

## Hydroxyphenylazopyrimidines: Characterization of the Active Forms and Their Inhibitory Action on a DNA Polymerase from *Bacillus subtilis*

(6-(*p*-hydroxyphenylazo)-uracil/6-(*p*-hydroxyphenylazo)-isocytosine/hydrazino forms)

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**ABSTRACT** The active forms of 6-(*p*-hydroxyphenylazo)-uracil and 6-(*p*-hydroxyphenylazo)-isocytosine were isolated and identified as their respective *hydrazino* derivatives. These arylhydrazino pyrimidines selectively inhibited a chromatographically distinct DNA polymerase from *Bacillus subtilis*. The actions of the reduced drugs on this polymerase were identical to those observed on ATP-dependent DNA synthesis in toluene-treated cells; dGTP competitively antagonized the inhibitory activity of the uracil derivative, and dATP competitively antagonized that of the isocytosine derivative. Analysis of the interactions of the arylhydrazinopyrimidines and nucleic acid bases by nuclear magnetic resonance suggested that hydroxyphenylhydrazino-uracil and hydroxyphenylhydrazino-isocytosine pair in a novel manner with, respectively, cytosine and thymine. A mechanism of inhibitor action, involving binding of the reduced drugs to enzyme and the pyrimidines of DNA template, is proposed.

The arylazopyrimidines, 6-(*p*-hydroxyphenylazo)-uracil (HPUra) and 6-(*p*-hydroxyphenylazo)-(2-amino,4-keto-pyrimidine (HPIsocytosine), selectively inhibit the semiconservative replication of DNA of *Bacillus subtilis* by inhibiting the function of a specific DNA polymerase (1-3). It now seems clear that these arylazopyrimidines do not inhibit DNA synthesis in drug-sensitive systems unless they are first reduced, either by metabolism, for toluene-treated cells (4, 5) or chemically, for cell-free preparations (2, 3). In this paper we present evidence that the active, reduced forms of HPUra and HPIsocytosine are their respective *hydrazino* derivatives. We also propose, on the basis of an analysis of novel base-pairing properties of these compounds and an examination of their action on a distinct DNA polymerase of *B. subtilis*, a mechanism of action that can account for their ability to antagonize the function of purine deoxyribonucleotides as substrates in replicative DNA synthesis (5).

### MATERIALS AND METHODS

**Chemicals.** 6-(Hydrazino)-uracil and arylazopyrimidines were generously provided by Dr. Bernard Langley of the Imperial Chemical Industries, Ltd. HPUra, HPIsocytosine, and 6-(phenylazo)-uracil (PUra) were purified by chromatography on silicic acid (6). [<sup>14</sup>C]HPUra was synthesized by reacting 6-(hydrazino)-uracil with [<sup>14</sup>C]benzoquinone (New England Nuclear Corp.) in ethanolic HCl according to methods established by Langley (personal communication).

Abbreviations: HPUra, 6-(*p*-hydroxyphenylazo)-uracil; HPIsocytosine, 6-(*p*-hydroxyphenylazo)-2-amino,4-keto-pyrimidine; PUra, 6-(phenylazo)-uracil; DMSO-d<sub>6</sub>, deuterated dimethylsulfoxide.

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**Preparation and Chromatography of Metabolites of [<sup>14</sup>C] HPUra.** *B. subtilis* NB841 (3) was grown and treated with 1% toluene, as described by Matsushita *et al.* (7). Standard incubation mixtures (0.5 ml) contained, in addition to [<sup>14</sup>C]HPUra, 70 mM potassium phosphate buffer (pH 7.4), 13 mM MgSO<sub>4</sub>, and 10<sup>9</sup> toluene-treated cells per ml. Incubation was terminated by chilling in ice, and all subsequent procedures were performed under nitrogen at 0-4°. Chilled mixtures were freed of cells by centrifugation at 15,000 × *g* for 3 min. The clear supernatant, containing more than 99% of input radioactivity, was applied to a column (0.65 × 6.5 cm; 2-ml bed volume) of DEAE-Sephadex (Pharmacia; A-25; HCO<sub>3</sub><sup>-</sup> form) that had been washed with 20 volumes of 5 mM triethylammonium bicarbonate buffer (pH 5.5) thoroughly purged with gaseous CO<sub>2</sub>. The column was eluted with the same buffer at a rate of 0.25 ml/min, and twenty 1-ml fractions were collected. The elution buffer then was changed to 1 M triethylammonium bicarbonate (pH 7.7), and 15 more fractions were collected. This method of elution resulted in the quantitative recovery of radioactivity applied to the column. One-half ml of each fraction was applied to 25-mm glass-fiber discs, which were dried at 80° and counted by liquid scintillation spectrometry.

**Preparation and Reduction of Arylazopyrimidines.** 2 ml of a fresh 1 M solution of sodium dithionite were mixed at 25° with 10 ml of 20 mM arylazopyrimidine in 50 mM NaOH. The solution, which turned colorless immediately, was cooled in ice to 0° and mixed with 0.2 ml of glacial acetic acid. The solution was purged with nitrogen and left in ice to promote precipitation of the reduced azopyrimidine. When precipitation was complete, the faint pink material was harvested by centrifugation and washed three times by centrifugation in 5 ml of cold H<sub>2</sub>O, which had been purged with nitrogen. The precipitate was dried at 50° under a stream of nitrogen and stored under nitrogen at 4°.

**Nuclear Magnetic Resonance (NMR) Analysis.** Compounds were weighed, dissolved in a known volume of dimethylsulfoxide-d<sub>6</sub> containing 1% tetramethylsilane as internal standard (DMSO-d<sub>6</sub>; Norell Chemical Corp.), and transferred to an NMR tube. Spectra were determined at 60 MHz on a JEOL C60-HL spectrometer, external lock-mode, field sweep. Hydrogen-bonding studies were made by stepwise addition of known amounts of bases to 50 mM solutions of reduced arylazopyrimidines in DMSO-d<sub>6</sub>.

**Purification and Assay of *B. subtilis* DNA Polymerases.** All buffers contained 20% (v/v) glycerol and 5 mM 2-mercapto-

ethanol, and purification procedures were done at 0–4°. Protein was estimated by the method of Lowry *et al.* (8). 5 g of packed *B. subtilis* NB841 were suspended in 10 ml of a buffer consisting of 20 mM Tris-acetate (pH 8.2)–10 mM magnesium acetate–0.5 mM EDTA, and ruptured in a French pressure cell. The lysate was freed of debris by centrifugation at 17,000  $\times g$  for 30 min, and the supernatant was further centrifuged for 3 hr in a Spinco 50.1 rotor at 42,000 rpm. The high-speed supernatant (10 ml; 20 mg of protein per ml) was diluted with 20 ml of 15 mM potassium phosphate buffer (pH 7.4) containing 300 mM  $(\text{NH}_4)_2\text{SO}_4$  and passed at a rate of 0.5 ml/min through a 13  $\times$  1.65 cm column of DEAE-cellulose (DE 52, Whatman) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 200 mM  $(\text{NH}_4)_2\text{SO}_4$ . The first 15 ml of eluant was discarded; the subsequent 45 ml, containing 85% of input protein, was mixed with 22 g of ground  $(\text{NH}_4)_2\text{SO}_4$  and stirred for 90 min. The precipitated protein was harvested by centrifugation, dissolved in 6 ml of 10 mM potassium phosphate (pH 6.5), and freed of  $(\text{NH}_4)_2\text{SO}_4$  by passage through Sephadex G-25 in the same buffer.

DNA polymerase was assayed in 0.05 ml of an incubation mixture containing 30% glycerol (v/v), 15 mM magnesium acetate, 100  $\mu\text{M}$  calf-thymus DNA treated with DNase-exonuclease III (9), 25  $\mu\text{M}$  each of dATP, dCTP, and dGTP, and 10  $\mu\text{M}$  [ $^3\text{H}$ ]TTP (400 cpm/pmol). Samples were incubated at 30° for 3 min, and the reaction was terminated by addition of 0.5 ml of 0.5 M NaOH containing 40  $\mu\text{g}/\text{ml}$  of denatured salmon-sperm DNA. After 5 min at 42°, the samples were chilled in ice and mixed with 0.5 ml of cold 20% trichloroacetic acid. The processing and counting of the acid-insoluble material have been described (3).

## RESULTS

**Isolation of the Active Reduced Metabolite of HPUra.** Earlier studies (4) with toluene-treated *B. subtilis* suggested that arylazopyrimidines were converted to active forms through NADPH-dependent metabolism. We explored this possibility by chromatographing on DEAE-Sephadex the soluble portions of incubation mixtures containing appropriate mixtures of toluene-treated *B. subtilis*, NADPH, and [ $^{14}\text{C}$ ]HPUra. The results of this study (Fig. 1A, B, and C) demonstrate that toluene-treated bacteria catalyze a NADPH-dependent conversion of HPUra to a new, chromatographically distinct species. Fig. 1C depicts the chromatogram of a mixture incubated with NADPH in the *absence* of bacteria and demonstrates a species with chromatographic behavior typical of native HPUra, which elutes from the column as a single red band after the change (arrow) of eluting buffers. Fig. 1A depicts the chromatographic behavior of HPUra after incubation with cells in the *absence* of NADPH. In this experiment, as in Fig. 1C, HPUra remained unchanged. However, as shown by Fig. 1B, incubation of HPUra with cells in the *presence* of NADPH resulted in the conversion of more than 80% of the drug to a chromatographically distinct species.

The properties of the major drug metabolite suggested that it was simply a reduced derivative of HPUra. The metabolite, as it emerged from columns, was colorless; however, when aerated or exposed briefly to alkaline pH, this material regained a red color, an absorption spectrum, and chromatographic properties identical to those of native HPUra. Further, a colorless species of drug with identical properties could

be generated simply by treating HPUra with the reducing agent, sodium dithionite (Fig. 1D).

Separate experiments, the results of which are not shown, indicated that the major metabolite of HPUra and the dithionite-treated derivative were, indeed, active drug forms. Material of both types was purified by chromatography as described in Fig. 1C and D and tested under anaerobic conditions for their ability to inhibit ATP-dependent DNA synthesis in toluene-treated *B. subtilis* in the *absence* of NADPH. In each case, the material was fully potent in these conditions, whereas native HPUra was inactive.

**Relationship of Drug Action to the Purine Substrate Requirement of a Specific DNA Polymerase.** On the basis of studies with toluene-treated preparations (5) and crude extracts (3) of *B. subtilis*, we have postulated that HPUra, and HPIsocytosine, in active form, inhibit DNA replication by interfering with the interaction of a replication-specific DNA polymerase and its purine deoxyribonucleotide substrates. Recently, Bazill and Gross (2) have strengthened this hypothesis considerably by isolating, from DNA polymerase I-deficient *B. subtilis*, three chromatographically distinct species of DNA

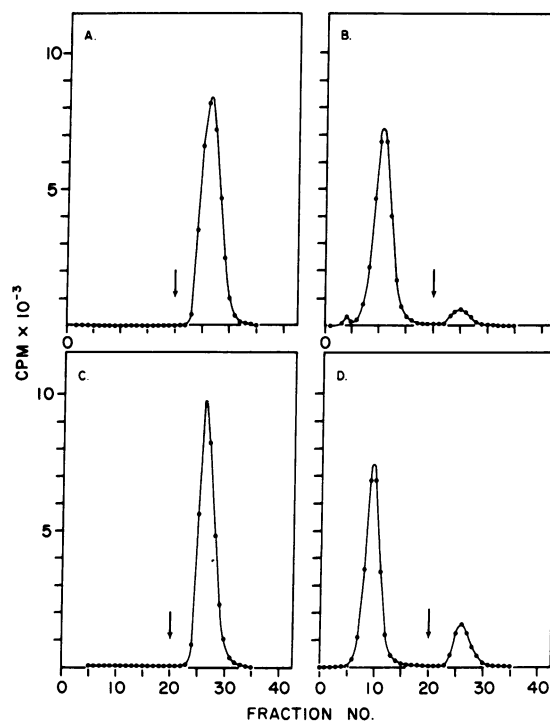


FIG. 1. DEAE-Sephadex chromatography of the products of cell metabolism and dithionite reduction of [ $^{14}\text{C}$ ]HPUra. A and B) Cell metabolites. 50 nmol of [ $^{14}\text{C}$ ]HPUra ( $1.2 \times 10^6$  cpm/ $\mu\text{mol}$ ) were incubated for 12 min at 37° with toluene-treated bacteria in a standard incubation mixture in the absence (A) and the presence (B) of 3 mM NADPH. The soluble portions of each extract were prepared and chromatographed; arrows indicate change of eluting buffer. (C) Control. Same as B, but with no cells. (D) Product of dithionite reduction. 50 nmol of [ $^{14}\text{C}$ ]HPUra (same specific activity as in A, B, and C) were dissolved in 80  $\mu\text{l}$  of 10 mM NaOH and mixed under nitrogen with 20  $\mu\text{l}$  of 25 mM sodium dithionite. The solution, which became colorless immediately, was diluted by the addition of 0.4 ml of a nitrogen-purged, ice-cold incubation mixture containing components at 1.25-times standard concentration and processed.

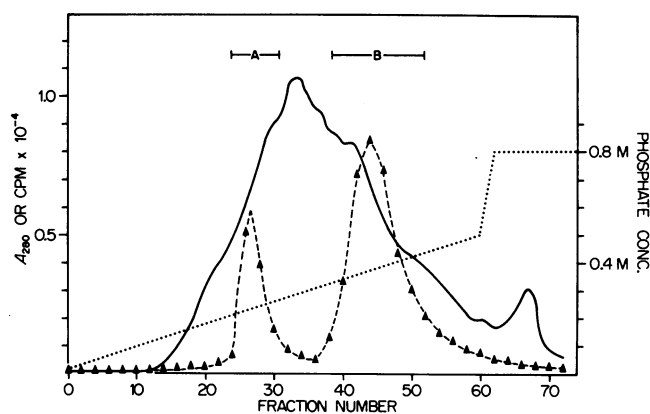


FIG. 2. Separation of *B. subtilis* DNA polymerases by DEAE-cellulose chromatography. Concentrated, DNA-free extract from 5 g of *B. subtilis* NB841 was adsorbed to a  $9 \times 1.65$  cm column of DEAE-cellulose and eluted with a linear gradient (0.01 to 0.5 M; volume, 150 ml; 0.5 ml/min) of potassium phosphate (pH 6.5) followed by 0.8 M potassium phosphate (pH 6.5). 10- $\mu$ l samples of fractions were taken for assay of DNA polymerase. (—), absorbance at 280 nm; ( $\blacktriangle$  -  $\blacktriangle$ ), DNA polymerase activity; ( $\cdots$ ), phosphate concentration.

polymerase, of which two were inhibited by dithiothreitol-treated HPURa.

We also have isolated DNA polymerases from a DNA polymerase I-deficient strain of *B. subtilis*. In contrast to Bazill and Gross, we found, by chromatography of DNA-free extracts on DEAE-cellulose (Fig. 2), two distinct enzymes, A and B, of which only one (B) was sensitive to the drug. The effects of reduced azopyrimidines on the sensitive B enzyme—in particular their susceptibility to antagonism by specific purine deoxyribonucleotides—indicate that these inhibitors acted on the isolated DNA polymerase in a manner identical to that observed (5) on ATP-dependent DNA synthesis in the environment of the toluene-treated cell. The results with the B enzyme are summarized in Fig. 3.

Fig. 3A depicts the effect of native and dithionite-treated HPURa. Native drug, even at high concentration, did not inhibit the activity of fraction B. In contrast, dithionite-treated HPURa clearly inhibited enzyme activity and did so with a potency entirely comparable to that observed on ATP-dependent DNA replication in toluene-treated cells (4). The inhibitory effect of reduced HPURa was antagonized specifically by increasing the concentration of dGTP in incubation mixtures. A more thorough, kinetic analysis of the drug-nucleotide antagonism with a preparation of the B enzyme further purified by phosphocellulose chromatography (details of purification to be published) strongly suggested that dGTP and reduced HPURa compete for access to the active site of the polymerase.

Fig. 3B depicts experiments done with HPIsocytosine, the 2-amino analog of HPURa. HPIsocytosine also inhibits DNA synthesis in intact and toluene-treated *B. subtilis* (5); however, its activity in toluene-treated *B. subtilis*, unlike that of HPURa, is antagonized by dATP and not by dGTP. The effects of HPIsocytosine on the (B) polymerase were entirely analogous to those obtained with HPURa; dithionite treatment was required to generate its inhibitory activity, and high concentrations of dATP specifically reduced its inhibitory potency.

**NMR Analysis of the Structure of Reduced Drugs.** The above results indicated that the active form of HPURa and, by analogy, that of HPIsocytosine were metabolic reduction products that could be synthesized simply by treatment of their native forms with sodium dithionite. The observation that the products reverted readily to native drug forms suggested that the active species of HPURa and HPIsocytosine were their hydrozino derivatives. This suggestion was confirmed by NMR analysis of dithionite-treated drugs.

The NMR data for the native and reduced forms of HPURa, HPIsocytosine, and PURa are given in Table 1. The upfield shifts of proton resonances and the appearance of three new resonance peaks clearly indicated that dithionite reduced the azo groups of all three compounds. The most prominent shifts in the dithionite-treated drugs were those of the C-5 protons; this was expected from consideration of the proximity of electron-donating NH-groups to the respective pyrimidine rings and from the observation (not tabulated) of a similar resonance for the C-5 protons of 6-(amino)-uracil (4.5 ppm) and 6-(hydrazino)-uracil (4.67 ppm).

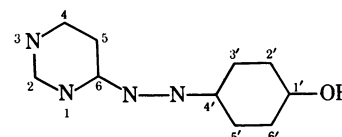
TABLE 1. NMR spectra in  $DMSO-d_6$

Compound*	$\delta_{1-H}$ †	$\delta_{3-H}$	$\delta_{5-H}$	$\delta_{6-NH}$
6-( <i>p</i> -Hydroxyphenylazo)-uracil	11.40	11.40	6.10	—
6-( <i>p</i> -Hydroxyphenylazo) <i>iso</i> -cytosine‡	—	(n.o.)	5.82	—
6-( <i>p</i> -Hydroxyphenylhydrazino)-uracil	10.20	10.35	4.67	8.22
6-( <i>p</i> -Hydroxyphenylhydrazino) <i>iso</i> -cytosine§	—	9.90	4.68	8.05
6-(Phenylazo)-uracil	11.26	11.50	6.28	—
6-(Phenylhydrazino)-uracil	10.30	10.30	4.58	8.32

	$\delta_{1'-OH}$	$\delta_{2',6'-H}$	$\delta_{3',5'-H}$	$\delta_{4'-NH}$
6-( <i>p</i> -Hydroxyphenylazo)-uracil	(n.o.)	7.06( <i>d</i> ) ( <i>J</i> = 9 Hz)	8.00( <i>d</i> )	—
6-( <i>p</i> -Hydroxyphenylazo) <i>iso</i> -cytosine‡	(n.o.)	7.06( <i>d</i> ) ( <i>J</i> = 9 Hz)	7.92( <i>d</i> )	—
6-( <i>p</i> -Hydroxyphenylhydrazino)-uracil	8.92	6.72( <i>s</i> )	6.72( <i>s</i> )	7.45
6-( <i>p</i> -Hydroxyphenylhydrazino) <i>iso</i> -cytosine§	8.74	6.63( <i>s</i> )	6.63( <i>s</i> )	7.22
6-(Phenylazo)-uracil	—¶	—	—	—
6-(Phenylhydrazino)-uracil	—	—	—	7.83

\*The numbering system used is:



† Chemical shifts in ppm ( $\delta$ ) downfield from internal tetramethylsilane; (n.o.), not observed; (*d*), doublet; (*s*), singlet.

‡  $\delta_{2-NH_2}$  = 7.03 ppm.

§  $\delta_{2-NH_2}$  = 6.27 ppm.

¶  $\delta_{ArH}$  = 7.92 ppm (complex multiplet).

||  $\delta_{ArH}$  = 6.97 ppm (complex multiplet).

Dithionite treatment also resulted in the spectral equivalence of the ring protons of the *p*-hydroxyphenyl groups. This phenomenon, also observed for the ring protons of *p*-aminophenol (singlet at 6.53 ppm), was apparently derived from the similar electronic properties of the *p*-OH and *p*-NH<sub>2</sub> groups.

Dithionite-treated HPURa and HPIsocytosine displayed three slightly broadened resonance peaks in the region of 7–9 ppm; these peaks were assigned to the protons of the 6-NH, 1'-OH, and 4'-NH moieties. Identification of the 1'-OH peak in each compound was based on its absence from the spectrum of reduced PUra. Of the remaining two peaks, that further downfield in reduced HPURa (8.22 ppm) and HPIsocytosine (8.05 ppm) was assigned to their respective 6-NH protons on the basis of specific shifts that occurred in hydrogen bonding interactions with cytosine and thymine (see results below). Two NH peaks were evident in the spectra of dilute solutions of reduced HPURa and PUra; the upfield peaks were assigned to 1-H based on hydrogen-bonding shifts.

**NMR Analysis of Hydrogen-Bonding of Reduced Drugs with DNA Bases.** The possibility of hydrogen-bonding interactions between the reduced drugs and nucleic acid bases was investigated by examination, in separate experiments, of the effects of cytosine, guanine, thymine, and adenine, their nucleosides, and their 2'-deoxyribonucleoside 5'-triphosphates on the NMR spectra of drug solutions in DMSO-*d*<sub>6</sub>. DMSO was chosen as the solvent, since it readily dissolved reduced drugs and was known to be a suitable solvent for study of hydrogen-bonding between compounds such as guanosine and cytidine (10–12). DMSO also does not readily permit base-stacking or formation of larger hydrogen-bonded complexes (12).

In all experiments, addition of bases and their derivatives to solutions of reduced drugs broadened the 1'-OH resonance peaks. This broadening apparently resulted from the facile exchange of the 1'-OH proton with the amino, hydroxyl, and phosphate groups of the added derivatives. Indeed, the most pronounced broadening of the 1'-OH peak occurred with addition of nucleoside triphosphates or phosphate salts such as (Na<sub>2</sub>)HPO<sub>4</sub> (results not shown).

TABLE 2. NMR studies of base-pairing in DMSO-*d*<sub>6</sub>

Substrate	Added base	$\Delta_{HB}^*$ (ppm)	$K^{20^\circ}$ (mol <sup>-1</sup> )	$\Delta H^\ddagger$ (kcal mol <sup>-1</sup> )
Reduced HPURa	Cytosine	1-H: 2.33	5.9	-4.1
		6-NH: 2.04		
Reduced HPURa	2'-Deoxycytidine	6-NH: 2.02	2.7	-3.8
2'-Deoxyguanosine	Cytosine	1-H: 2.10	5.2	-4.9
		2-NH <sub>2</sub> : 1.33		
Reduced HPIsocytosine	Thymine	2-NH <sub>2</sub> : 1.18	0.1 ± 0.05	—
		6-NH: 2.67		
Guanosine	Cytidine	1-H: 2.76	3.7 (32°)	-5.8†
		2-NH <sub>2</sub> : 1.37		

\*  $\Delta_{HB}$  (substrate) =  $\delta_{\text{complex}} - \delta_{\text{free}}$ .

† Calculated by plotting  $\ln K$  against  $1/T$  (°K) where the slope is  $-\Delta H/R$  over the range 20–55°.

‡ From ref. 12.

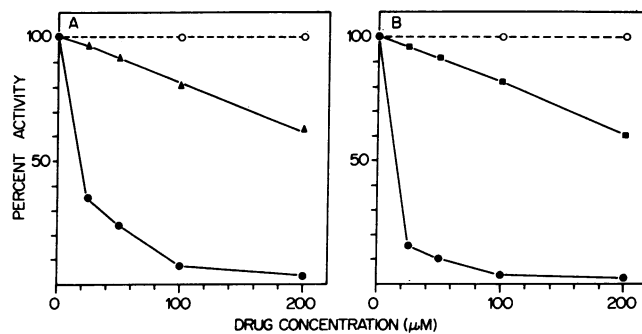


FIG. 3. Effect of native and reduced arylazopyrimidines on the DNA polymerase activity of DEAE-cellulose fraction B. The B pool of DEAE-cellulose eluate (see Fig. 2) was concentrated by precipitation in 90% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 15 μg of protein were used for assay; 100% activity represented incorporation of 49 pmol of [<sup>3</sup>H]TMP into acid-insoluble product. HPURa and HPIsocytosine were reduced just before use by mixing drug solutions (2.5–20 mM; in 50 mM NaOH) with an equal volume of fresh 100 mM sodium dithionite. Dithionite, at the concentrations present during assay, had no effect on enzyme activity. (A) Effect of HPURa. (B) Effect of HPIsocytosine. ○—○, native drug; ○—○, reduced drug in standard conditions; ▲—▲, reduced drug plus 1 mM dGTP; ■—■, reduced drug plus 1 mM dATP.

**Interaction of Cytosine with Reduced HPURa.** Of the four bases and their derivatives used, only those containing cytosine caused significant shifts of peaks in the spectrum of reduced HPURa. Addition of either cytosine or deoxycytidine caused a downfield shift of the 6-NH and the 1-H resonances; the extent of these shifts was directly proportional to the amount of base added. The shifts of the 1-H peaks were difficult to quantitate at high base concentration, since this peak broadened and became barely discernible above background. Nevertheless, the lower limits for H-bonding shifts ( $\Delta_{HB}$ ) and for association constants ( $K$ ) for base-pairing could be determined; this was done by addition of a 4–8 molar excess of base to dilute drug solutions and plotting the reciprocal of the induced shifts in Hz against the reciprocal of base concentration (B), according to Mathur *et al.* (13). From the plot intercept ( $1/\Delta_{HB}$ ) and the slope ( $1/K\Delta_{HB}$ ),  $\Delta_{HB}$  and  $K$  were obtained; their values are given in Table 2, along with values obtained from a similar study of base-pairing between guanosine and cytosine. Literature values (12) for pairing interactions between guanosine and cytidine are also included for comparison.

The magnitude of  $K$  for binding of cytosine both to reduced HPURa and to deoxyguanosine were clearly comparable, indicating that the reduced drug can readily mimic guanine in its capacity to pair strongly with cytosine.

**Interaction of Thymine with Reduced HPIsocytosine.** Studies identical to those discussed above for reduced HPURa were performed with reduced HPIsocytosine. Of the bases tested, only thymine bound to this drug in a manner analogous to that observed for cytosine and reduced HPURa, i.e., through interaction with the 6-NH proton. The observed shifts of the 2-NH<sub>2</sub> and 6-NH protons were small in comparison to those observed upon addition of cytosine to reduced HPURa; however, the calculated  $\Delta_{HB}$  values for these protons were of comparable magnitude (see Table 2). The  $K$  value for reduced HPIsocytosine and thymine ( $0.1 \pm 0.05$  mol<sup>-1</sup>) was smaller

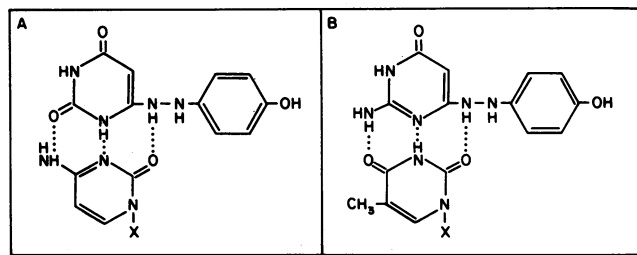


FIG. 4. Proposed mechanism of pyrimidine-arylhydrazino-pyrimidine pairing. (A) Cytosine:hydroxyphenylhydrazino-uracil. (B) Thymine:hydroxyphenylhydrazino-isocytosine.

than that found for HPURa and cytosine and, therefore, was difficult to determine with the same accuracy.

**Proposed Mechanism of Drug:Base Pairing.** The hydrogen-bonding interactions of reduced HPURa and cytosine and reduced HPIsocytosine and thymine suggested specific, inhibitor-base-pairing interactions involving three hydrogen bonds (Fig. 4). The observed values of  $K$  and  $\Delta H$  (see Table 2), particularly the  $K$  value for thymine and reduced HPIsocytosine, were low for pairs involving three bonds; this, however, was not unexpected in DMSO, since this solvent is known to compete for hydrogen-bonding of nucleic acid bases (11, 12). Indeed, the inordinately low value of  $K$  obtained for reduced HPIsocytosine and thymine might have derived from a greater capacity of this drug, through its 2-NH<sub>2</sub> group, to bind to DMSO.

#### DISCUSSION

Collectively, the above data clarify several features of the mechanism by which 6-(aryldiazinyl)-pyrimidines inhibit replicative DNA synthesis. First, they support the suggestion of experiments reported elsewhere (2, 3, 5) that the native arylazopyrimidines are not inhibitory *per se*, but require activation by reduction, either metabolically or chemically. Second, the chemical and structural properties (see Table 1) of the active drug species clearly identify them as the arylhydrazino derivatives. Third, the effects of the arylhydrazinopyrimidines on a chromatographically distinct species of DNA polymerase (Fr. B enzyme, Figs. 2 and 3) strongly suggest that this enzyme is specifically involved in replicative DNA synthesis and is, indeed, the site of drug action in intact and toluene-treated *B. subtilis*. The mechanism of drug action at the level of the B polymerase is clearly similar to that observed (5) on ATP-dependent DNA replication in the environment of the toluene-treated cell. In both systems, dGTP specifically and competitively antagonized the inhibitory activity of reduced HPURa; similarly, dATP antagonized the activity of reduced HPIsocytosine.

The mechanism by which the arylhydrazinopyrimidines inhibit the B polymerase is not clear. Kinetic analyses with a purified preparation of this enzyme suggest a mechanism in which the drugs and purine deoxyribonucleotides compete for a common site. This site could be identical with the active substrate site of the enzyme, an area of the primer:template DNA proximal to this active site, or a site generated by the interaction of enzyme and its primer:template.

At present, we favor a mechanism of inhibitor action in which drug interacts with both the enzyme and pyrimidine residues in template DNA, thus denying purine substrates access to the active triphosphate binding site. The results of the base-pairing studies (see Table 2) clearly support such a mechanism. Reduced HPURa, which apparently mimics dGTP, can pair specifically (see Fig. 4A for proposed mechanism) with cytosine, the normal guanine complement. Similarly, reduced HPIsocytosine, which mimics dATP, apparently binds in an analogous manner with thymine (Fig. 4B). A mechanism of this type may be resolved by studying what specific effects, if any, reduced HPURa and reduced HPIsocytosine have on the ability of purified fraction B enzyme to use template:primer combinations such as poly(dT):oligo(dA) and poly(dC):oligo(dG).

The unique drug sensitivity of the fraction B polymerase of *B. subtilis* and, presumably, of similar enzymes from other Gram-positive bacteria, is an intriguing property. Equally intriguing is the drug resistance of DNA polymerases I (14), II, and III (2) of *E. coli*, and the fraction A polymerase and DNA polymerase I of *B. subtilis* (15, 16). Indeed, the response of any of the above polymerases to reduced arylazopyrimidines would be difficult to predict on the basis of their template and substrate requirements and their catalytic properties. Thus far, the results of preliminary studies of the properties of purified B enzyme have not revealed any obvious basis for its response to reduced HPURa and HPIsocytosine. Clearly the best tools for probing this unique property seem to be the inhibitors themselves.

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