Further Studies on the Mechanism of Action of Hydroxyurea

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SUMMARY

Addition of the four deoxyribonucleosides in vitro to 6C3HED mouse ascites tumor stimulates 32P incorporation into DNA and depresses incorporation into RNA at concentrations greater than 10−4 M. The four ribonucleosides stimulate incorporation into both DNA and RNA. Hydroxyurea blocks the ribonucleoside stimulation of 32P incorporation into DNA but not RNA. The inhibition of 32P incorporation into DNA by hydroxyurea is not prevented by the four deoxyribonucleosides in vitro when added either initially or at intervals throughout the period of incubation. Studies in vivo also indicate the failure of deoxyribonucleosides to prevent inhibition induced by hydroxyurea. The failure of deoxyribonucleosides to prevent the hydroxyurea effect cannot be explained on the basis of the failure of the added nucleosides to be incorporated into DNA or on the basis of their conversion to ribonucleosides and secondary inhibition of DNA synthesis. It would appear, on the basis of these data, that in this system the primary site of action of hydroxyurea is not at the level of conversion of ribonucleotides to deoxyribonucleotides and, therefore, additional sites of action should be investigated.

INTRODUCTION

Hydroxyurea was synthesized by Dresler and Stein in 1869 (6). It was first employed in biologic studies in 1928 by Rosenthal et al. (21), who demonstrated that the drug produced a megaloblastic picture similar to that of pernicious anemia, as well as a depression in leukocyte formation. In 1960, antitumor activity against Sarcoma 180 was reported in animal-screening studies (23). The initial clinical studies of hydroxyurea demonstrated bone marrow depression, megaloblastosis, and objective improvements in a variety of solid tumors, as well as acute and chronic leukemia (2-4, 7). Although occasionally useful clinical responses may be observed in solid tumors, its use in such tumors has generally been disappointing. It has not proven superior to other agents in acute leukemia (7). However, it would appear to be as good as busulfan in the treatment of chronic myelogenous leukemia and clearly has a role in the management of some patients with this disease (12, 24).

Hydroxyurea is a hydroxamic acid. A number of natural products contain one or more oxidized peptide (amide) bonds and are classified as hydroxamic acids. These substances have been found to possess numerous biologic activities including actions as growth factors, antibiotics, antibiotic antagonists, tumor inhibitors, and cell division factors. Naturally occurring monohydroxamic acids include hadacine (antitumor agent), aspiragolic acid and its derivatives (antibiotics), actinomycin (antibiotic), and seizokinen (cell division factor). Naturally occurring dihydroxamic acids include mycoebaetims P and T, which are growth factors for *Mycobacterium philoi* and *Mycobacterium tuberculosis*, as well as the antibiotic myceliamide. Naturally occurring trihydroxamic acids include the antibiotics ferrimycin, succinamycin, danamycin, albomyein, and griesen; the antibiotic antagonists ferrihexan, ferricactin, ferrirubin, and ferrirubin; and the growth factors coprogen, ferriochrome, and the ferrochelamine series (18). Artificial monohydroxamic acids include hydroxyurea, hydroxyurethan, acetohydroxamic acid, and their derivatives. In spite of the multiple functions of the hydroxamic acids as growth stimulators and growth inhibitors, it has as yet not been possible to relate these effects to a single molecular event.

The clinical observation of megaloblastosis suggested quite early that hydroxyurea inhibited DNA synthesis (2). Numerous studies confirmed that DNA synthesis was inhibited in various systems including HeLa cells (32), ascites tumor (10, 29), regenerating liver (22, 31), and bacteria (20). A number of hydroxamic acids have been found to inhibit DNA synthesis (11, 33). Hydroxyurea and hydroxyurethan have an equal potency on a molar basis (19, 30). Hydroxyurea has been shown to inhibit histone synthesis as well as DNA synthesis (28, 31).

Demain (5) has presented evidence to suggest that the antibiotic hadacine, a monohydroxamic acid, inhibits the enzyme adenylsuccinate synthetase which is involved in the de novo synthesis of adenine. Vogler et al. (26) observed that hydroxyurea reverses the orotic aciduria induced by azauridine in human subjects and postulated that hydroxyurea blocks pyrimidine synthesis at some step prior to orotic acid formation. Frenkel et al. (8, 9) suggested that hydroxyurea blocks the conversion of ribonucleotides to deoxyribonucleotides. Hydroxyurea does not inhibit incorporation of thymidine-14C into DNA in a cell-free system containing the deoxyribonucleotides and DNA primer (32). The failure of hydroxyurea to block incorporation of thymidine into DNA in a cell-free system containing DNA polymerase and the three deoxyribonucleotide triphosphates suggests that hydroxyurea does not act at the level of DNA polymerase (32). Action upon thymidylate kinase has also been rendered unlikely by the observation that normal phosphorylation of thymidine occurs in the presence of hydroxyurea (32). The phosphorylation of deoxyribonucleotide diphosphates to...
the triphosphate level by their respective kinases has been studied and found to be normal (1).

Addition of various deoxyribonucleosides has been observed to exert a protective effect against hydroxyurea. Mohler (16) obtained partial protection with pyrimidine deoxyribonucleosides in Chinese hamster cells but no protection when the HeLa cells were used. Addition of the three deoxyribonucleosides AdR, GdR, and CdR can partially correct the inhibition of thymidine incorporation into DNA produced by hydroxyurea (34). This incomplete reversal of hydroxyurea effect has been observed by others and has raised a serious question that hydroxyurea does indeed act at the reductive step (19, 27). The effect of added ribonucleosides and deoxyribonucleosides alone or in combination appears to be somewhat variable and dependent upon the tissue and conditions of the experiment. Inhibition of DNA synthesis may lead to accumulation of precursors which produce a feedback inhibition of ribonucleotide reductase. Such inhibition has been reported (15, 17), and this complex reductive step is known to be influenced by multiple feedback control mechanisms (14).

Because of these complexities, Young et al. (34) studied the ova of *E. para*ma, an echinoderm species, which contain quantities of acid soluble deoxyribonucleotides. Addition of hydroxyurea to this system caused no inhibition of DNA synthesis during the first few divisions. The authors interpreted this to indicate that hydroxyurea does not affect phosphorylation or DNA assembly, but rather acts at the level of conversion of ribonucleotide to deoxyribonucleotide.

Adams and Lindsay (1) reported complete reversal of hydroxyurea effect in mouse fibroblast cells by addition of the four deoxyribonucleosides and suggested that failure of reversal in other systems might be due to either failure of deoxyadenosine triphosphate production from added deoxyadenosine or to dilution of the isotopic precursor used.

It would appear on the basis of these reported observations that the effect of added deoxyribonucleosides may depend upon the size of the nucleotide pools in tissue under study and the isotopic precursor used. Therefore, it is essential that any evaluation of the effect of added deoxyribonucleosides upon the hydroxyurea-induced inhibition of DNA synthesis takes into consideration the effect of different concentrations of deoxyribonucleosides on the tissue under study.

This study was therefore designed to evaluate the effect of addition of the four deoxyribonucleosides in various concentrations upon DNA and RNA synthesis, as well as their effect upon the hydroxyurea-induced inhibition of DNA synthesis. The isotope $^{32}$P was selected to measure nucleic acid synthesis to avoid the problems encountered in pool size and isotope dilution when one of the added nucleosides is at the same time the isotope used to evaluate nucleic acid synthesis.

**MATERIAL AND METHODS**

The tissue used for these studies was the 6C3HED mouse ascites tumor. For in *vitro* studies, the ascites tumors from several animals were pooled in the desired volume in a flask at 4°C, and $^{32}$P as the sodium phosphate was added in an amount of 1 mc for each 20 ml of tumor fluid. One-ml portions of the tumor containing the isotope were added to test tubes containing appropriate concentrations of hydroxyurea, the ribonucleosides (guanosine, adenosine, cytidine, and uridine), or the deoxyribonucleosides GdR, AdR, CdR, and thymidine dissolved in physiologic saline. Equal volumes of physiologic saline were used for control tubes. The tubes were incubated with shaking at 37°C in a water bath for the desired time, following which they were removed and immersed in an ice bath; the reaction was terminated by addition of 5 ml of 5% TCA. The specific activity of acid-soluble phosphorus, RNA, and DNA was determined by methods previously described (27). In some instances, the ascites tumor fluid was incubated in 50-ml Erlenmeyer flasks with shaking at 37°C in a volume of approximately 10 ml in order to permit addition of deoxyribonucleosides at preselected intervals throughout the incubation, as well as the removal of 1-ml samples of tumor from the incubation flask at appropriate time intervals. For *in vivo* experiments, animals carrying seven-day tumor were injected intraperitoneally with 100 µc of $^{32}$P as the sodium phosphate dissolved in approximately 0.2 ml of physiologic saline. Hydroxyurea, the ribonucleosides, and the deoxyribonucleosides were dissolved in physiologic saline, such that the appropriate dosage would be contained in 0.2 ml, and were injected intraperitoneally at the time of administration of the isotope. The animals were sacrificed by neck fracture, and the ascites tumor was drained into a test tube in an ice bath containing 5 ml of 5% TCA. The nucleic acid fractions were isolated and their specific activity determined as described above. For *in vivo* experiments, relative specific activity was calculated as previously described (29).

Relative specific activity is the specific activity of the nucleic acid expressed as a percentage of the specific activity of the acid-soluble phosphate for an individual sample.

**RESULTS**

When hydroxyurea is added to tumor cells in *vitro* in concentrations from 1 to 100 µg/ml, increasing degrees of inhibition of $^{32}$P incorporation into DNA are observed with very little influence upon incorporation into RNA except at the highest concentration used (Chart 1).

When various concentrations of added deoxyribonucleosides varying from $10^{-3}$ M to $10^{-8}$ M were studied at 30 minutes in *vitro*, there was clearly a stimulation of $^{32}$P incorporation into DNA at the higher concentrations and an inhibition of $^{32}$P incorporation into RNA at these concentrations (Table 1). At concentrations of $10^{-6}$ M or less, the incorporation of $^{32}$P into DNA and RNA does not differ from control values, whereas, at concentrations of $10^{-4}$ M or greater, incorporation of $^{32}$P into DNA is stimulated and incorporation into RNA is inhibited.

When the effect of hydroxyurea is studied in a system in which the four deoxyribonucleosides are added in a concentration which produces a stimulation of DNA synthesis, that is, $10^{-3}$ M, no significant correction of the inhibition induced by hydroxyurea is accomplished by added deoxyribonucleosides (Table 2).
A similar effect may be seen in vivo (Table 3). Injection of deoxyribonucleosides does not correct the inhibition induced by hydroxyurea seen at 30 minutes, even though the amount of deoxyribonucleosides injected was sufficient to markedly increase incorporation of isotope into DNA. Injection of ribonucleosides in a concentration sufficient to produce stimulation of $^3$P incorporation into DNA fails to correct hydroxyurea inhibition in vivo at one hour. When the effect of injected deoxyribonucleosides in vivo at one hour is evaluated, the results are quite comparable to those seen at 30 minutes. It is noteworthy that inhibition of RNA synthesis by added deoxyribonucleosides was not seen in these in vivo experiments. The significance of this observation remains obscure.

Because it has been suggested that added deoxyribonucleosides are rapidly cleaved to sugar and free base, the experiment illustrated in Chart 2 was conducted in vitro with addition of the four deoxyribonucleosides at 0, 5, 10, 15, and 20 minutes. The amount of deoxyribonucleosides added was such that the initial concentration of each was $3.3 \times 10^{-4}$ M. A sufficient amount of each deoxyribonucleoside was added at each of the indicated time points to bring the concentration back to this level, assuming complete destruction or utilization of the deoxyribonucleosides in the preceding five minute interval.

![Chart 1. Incorporation of $^3$P into DNA and RNA in vitro as % control in 6C3HED ascites tumor at 1 hour in the presence of various concentrations of hydroxyurea. Each point represents the mean of two experimental samples expressed as a % of the mean of four control samples.](image)

### Table 1

<table>
<thead>
<tr>
<th>Concentration of deoxyribonucleosides (M)</th>
<th>DNA specific activity</th>
<th>RNA specific activity</th>
<th>DNA, % of control</th>
<th>RNA, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.56 ± 1.96</td>
<td>65.9 ± 14.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>9.16 ± 1.36</td>
<td>67.2 ± 9.52</td>
<td>96</td>
<td>102</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>9.73 ± 0.405</td>
<td>66.2 ± 4.72</td>
<td>102</td>
<td>100</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>9.74 ± 1.95</td>
<td>56.8 ± 10.3</td>
<td>102</td>
<td>86</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>14.9 ± 2.41</td>
<td>61.2 ± 13.4</td>
<td>156</td>
<td>93</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>18.4 ± 4.58</td>
<td>53.4 ± 4.33</td>
<td>192</td>
<td>81</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>17.6 ± 1.38</td>
<td>51.6 ± 1.89</td>
<td>184</td>
<td>78</td>
</tr>
</tbody>
</table>

DNA and RNA specific activity after 30 minutes of incorporation in vitro with the four deoxyribonucleosides 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, and thymidine each in the concentration indicated. Acid-soluble phosphate specific activity was 1940 cpm/ microgram. Data are expressed as the mean ± standard deviation of eight controls and four samples for each concentration.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>Hydroxyurea</th>
<th>Deoxyribonucleosides</th>
<th>Hydroxyurea deoxyribonucleosides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>11.8</td>
<td>7.33</td>
<td>20.0</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.765</td>
<td>0.15</td>
<td>1.90</td>
<td>1.27</td>
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<tr>
<td>RNA Mean</td>
<td>45.7</td>
<td>42.9</td>
<td>36.4</td>
<td>35.2</td>
</tr>
<tr>
<td>S.D.</td>
<td>4.78</td>
<td>2.03</td>
<td>7.51</td>
<td>2.95</td>
</tr>
</tbody>
</table>

Effect of the four deoxyribonucleosides 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, and thymidine in a concentration of $10^{-3}$ M on hydroxyurea inhibition of radiophosphorus incorporation into DNA and RNA in vitro at 30 minutes. Hydroxyurea was added in a concentration of 25 micrograms per ml. Acid-soluble phosphate specific activity was $2.18 \times 10^3$ cpm per microgram. Mean and standard deviation of three samples are expressed as cpm per microgram of nucleic acid phosphorus.
Mechanism of Hydroxyurea Action

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hydroxyurea</th>
<th>Nucleosides</th>
<th>Hydroxyurea plus Nucleosides</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
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<td></td>
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<tr>
<td>DNA</td>
<td>0.378</td>
<td>0.094</td>
<td>0.536</td>
<td>0.120</td>
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<tr>
<td>Mean</td>
<td>0.0637</td>
<td>0.0219</td>
<td>0.0679</td>
<td>0.0159</td>
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<tr>
<td>RNA</td>
<td>1.48</td>
<td>1.21</td>
<td>1.48</td>
<td>1.40</td>
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<tr>
<td>Mean</td>
<td>0.237</td>
<td>0.240</td>
<td>0.146</td>
<td>0.237</td>
</tr>
<tr>
<td><strong>Experiment II</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>1.18</td>
<td>0.411</td>
<td>1.84</td>
<td>0.246</td>
</tr>
<tr>
<td>Mean</td>
<td>0.284</td>
<td>0.119</td>
<td>0.428</td>
<td>0.0821</td>
</tr>
<tr>
<td>RNA</td>
<td>4.62</td>
<td>3.94</td>
<td>6.74</td>
<td>5.23</td>
</tr>
<tr>
<td>Mean</td>
<td>1.40</td>
<td>1.35</td>
<td>1.52</td>
<td>1.36</td>
</tr>
<tr>
<td><strong>Experiment III</strong></td>
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<td></td>
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<tr>
<td>DNA</td>
<td>0.758</td>
<td>0.375</td>
<td>1.37</td>
<td>0.473</td>
</tr>
<tr>
<td>Mean</td>
<td>0.103</td>
<td>0.0382</td>
<td>0.127</td>
<td>0.0965</td>
</tr>
<tr>
<td>RNA</td>
<td>3.22</td>
<td>3.49</td>
<td>4.05</td>
<td>4.03</td>
</tr>
<tr>
<td>Mean</td>
<td>0.389</td>
<td>0.590</td>
<td>0.964</td>
<td>0.801</td>
</tr>
</tbody>
</table>

Effect of injected nucleosides on the hydroxyurea inhibition of radiophosphorus incorporation into DNA and RNA in vivo. Hydroxyurea dose was 1.5 mg per mouse. Mean and standard deviation are expressed as relative specific activity. In Experiment I, 0.3 micromoles of 2'-deoxyguanosine, 2'-deoxyadenosine, thymidine, and 2'-deoxycytidine was injected 30 minutes before sacrifice (n = 10). In Experiment II, 1.2 micromoles of adenosine, guanosine, uridine, and cytidine was injected 60 minutes before sacrifice (n = 7). In Experiment III, 2.0 micromoles of 2'-deoxyguanosine, 2'-deoxyadenosine, thymidine, and 2'-deoxycytidine was injected 60 minutes before sacrifice (n = 5).

DISCUSSION

It is apparent from a review of the literature that addition of ribonucleosides or deoxyribonucleosides may produce a variable effect depending upon the tissue under study. The data presented above indicate that, in 6C3HED ascites tumor in vitro, the addition of the four deoxyribonucleosides stimulates DNA synthesis and inhibits RNA synthesis, as measured by radiophosphorus incorporation, when these deoxyribonucleosides are added in a concentration greater than $10^{-4}$ M. When injected in vivo, the deoxyribonucleosides produce a stimulation of DNA synthesis. In these experiments, it appears that the concentration of the four deoxyribonucleosides limits the rate of DNA synthesis. A failure of other systems to show such stimulation may indicate that other factors are rate limiting, e.g., energy-generating systems. The inhibition of radiophosphorus incorporation into RNA by addition of the four deoxyribonucleosides implies that either there is competition for the kinases necessary to elevate the ribonucleosides to the triphosphate level, or that feedback inhibition by the deoxyribonucleosides produces a decreased de novo production of the ribonucleoside precursors of RNA. Significant amounts of the added deoxyribonucleosides are probably not converted to the free base form and subsequently metabolized to the ribonucleosides, since concentrations of the four ribonucleosides which are less than the concentration of the added deoxyribonucleosides produce a stimulation of RNA synthesis. The fact that availability of deoxyribonucleosides appears to be rate limiting in DNA synthesis under these conditions renders this system particularly desirable for a study of the effect of these metabolites as inhibitors of the hydroxyurea-produced inhibition of DNA synthesis.

The failure of added deoxyribonucleosides to correct the hydroxyurea effect presents a serious objection to the postulated mechanism of hydroxyurea action by inhibition of conversion of ribonucleotides to deoxyribonucleotides. It has been suggested...
therefore, that the failure of deoxyribonucleosides to correct the inhibition produced by hydroxyurea cannot be explained by postulating a second and alternate inhibition of DNA synthesis by the added deoxyribonucleosides being cleaved and the bases incorporated into ribonucleotides, or by suggesting that the added deoxyribonucleosides are not available for DNA synthesis.

Failure of added deoxyribonucleosides to reverse the hydroxyurea effect in this system may not be ascribed to failure of deoxyadenosine triphosphate formation or isotope dilution as had been suggested (1), since DNA synthesis is stimulated by such addition and the isotope used was $^{32}$P rather than thymidine. Such stimulation has been observed by others (19).

These data suggest that, in the system under study, hydroxyurea does not act primarily by inhibition of the conversion of ribonucleotides to deoxyribonucleotides. This should not be interpreted as indicating that hydroxyurea does not inhibit ribonucleotide reductase. Indeed, that this is probably one site of action is supported by the observation (Table 3) that hydroxyurea blocks the ribonucleoside stimulation of DNA synthesis. These data do suggest, however, that multiple sites of action may be involved and that additional inhibitory actions should be investigated.

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