of isocyanates and isothiocyanates was condensed with $HONH_2$ to yield the

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TABLE I

N-SUBSTITUTED *N'*-HYDROXYUREAS AND -*N'*-HYDROXYTHIOUREAS

	——RNHCONHOH——			——RNHCSNHOH——		
R Group	Mp, °C	% yield[a]	Formula[b]	Mp, °C	% yield[a]	Formula[b]
Me	129–131[c]	57	$C_2H_6N_2O_2$	101–102[d]	14	$C_2H_6N_2OS$
Et	126–127[e]	62	$C_3H_8N_2O_2$	105–106[f]	54	$C_3H_8N_2OS$
n-Pr	128–129[g]	45	$C_4H_{10}N_2O_2$			
n-Bu	129–131[h]	28	$C_5H_{12}N_2O_2$	107–108[i]	55	$C_5H_{12}N_2OS$
t-Bu				110–111	31	$C_5H_{12}N_2OS$
n-Hex	131–132	38	$C_7H_{16}N_2O_2$			
C_6H_{11}	116–118[j]	66	$C_7H_{14}N_2O_2$	109–110	55	$C_7H_{14}N_2OS$
C_6H_5	155–157[k]	52	$C_7H_8N_2O_2$	103[l]	18	$C_7H_8N_2OS$
α-$C_{10}H_7$	174–175	23	$C_{11}H_{10}N_2O_2$			
CH_2=$CHCH_2$	130–132	36	$C_4H_8N_2O_2$	100–102	26[m]	$C_4H_8N_2OS$

[a] Yields given are based on highly purified products; essentially quantitative yields on crude products were obtained. [b] All compounds except MeNHCONHOH, were analyzed for C, H, N. This compound was analyzed for C, H only. Analytical results were within ±0.4% of the theoretical values. [c] L. Francesconi and A. Parrozzani, *Gazz. Ital.*, **31,** 334 (1901), mp 127°, through Beilstein Band IV, p 70. [d] C. Kjellin and K. G. Knylenstjerna, *Justus Liebigs Ann. Chem.*, **298,** 117 (1897), reported mp 101°. [e] Reported mp 129°, footnote *c*, through Beilstein, Band IV, p 117. [f] Reported mp 109°, footnote *d*. [g] Reported mp 126°, G. Zinner, R. O. Weber, and W. Ritter, *Arch. Pharm.*, **298** (12), 869 (1965). [h] German Patent 11,131,655; *Chem. Abstr.*, **57,** 13628e (1962), reported mp 123–125° dec. [i] Reported mp 106°, G. Zinner and R. O. Weber, *Pharmazie*, **21,** 23 (1966). [j] Reported mp 112–113° dec, footnote *h*. [k] Reported mp 144°, C. Kjellin, *Ber.*, **26,** 2384 (1893). [l] Reported in footnote *h*, but no melting point recorded. [m] Reported mp 85–120°, footnote *d*.

TABLE II

INHIBITORY LEVELS OF SOME *N*-SUBSTITUTED *N'*-HYDROXYTHIOUREAS TO MICROBIAL GROWTH[a]

	Concentration required, μg/ml, for complete growth inhibition[b]			
Substituent group	*E. coli* 9723[c]	*L. arabinosus* 17-5[c]	*L. dextranicum* 8086	*S. faecalis* 8043
Me	>60[d]	0.6	2	2
Et	>60	2	2	2
n-Bu	>60	2	2	2
t-Bu	>60	2	2	0.6
Allyl	>60	2	2	2
C_6H_{11}	20	0.6	2	2
C_6H_5	>60	0.6	2	6

[a] Unless otherwise noted, the corresponding *N*-substituted *N'*-hydroxyureas and *N*-substituted *N'*-ureas were essentially noninhibitory at their solubility limits, *i.e.*, about 2000 μg/ml. [b] The assay media for each organism are described in the Experimental Section. [c] *N*-Ph- and *N*-naphthyl-*N'*-hydroxyurea toxic at 2 mg/ml. [d] Limit of solubility. [e] Hydroxyurea, *N*-Ph- and *N*-*n*-hexyl-*N'*-hydroxyurea toxic at 200 μg/ml.

TABLE III

REVERSAL OF TOXICITY OF *N*-METHYL-*N'*-HYDROXYTHIOUREA TO GROWTH OF *Lactobacillus arabinosus* 17-5 BY VARIOUS MERCAPTO COMPOUNDS

N-Methyl-*N'*-hydroxythiourea, μmol	Supplement to general assay medium: None	0.25 μmol: L-Cy[a]	0.25 μmol: DL-Cy[b]	0.25 μmol: DL-Hcy[c]	2.5 μmol: L-Cy	2.5 μmol: DL-Cy	2.5 μmol: DL-Hcy
	Growth, per cent transmission[d]						
0	32	30	28	31	23	25	25
0.01	40						
0.025	55	35	30	30			
0.05	85	45	54	44			
0.1		84	87	82			
0.25					27	23	21
0.5					75	77	86
1.0					95	95	93

[a] L-Cysteine. [b] DL-Cysteine. [c] DL-Homocysteine. [d] Determined on a Bausch and Lomb Spectronic 20 at 600 mμ.

corresponding hydroxyurea or hydroxythiourea derivatives, respectively. The *N*-substituted *N'*-hydroxy-

$$RN{=}C{=}X + NH_2OH \longrightarrow RNHC(=X)NHOH \quad (X = S \text{ or } O)$$

urea analogs were stable to both moisture and light; however, the corresponding hydroxythiourea derivatives decomposed within a few weeks unless they were maintained under anhydrous conditions at about −15°.

Each of these derivatives was examined for inhibition of microbial growth using *E. coli* 9723, *L. arabinosus* 17-5, *L. dextranicum* 8086, and *S. faecalis* 8043. For *E. coli* no significant inhibition was observed; however, in *L. arabinosus*, *L. dextranicum*, and *S. faecalis* the *N*-substituted *N'*-hydroxythioureas were toxic to growth at about 2 μg/ml (Table II); whereas, the corresponding *N*-substituted *N'*-hydroxyureas were either noninhibitory or were toxic only at the limit of their solubility in the assay medium, *ca.* 2 mg/ml.

In an effort to determine if naturally occurring products might reverse these toxicities, supplements of vitamins, purines, and pyrimidines were added to culture tubes concurrently with the hydroxythioureas and these metabolites were found to be ineffective. However, an amino acid mixture did induce a slight reversal of toxicity, and peptone proved to be an effective source of reversing agent(s). Subsequently, a complete reversal of toxicity was observed when cysteine alone was added aseptically to the assay medium. The relative amounts of cysteine required to reverse various inhibitory concentrations of *N*-methyl-*N'*-hydroxythiourea in *L. arabinosus* were examined in detail, and these data are presented in Table III.

It should be noted from Table III that the ratio of reversing agent concentration to *N*-methyl-*N'*-hydroxythiourea concentration which is required for reversal of toxicity results in an inhibition index (the ratio of the concentration of inhibitor to the concentration of substrate necessary for a defined degree of inhibition of a biological process) of 0.2 which is inconsistent with a direct competition for enzyme sites. A reasonable interpretation might be that the biochemical response involves a chemical destruction of the inhibitor to produce a response similar to a "precursor effect."[19] This

[19] R. J. Williams, R. E. Eakin, E. Beerstecher, Jr., and W. Shive, "The Biochemistry of the B Vitamins," Reinhold, New York, N. Y., 1950, pp 443–480.

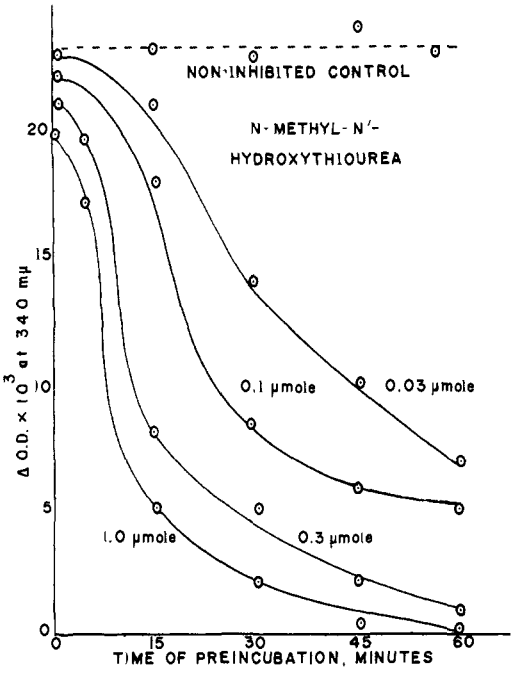

Figure 1.—Effect of preincubation of yeast alcohol dehydrogenase in the presence of increasing concentrations of *N*-methyl-*N'*-hydroxythiourea on its enzymic activity for varying periods of time. Activity determined as ΔOD response between 30 and 45 sec after start of the reaction; basal incubation mixture: 1000 μmol of ethanol, 2 μg of treated enzyme, 0.125 μmol of NAD.

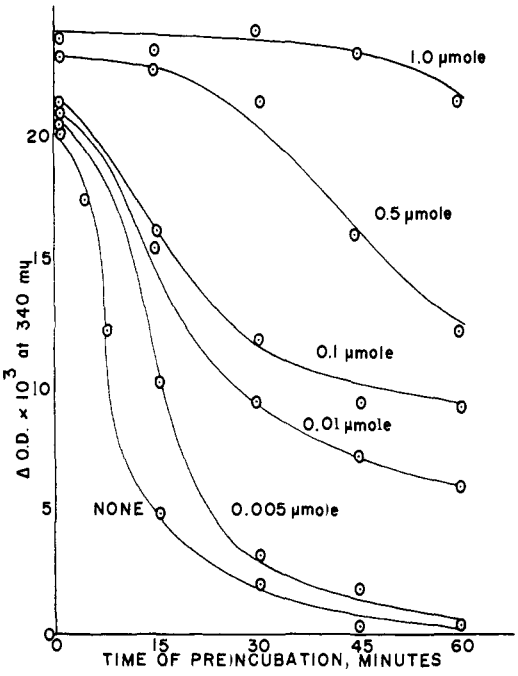

Figure 2.—Reversal of toxicity of *N*-methyl-*N'*-hydroxythiourea on yeast alcohol dehydrogenase enzyme by increasing supplements of L-cysteine. The enzyme was preincubated in the presence of 1 μmol of the inhibitor and the indicated concentrations of L-cysteine for varying periods of time. Activity of the enzyme was then determined between 30 and 45 sec after start of the reaction; basal incubation mixture: 1000 μmol of ethanol, 2 μg of treated enzyme, 1 μmol of *N*-methyl-*N'*-hydroxythiourea, and 0.125 μmol of NAD.

concept is consistent with the fact that the same degree of microbial growth is observed when a given inhibitory level of *N*-methyl-*N'*-hydroxythiourea is reversed by racemic mixtures of SH reagents. For example, molecular equivalents of L-cysteine, DL-homocysteine, and DL-cysteine are equally effective in reversing the toxicity of the methyl derivative over a tenfold range of concentrations as indicated in Table III. The same type of response was observed in all of the other hydroxythiourea derivatives.

Subsequently, a study of the chemical reactions between the *N*-substituted *N'*-hydroxythioureas and SH compounds was undertaken. For example, cysteine and *N*-cyclohexyl-*N'*-hydroxythiourea were allowed to react in phosphate buffer, pH 8.3, at room temperature for 24 hr. The principle products of the reaction were cystine and *N*-cyclohexylthiourea indicating an oxidation reduction had occurred. Since *N*-cyclohexylthiourea is not inhibitory, these data in conjunction with the reversal data on microbial inhibition (Table III) further suggest that the toxic structure is degraded *in vivo* by sulfhydryl containing compounds.

In an effort to determine if such a SH interaction would result in protein denaturation, an enzymic system (alcohol dehydrogenase) was chosen for *in vitro* studies. The biological activity of this protein was found to be progressively inactivated by preincubation with increasing concentrations of the hydroxythiourea derivatives (Figure 1). A series of studies was then conducted in which supplements of mercapto compounds including cysteine were preincubated concurrently with various levels of inhibitor and enzyme, and the rate of dehydrogenase activity was determined. As indicated in Figure 2, increasing levels of L-cysteine do negate the inhibitory effect of *N*-methyl-*N'*-hydroxythiourea. However, once the enzyme is inactivated by preincubation with an *N*-substituted *N'*-hydroxythiourea compound alone, it cannot be reactivated by the addition of cysteine even in high concentrations. Further, preincubation of the enzyme in the presence of cysteine prior to the addition of inhibitor affords no more protection to the enzyme than when the inhibitor and cysteine are added concurrently. Supplements of mercaptoacetic acid produced comparable physiological effects which suggest that the presence of exogenous SH groupings alone may be negating the inhibitory properties of the *N*-substituted *N'*-hydroxythiourea derivatives. The sigmoidal shape of the curves in Figures 1 and 2 may be explained by assuming a series of reactions in which the *N*-substituted *N'*-hydroxythiourea derivative initially oxidizes exposed SH groupings on the protein molecule; after which, following a partial unfolding, a reaction then occurs with internal SH groups.

It is interesting to note that the presence of even 0.01 and 0.1 μmol of cysteine provided a considerable amount of protection to the enzyme at an inhibitor concentration of 1.0 μmol. These effects are probably due to a greater rate of reaction between the highly soluble low molecular weight SH compounds and *N*-substituted *N'*-hydroxythioureas than is possible for the more sterically hindered SH groups attached to large protein molecules.