Effect of Hydroxyurea on *Pseudomonas aeruginosa*

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SUMMARY

Hydroxyurea was shown to inhibit the growth of species of *Pseudomonas*. The growth of fungi, yeasts, and other bacteria was unaffected. Inhibition of the growth of *Ps. aeruginosa* was accompanied by marked elongation of individual cells. Electron microscopy revealed that nuclear material of inhibited cells was either virtually absent or coalesced into electron-dense particles. Chemical analyses showed that hydroxyurea caused alterations in the nucleic acid and ribonucleotide patterns, the most consistent being a reduction of the DNA/RNA ratio. It was concluded that the drug inhibits cell division through interference with DNA metabolism while inhibiting cell growth to a lesser extent.

Following observations of the favorable responses of a number of experimental tumors to hydroxyurea, a series of clinical trials was begun in 1960 as a Phase I evaluation (7). The drug was given to 143 patients by the Southwest Cancer Chemotherapy Study Group at dosages ranging from 1 to 80 mg/kg/day. It was established that the maximum tolerated dose was 40-60 mg/kg/day by the oral route. Toxic responses consisted of skin rashes, elevated BUN, vomiting, alopecia, and cardiac arrhythmias, but the limiting factor in therapy was considered to be bone marrow depression. In an addendum to that report, the writers noted that, in the initial stage of a subsequent Phase II study with the drug, onset of leukopenia was delayed, thrombocytopenia was more of a problem, and anemia was not so severe, in comparison with the Phase I study. It was also apparent that older patients incurred more toxic effects than did younger patients. An alteration of the drug seemed a possible explanation of the divergent results.

Adamson, Ben, and Rall (1) confirmed the previously reported activity of hydroxyurea against advanced leukemia L1210 and showed that urea, isohydroxyurea, hydroxylamine, semicarbazide, and aminoiminomethane sulfonic acid were ineffective in treatment of this experimental tumor.

Evidence has been presented recently that 1–10 per cent of the administered dose of hydroxyurea may be converted to hydroxylamine (5). This was based on the finding of acetylhydroxamic acid in the blood of three patients receiving hydroxyurea. It was suggested that the acetylhydroxamic acid which was detected was one of the products of the cleavage of acetyl coenzyme A by hydroxylamine formed from hydroxyurea, and this action was proposed as at least one facet of the mechanism of leukopenic action of the drug.

As part of a study of the pharmacology of a number of hydroxamic acids, an investigation of possible antimicrobial properties of hydroxyurea was begun in this laboratory soon after the report of the Phase I study appeared (7). It was noted that the growth of a number of *Pseudomonas* species was inhibited by the drug, and the following is a report of this study.

MATERIALS AND METHODS

Hydroxyurea was synthesized by the Hynes Chemical Research Corporation, Durham, North Carolina, and different lots melted in the range of 146° C.–147° C. A typical elemental analysis showed: C, 15.95 per cent; H, 5.43 per cent; N, 36.62 per cent. Calculated for CH₂N₂O₉: C, 15.79 per cent; H, 5.29 per cent; N, 36.84 per cent. All other chemicals used were reagent grade from commercial sources, unless indicated otherwise. All *Pseudomonas* species either were stock laboratory strains, which had been maintained in this laboratory for months, or were fresh isolates obtained from the clinical bacteriology laboratory at the Durham Veterans Administration Hospital. Each recently isolated strain was inoculated into the appropriate media to differentiate *Ps. aeruginosa* from other *Pseudomonas* species (2). All subsequent tests were done in tryptic soy medium.

Photomicrographs of gram-stained preparations were prepared with the use of a Leitz Wetzlar apparatus. Electron micrographs of whole bacteria were obtained after air-drying small drops of washed cell suspensions on Formvar-coated grids followed by shadowing with platinum at an angle of approximately 45°. To obtain thin sections, cells were fixed by the method of Kellenberger, Ryter, and Séchaud (6). Uranyl acetate was used as an electron stain in the first washings in water.
TABLE 1

EFFECT OF HYDROXYUREA ON TOTAL PROTEIN AND NUCLEIC ACIDS OF *PSEUDOMONAS AERUGINOSA*

Figures in "per cent change" column indicate the per cent alteration induced by the drug.

See text for experimental details.

<table>
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<tr>
<th>Experiment</th>
<th>Total Protein (mg.)</th>
<th>DNA Protein</th>
<th>RNA Protein</th>
<th>RNT* Protein</th>
<th>DNA RNA</th>
<th>DNA RNT*</th>
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<td></td>
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<td>.045</td>
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* Acid-soluble ribonucleotide fraction.

Preceding dehydration in a graded alcohol series. Specimens were embedded in Araldite and polymerized at 60° C. Sections were cut on a Porter-Blum microtome with a diamond knife and examined in an RCA EMU3-F electron microscope with a 50-keV accelerating voltage through a 25-m objective aperture.

Ascending paper chromatography was done in 90 per cent butanol saturated with water; 1 per cent ferric chloride in 0.1 N hydrochloric acid was used to detect hydroxyamic acids on the developed sheets. Hydroxylamine was measured by the method of Csáky (4). All hydroxyurea solutions were sterilized by Millipore filtration. Initial screening against a representative variety of gram-positive and gram-negative bacteria, yeasts, and fungi was done on seeded agar plates by the crossed filter paper-strip method and in tryptic soy broth. This particular strain was a recent isolate of *P. aeruginosa* strain 59 was inoculated into 100 ml. Erlenmeyer flasks containing 200 ml. of tryptic soy broth. Each determination was done in triplicate, and the average deviations in optical density readings among the three, obtained with a Coleman Junior spectrophotometer, never exceeded 5 per cent.

Protein content of the cultures in experiments A through F was determined by the biuret method (see [3, pp. 450–51]). A standard curve relating optical density to mg. protein was prepared in each experiment with the use of crystalline bovine albumin. A slight turbidity remained in the culture samples after 30 minutes' incubation with the biuret reagent; consequently, duplicate samples were set up with a biuret reagent which contained no copper sulfate. The optical density readings thus obtained were subtracted from the readings of the samples containing the complete reagent.

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RESULTS

Preliminary assays of hydroxyurea against a number of bacteria, yeasts, and fungi, by the saturated filter paper disc method on seeded agar plates, showed that only species of Pseudomonas were inhibited. The two strains tested in the original assay were both *Ps. aeruginosa*; consequently, a number of other isolates of Pseudomonas were collected from specimens which had been sent to the clinical bacteriology laboratory, and the activity of hydroxyurea against each of these was determined. Activity was demonstrated against all isolates, both *Ps. aeruginosa* and other species of Pseudomonas. Quantitative assays in broth showed only slight differences in sensitivity among eleven strains.

A peculiar type of dose-response curve was noted in the quantitative assays. Following a slight stimulation at about 0.0005 M hydroxyurea, the curve decreased sharply, with 50 per cent inhibition occurring usually at a concentration of about 0.0025 M. The curve then leveled off and did not reach 100 per cent inhibition even at drug concentrations up to 0.01 M (Chart 1). Such a curve may be expected in any of a number of circumstances, principal among which would be (a) slow transformation of a noninhibitory compound to an inhibitory one or (b) utilization by the cells of a pre-existing metabolite(s) the formation of which is blocked by the inhibitor, with cessation of growth occurring only after exhaustion of the pre-existing material. In the former case a possible breakdown product of hydroxyurea would be hydroxylamine. It can be seen from Chart 1 that 50 per cent inhibition would be effected by a hydroxylamine concentration of about 0.0002 M or approximately 8 per cent of the hydroxyurea concentration which reduced growth by 50 per cent.

To attempt to determine the rate of formation of hydroxylamine from hydroxyurea, 1000 μg/ml hydroxyurea was added to sterile tryptic soy broth, and the broth was analyzed for hydroxylamine at intervals over a period of 72 hours. There was a gradual increase in hydroxylamine concentration over the period of observation, but owing to difficulties encountered in measuring hydroxylamine in this broth the absolute amount converted could not be determined. However, it appeared that not over 0.03 per cent of the initial amount of hydroxyurea was converted per hour, a rate which theoretically would be inadequate to account for the observed inhibition if this were the sole mechanism. To determine whether the rate of breakdown to hydroxylamine was approximately the same order of magnitude in the presence of cells as compared with the rate in sterile broth, an attempt was made to measure bound hydroxylamine at intervals after the addition of hydroxyurea to a log phase culture of *Ps. aeruginosa*. These experiments were not successful, however, since the treatment required to release bound hydroxylamine (4) hydrolyzed some of the hydroxyurea with resultant falsely high values.

To obtain more direct evidence whether nonenzymatic conversion of hydroxyurea to hydroxylamine could account for the particular type of dose-response curve obtained, three identical sets of culture tubes were set up containing graded concentrations of hydroxyurea up to 0.01 M. *Ps. aeruginosa* was inoculated into one set of tubes immediately after preparation and into each of the others 24 and 48 hours afterward. Growth was assessed by optical density measurements 18 hours after inoculation. No differences whatsoever were evident in the three dose-response curves, indicating no significant formation of hydroxylamine during pre-incubation of the drug in sterile broth. Similarly, filter paper strips impregnated with each compound gave different appearances on agar plates seeded with *Ps. aeruginosa*. After 24 hours’ incubation inhibition was noted on those plates containing strips impregnated with 0.6 per cent hydroxylamine or higher. The inhibitory zone was 6 mm. wide at a 1.0 per cent hydroxylamine concentration. On plates containing strips saturated with 1.0 per cent hydroxyurea, the zone of inhibition was wider (10 mm.) and was surrounded by a zone of enhanced pigment production. No such enhanced pigment production occurred on the plates containing hydroxylamine.

Ascending paper chromatography of the supernatant solutions from cultures of *Ps. aeruginosa* after incubation with hydroxyurea for up to 72 hours did not reveal a spot corresponding to acetohydroxamic acid. A mechanism of action other than that proposed by Fishbein and Carbon (5), therefore, appears probable.

Examination of gram-stained preparations of cells of *Ps. aeruginosa* grown in the presence of hydroxyurea showed a striking difference in appearance as compared with control cells. Whereas control cells were approximately 2.5 μ in average length, drug-treated cells were up to 30 times as long (Figs. 1, 2). The same change was induced by the addition of hydroxyurea to a log phase culture followed by further incubation for 18 hours. Cells grown in sublethal concentrations of hydroxylamine, or
cells in log phase to which hydroxyxylamine was added at lethal concentrations, did not show any change in average length. These observations prompted the electron microscopic study of changes induced by hydroxyurea, results of which are shown in Figures 3 through 7. Shadowed specimens of whole cells, negatively stained in Figures 3 and 4, revealed a similar gross morphology in control and treated cells except for differences in length. Occasional electron-dense spherical bodies were seen in drug-treated cells, but the nature of these organelles was not readily apparent. Thin sections of fixed cells embedded in Araldite are shown in Figures 5—7. A control cell of Ps. aeruginosa (Fig. 5) showed the customary features of gram-negative bacteria. Two triple-layered membranes were resolved at the periphery, enclosing cytoplasm which was predominantly granular. The electron-thin nuclear material occupied a large portion of the cytoplasm and was primarily fibrillar. Cells grown in the presence of hydroxyurea showed two predominant changes, in addition to their greater length. Numerous cells were observed which displayed little or no typical nuclear material (Fig. 6). The remainder of the cytoplasm of such cells tended to be more discretely granular than that of control cells. The other principal change is shown in Figure 7. In this section a granular cytoplasm is evident, but the nuclear zone shows a coalescence of material with a marked electron density.

The increased length of drug-treated cells and the cytological aberrations observed in thin sections suggested a mode of action in which cell growth is affected slightly or not at all, whereas cell division is inhibited. Since this was considered to be indicative of an alteration in nucleic acid metabolism, control and drug-treated cells were analyzed for ribonucleic acid (RNA), deoxyribonucleic acid (DNA), soluble ribonucleotides (RNT), and total protein. The results of six such experiments are shown in Table 1. In five of the six experiments the DNA/protein ratio was reduced by 33—48 per cent. The RNA/protein ratio was enhanced markedly in Experiments A and B and was unchanged or slightly increased in Experiments C through F (in Experiments A and B cells were in contact with hydroxyurea from the time of inoculation of the cultures; in Experiments C through F hydroxyurea was added to a log phase culture and incubation continued for 17—19 hours). The net result in each experiment, however, was a reduction of the DNA/RNA ratio, with the decrease ranging from 38 to 61 per cent. The DNA/RNT ratio was likewise decreased in each case, but to a lesser degree.

Attempts to reverse the inhibitory action of hydroxyurea by the crossed filter paper method were inconclusive; suggestive reversal was observed occasionally with cobalt, cyanocobalamin, and thymidine up to 1 per cent, but reproducibility was poor. Further studies are under way to attempt to determine the mode by which the DNA/RNA ratio is altered by the drug.

**DISCUSSION**

One of the most interesting aspects of hydroxyurea is the relative simplicity of the molecule as compared with other compounds currently in use in therapy of neoplastic diseases. It appears, however, that this simplicity of structure is not accompanied by chemical stability. This fact may lead to dissimilarities in results of future clinical testing, as it possibly has already done in the Phase I and Phase II evaluations (7). It is reported in the N.C. C.S.C. data sheet that the compound has a melting point of 140° C. Most batches of the drug used in these studies, synthesized by Hynes Chemical Research Corp., melted at 146° C—147° C after several recrystallizations from absolute ethyl alcohol. It was noted that a few batches obtained from that source melted at a lower temperature, and this lower melting point was consistently accompanied by a larger temperature range over which melting occurred, suggestive of the presence of some impurity. These batches, which were not used in the present study, did not improve upon further crystallization.

The data of Fishein and Carbone (5) on conversion of hydroxyurea to acetohydroxamic acid through hydroxylamine as intermediate are highly convincing, and their hypothesis for the mechanism of action of the drug is theoretically sound. However, no evidence could be obtained for a similar mechanism of action against Ps. aeruginosa. Further, the data of Adamson, Ben, and Rall (1) do not support the hypothesis as applied to experimental leukemia L1210. Fishein and Carbone (5) considered it surprising that such a general inhibition of cell metabolism by hydroxylamine through a diminution of oxidative phosphorylation and consequential lowering of adenosine triphosphate concentrations should be effective in chronic myelogenous leukemia.

In the present study nonenzymatic conversion of hydroxyurea to hydroxylamine does not account for the inhibition of Ps. aeruginosa, but a nonenzymatic conversion would not be compatible with a specificity against only species of Pseudomonas, of all those tested. The difficulties encountered in measuring bound hydroxylamine in the presence of hydroxyurea precluded a study of any possible enzymatic breakdown of the drug. The ineffectiveness of hydroxylamine in inducing elongation of bacterial cells, and the inability to demonstrate acetohydroxamic acid in cultures to which hydroxyurea was added, are evidence of another mode of action.

**REFERENCES**


Fig. 1.—Gram-stained preparation of control cells of Ps. aeruginosa. The eleven isolates examined were identical in appearance. X 3,150.

Fig. 2.—Gram-stained preparation of Ps. aeruginosa grown in 0.013 M hydroxyurea (1000 µg/ml). The increased length was characteristic of all eleven isolates. X 3,150.

Fig. 3.—Control cell of Ps. aeruginosa shadowed with platinum. X 17,500.

Fig. 4.—Shadowed preparation of Ps. aeruginosa grown in 0.013 M hydroxyurea. X 17,500.
Fig. 5.—Thin section of control cell of *Ps. aeruginosa*. The electron-thin nuclear material with its fibrillar inclusions occupies a large portion of the cell. × 65,000.

Fig. 6.—Thin section of *Ps. aeruginosa* grown in 0.013 M hydroxyurea. Nuclear material is markedly diminished. × 65,000.

Fig. 7.—Same conditions as in Figure 6. This section shows coalescence of electron-dense material in the nuclear zones. × 70,000.
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