Inhibition of DNA Synthesis in HeLa Cells by Hydroxyurea

S. E. PFEIFFER and L. J. TOLMACH

Committee on Molecular Biology, and the Edward Mallinckrodt Institute of Radiology, School of Medicine, Washington University, St. Louis, Missouri

Summary

Hydroxyurea is a highly specific, rapidly acting inhibitor of DNA synthesis in HeLa S3 cells. It is without detectable effect on cells that are not synthesizing DNA. Incubation in inhibitory concentrations for as long as 19 hr does not kill cells in any phase of the division cycle. Rapid and complete reversal is accomplished simply by removing the drug from the culture medium.

Introduction

The introduction of hydroxyurea as a potentially useful agent in leukemia chemotherapy (e.g., Ref. 21) has led to several studies of its effects at the cellular level. Mohler (8) found that this drug both inhibits division and causes death of Chinese hamster cells and that it inhibits division of HeLa S3 cells as well. Young and Hodas (24) reported that synthesis of DNA in HeLa cells is inhibited by hydroxyurea, that neither RNA nor protein synthesis is inhibited, and that the inhibition of DNA synthesis is readily reversible 30 min after the initiation of treatment. They suggested, as did Frenkel et al. (2), that inhibition of DNA synthesis arises from interference with ribonucleotide diphosphate reduction. Finally, Sinclair (18) has recently reported that the toxicity of hydroxyurea for Chinese hamster cells is restricted to cells in the DNA-synthesizing (S) phase of the division cycle. He also found that the drug is without effect on the progression of these cells through the non-DNA-synthesizing portions of the cycle, so that cells incubated in its presence accumulate at the end of the pre-DNA-synthetic (G1) phase. This accumulation, together with the killing of the S-phase cells, suggested that hydroxyurea might be a useful agent for producing synchronous populations of cells.

The generality of the foregoing effects has not, however, been widely tested; indeed, Mohler (8) reported that while thymidine prevents the inhibitory action of hydroxyurea on Chinese hamster cells, it is without effect on hydroxyurea-inhibited HeLa S3 cells. It would appear, therefore, that different mammalian cell types may respond in qualitatively different ways to the introduction of hydroxyurea as a potentially useful agent for proliferation of mammalian cells.

Materials and Methods

HeLa S3 cells were grown in Medium N16HHF by conventional methods (3). Synchronization was carried out by the mitotic cell selection procedure (20), using a modified medium (N16FCF) containing 10% calf and 5% fetal calf sera in place of the human and horse sera, in order to achieve stronger adherence of interphase cells to the growth vessel (12, 15); in addition, calcium was eliminated until the time of collection to improve the yield (16). All experiments were carried out in Medium N16FCF unless otherwise noted and were timed from collection of mitotic cells.

The rates of DNA, RNA, and protein synthesis were determined from measurements of the incorporation of radioactive precursors. Small volumes (10-20 µl) of ³H-labeled thymidine (0.05 µc; 33 mc/m mole), uridine (0.1 µc; 230 mc/m mole), or leucine (0.2 µc; 198 mc/m mole) were added to 1 ml of culture medium for pulse (10-30 min) labeling of DNA, RNA, or protein, respectively. Additional unlabeled thymidine (10⁻⁴ m) was added for continuous labeling of DNA. At the end of the labeling period the growth medium was withdrawn and the cultures were quickly rinsed twice with buffered saline and fixed with acetic acid-ethanol (1:3) for at least 30 min. Cultures labeled with uridine-¹⁴C were fixed as above and washed twice with 0.2 N perchloric acid for at least 1 hr/wash, all at 4°C. Finally, the culture dishes were rinsed with 70% ethanol and air dried. The bottoms were punched out for counting in a low-background Geiger counter.

Cell division was monitored by repeated microscopic observations of delineated fields (7). The mean doubling time of the cells in Medium N16FCF was about 24 hr. Cell survival was determined by scoring colonies containing 50 or more cells after 10-14 days of incubation.

Hydroxyurea, obtained originally from Miss Barbara Stearns of The Squibb Institute for Medical Research, was dissolved in double distilled water and stored at -20°C until just before use. A calculated volume of a 100-fold concentrated stock solution was added to a culture to obtain the concentration desired. Additional unlabeled thymidine (0.05 µc; 33 mc/m mole), uridine (0.1 µc; 230 mc/m mole), or leucine (0.2 µc; 198 mc/m mole) were added to 1 ml of culture medium for pulse (10-30 min) labeling of DNA, RNA, or protein, respectively. Additional unlabeled thymidine (10⁻⁴ m) was added for continuous labeling of DNA. At the end of the labeling period the growth medium was withdrawn and the cultures were quickly rinsed twice with buffered saline and fixed with acetic acid-ethanol (1:3) for at least 30 min. Cultures labeled with uridine-¹⁴C were fixed as above and washed twice with 0.2 N perchloric acid for at least 1 hr/wash, all at 4°C. Finally, the culture dishes were rinsed with 70% ethanol and air dried. The bottoms were punched out for counting in a low-background Geiger counter.

Materials and Methods

HeLa S3 cells were grown in Medium N16HHF by conventional methods (3). Synchronization was carried out by the mitotic cell selection procedure (20), using a modified medium (N16FCF) containing 10% calf and 5% fetal calf sera in place of the human and horse sera, in order to achieve stronger adherence of interphase cells to the growth vessel (12, 15); in addition, calcium was eliminated until the time of collection to improve the yield (16). All experiments were carried out in Medium N16FCF unless otherwise noted and were timed from collection of mitotic cells.

The rates of DNA, RNA, and protein synthesis were determined from measurements of the incorporation of radioactive precursors. Small volumes (10-20 µl) of ³H-labeled thymidine (0.05 µc; 33 mc/m mole), uridine (0.1 µc; 230 mc/m mole), or leucine (0.2 µc; 198 mc/m mole) were added to 1 ml of culture medium for pulse (10-30 min) labeling of DNA, RNA, or protein, respectively. Additional unlabeled thymidine (10⁻⁴ m) was added for continuous labeling of DNA. At the end of the labeling period the growth medium was withdrawn and the cultures were quickly rinsed twice with buffered saline and fixed with acetic acid-ethanol (1:3) for at least 30 min. Cultures labeled with uridine-¹⁴C were fixed as above and washed twice with 0.2 N perchloric acid for at least 1 hr/wash, all at 4°C. Finally, the culture dishes were rinsed with 70% ethanol and air dried. The bottoms were punched out for counting in a low-background Geiger counter.

Cell division was monitored by repeated microscopic observations of delineated fields (7). The mean doubling time of the cells in Medium N16FCF was about 24 hr. Cell survival was determined by scoring colonies containing 50 or more cells after 10-14 days of incubation.

Hydroxyurea, obtained originally from Miss Barbara Stearns of The Squibb Institute for Medical Research, was dissolved in double distilled water and stored at -20°C until just before use. A calculated volume of a 100-fold concentrated stock solution was added to a culture to obtain the concentration desired. Additional unlabeled thymidine (0.05 µc; 33 mc/m mole), uridine (0.1 µc; 230 mc/m mole), or leucine (0.2 µc; 198 mc/m mole) were added to 1 ml of culture medium for pulse (10-30 min) labeling of DNA, RNA, or protein, respectively. Additional unlabeled thymidine (10⁻⁴ m) was added for continuous labeling of DNA. At the end of the labeling period the growth medium was withdrawn and the cultures were quickly rinsed twice with buffered saline and fixed with acetic acid-ethanol (1:3) for at least 30 min. Cultures labeled with uridine-¹⁴C were fixed as above and washed twice with 0.2 N perchloric acid for at least 1 hr/wash, all at 4°C. Finally, the culture dishes were rinsed with 70% ethanol and air dried. The bottoms were punched out for counting in a low-background Geiger counter.

Results

CONCENTRATION DEPENDENCE OF INHIBITION. The inhibition of DNA synthesis as a function of hydroxyurea concentration was determined by measuring thymidine-¹⁴C incorporation in a series of randomly dividing cultures over a 3-hr period in the

1This investigation was supported by USPHS Research Grant No. CA-04483 from the National Cancer Institute.

1Trainee supported by USPHS Training Grant 5T1 GM-714. Received May 27, 1966; accepted August 18, 1966.

124 CANCER RESEARCH VOL. 27

Received May 27, 1966; accepted August 18, 1966.
Inhibition of DNA Synthesis

Chart 1. Rate of thymidine-\(^{14}\)C incorporation (% of untreated control) into randomly dividing HeLa cell cultures as a function of hydroxyurea concentration (see text for details of the method).

Chart 2. Effect of increasing concentrations of hydroxyurea on cellular progression of synchronous cultures of HeLa cells (2 experiments combined) as examined by the continuous incorporation of thymidine-\(^{14}\)C into DNA (open symbols) and cell division (closed symbols; 100-160 cells present at 7 hr). Hydroxyurea concentrations: 0 mM (○), 0.025 mM (△), 0.25 mM (○), 1 mM (▽), and 5 mM (□).

Presence of the drug. Semilogarithmic plots of the cpm incorporated as a function of the duration of exposure to hydroxyurea and thymidine-\(^{14}\)C were drawn, the exponential rate constant for each concentration, relative to that for the untreated control, being taken as a measure of the degree of inhibition of DNA synthesis (Chart 1). The 50% inhibition concentration of \(5 \times 10^{-4}\) M is close to that found by Young and Hodas (24) for HeLa cells \((1 \times 10^{-4}\) M\), but is less than Mohler (8) found for Chinese hamster cells \((6.5 \times 10^{-4}\) M\) or Yarbro et al. (23) found for ascites tumor cells \((about 3 \times 10^{-4}\) M\). At about 1 mM the rate of in-
Inhibition of DNA Synthesis

corporation is reduced to 2% of that in untreated cultures; it remains constant at that level to at least 5 mM. In subsequent experiments in which maximal inhibition was desired, 2.5 mM hydroxyurea was used.

Chart 2 shows that when increasing concentrations of hydroxyurea are added to synchronous cultures immediately after the harvest of mitotic cells, the subsequent rate of DNA synthesis is correspondingly reduced, as would be predicted from the data of Chart 1. As expected, the nearly complete inhibition of DNA synthesis at concentrations above 1 mM prevents cell division; no division can be detected by 25 hr, while 80% of the cells in untreated cultures have divided by this time.

**Restriction of Inhibitory Action to the S Phase.** Because of experimental uncertainty arising from the low levels of thymidine-14C incorporated at early times, it is difficult to determine from the data of Chart 2 whether hydroxyurea has any effect on the progression of cells through G1. To test this point, synchronous cells were treated continuously with 2.5 mM hydroxyurea from 0 to 5 hr, i.e., before the normal start of the S phase (Chart 3A). There was no lag in the onset of DNA synthesis, nor any delay in progression through the S phase or cell division; treatment confined to G1 is without detectable effect on cell progression.

In a similar experiment, hydroxyurea was added to synchronous cells 18 hr after collection, when the majority were in the post-DNA-synthetic (G2) phase. Subsequent monitoring of cell division (Chart 3B) showed that cells in the treated culture began to divide at the same time as in the control culture and that division proceeded at the control rate for about 4 hr, the duration of G1 for these cells (20). The division rate then declined in the treated cultures, finally reaching zero when 54% of the cells had divided; in contrast, more than 90% of the control cells divided. An estimate of the number of cells still in the S phase at 18 hr was made from the ratio of the rate of DNA synthesis at this time relative to the rate 14 hr after collection, when DNA was being synthesized most rapidly. The ratio indicates that approximately 40% of the cells were still synthesizing DNA at the time of addition of hydroxyurea. This value is in agreement with the fraction of cells, relative to control, that did not divide (0.4). Thus, it appears that the progression of cells in G2, as in G1, is not affected by 2.5 mM hydroxyurea. Since the cells proceeding to mitosis divided normally, the same conclusion can be drawn for progression through mitosis.

**Specificity of Action.** If the rate of uridine incorporation into RNA is measured in hydroxyurea-treated synchronous G1 cells, little or no inhibition of RNA synthesis can be detected. Similarly, no inhibition of leucine incorporation into protein can be detected under these conditions. Table 1 presents data illustrating this for cells to which hydroxyurea was added at 3.75 hr and which were tested at later times. Analogous results were obtained with randomly dividing cultures in which DNA synthesis was inhibited 98% by addition of the drug and tested immediately thereafter.

It would appear from these results, from the report of Young and Hodas (24), and from the restriction of the inhibitory action of hydroxyurea to the S phase, that the drug affects only DNA synthesis. However, as will be described elsewhere, treatment with hydroxyurea severely inhibits the increase in the rate of RNA synthesis that normally occurs early in the S phase of this system. Nevertheless, as the rate of RNA synthesis is not decreased, it is likely that the effect is an indirect consequence of the inhibition of DNA synthesis; a similar effect on acceleration of RNA synthesis is brought about by other DNA inhibitors as well (Pfeiffer, unpublished data).

**Speed of Action, Reversibility, and Toxicity.** It is often desirable that an inhibitor act rapidly and be highly (and, preferably, rapidly) reversible. In addition, if the studies in which it is to be used involve cell survival (colony-forming ability) measurements, the temporary inhibition must cause little or no cell killing.

The speed with which hydroxyurea inhibits DNA synthesis is shown in Chart 3C. When added to synchronous cells synthesizing DNA at the peak rate, maximal inhibition occurs within the time required for experimental manipulation of the cultures.

Reversibility was examined by exposing mitotic cells to hydroxyurea for 16.6 hr and then removing the inhibitor. Chart 3D shows the effect on both DNA synthesis and cell division. The following observations are pertinent: (a) DNA synthesis (closed circles) resumes immediately after the drug is removed, even after a period of inhibition much longer than that studied by Young and Hodas (24); i.e., reversal is rapid. (b) DNA synthesis proceeds more rapidly in the treated culture than in the control (open circles), but the areas under the curves are the same within the limits of accuracy of the measurements; this

<table>
<thead>
<tr>
<th>PRECURSOR</th>
<th>TIME AFTER COLLECTION (hr)</th>
<th>RATE OF INCORPORATION (cpm)</th>
<th>CONTROL</th>
<th>TREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine-14C</td>
<td>6</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Uridine-14C</td>
<td>6</td>
<td>46</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>55</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

* 2.5 mM hydroxyurea was added at 3.75 hr.

**TABLE 1**

<table>
<thead>
<tr>
<th>PRECURSOR</th>
<th>TIME AFTER COLLECTION (hr)</th>
<th>RATE OF INCORPORATION (cpm)</th>
<th>CONTROL</th>
<th>TREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine-14C</td>
<td>6</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Uridine-14C</td>
<td>6</td>
<td>46</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>55</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

* 2.5 mM hydroxyurea was added at 3.75 hr.

b cpm/culture after 20 (leucine)- or 10 (uridine)-min pulses with labeled precursor.

---

† Autoradiographic determination of the fraction of thymidine-labeled cells at 18 hr would give a direct measure of the fraction of cells in S. Since the rate of incorporation of thymidine-14C by the culture is the product of the fraction of labeled cells and rate of synthesis per cell, the use of this parameter tends to underestimate the fraction of cells still in S (see Ref. 20, Fig. 6).

Chart 3. Rate of DNA synthesis (circles; thymidine-14C incorporation, 30-min pulses) and relative cell number (squares; 100-200 cells present at the first reading) versus time after collection of mitotic cells in control (open symbols) and hydroxyurea-treated (closed symbols; 2.5 mM) cultures of synchronous HeLa S3 cells. Hydroxyurea was present: A, 0-5 hr; B, continuously from 18 hr; C, continuously from 12 hr; and D, 0-16.6 hr.
indicates that delaying the onset of DNA synthesis does not affect the total amount of DNA synthesized. (c) The fraction of cells ultimately dividing (squares) is nearly the same in both cultures (control, 93%; treated, 90%). Observations (b) and (c) indicate that reversal of inhibition is complete on removal of the drug.

The increased rate of DNA synthesis observed in Chart 3D is due to 2 factors. First, an increase in the rate of DNA synthesis in the culture as a whole may result from an increase in the degree of synchrony arising from the accumulation of cells at the end of G1 in the presence of the inhibitor. Sinclair has demonstrated such behavior conclusively by autoradiography in Chinese hamster cells (18) treated with hydroxyurea, and, in fact, synchonization has been effected or enhanced with a number of DNA inhibitors in a variety of systems. Second, there is actual shortening of S for at least some cells. Treated cells began to divide as early as 8 hr after reversal of inhibition, whereas the minimum duration of S plus G2 in the untreated cells was 10 hr. (Since the duration of S plus G2 is quite constant among the cells of a given population (19, 20), it is presumed that the first cells to divide were the first to commence DNA synthesis.) Thus, at least the most rapidly progressing cells which have undergone temporary inhibition at the end of G1 appear to traverse the S and G2 phases more quickly than do untreated cells. This is not unexpected, inasmuch as the rate of DNA synthesis is faster after reversal of inhibition, while the amount of DNA synthesized is the same. Similar behavior has been noted by others in experiments in which DNA synthesis was delayed by treatment with different inhibitors (17, 22). Such distortion of the normal rate of cell progression must be taken into consideration when methods of synchronization based on inhibition of DNA synthesis are employed.

The toxicity of hydroxyurea was examined under a wide variety of conditions: randomly dividing cells, or synchronous cells in either S or G1, were exposed to hydroxyurea for periods ranging from 0.5 to 31.5 hr (Chart 4). Only after inhibition for more than 19 hr is the plating efficiency definitely reduced, in marked contrast to the reported behavior of Chinese hamster cells (8, 18). This delayed effect may be attributed to the long-term inhibition of DNA synthesis rather than to a specific effect of hydroxyurea; the effect of long-term inhibition with fluorodeoxyuridine has been examined in detail by others (17).

The foregoing plating efficiency measurements were carried out in Medium N16FCF, which is known to contain thymidine (presumably as a component of the fetal calf serum). Since Mohler had reported (8) that thymidine protects Chinese hamster cells from the inhibitory action of hydroxyurea, we examined the possibility that it might protect HeLa cells from loss of reproductive capacity, even though the experiments reported here show that it is unable to prevent inhibition of DNA synthesis by hydroxyurea in this cell type.4 When plating efficiencies were measured in synchronous populations growing either in Medium N16HHF (which lacks significant amounts of thymidine) or in N16HHF with 10^{-8} M thymidine added no cell killing was found after treatment for 4.5 hr during the S

---

4 In Mohler's experiments (8), thymidine appeared to protect Chinese hamster cells more against loss of viability than against inhibition of division.
phase (Chart 4, triangles). It may be concluded, therefore, that HeLa cells respond differently from Chinese hamster cells to treatment with hydroxyurea, in regard not only to toxicity during S but also to the effect of thymidine (8, 18).

Discussion

The present experiments confirm the report of Young and Hodas (24) concerning the specific inhibition by hydroxyurea of DNA synthesis in HeLa cells. In addition, information is presented regarding the absence of inhibitory effects on cells in phases of the division cycle other than S, the speed with which inhibition and reversal of this inhibition occurs, and the length of time that inhibition may be sustained before cell killing is observed. In this last respect these results with HeLa cells are in marked contrast to the killing effect of the drug observed in Chinese hamster cells (8) inhibited in the S phase of the division cycle (18). Whereas Sinclair (18) found that exposure of S-phase cells to hydroxyurea for 1 hr was sufficient to kill 80-90% of the population, Chart 4 indicates that HeLa cells can withstand up to 19 hr of exposure to the drug without significant toxic effects. Such divergent behavior between these 2 cell strains is unexplained. However, considerations of such differences are of possible significance in the application of hydroxyurea to tumor therapy.

In conclusion, hydroxyurea appears to closely resemble fluorodeoxyuridine (17, 22), deoxyadenosine (5, 6, 10, 13), and high concentrations of thymidine (5, 14) in being a rapidly acting inhibitor of DNA synthesis in animal cells; however, it possesses a particularly useful combination of properties for experimental studies. Thus, unlike inhibition by fluorodeoxyuridine, the action of hydroxyurea is readily reversed simply by removing the drug from the culture medium, and hydroxyurea has the added advantage of exerting its inhibitory effect in the presence of concentrations of thymidine that reverse the action of fluorodeoxyuridine. Further, the inhibitory action of hydroxyurea is specific for DNA synthesis, whereas thymidine at high concentrations also inhibits incorporation of uridine (4, 9, 11) or cytidine (1) into RNA. Thus, hydroxyurea would appear to be an important addition to the list of agents available for the study of growth dynamics in animal cells.6

References


* Some of the data presented in Charts 2 and 4 are from experiments performed by Dr. Robert A. Phillips and Dr. Barbara G. Weiss.
Inhibition of DNA Synthesis in HeLa Cells by Hydroxyurea

S. E. Pfeiffer and L. J. Tolmach