

# Differential Effects of Hydroxyurea on Survival of Proliferating Cells *in Vivo*<sup>1</sup>

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## SUMMARY

A single injection of hydroxyurea inhibits DNA synthesis in continuously dividing cells (crypt cells of the small intestine, germinal centers of lymph follicles, basal layer of the tongue epithelium) and in quiescent tissues that are stimulated to synthesize DNA and divide by an appropriate stimulus (regenerating liver after partial hepatectomy, folic acid-stimulated kidney, isoproterenol-stimulated salivary gland). However, whereas a single injection of hydroxyurea produces extensive necrosis in the DNA-synthesizing cells of continuously dividing tissues, the same dose is ineffective in producing necrosis in the DNA-synthesizing cells of stimulated tissues. This differential action of hydroxyurea seems therefore capable of distinguishing DNA-synthesizing cells from different types of tissues.

## INTRODUCTION

It has been shown by Philips *et al.* (12) that hydroxyurea damages irreversibly the DNA-synthesizing cells of the crypts of the small intestine, although it has no such effect on DNA-synthesizing cells of regenerating liver (13). Crypt cells of the small intestine are continuously dividing cells, while liver cells are quiescent cells that are stimulated to enter DNA synthesis and mitosis only by the stimulus of partial hepatectomy (6). The purpose of these experiments was to determine whether this difference in sensitivity to hydroxyurea could be extended to other continuously dividing cells and to other models of stimulated DNA synthesis. The damage produced by hydroxyurea in DNA-synthesizing cells was studied in the following tissues: (a) the crypts of the small intestine, the basal layer of the tongue epithelium, and the germinal centers of lymph follicles, which represent continuously dividing tissues (7), and (b) the folic acid-stimulated kidney (15), the isoproterenol-stimulated salivary gland (1, 2), and the regenerating liver, which represent models of quiescent tissues that can be stimulated to enter DNA synthesis and mitosis by an appropriate

stimulus. The results, both in rats and mice, confirm that continuously dividing cells, while in DNA synthesis, are much more easily killed by hydroxyurea than DNA-synthesizing cells from stimulated tissues.

## MATERIALS AND METHODS

**Animals.** Male A mice, bred at the Fels Research Institute, were used when 4-5 months old and weighing 30-33 gm. The rats were male white Wistar (Carworth Farms) weighing 180-250 gm. All animals were given food and water *ad libitum*.

**Reagents.** Folic acid (Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in sodium carbonate to a concentration of 60 mg/ml and was injected intraperitoneally. Isoproterenol [ $\alpha$ (isopropylaminomethyl)-protocatechyl alcohol], purchased from Winthrop Laboratories, New York, was dissolved in distilled water (150 mg/ml) and injected intraperitoneally. Hydroxyurea (Nutritional Biochemicals Corp. and from the Cancer Chemotherapy National Service Center) was dissolved in 0.9% NaCl (90 mg/ml) and was also injected intraperitoneally. Thymidine-methyl-<sup>3</sup>H, 6.7 c/mmole (New England Nuclear Corp.) was dissolved in sterile water (50  $\mu$ c/ml) and injected subcutaneously in the back. All other chemicals were of reagent grade.

**Experimental Procedures.** The animals were killed by cervical dislocation (mice) or by decapitation (rats). The tissues were fixed in calcium-formalin, embedded, and the sections autoradiographed by the method previously described (4). Eastman Kodak NTB emulsion was used, and the exposure time was 25 days. After photographic processing, the sections were stained with hematoxylin and eosin.

**Counting of Cells and Determination of Cell Damage.** The percentage of cells labeled by thymidine-<sup>3</sup>H was determined in: (a) the lining epithelium of the crypts of the jejunum; (b) the cells of the kidney tubules; (c) the acinar cells of the parotid gland; and (d) the basal layer of the surface epithelium of the tongue. The percentage of labeled cells in these four types of cells was determined in all control animals receiving saline alone, in jejunal and tongue epithelium in animals injected with saline and hydroxyurea, in the renal tubules in animals receiving folic acid, and on the acinar cells of the parotid gland in animals injected with isoproterenol. A minimum of 1,000 cells was counted for each animal. No attempt was made to estimate the percentage of labeled cells in lymph follicles because of uncertainties in cell classification. Necrotic

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cells (i.e., cells showing dissolution of the nuclear membrane and disruption of the nuclear material) could be counted with reasonable accuracy in the crypts of the jejunum, the basal layer of the tongue epithelium, and the acinar cells of the parotid gland (no necrotic cells were found in the kidneys). The recognition of necrotic cells was made easier by prelabeling with thymidine-<sup>3</sup>H and autoradiography, the necrotic nuclei showing clumps of labeled chromatin scattered within or even without the cell boundaries. The number of necrotic cells in the lymph follicles was not determined, but the germinal centers were simply classified as necrotic or nonnecrotic.

**Determination of Specific Activity of DNA.** In Experiment 2 in which the specific activity of parotid gland DNA or kidney DNA was measured, the animals were killed 30 min after a subcutaneous injection of thymidine-<sup>3</sup>H. The parotid glands and the kidneys were homogenized in 0.25 M sucrose containing 5% citric acid, and the specific activity of DNA was determined as described previously (9).

## RESULTS

**Experiment 1.** Four groups of 6 mice each were treated as indicated in the first column of Table 1. After being injected with thymidine-<sup>3</sup>H, each group was divided into two subgroups: one-half received hydroxyurea, the other half an equal volume of saline. All animals were killed 3 hr after hydroxyurea (or saline), and autoradiographs of selected tissues were made. The results are shown in Table 1. Kidneys and parotid glands from control animals not injected with either folic acid or isoproterenol are not included in Table 1, but they showed the usual low percentage of labeled cells previously reported in the literature, i.e., 0.2% for the cells of the kidney tubules (12) and 0.1% for the acinar cells of the parotid gland (4). Column 3 of Table 1 shows that folic acid was effective in stimulating DNA synthesis in the kidney, and isoproterenol in stimulating DNA synthesis in the parotid.

Necrotic cells in large number could be found in the crypts of the jejunum and among the labeled cells of the basal layer of the tongue epithelium. Only an occasional labeled cell in the parotid was necrotic; unlabeled cells in the parotid and kidney cells (labeled or unlabeled) did not show any detectable damage.

**Experiment 2.** Although the inhibitory effect of hydroxyurea on DNA synthesis has been established in both small intestine (12) and regenerating liver (13), we felt that such inhibition ought to be proved also for the folic acid-stimulated kidney and the isoproterenol-stimulated salivary gland. Table 2 shows that this is the case: hydroxyurea effectively inhibits DNA synthesis in kidneys stimulated by folic acid and in parotid glands stimulated by isoproterenol.

**Experiment 3.** An objection may be made that a 3-hr interval between hydroxyurea and killing might not be sufficient for cell necrosis to develop and become histologically visible. For this purpose, mice injected with isoproterenol or folic acid were given hydroxyurea, respectively, 26 and 40 hr later. They were then divided into groups that were killed 6, 12, 24, and 48 hr after hydroxyurea. No necrosis was ever found in the kidney cells of the folic acid-injected mice. About 2% of the parotid cells from isoproterenol-injected mice were found to be necrotic 6 hr after hydroxyurea, but apparently the necrotic cells must have been quickly eliminated because, at later times, no necrotic cells were found.

**Experiment 4.** A group of mice was injected intraperitoneally with 0.25 mg of isoproterenol/gm body weight, an amount that is known to produce a marked increase in DNA synthesis in the salivary glands of both rats (1) and mice (2), with onset at about 20 hr and peak at 26–28 hr. Twenty-six hr after this first injection of isoproterenol, the animals were divided into two groups: one-half received saline, the other half hydroxyurea. At 48 hr, all mice received a second injection of isoproterenol, which causes a second wave of DNA synthesis (16), followed by thymidine-<sup>3</sup>H 26 hr later. The specific activity of salivary gland DNA was the same in both groups of animals (saline group, 2900, 3600, 4000 cpm/mg DNA; hydroxyurea group, 4000, 4000, 3400 cpm), confirming that the damage done by hydroxyurea to DNA-synthesizing cells (26 hr after the first injection), if any, is transitory or of modest proportions.

**Experiment 5.** This was essentially the same as Experiment 1, except that rats were used instead of mice and the doses of hydroxyurea, folic acid, and isoproterenol were slightly decreased. The results (Table 3) confirm the results obtained in mice, namely, that hydroxyurea produced necrosis in continuously dividing cells (crypts of the jejunum and germinal cen-

Table 1

Treatment	Tissue	Labeled cells per thousand	Necrosis
Saline only	Jejunum	332 ± 28	No necrosis
Hydroxyurea only	Jejunum		50–60% necrosis of crypts; no labeled cells intact
Saline only	Tongue	75 ± 7	No necrosis
Hydroxyurea only	Epithelium		45% of labeled cells necrotic
Folic acid + saline <sup>a</sup>	Kidney	93 ± 5	No necrosis
Folic acid + hydroxyurea <sup>a</sup>	Kidney		No necrosis
Isoproterenol + saline <sup>b</sup>	Parotid	207 ± 27	No necrosis
Isoproterenol + hydroxyurea <sup>b</sup>	Parotid		1–4% of labeled cells necrotic

Effect of hydroxyurea on mouse cells in DNA synthesis. All mice were injected with 0.3  $\mu$ C/gm body wt. of thymidine-<sup>3</sup>H and, 30 min later with hydroxyurea (1.5 mg/gm body wt.) or saline. All animals were killed 3 hr after hydroxyurea or saline. All injections were intraperitoneal.

<sup>a</sup>Folic acid, 0.25 mg/gm body wt., 26 hr before hydroxyurea.

<sup>b</sup>Isoproterenol, 0.25 mg/gm body wt., 44 hr before hydroxyurea.

Table 2

Treatment	dpm/mg DNA <sup>a</sup>	
	Parotid gland	Kidney
None	450 ± 120	50 ± 18
Folic acid + saline		7250 ± 660
Folic acid + hydroxyurea		Background level
Isoproterenol + saline	5114 ± 500	
Isoproterenol + hydroxyurea	Background level	

Effect of hydroxyurea on DNA synthesis in stimulated parotid gland and kidney. All mice were injected with 0.03  $\mu$ g/gm body wt. of thymidine-<sup>3</sup>H, 30 min before killing. Hydroxyurea (1.5 mg/gm body wt. in saline) or saline were given 30 min before thymidine-<sup>3</sup>H. Folic acid (0.25 mg/gm body wt.) was injected 42 hr before hydroxyurea and isoproterenol (0.25 mg/gm body wt.) 25 hr before hydroxyurea. All injections were intraperitoneal.

<sup>a</sup>Mean ± standard deviations.

ters of the lymph follicles) but not in cells stimulated to enter DNA synthesis from a quiescent state (folic acid-stimulated kidney cells and isoproterenol-stimulated salivary glands).

**Experiment 6.** Groups of 2 rats each were injected with hydroxyurea (200 mg per kg body weight) at 4, 8, 16, 20, or 24 hr after being subjected to partial hepatectomy (about 67%) (8) and were killed 24 hr later. This 24-hr interval is sufficient to allow ready histologic identification of dead liver parenchymal cells. Careful study of many sections of each liver failed to reveal any evidence of liver cell necrosis, even in those animals which received hydroxyurea during the period of DNA synthesis (16, 20, and 24 hr). Similar doses regularly induce intestinal crypt cell necrosis and cell death in many cells of the germinal centers of lymphoid tissue in rats.

In order to check further the results with the liver, a portion of the experiment was repeated with the added feature of prelabeling the regenerating liver cells with thymidine-methyl-<sup>3</sup>H (1  $\mu$ g/gm body weight) at 17 hr after partial hepatectomy. The hydroxyurea (250 mg/kg body weight) or saline were injected at 18 hr, and the animals (2 per group) were killed 18 hr later, i.e., 36 hr after operation. Again, no differences were noted between the saline- and the hydroxyurea-treated animals except for the presence of more mitotic figures in the latter group (presumably due to the delay in the completion of the cell cycle caused by interruption of the S phase by the inhibi-

tion of DNA synthesis). No labeled or unlabeled cells were necrotic.

## DISCUSSION

It is evident from the results of this study that proliferating cells respond quite differently to the same inhibitor of DNA synthesis, hydroxyurea (even though the degree of inhibition is comparable). Although measurement of DNA synthesis in the intestine and liver was not done in this study, it is apparent from the literature that these tissues show almost complete inhibition of DNA synthesis with doses of hydroxyurea similar to those used in this study (12, 13). Unpublished work in our laboratories with many animals has confirmed these findings. Therefore, it is evident that it is not the variation in response to hydroxyurea with respect to its effect on DNA synthesis but rather some other facet of cell organization which must underlie the difference in the response patterns of the different cells to this inhibitor.

In addition, variation in the number of cells in DNA synthesis cannot be held responsible for the difference between continuously dividing and stimulated cells. The fraction of cells that enter DNA synthesis after the appropriate stimulation has been found to be 90 percent in the regenerating liver after partial hepatectomy (14), 60 percent in the parotid acinar cells after isoproterenol (16), and 20 percent in the renal tubular cells after folic acid (5). In all cases, if cells in DNA synthesis were sensitive to the necrotizing action of hydroxyurea, necrosis should be easy to detect.

An important consideration relevant to the eventual elucidation of the molecular events is whether the differences observed in this study are unique to hydroxyurea or are more general and are seen with other compounds interfering with DNA metabolism. No final answer on this question is available. However, in unpublished work of the authors, a similar response pattern has been seen with arabinosylcytosine (cytosine arabinoside, ara-C) and with the nitrogen mustard 2,2'-dichloro-N-methyldiethylaniline (HN2) in respect to intestinal crypt cells, lymphoid tissue, and regenerating liver. These two agents readily induce crypt cell and selected lymphoid tissue necrosis without any evidence from autoradiographic and regu-

Table 3

Treatment	Tissue	Labeled cells per thousand	Necrosis
Saline only	Jejunum	293	No necrosis
Hydroxyurea only	Jejunum	289	10–15% necrotic crypt cells; labeled cells often fragmented
Saline only	Germinal centers		No necrosis
Hydroxyurea only	Germinal centers		Central necrosis, modest
Folic acid + saline <sup>a</sup>	Kidney	3	No necrosis
Folic acid + hydroxyurea <sup>a</sup>	Kidney	43	No necrosis
Isoproterenol + saline <sup>b</sup>	Submandibular gland	1	No necrosis
Isoproterenol + hydroxyurea <sup>b</sup>	Submandibular gland	122	No necrosis

Effect of hydroxyurea on rat cells in DNA synthesis. All rats were injected with 0.25  $\mu$ g/gm body wt. of thymidine-<sup>3</sup>H and, 30 min later, with hydroxyurea (0.15 mg/gm body wt.) or saline. All animals were killed 3.5 hr after hydroxyurea or saline. All injections were intraperitoneal.

<sup>a</sup>Folic acid, 0.15 mg/gm body wt., 41.5 hr before hydroxyurea.

<sup>b</sup>Isoproterenol, 0.15 mg/gm body wt., 24.5 hr before hydroxyurea.

lar histologic study of cell death in regenerating liver cells. Thus, it is very probable that the varying patterns seen in response to hydroxyurea are not an exclusive function of this agent but are related to some basic difference in metabolic organization.

One might argue that the regenerating liver and the proliferating salivary acinar and renal tubular cells would be just as responsive to an interference with DNA metabolism as are the crypt and lymphoid cells if the agent were administered at some other time during the S phase. Although no data on this point are available for the salivary gland and kidney, recent data obtained in one of our laboratories, with the regenerating liver and ara-C, do not favor this alternate explanation. When ara-C (250 mg/kg body weight per dose) was administered intraperitoneally repeatedly every 3 hr from 13 to 25 hr after partial hepatectomy, no evidence for cell death and necrosis of prelabeled (with thymidine-methyl-<sup>3</sup>H) or unlabeled cells was observed. Thus, it must be tentatively concluded that the differences observed between the different cell types in the present study are probably not due to some trivial difference in timing, etc., but they reflect more fundamental properties of the cells. In addition, the cell cycles of the isoproterenol-stimulated parotid cells (3) or of the folic acid-stimulated kidney cells (5) are quite similar to the cell cycle of intestinal crypt cells (10).

The most obvious difference between the tissues studied is their physiologic behavior. The intestinal crypt cells and the basal cells of the tongue are continually dividing under physiologic circumstances, while the majority of the liver, kidney, and salivary gland cells do so only in response to stimuli not normally operating to any significant degree in the usual animal. The lymphoid tissue is difficult to characterize. Mitotic figures are regularly observed in lymphoid tissue in most animals. Therefore, cells in this tissue fall into the first category. However, whether this continual proliferation is due to the ever present antigenic stimuli or due to some other physiologic need is not clear.

An important implication of the results of this study concerns the mechanism of cell death induced by agents that interfere with DNA metabolism. It is evident that inhibition of DNA synthesis *per se* in a proliferating cell is not the immediate cause of cell death but rather some metabolic event or events which are somehow geared to the synthesis of DNA. A knowledge of the nature of such coupled reactions and the reason for their apparent absence in some cells would lead to new insight into the important subject of intracellular metabolic organization as a basis for understanding varying response patterns of cells.

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