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6-(*p*-Hydroxyphenylazo)-uracil: A Selective Inhibitor of Host DNA Replication in Phage-Infected *Bacillus subtilis*

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Abstract. The azopyrimidine, 6-(*p*-hydroxyphenylazo)-uracil, inhibits the replication of bacterial DNA selectively, completely, and reversibly, and has no apparent effects on the metabolism of other cellular macromolecules thus far examined. The mechanism of its action has been investigated in uninfected and phage-infected *Bacillus subtilis*, and the compound appears to be specific for a host function. In cells infected with virulent phage the synthesis of phage DNA proceeds normally, while residual host DNA synthesis is completely blocked. The drug-sensitive host site retains its sensitivity even after partial disruption of the cell by lysozyme treatment.

The mechanisms by which the bacterial chromosome replicates are poorly understood. The energy[1] and substrate requirements for DNA replication, the components of the replication apparatus, and their structure and function are essentially unexplained. The function of enzymes such as polynucleotide ligase[2,3] and DNA polymerase[4] are unclear. The polymerase appears well suited for DNA repair[5]; but its role in replication has been questioned.[6] The recent discovery[6] of a viable, UV-sensitive mutant of *Escherichia coli* remarkably deficient in soluble DNA polymerase activity has added further uncertainty. The isolation from disrupted *E. coli* of ATP-stimulated particulate preparations[7–9] that polymerize 5′-deoxyribonucleotides also suggests the existence of a novel replicating system, or at least the operation of a DNA polymerase in a unique structural or functional state not observed previously *in vitro*.

It seems clear that the characterization of a process as complex as bacterial DNA replication will require experimental approaches which minimally disturb the bacterium's structure and function; one way to accomplish this is to use a site-specific probe or inhibitor. The application of rifampicin[10] and actinomycin D[11] to the characterization of the process of cellular RNA synthesis stands as an excellent example.

Earlier studies[12,13] suggested that a specific probe for the study of bacterial DNA replication may exist among a group of 6-substituted azopyrimidines (B. Langley, personal communication) known to be selectively toxic for gram-positive bacteria (A. R. Martin, personal communication). One of these, 6-(*p*-hydroxyphenylazo)-uracil (HPUra), has been found to inhibit specifically the synthesis of DNA. This paper describes its effects on the synthesis of DNA by *Bacillus subtilis* and by *B. subtilis* infected with virulent bacteriophage.

HPUra does not inhibit DNA synthesis by the bacteriophage. The compound may be useful both *in vivo* and *in vitro* for the examination of the process of bacterial DNA replication and the components of the replication apparatus.

Materials and Methods. Bacteria, phage and media: *B. subtilis* 168 Ind⁻Thy⁻ (ref. 14) (W168^r) and phage SP3 were kindly provided by Dr. Mutsuko Nishihara. *E. coli* W3110 Thy⁻ and W3110 Thy⁻PolA⁻ (ref. 6) were gifts of Dr. John Cairns. SP3 was propagated on W168^r in TY broth[15] and titered on a nutrient agar (SP agar) described by Okubo *et al.*[16] Isotope incorporation by SP3-infected W168^r was studied in CY medium, a modified TY broth in which tryptone was replaced with an equal amount of powdered casein hydrolysate (Calbiochem). Most of the experiments on W168^r were done at 37°C in Spizizen's[17] synthetic medium containing 0.5% glucose and supplemented with 0.04% casein hydrolysate, 50 μg/ml L-tryptophan, and 20–40 μM thymidine. This medium will be referred to as S medium.

Materials: HPUra (U.K. patent 876,601) was synthesized and kindly provided by Dr. Bernard Langley of Imperial Chemical Industries, Ltd. It was purified to chromatographic homogeneity by methods described previously,[12] and stock solutions (10–20 mM) were prepared in 0.05 M NaOH. Radioactive compounds were purchased from New England Nuclear, and nucleosides, purines, and pyrimidines were purchased from P-L Biochemicals, Inc. Lysozyme was obtained from Worthington Biochemicals.

Determination of nucleic acids and protein: Log phase cells (3–5 mg dry weight) were rapidly chilled and harvested at 4°C on membrane filters, washed with cold 0.9% saline, and resuspended in cold 0.5 M perchloric acid for 30 min at 4°C. The cells were washed free of soluble material by repeated centrifugation in cold 0.5 M perchloric acid and finally digested in 1 ml of 0.5 M perchloric acid at 90°C for 30 min to hydrolyze nucleic acids. After centrifugation the clear supernate was assayed for ribose and deoxyribose by an orcinol[18] and a diphenylamine[19] method. The absolute amounts of cellular nucleic acids were estimated against hot perchloric acid hydrolysates of purified yeast RNA (Calbiochem) or calf thymus DNA (Worthington) as standards.

The pellet insoluble in hot perchloric acid was digested in 1.5 ml of 2 M NaOH at 50°C for 2 hr. After removal of a slight precipitate by centrifugation, the clear digest was used for the estimation of cellular protein.[20]

Dry cell mass was determined by a membrane filter technique described previously.[12] Incorporation of radioisotopes into various cell fractions was assayed by a membrane filter fractionation technique[21] employing liquid scintillation counting.[12]

Results. Specific inhibition of DNA synthesis: Fig. 1 depicts the effects of increasing concentrations of HPUra on the incorporation of [^{14}C]thymidine into the cold trichloroacetic acid (TCA)-insoluble, alkali-stable portion of *B. subtilis* W168^r. Uptake is immediately inhibited by HPUra and the extent of inhibition is related to its concentration. Concentrations of 40 μM and higher completely block uptake of isotope.

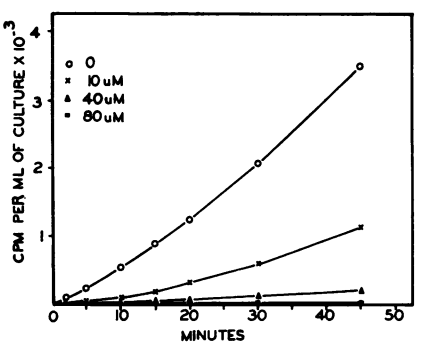


FIG. 1. HPUra inhibition of thymidine incorporation by W168^r. At time zero a tracer amount of [^{14}C]thymidine and HPUra (concentrations given on the graph) or diluent were added to a log phase culture of W168^r (10^8 cells/ml) growing at 37°C in S medium containing 40 μM thymidine (2×10^6 cpm/μmol). Radioactivity insoluble in cold trichloroacetic acid (TCA) was determined. The radioactivity incorporated remained insoluble in cold TCA after digestion with 0.5 M NaOH, and more than 99.5% of the label was solubilized by digestion in 5% TCA at 90°C for 15 min.

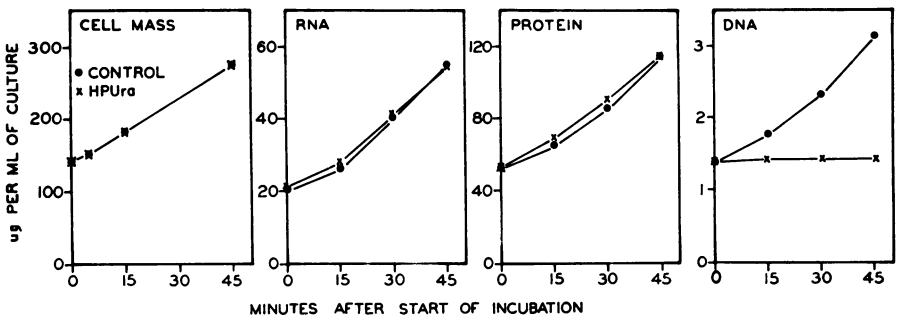


FIG. 2. Cell growth and synthesis of macromolecules in *B. subtilis* W168^r treated with HPUra. At time zero HPUra (final conc, 75 μM) or diluent was added to log phase cultures growing under the conditions described in Fig. 1, ●, Control; ×, HPUra.

The results shown in Fig. 2 (DNA) indicate that HPUra not only inhibits the uptake of thymidine by *B. subtilis* but actually prevents the net synthesis of DNA. Fig. 2 also demonstrates the specificity of the inhibition. Cell mass and RNA and protein content of the HPUra-treated culture increase at rates identical with those of the control, and there results an example of unbalanced growth[22] typical of bacteria specifically unable to replicate their DNA.

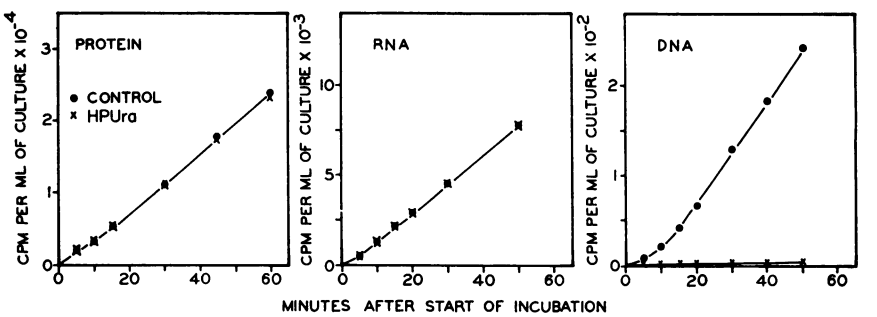


FIG. 3. Effect of HPUra on the incorporation of radioactivity into RNA, DNA, and protein by W168^r. As in Fig. 2, except that radioisotopes were added along with HPUra. (*Left*). Incorporation of [U-^{14}C]L-leucine (250 μCi/μmol, final concn, 50,000 cpm/ml) into material insoluble in hot TCA. In (*center*) and (*right*), [8-^{14}C]adenine (3.3 × 10^5 cpm/μmol, 0.2 mM) was used. Incorporation into the alkali-soluble (*center*) or alkali-stable (*right*) portion of the cold TCA-insoluble material. ●, Control; ×, HPUra.

Fig. 3 shows the specificity of the effect of 75 μM HPUra by more sensitive isotopic methods. The incorporation of [^{14}C]leucine into hot TCA-insoluble material is unaffected; thus the synthesis of bulk protein proceeds normally. Incorporation of [^{14}C]adenine into RNA is unaffected, while incorporation into DNA is completely blocked.

Experiments similar to those described in Fig. 3, but done with [^{14}C]acetate, [^{32}P]phosphate, and [^{14}C]alanine, indicate that concentrations of HPUra sufficient to block DNA synthesis completely have no effect on the incorporation of isotope into lipid, phospholipid, or cell wall components of W168^r.

Reversibility of the effect of HPUra: Earlier experiments with *Streptococcus fecalis*[12] suggested that the effect of HPUra on DNA synthesis was reversible. However, in those experiments inhibited cells were freed of drug and examined only for their ability to incorporate labeled thymine; the experiments did not indicate whether the inhibition of *net* DNA synthesis was reversible. Fig. 4

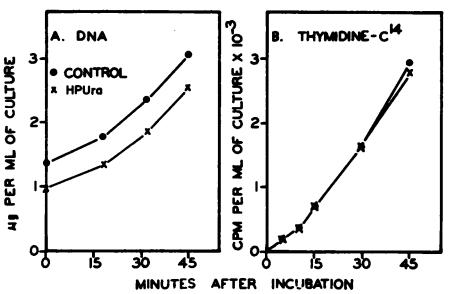


FIG. 4. Reversal of HPUra inhibition of DNA synthesis. HPUra (final conc, 75 μM) or diluent was added to identical log phase cultures of W168[r] (5 × 10^7 cells/ml) growing at 37°C. After incubation for 20 min, samples of each culture were rapidly harvested at 37°C on membrane filters, washed, resuspended in fresh warm S medium (thymidine, 40 μM) at a concentration of 150 μg/ml (dry weight), and incubated at 37°C. [^{14}C]Thymidine (final sp act 10^6 cpm/μmol) was added to small portions of each culture and after the times shown, DNA content (*A*) and radioactivity insoluble in cold TCA (*B*) were determined.

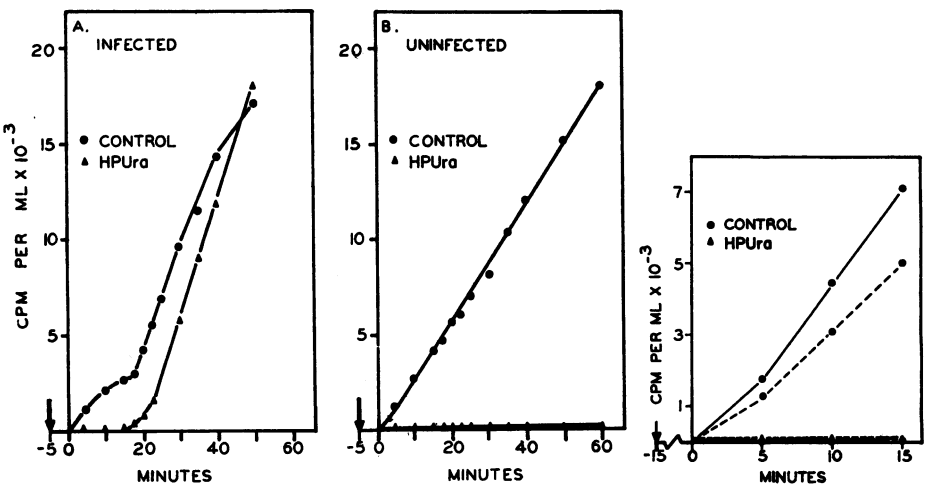
indicates that in *B. subtilis* W168[r], reversal applies to *both* the uptake of thymidine and the net synthesis of DNA. In this experiment cells were freed of drug after a 20-min exposure to a concentration of 75 μM, which completely inhibited DNA synthesis.

Lack of effect of HPUra on development of phage SP3 and synthesis of phage DNA: The specificity of HPUra for a bacterial function is demonstrated by the experiment described in Fig. 5, using W168[r] and the thymine-containing bacteriophage, SP3. Cultures of log phase cells were incubated with or without HPUra and then infected with SP3 in the presence of radioactive thymidine.

In uninfected cultures (*B*) HPUra completely prevented uptake of label. In infected cultures (*A*) the picture was different. In the control, isotope was incorporated at a progressively decreasing rate, tending to plateau between 15 and 17 min. Thereafter, the rate of incorporation increased sharply. This biphasic curve for thymidine uptake is typical for the uptake of DNA precursors by cultures of phage-infected *B. subtilis*.[23] The first part represents host cell DNA synthesis, which is gradually shut off after phage infection. The second part represents the synthesis of phage DNA. In the presence of HPUra the initial uptake of isotope into host DNA was completely inhibited, as in uninfected cultures, but the second phase of uptake into phage DNA is apparently unaffected.

The above observation suggested that HPUra, although fully inhibitory for host cell DNA synthesis, had no effect on the replication of phage DNA. This conclusion was supported by another experiment (not shown). In this study, log phase cultures were incubated with or without HPUra (400 μM) as in the previous experiment and then infected with SP3 at a multiplicity of infection of 10. After separation from unadsorbed phage by rapid centrifugation, the cells were resuspended in their respective media. Incubation was continued, and samples were removed at intervals, rapidly diluted, and plated (see *Methods*).

HPUra was present during the entire process of adsorption, infection, and cell lysis. Nevertheless, the curve depicting plaque-forming units (average burst, 200 pfu per infectious center) as a function of time of incubation was superimposable on that of the control; thus HPUra had no effect on the development and expression of biologically active phage and its DNA.



(*Left*) FIG. 5. HPUra inhibition of host cell incorporation of thymidine in SP3-infected and uninfected *B. subtilis*. At minus 5 min (*arrow*) HPUra (final conc, 400 μM) or diluent was added to identical log phase cultures of W168^r (5×10^7 cells/ml) growing at 37°C in CY medium containing 25 μM thymidine. At time zero the cultures were divided into two portions, each receiving phage (multiplicity of infection 10; (*A*) or phage diluent (*B*) and [^{14}C]thymidine (final sp act 3×10^6 cpm/μmol). Samples were then removed for analysis of incorporation of radioactivity into cold TCA-insoluble material.

(*Right*) FIG. 6. HPUra inhibition of thymidine incorporation by protoplasts in osmotically-stabilized S medium. Lysozyme was added (*arrow*) to a concentration of 300 μg/ml to a log phase culture of W168^r (4×10^7 viable cells/ml) growing at 37°C in S medium containing 20 μM thymidine and 0.5 M sucrose. After 15 min (time zero), when microscopic examination revealed that protoplast formation was essentially complete (<500 osmotically-stable, viable cells per ml), [^{14}C]thymidine and HPUra (final conc, 75 μM) or diluent were added and samples were analyzed for cold acid-insoluble radioactivity. Lysozyme-treated cultures, - - -; identical cultures minus lysozyme, ——.

Essentially identical results have been obtained in *B. subtilis* using bacteriophage SPO2C$_1$, a phage with DNA which contains thymine, and for SP8, a phage in which DNA thymine is replaced by hydroxymethyluracil. Accordingly, the lack of HPUra effect on phage DNA synthesis is not unique to SP3, nor is it limited solely to phages containing thymine.

Inhibition of protoplast DNA synthesis: Attempts were made to examine the importance of the physical integrity of *B. subtilis* in determining its sensitivity to HPUra. The initial approach utilized protoplasts to examine the influence of the rigid confines of the cell wall (Fig. 6). Protoplasts were formed directly in log phase cultures[24] of *B. subtilis* W168^r growing in S medium containing sucrose and assayed for their ability to incorporate thymidine in the presence and absence of HPUra.

The data of Fig. 6 show that protoplasts incorporate thymidine with an efficiency close to that of control cells. It is also clear that HPUra inhibits incorporation as efficiently as in control cells. Furthermore, results of other studies (not shown), similar to those depicted in Fig. 6 but done with [^{14}C]adenine, indicate that the differential effect of HPUra on the synthesis of DNA and RNA by protoplasts is as clearly defined as in normal cells.

Discussion. The toxicity of HPUra for *B. subtilis* clearly derives from its ability to inhibit selectively the replication of DNA. This observation extends earlier findings with *S. fecalis*[12,13] and suggests that the action of HPUra is uniform within the broadly defined class of gram-positive bacteria. As yet there has been no indication in *B. subtilis* of an effect of HPUra on the synthesis of cellular macromolecules other than DNA (Figs. 2 and 3). Nor does HPUra selectively inhibit the complex, cyclic process of initiation[25] of bacterial DNA replication; the onset of inhibition is too rapid (Fig. 1) and the extent of inhibition too complete to be consistent with such an action. These results suggest rather that HPUra, or possibly a related active metabolite, interacts with a specific site or function directly involved in the process of DNA replication.

The results of the phage experiments also suggest that the HPUra-sensitive site is unique to bacteria, or at least is not present in the processes responsible for phage DNA synthesis. Otherwise, the synthesis of phage SP3 DNA would not have been expected to proceed normally when the replication of host cell DNA was completely blocked by drug as shown in Fig. 5. Nalidixic acid, another specific inhibitor of bacterial DNA synthesis[26], also has selectivity for host cell DNA replication in phage-infected *B. subtilis*.[27] However, the selectivity of nalidixic acid is not entirely complete, since it both inhibits and delays the onset of phage DNA synthesis.[27]

The results in Fig. 4 show that removal of HPUra from drug-treated cultures of *B. subtilis* reverses the inhibition of DNA synthesis. Although this experiment was done on cells grown in HPUra for only 20 min (approximately 0.6 generation time), the ability to reverse the effects on DNA synthesis remains for periods approaching 2 generation times in S medium.

HPUra inhibits the division of *B. subtilis* as it does that of *S. fecalis*.[12] It is also possible to reverse this effect by removal of HPUra as described in Fig. 4, provided that exposure to drug is limited to 1 generation time or less. Longer exposure to HPUra results in a progressive loss of viability, possibly related to unbalanced growth[22] or induction of defective bacteriophage[28] carried by the W168 strains of *B. subtilis*.

The specificity of HPUra for gram-positive bacteria is striking. Large doses are virtually nontoxic to laboratory animals (B. Langley and A. R. Martin, personal communication), and high concentrations have little apparent effect on the growth of eukaryotes such as *Tetrahymena* (L. Bolund, personal communication) or *Saccharomyces* in fermentable and nonfermentable media. Gram-negative bacteria—particularly *E. coli* strains—are remarkably resistant to HPUra. Concentrations as high as 2mM do not appear to affect the growth of strains B, C, CR, W3110 Thy$^-$, and W3110 Thy$^-$ PolA$^-$ (unpublished data; and O. Karlström, personal communication). The growth of phage T4 (O.

Skold, personal communication), and ϕX174 (D. Brown, personal communication) are also refractory to HPUra treatment. The reason for the resistance of *E. coli* to HPUra is not clear. It is possible that permeability may play a role, as in the case of the resistance of *E. coli* to actinomycin.[29]

HPUra seems to induce blockage of DNA replication at the polymerization step. The results of earlier work with *S. fecalis*[12] and the phage experiments of Fig. 5 suggest that the potential precursors of DNA—such as cellular 5′-deoxyribonucleotide triphosphates—are not limited to HPUra treatment. Accordingly, the effects of HPUra on potential components of the replicating system have been examined. Extensive studies on DNA synthesis by crude, cell-free, and particulate preparations of *S. fecalis* have not revealed any inhibitory effect of HPUra on the incorporation of deoxyribonucleotide triphosphates.[13] Similarly, concentrations of HPUra as high as 2 mM have no effect on the activity of a highly purified preparation (phosphocellulose fraction) of *E. coli* DNA polymerase[13] and *E. coli* polynucleotide ligase (R. Gumport, personal communication). HPUra does not appear to have a high affinity for native or denatured DNA. The spectra of HPUra and DNA are unchanged in mixtures,[13] and dialysis of DNA against ^{14}C-labeled and unlabeled HPUra also shows no evidence of binding (ref. 13 and unpublished observations).

Studies of the effects of HPUra on DNA synthesis by partially disrupted bacteria have given equivocal results. The selective inhibition of DNA synthesis by HPUra in *B. subtilis* protoplasts (Fig. 6) indicates that the integrity of the cell wall can be violated and the cell morphology partially disrupted without destroying the sensitivity of the drug-sensitive site. However, treatment of *B. subtilis* with toluene[9] to permit incorporation of deoxyribonucleotide 5′-triphosphates into DNA appears to result in a dramatic reduction of HPUra sensitivity (unpublished observations). The synthesis by toluenized cells is only partially inhibited, even by concentrations of HPUra 10–20 times higher than necessary in normal cells. The significance of this observation and its relevance to DNA replication in the untreated cell are at present unclear. It is to be hoped that isolation of resistant mutants and further study of the effects of HPUra on DNA replication in the whole cell will complement these and other *in vitro* approaches to provide a clearer understanding of the drug-sensitive site.

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Abbreviations: HPUra, 6-(*p*-hydroxyphenylazo)-uracil; TCA, trichloroacetic acid.

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