Hydroxyurea Derivatives: 1-Hydroxybiuret and 3-Hydroxybiuret* (32907)

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Recent reports from this laboratory have described selective inhibition by certain hydroxyurea (HU) derivatives of DNA synthesis in microbial and tumor cell test systems (1–3). To assess additional alkyl hydroxamic acids as selective inhibitors in these systems, 1-hydroxybiuret (1-HB) and 3-hydroxybiuret (3-HB) (Fig. 1) were synthesized and tested; a report follows.

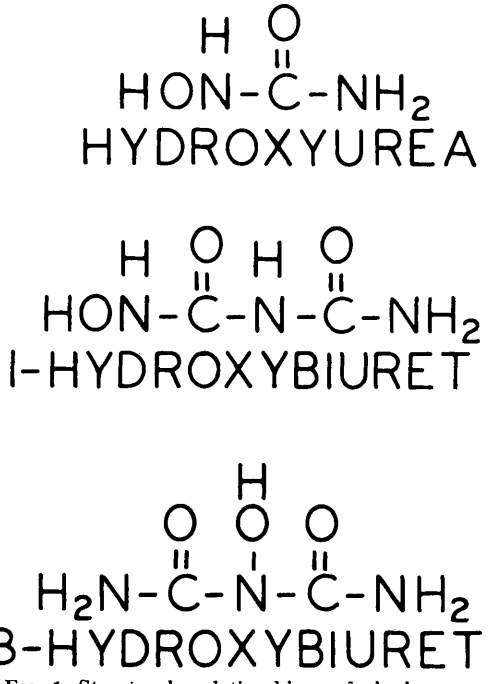

FIG. 1. Structural relationships of hydroxyurea (HU), 1-hydroxybiuret (1-HB), and 3-hydroxybiuret (3-HB).

Materials and Methods. The 1-HB was prepared by reacting equimolar quantities of methyl allophanate and hydroxylamine in anhydrous methanol using NaOH as the base. After the solution stood for several days at room temperature, it was neutralized with gaseous HCl, filtered, and evaporated to dryness to yield a white amorphous solid which gave a $FeCl_3$ test result similar to that of HU. The 3-HB was prepared by the method of Exner (4). The NCTC-109 tissue culture medium was from Microbiological Associates, and radioisotopes were from New England Nuclear Corporation. Other chemicals were of reagent grade from various commercial sources. BALB/c mice from Dublin Laboratory Animals, Dublin, Va., were used to propagate the Ehrlich ascites tumor strain obtained from Microbiological Associates. *Pseudomonas* strains were recent isolates from the diagnostic bacteriology laboratory of the Medical College of South Carolina.

Tumor cells were removed from mice 5–10 days after inoculation, washed twice with NCTC-109 medium, and resuspended to a 1% (v/v) concentration in this medium. Twenty-five-ml Erlenmeyer flasks or 20 mm outside diameter test tubes served as reaction vessels and were maintained at 37°C on a Dubnoff metabolic shaker or in a stationary water bath with gentle agitation. Each reaction mixture consisted of 4.5 ml of a 1% cell suspension, 0.5 ml of 0.9% saline with or without the test compound at 10 times the final concentration, and 0.5 ml of 0.9% saline with the appropriate isotopic precursor. This latter volume contained thymidine-^{3}H (10μC), uridine-^{3}H (1μC), or leucine-^{14}C (1μC) to measure synthesis of DNA, RNA, or protein, respectively. To stop the reactions, 4-ml samples were added to 4 ml of cold 10% trichloroacetic acid(TCA) at indicated times. The insoluble precipitate was washed three times with cold 5% TCA by sedimentation and resuspension, and finally suspended in 0.5 ml of methanol. Two ml of 1*M* hydroxide of Hyamine were added and the contents of each tube were transferred quantitatively to 15 ml of liquid

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scintillation phosphor (PPO-POPOP, Packard Instrument Co.) in toluene. Radioactivity of each sample was measured with a Mark I liquid scintillation spectrometer (Nuclear Chicago Corporation).

Results. Typical dose response relationships of DNA synthesis in ascites tumor cells to increasing concentrations of HU and the biuret derivatives are shown in Fig. 2. The

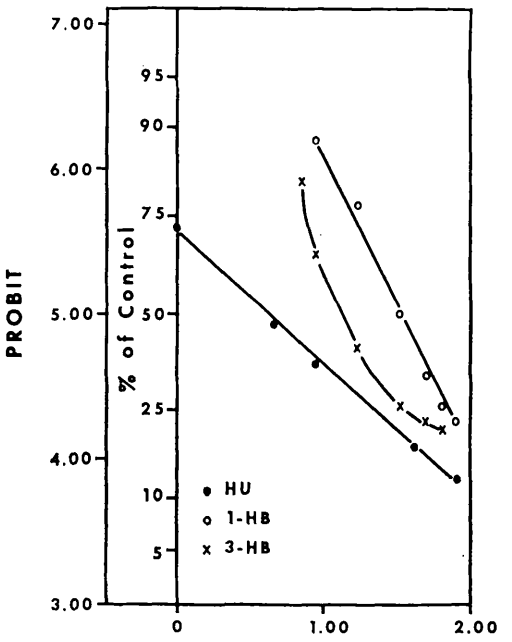

FIG. 2. Response of the rate of DNA synthesis in Ehrlich ascites tumor cells to various concentrations of HU, 1-HB, and 3-HB. Experimental details are given in the text. The period of isotope labeling was 30 min. Abscissa: log ($M \times 10^2$).

concentrations of HU, 1-HB, and 3-HB conferring 50% inhibition were approximately 4.5 $\times$ 10^{-5} M, 4 $\times$ 10^{-4} M, and 1.5 $\times$ 10^{-4} M, respectively. Linearity of response was achieved by plotting the probit of percentage of control against the logarithm of the concentration in the case of HU and 1-HB, but the response was consistently nonlinear in the case of 3-HB.

To determine the pattern of onset of inhibition of DNA synthesis, cells were exposed to each inhibitor for intervals varying from 1 min to 2 hours, following which thymidine-^{3}H was added. Twenty min later the reaction was terminated by TCA and the insoluble material was processed for measurement of isotope activity. Over 50% inhibition of the rate of DNA synthesis was obtained after only 1-min exposure of the cells to each compound at 10^{-3} M; further exposure up to 2 hours enhanced this depression by only approximately 25% (Fig. 3).

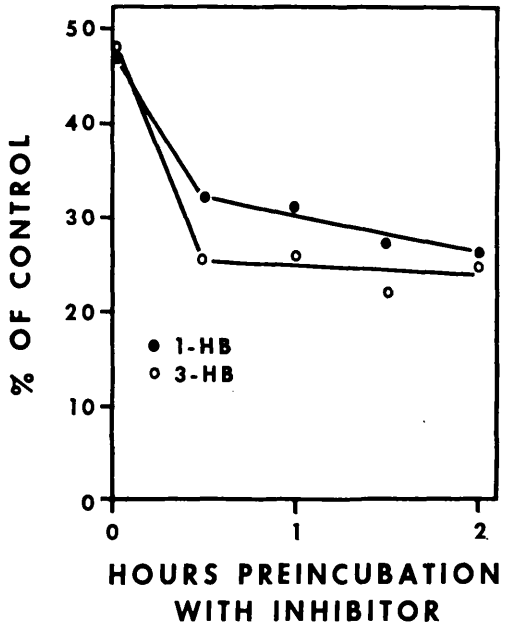

FIG. 3. Onset of inhibition of DNA synthesis in Ehrlich ascites tumor cells upon incubation with 1-HB and 3-HB for various intervals. Following exposure of the cells to each inhibitor for the periods indicated on the abscissa, thymidine-^{3}H was added to each reaction vessel. The reaction was terminated by acid precipitation after 20-min incubation with the isotope, and the rate of DNA synthesis was assessed as described in the text. The final concentration of each inhibitor was 10^{-3} M.

To determine if selectivity of action against DNA synthesis could be shown, the rates of synthesis of DNA, RNA, and protein were measured simultaneously with and without 1-HB and 3-HB at 10^{-3} M. Selective inhibition of DNA synthesis by 1-HB and 3-HB was evident almost immediately after addition of the compounds to the cells (Fig. 4) and was depressed 50% by 10^{-3} M 1-HB and 71% by the same concentration of 3-HB. The RNA synthesis was unaffected by 1-HB, and protein synthesis was depressed approximately 16%. The rate of RNA synthesis was

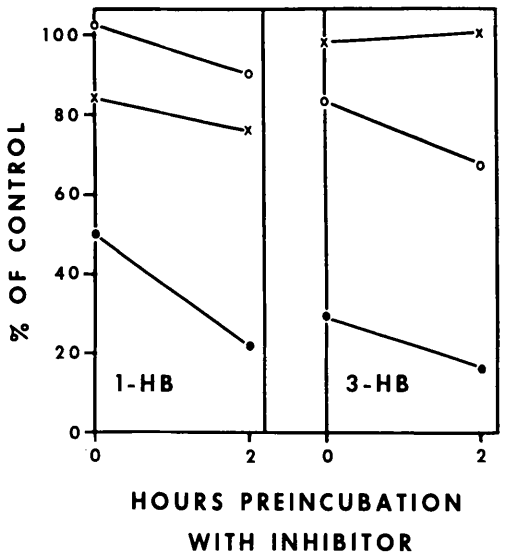

FIG. 4. Selectivity of inhibition by 1-HB and 3-HB of DNA synthesis in Ehrlich ascites tumor cells following 0 and 2-hour exposure of the cells to each compound. The DNA (●), RNA (○), and protein (x) synthesis rates were assessed by the incorporation into the acid-insoluble cell fraction of thymidine-^{3}H, uridine-^{3}H, and L-leucine-^{14}C, respectively. Each inhibitor was at a final concentration of 10^{-3} *M*, and the isotope labeling period was 30 min.

reduced 17% by 3-HB, with no effect on protein synthesis. When the cells were preincubated with each compound at 10^{-3} *M* for 2 hours prior to the addition of isotopes, the rate of DNA synthesis as assessed by the 30-min labeling period was inhibited approximately 80% by both compounds; RNA synthesis was depressed 10% by 1-HB, and 33% by 3-HB. Protein synthesis was reduced 24% by 1-HB, but again was unaltered by 3-HB.

To determine if 1-HB or 3-HB tends to depolymerize the DNA molecule to an acid soluble state, 80 ml of a 1% tumor cell suspension in NCTC-109 medium were incubated with thymidine-^{3}H (16 μC) at 37°C for 20 min, washed 3 times with cold medium to remove unincorporated isotope, and finally suspended in 80 ml of this medium. A 9-ml aliquot was transferred to a test tube containing 1 ml of 0.9% saline, and 2-ml samples from the latter suspension were added to 2 ml of 10% TCA at 0°C (zero-time controls). From the remaining washed cell suspension, 4.5 ml were added to tubes containing 0.5 ml 0.9% saline with or without the test compound at 10^{-2} *M*. After 1.5 and 3 hours incubation at 37°C, 2-ml samples were removed and processed for radioactivity assay as above. No diminution by either compound of the amount of the ^{3}H-labeled, acid-insoluble DNA was observed.

Antagonism by deoxyribosides of the inhibitory action of HU on HeLa and mouse fibroblast cells has been demonstrated (5,6). Such findings are interpreted as evidence of interference by HU with the bioreduction of ribonucleotides to deoxyribonucleotides. The possibility that deoxyribosides may antagonize the action of 1-HB or 3-HB was investigated by incubating 4.5 ml of a 1% cell suspension with 0.5 ml of a deoxyriboside mixture (deoxycytidine, 10^{-4} *M*; deoxyguanosine, 2×10^{-3} *M*; deoxyadenosine, 7×10^{-3} *M*) in NCTC-109 medium for 10 min at 37°C prior to the addition of 1-HB or 3-HB at 10^{-3} *M* final concentration. Controls received only the appropriate volume of medium without supplemental deoxyribosides. Antagonism of the inhibitory action of HU was also measured as a positive control. After 30-min continued incubation at 37°C, thymidine-^{3}H was added; 4-ml samples were removed after a 30-min labeling period and processed for radioactivity assay. The action of HU, 1-HB, and of 3-HB on DNA synthesis was shown to be reversed virtually 100% by the mixture of deoxyribosides, suggesting similar mechanisms of action of the 3 compounds (Table I).

TABLE I. Effect of a Mixture of Deoxyadenosine (7×10^{-3} *M*), Deoxyguanosine (2×10^{-3} *M*), and Deoxycytidine (10^{-4} *M*) (Deoxyribosides, dR) on the Inhibitory Action of Hydroxyurea (HU), 1-Hydroxybiuret (1-HB), and 3-Hydroxybiuret (3-HB) on DNA Synthesis in Ehrlich Ascites Tumor Cells.

	% of control DNA synthesis rate[a]	
Inhibitor (10^{-3} *M*)	Inhibitor alone	Inhibitor + dR
HU	16	103
1-HB	21	98
3-HB	15	102

[a] Values given are averages of 2 experiments.

To determine the extent to which the inhibitory effect of 1-HB and 3-HB on DNA synthesis may be reversible upon removal of the inhibitor, tumor cells were preincubated for 30 min with and without each inhibitor. Those preincubated without 1-HB or 3-HB were washed 3 times with fresh medium; those preincubated with each inhibitor were divided into 2 equal aliquots, one of which was washed 3 times with fresh medium without the inhibitor, while the other was washed with medium containing 1-HB or 3-HB, as appropriate, at the same concentration as was present during the 30-min preincubation period. Thymidine-^{3}H was then added and after 20- and 40-min continued incubation, the rate of DNA synthesis was assessed as previously. Removal of either inhibitor partially abolished the inhibition conferred by 1-HB and 3-HB at 10^{-3} *M* (Fig. 5), but restoration to control values was not obtained.

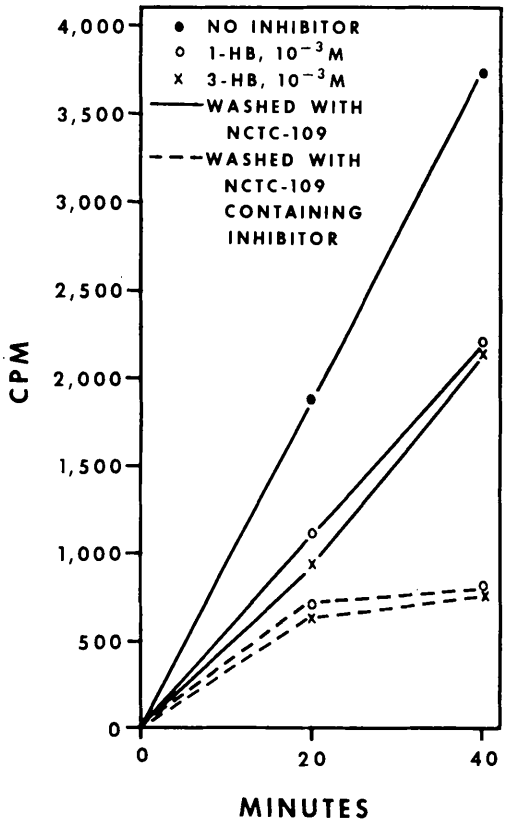

FIG. 5. Partial reversal of the inhibitory action of 1-HB and 3-HB on DNA synthesis in Ehrlich ascites tumor cells upon washing the cells with fresh medium. Experimental details are given in the text.

When Eagle's minimum essential medium with Hanks' balanced salt solution (Microbiological Associates) was substituted for NCTC-109 medium, no inhibition of DNA synthesis by either 1-HB or 3-HB could be demonstrated.

Five different strains of *Pseudomonas* were incubated at 37°C for 18 hours in tryptic soy broth with or without HU, 1-HB, or 3-HB at 10^{-2} *M*. The optical density of each culture tube was determined at 640 mμ with a Coleman spectrophotometer and compared with duplicate control tubes without HU, 1-HB, or 3-HB. All strains were inhibited by HU and 3-HB; each of these compounds at 10^{-2} *M* conferred from 40% to 90% inhibition of the several strains tested. At this same concentration 1-HB was not inhibitory. Light microscopic examination of cells stained with gentian violet revealed a similar type of cell elongation in the presence of HU and 3-HB as had been observed with HU (7) and certain of its derivatives (2,3), indicative of unbalanced growth (Fig. 6). Morphology of the cells grown in the presence of 1-HB appeared unaltered.

Discussion. The synthesis and preliminary pharmacological evaluation of 1-HB and 3-HB were undertaken not only because each is structurally related to HU and consequently was expected to show some inhibitory activity as such, but also because enzymatic hydrolysis of the single ureido group (-$CONH_2$) of 1-HB, or of one of the two such groups of 3-HB, would yield HU. On the basis of experiments herein reported, it seems evident that such an hydrolysis was not necessary for selective action against DNA synthesis in the *in vitro* system used, since both compounds had a virtually immediate onset of action. Even though somewhat greater inhibition of DNA synthesis was obtained following 30-min incubation of the cells with each inhibitor, this may have been a manifestation of the rate of penetration into the cells or of some other factor quite unrelated to hydrolysis. The possibility that such an hydrolysis may occur in the intact animal seems worthy of investigation, however, since

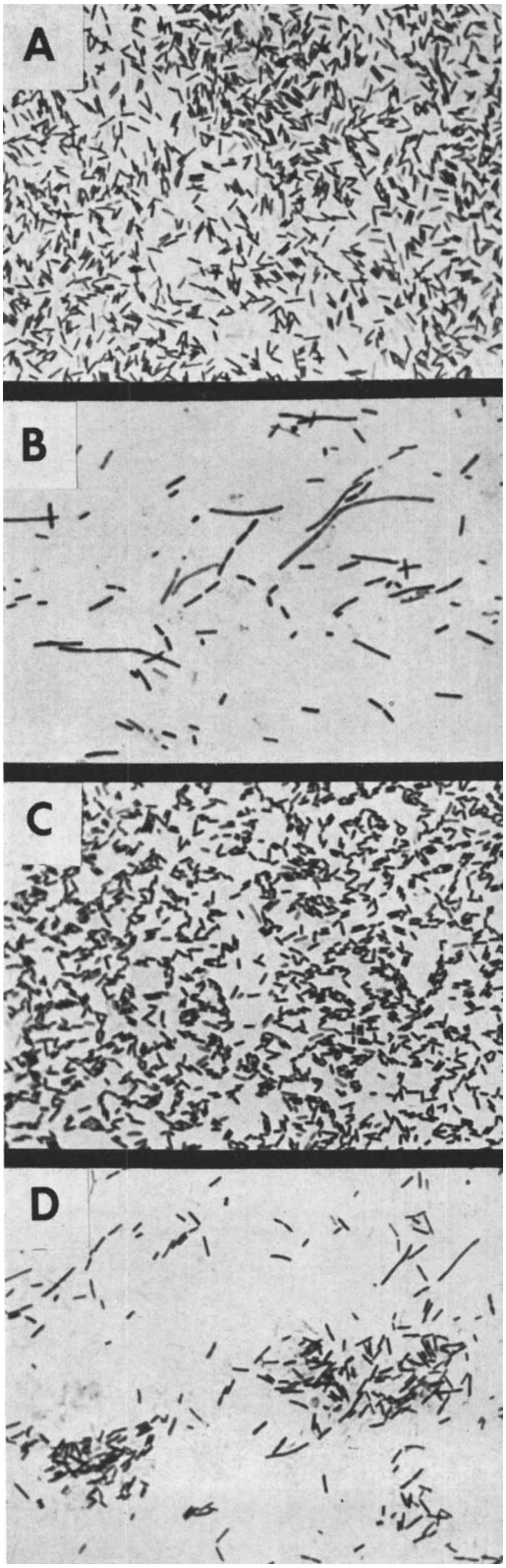

FIG. 6. Photomicrographs of a typical wild strain of *Pseudomonas* after growth for 18 hours in tryptic soy broth containing no supplement (A), hydroxyurea (B), 1-hydroxybiuret (C), and 3-hydroxybiuret (D). Each hydroxamate was at a final concentration of 10^{-2} *M*.

such a transformation, i.e., the metabolic conversion of a selective inhibitor of DNA synthesis to another compound with substantially identical pharmacological properties, could appreciably prolong the pharmacological half-life of the regimen.

The lack of inhibitory action of either 1-HB or 3-HB against DNA systhesis when Eagle's minimum essential medium with Hanks' balanced salt solution was substituted for the more complex NCTC-109 medium was entirely unexpected, since HU has virtually equal *in vitro* activity in both media (unpublished data). The recently noted lack of activity of acetoxyoxamide in Eagle's medium (8) is not a strict parallelism, since this compound is inhibitory to DNA synthesis only after a prolonged lag period which presumably corresponds to the time required for hydrolysis of the acetyl group to yield the active hydroxamate containing an unsubstituted proton (2). No such lag period was evident in the onset of action of either biuret derivative. Complex formation between 1-HB or 3-HB and some constituent of the tissue culture medium may be essential to obtain inhibition, however, this hypothesis has not been investigated.

Summary. 1-Hydroxybiuret (1-HB) and 3-hydroxybiuret (3-HB) were shown to be inhibitors of DNA synthesis in an *in vitro* Ehrlich ascites tumor test system in NCTC-109 medium. Inhibition was manifest almost immediately upon addition of either compound to the cells. The rates of synthesis of RNA and protein were not substantially altered by either compound. Removal of each inhibitor by washing the cells did not completely restore the rate of DNA synthesis to its control value. The inhibitory action of each against DNA synthesis was antagonized by a mixture of deoxyribosides, suggesting that inhibition of ribonucleotide bioreduction may be a major mode of action, similar to that of hydroxyurea. Morphological evidence indicated that 3-HB induced unbalanced growth in several strains of *Pseudomonas*, but

1-HB was devoid of activity in the microbial system.

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Toxic Effects Induced in Rabbits by Extracellular Products and Sonicates of Group A Streptococci* (32908)

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It has been previously shown (1, 2) that myocardial, hepatic, and diaphragmatic lesions were induced in rabbits following the intratonsillar injection of living group A streptococci or of some streptococcal extracellular products (SEP). The myocardial lesions were characterized by muscle necrosis and by infiltration with inflammatory cells. In some cases giant-cell granulomas containing clumps of calcified tissue were found. The liver lesions were generally similar to those found in the heart, the large areas of necrosis also containing many foreign body giant cells surrounding clumps of amorphous basophilic material. In many of the rabbits injected with SEP there was also a marked increase in serum concentration of glutamic-oxalacetic transaminase (GOT), sorbitol dehydrogenase (SOD), and total serum lipids within 24 hours of the administration of SEP, indicating that tissue damage occurred soon after the injection.

The purpose of the present work is to study further the toxic effects of SEP in rabbits. Tissue damage induced in rabbits by group A streptococcal sonicates will also be described.

Materials and Methods. Bacterial strains. Group A streptococcus, type 6, was obtained from the Central Government Laboratories, Ministry of Health, Jerusalem. Streptococcus mitis was isolated from human saliva following growth on a mitis-salivarius medium (Difco). The streptococci were cultivated in brain heart infusion broth (Difco), harvested from the logarithmic phase of growth, washed in saline buffered with 0.025 *M* phosphate, pH 7.4, resuspended in buffer and kept at 4°C.

Streptococcal extracellular products (SEP). A lyophilized preparation of culture supernates obtained from a type 4 streptococcus grown in a chemostat was employed. The SEP contained at least 12 antigens as determined by immunoelectrophoretic analysis using rabbit antiserum. A detailed description of the method of preparation of SEP, its antigenic composition and enzyme content is given elsewhere (2).

Preparation of streptococcal sonicates. Washed streptococci obtained from 2 liters of culture were subjected to sonic oscillation in a 9 Kc Raytheon sonic oscillator for 90 min. Following the disruption, the bacterial debris was removed by centrifugation at 36,000*g* for 30 min at 4°C. The supernatant fluid was dialyzed overnight against 0.01 *M* phosphate buffer, pH 7.4, passed through a Millipore filter (0.22 μ) and kept at —25°C.

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