Irreversible Inhibition of Dehalogenation of 5-Iodouracil by 5-Diazouracil and Reversible Inhibition by 5-Cyanouracil

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SUMMARY

The dehalogenation of 5-iodouracil was studied in vitro, and two inhibitors of the reaction were found. One of these, 5-cyanouracil, previously reported to inhibit dihydrouracil dehydrogenase, was shown in this study to inhibit dehalogenation. The second inhibitor, 5-diazouracil, was shown to be an irreversible inhibitor of dehalogenation, which appears to act at the substrate binding site. Diazouracil was shown to be an irreversible inhibitor of dehalogenation, which appears to act at the substrate binding site. Diazouracil was shown to be an irreversible inhibitor of dehalogenation, which appears to act at the substrate binding site. Diazouracil was shown to be an irreversible inhibitor of dehalogenation, which appears to act at the substrate binding site. Diazouracil was shown to be an irreversible inhibitor of dehalogenation, which appears to act at the substrate binding site. 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RESULTS

Characteristics of the Dehalogenation Reaction. The time course of dehalogenation of $^{125}\text{I}$U is shown in Chart 1. Addition of more protein preparation after dehalogenation has ceased (at 45 min) results in restoration of the initial rate (8 nmoles/h/mg of protein). Omission of TPNH from the reaction mixture diminished dehalogenation activity 10-fold. After dialysis of the enzyme preparation for 18 hr against 0.1 M phosphate buffer (pH 7.4), there was no dehalogenation activity in the absence of TPNH; full activity was restored by TPNH addition. This requirement for TPNH is consistent with the involvement of dihydrouracil dehydrogenase in the dehalogenation of IU.

Further evidence that suggests the involvement of dihydrouracil dehydrogenase was obtained by testing known substrates of the enzyme as inhibitors of IU dehalogenation (Table 1). All of the pyrimidine bases tested are substrates for dihydrouracil dehydrogenase (7), and they all inhibit the dehalogenation of IU. The inhibitory activity of BUDR could arise as a result of cleavage of BUDR to BU (19). The reduced compounds (dihydrouracil and dihydrothymine) were not inhibitory. This result was expected due to their relatively low affinity for dihydrouracil dehydrogenase (9) and because they would be rapidly degraded in the crude assay mixture used (4).

After 20 min of incubation, paper chromatography of the reaction products on Whatman No. 1 filter paper in isopropyl alcohol:ethanol:water (85:85:30) and water-saturated colidine revealed only 2 peaks containing $^{125}\text{I}$ activity. One of these cochromatographed with IU (RF values of 0.60 and 0.75 in the 2 solvents, respectively), and the 2nd cochromatographed with sodium iodide (RF values of 0.45 and 0.65 in the 2 solvents, respectively). No $^{125}\text{I}$-dihydroiodouracil was observed, nor were any other iodinated products of pyrimidine catabolism observed.

Inhibition of $^{125}\text{I}$U Dehalogenation. Several plausible inhibitors were found not to inhibit IU dehalogenation. These included 5-trifluoromethyluracil, 5-aminouracil, 5-carboxyuracil, 5-carboxythymine, 5-nitouracil, 5-thiouracil, 5-hydroxymethyluracil, orotic acid, 6-azauracil, 6-azathymine, 6-methyluracil, and 2-thiouracil. Two compounds, 5-
Competition between inhibitors and substrate

The reaction mixture contained the indicated amount of 125IU, 0.05 μmole of inhibitor where indicated, and 0.1 ml of enzyme. The amount of 125I (nmoles) formed after 20 min of incubation is shown. Inhibitor 1 25 I (μmole) None 5-Diazouracil 5-Cyanouracil

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>125 I (μmole)</th>
<th>5-Diazouracil</th>
<th>5-Cyanouracil</th>
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<tr>
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<td>33</td>
<td>13</td>
</tr>
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<td></td>
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<td>37</td>
<td>25</td>
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<tr>
<td></td>
<td>1.0</td>
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Inhibitors of Dehalogenation of IU

Cyanouracil and 5-diazouracil were found to be effective inhibitors of dehalogenation (Chart 2).

Due to the high reactivity of the diazo group, we tested the possibility that 5-diazouracil might interact irreversibly with the enzyme. The results of preincubation of the inhibitors with the enzyme preparation before addition of substrate are shown in Table 2. Preincubation with 5-cyanouracil does not affect the extent of inhibition. However, preincubation with 5-diazouracil markedly increases inhibition. This indicates that 5-diazouracil may be an irreversible inhibitor. Irreversible inhibition is prevented if unlabeled IU is present during the preincubation period (Table 2). This result is consistent with competition between IU and 5-diazouracil for a common enzymatic binding site.

A further test of irreversible interaction between the enzyme and 5-diazouracil is illustrated in Chart 3. In this experiment, the concentrations of inhibitors and substrate were constant, while the amount of protein preparation in the reaction mixture was varied. Activity increases linearly with protein concentration for uninhibited enzyme and enzyme inhibited with 5-cyanouracil. However, when dehalogenation is inhibited with 5-diazouracil, the curve is biphasic. This indicates that the inhibitor is titrating the enzyme. At low enzyme concentrations, the enzyme has quantitatively reacted with 5-diazouracil. However, when we increase the amount of enzyme, we reach a point where the amount of inhibitor is no longer sufficient to bind all the enzyme present. Past this point, additional enzyme produces a linear increase in activity at a comparatively high rate. The results of the experiment further demonstrate the irreversibility of 5-diazouracil inhibition.

The irreversibility of 5-diazouracil inhibition was also tested by dialyzing the enzyme preparation after preincubation with 5-diazouracil. The enzyme preparation (0.1 ml) was incubated with 25 nmoles of 5-diazouracil under standard conditions in the absence of substrate. This preparation was then dialyzed for 24 hr against 0.1 M phosphate buffer (pH 7.4) and assayed under standard conditions. A dialyzed control preincubated without 5-diazouracil catalyzed the formation of 40 nmoles of 125I in 20 min, while the sample preincubated with 5-diazouracil catalyzed the formation of only 8 nmoles of 125I in the same time. Thus, dialysis did not reverse 5-diazouracil inhibition.

The effect of varying substrate concentration is illustrated in Table 3. Uninhibited enzyme is nearly saturated by 0.2 μmole of substrate. However, in the presence of either 5-diazouracil or 5-cyanouracil, enzyme activity is substantially increased by increasing concentrations of 125IU. These results are characteristic of a competitive inhibitor. However, a rigorous kinetic analysis could not be made since the crude enzyme preparation does not exhibit classical Michaelis-Menten kinetics.

Dehalogenation of 125IUDR. The results of studies with 125IUDR as a substrate for dehalogenation in the crude enzyme preparation are presented in Table 4. The fact that dehalogenation of the nucleoside is inhibited by IU, 5-cyano-uracil, and 5-diazouracil is consistent with degradation of 125IUDR to 125IUI, followed by dehalogenation of the free base.

Since dehalogenation is thought to occur via the free base, we determined the effect of trifluorothymidine, which inhibits nucleoside phosphorylase of Ehrlich ascites cells (14), on
dehalogenation of the nucleoside IUDR. It did not inhibit IUDR dehalogenation (Table 4). This may be accounted for by the fact that Ehrlich ascites has only a uridine phosphorylase (26), whereas rat liver has both uridine phosphorylase and thymidine phosphorylase (25,26); the latter may not be inhibited by trifluorothymidine.

Inhibition by 5-diazouracil In Vivo. A dose of 5 mg/kg of 5-diazouracil was administered by i.p. injection to Sprague-Dawley rats. Three hr after 5-diazouracil administration, the animals were sacrificed, their livers were removed, and enzyme was prepared in the standard manner. These preparations were then assayed for dehalogenation of $^{125}$IU and $^{125}$IUDR. Dehalogenation of the 2 substrates was inhibited 91 and 95%, respectively, indicating that 5-diazouracil is an effective inhibitor of dehalogenation in vivo.

DISCUSSION

The principal result of this study indicates that 5-diazouracil irreversibly inhibits IU dehalogenation. The fact that 5-diazouracil and IU appear to compete for a common enzymatic site suggests that 5-diazouracil may react irreversibly with an amino acid at the catalytic site. However, it is also possible that 5-diazouracil acts at another site on the enzyme in such a manner that binding of the inhibitor is interfered with by the binding of substrate.

In contrast to the irreversible inhibition by 5-diazouracil, 5-cyanouracil is a reversible inhibitor of dehalogenation. Its inhibition of IU dehalogenation is similar to the inhibition of uracil reduction catalyzed by dihydrouracil dehydrogenase previously reported by Dorsett et al. (7). One mechanism that might be proposed to account for this inhibitory activity is inhibition resulting from the release of cyanide. However, addition of 0.4 μmole of KCN to the reaction mixture does not affect dehalogenation activity.

Our results are consistent with the fact that degradation of the nucleoside to the free base occurs prior to dehalogenation as has been observed in vivo (16,17,21). The fact that trifluorothymidine, which inhibits uridine phosphorylase (14), did not effectively inhibit degradation of IUDR may be accounted for by the presence of thymidine phosphorylase in the liver homogenate. Dehalogenation of the free base is thought to proceed via reduction of the 5,6 double bond by the enzyme dihydrouracil dehydrogenase, followed by spontaneous elimination of the halide (2,20,21). The current findings that dehalogenation in vitro requires TPNH, is inhibited by substrates of dihydrouracil dehydrogenase, and is inhibited by 5-cyanouracil are consistent with this mechanism.

The irreversible inhibition of dehalogenation by 5-diazouracil may be relevant to a variety of chemotherapeutic situations involving fluorinated pyrimidines and to the use of IUDR and BUDR as radiosensitizing agents. The effectiveness of these compounds as tumor therapeutic agents has been studied by several workers (1,3,18,22—24). In these studies, the greatest success has been achieved when the problem of catabolism was circumvented by administration of BUDR directly to the tumor site by intraarterial infusion (1,22,23) or by i.p. injection in the case of an i.p. ascites lymphoma (18).

Administration of 5-diazouracil is expected to block degradation of BUDR at the level of the free pyrimidine, resulting in increased availability of the free base to tumor cells. Relative to the deoxynucleoside, the free base is a poor DNA precursor. However, if further degradation of the free pyrimidine is prevented, it may be utilized to a significant extent. Incorporation of bromouracil into DNA of Ehrlich ascites cells in vitro has been increased to that obtained with BUDR as a precursor by addition of deoxyinosine to the culture medium (10). Such a technique, combined with inhibition of dehalogenation, may be helpful in stimulating incorporation of the free pyrimidine in vivo.

Preliminary toxicity studies in collaboration with Dr. Wilhelmina Dunning indicate that administration of a single dose of 20 mg/kg or 4 daily doses of 5 mg/kg of 5-diazouracil together with up to 200 mg/kg of BUDR and 0.1 mg/kg of methotrexate is well tolerated by Fischer rats. In vivo studies to evaluate the possibility of using 5-diazouracil and its nucleoside derivatives to augment tumor radiosensitization and chemotherapy with halogenated pyrimidines are in progress.

REFERENCES

12. Greer, S., and Zamenhof, S. Effect of 5-Bromouracil in Deoxyribonucleic Acid of E. coli on Sensitivity to Ultraviolet Irradiation. In:
Abstracts of the American Chemical Society Meeting, Miami, Fla., April 1957, p. 3c.


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