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Comparison of Cytotoxicity of Hydroxyurea in Normal and Rapidly Proliferating Epidermis and Small Intestine in Mice[1]

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SUMMARY

Cytotoxicity of hydroxyurea has been studied in mouse epidermis and small intestine by determining the loss of thymidine-^{3}H-labeled DNA by both autoradiographic and biochemical methods. In the normal epidermis, hydroxyurea-induced cytotoxicity appears to be limited to some of the DNA-synthesizing cells, which are lost within 12 hr after hydroxyurea injection. Labeled cells that survive hydroxyurea treatment proceed through mitosis. A low dose of hydroxyurea, which inhibits DNA synthesis effectively for at least 1 hr, is not cytotoxic.

In rapidly proliferating epidermis 18 hr after an application of croton oil, the duration of the inhibition of DNA synthesis by a given dose of hydroxyurea is somewhat reduced, and the loss of prelabeled DNA is greatly reduced. In contrast, the extent of hydroxyurea-induced cytotoxicity in the rapidly regenerating small intestine 3 days after whole-body X-irradiation with 1000 R was only slightly less than that found under normal conditions. This difference between the number of cells killed by hydroxyurea in the two stimulated tissues may be related to the degree of increase in cell proliferation rate. The thymidine-^{3}H labeling index in croton oil-treated epidermis was increased more than 10-fold, while that in the small intestine of X-irradiated mice was increased less than 2-fold.

INTRODUCTION

Hydroxyurea promptly inhibits DNA synthesis, without affecting RNA or protein synthesis, when added to cultured mammalian cells or injected into experimental animals (18, 20). Both *in vivo* and *in vitro*, hydroxyurea is selectively toxic to cells synthesizing DNA (12, 16). The killing of cells in other phases of the cell cycle, observed in cell culture after extended treatment (16), has not been observed *in vivo* after a single injection of hydroxyurea, apparently because of the rapid loss of hydroxyurea from plasma and tissues (12).

The molecular mechanisms of action of hydroxyurea have not been fully elucidated. Hydroxyurea-induced cytotoxicity and inhibition of DNA synthesis can be partially reversed by addition of deoxyribonucleosides (9, 21); the reduction of ribonucleotides to deoxyribonucleotides is blocked by hydroxyurea via inhibition of ribonucleotide reductase (7).

Although hydroxyurea is toxic to DNA-synthesizing cells in the intestine (11), no cell killing was found in regenerating liver (14). This difference in cytotoxicity between cells in continuously dividing tissues and in quiescent tissues stimulated to divide has been studied in detail by Farber and Baserga (5). However, the cytotoxicity of hydroxyurea has not been determined in continuously dividing tissues stimulated to a more rapid rate of proliferation.

In this report, the cytotoxicity of hydroxyurea is compared in normal epidermis and in rapidly proliferating epidermis 18 hr after an application of croton oil. A similar comparison is made in the normal intestine and the rapidly regenerating intestine 3 days after whole-body X-irradiation. The loss of DNA-synthesizing cells from these tissues has been examined by autoradiographic and biochemical procedures.

MATERIALS AND METHODS

Materials. Female hairless (*hr/hr*) mice, 60 to 75 days old and weighing 20 to 25 g, were used in the experiments with epidermis. Female mice of the WLO strain, 70 to 90 days old and weighing 22 to 28 g, were used in the experiments with intestine.

Hydroxyurea was purchased from the Pierce Chemical Company, Rockford, Ill. Thymidine-methyl-^{3}H (6.7 Ci/mmoles) was obtained from the New England Nuclear Corporation, Boston, Mass. Solutions for injection were prepared in sterile 0.154 M NaCl solution.

Nucleic Acid Extraction. Nucleic acid hydrolysates were prepared from samples of intestine or acetic acid-separated epidermis pooled from groups of 4 mice (6). After the tissues were homogenized in ice water, the macromolecules were precipitated with perchloric acid, and the precipitates were washed with dilute perchloric acid and 96% ethanol. The nucleic acids were then hydrolyzed in 5% perchloric acid at 90° for 20 min.

Rate of DNA Synthesis. Mice were given i.p. injections of 30 μCi of thymidine-^{3}H 30 min before sacrifice. The specific activity of DNA at this time was used as an estimate of the rate of DNA synthesis. Aliquots of the nucleic acid hydrolysate prepared as described above were counted in a scintillation counter and used for DNA estimation by the diphenylamine reaction. The specific activity of DNA was expressed as cpm/μg DNA.

Cytotoxocity of Hydroxyurea. DNA-synthesizing cells were

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labeled by giving mice injections of thymidine-^{3}H. Thirty min later, the same mice were given injections of hydroxyurea or 0.154 M NaCl solution. In most experiments, mice were killed 24 hr later, and the fate of the labeled DNA was determined in one of two ways. (*a*) Autoradiographs were prepared after fixation of the tissue in 4% formalin, cutting at 4 μ, dipping in Kodak Nuclear Track Emulsion, and staining with hematoxylin. As a result of cell division, the number of labeled cells in epidermis and intestine should approximately double between a time soon after and 24 hr after thymidine-^{3}H injection. Cytotoxicity of hydroxyurea to DNA-synthesizing cells would reduce the number of labeled cells observed at 24 hr. In the normal epidermis or intestine, labeled cells are not lost from the tissues within 24 hr; the maturation times for cells in the epidermis and intestine are about 70 to 80 (17) and 46 hr (19), respectively. (*b*) In similar experiments, a nucleic acid hydrolysate was prepared and the specific activity of DNA was determined. In the normal epidermis or intestine, the specific activity of DNA remains constant from 30 min after thymidine-^{3}H injection until cells are lost after maturation. Cytotoxicity of hydroxyurea to cells in S phase would result in a reduction in the specific activity of DNA at 24 hr.

RESULTS

Experiments in Epidermis. Thirty min after thymidine-^{3}H injection, mice were given injections of either 0.154 M NaCl solution or 30 mg hydroxyurea. Groups of 8 mice were sacrificed at 3 and 24 hr, autoradiographs were prepared, and the labeling index was determined. In the NaCl solution-treated controls, the labeling index (number of labeled cells in 8 mm interfollicular epidermis) was increased from 31 ± 4.3 (S.D.) to 55 ± 11.9 between 3 and 24 hr. In the hydroxyurea-treated mice, the labeling index did not increase but rather decreased from 28 ± 7.4 to 19 ± 5.3 between 3 and 24 hr.

For determination of whether the labeled cells surviving 24 hr after hydroxyurea administration had divided, the number of silver grains over each labeled cell was counted. Three hr after hydroxyurea injection, the average grain count ± S.D. was 20.9 ± 2.9. By 24 hr, the grain count was reduced to 9.4 ± 1.6, indicating that the cells surviving hydroxyurea treatment have divided once. Histograms showing the distribution of grains are shown in Chart 1.

The cytotoxicity of hydroxyurea was also determined in mice treated with 0.5% croton oil 18 hr before administration of hydroxyurea or NaCl solution. At this time, the labeling index in the interfollicular epidermis was increased more than 10-fold. In the NaCl solution-treated controls, the labeling index increased from 411 ± 67.7 to 642 ± 71.0 between 3 and 24 hr. The lack of doubling probably indicates that many cells have divided by 3.5 hr after thymidine-^{3}H injection, due to a shortening of the duration of G_2 and mitosis (4) in croton oil-treated mice. Between 3 and 24 hr after hydroxyurea injection, the labeling index increased from 395 ± 73.6 to 496 ± 70.1. Thus, the cytotoxic effect of hydroxyurea was much less pronounced in croton oil-treated epidermis.

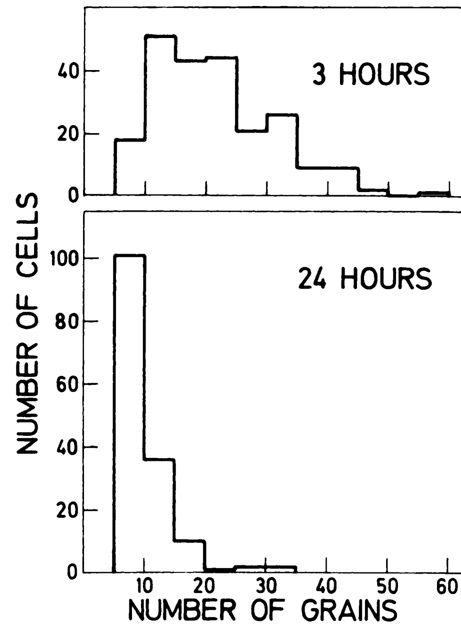

Chart 1. Distribution of grains over prelabeled epidermal cells 3 and 24 hr after hydroxyurea injection. Groups of 8 mice were given injections of 30 μCi thymidine-^{3}H 30 min before i.p. injection of 0.2 ml of a NaCl solution containing 30 mg hydroxyurea. Mice were killed 3 or 24 hr later, autoradiographs were prepared, and the number of grains over each labeled cell was determined. Cells with 0 to 5 grains were not counted; grain counts of 6 to 10, 11 to 15, etc., were pooled and are shown by the *bars* in the histograms.

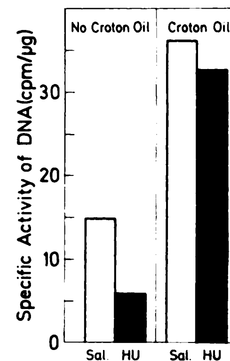

Chart 2. Effect of hydroxyurea on the specific activity of prelabeled epidermal DNA. Groups of 4 mice were given i.p. injections of 30 μCi thymidine-^{3}H 30 min prior to i.p. injection of 0.154 M NaCl solution (*Sal.*) or 30 mg hydroxyurea (*HU*). In the experiment at *right*, mice were also treated with 0.2 ml 0.5% croton oil at −18 hr. All animals were killed 24 hr after the injection of NaCl solution or hydroxyurea, and the specific activity of DNA (cpm/μg) was estimated.

The large reduction in the number of labeled cells 24 hr after hydroxyurea injection should be accompanied by a similar decrease in the specific activity of DNA isolated from skin. Twenty-four hr after hydroxyurea injection in normal, prelabeled mice, the specific activity of DNA was reduced to 5.8 cpm/μg, compared to a value of 11.1 at 3 hr. The reduction at 24 hr was greater than 60% when compared to NaCl solution-treated controls (Chart 2). In mice treated with croton oil, only a small reduction was found. These results

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agreed well with those obtained by counting labeled cells in autoradiographs; the biochemical technique was used in subsequent experiments.

The decreased toxicity of hydroxyurea in croton oil-treated epidermis suggested that the level of hydroxyurea reaching epidermal cells could be decreased, perhaps as a result of inflammation. However, 1 hr after injection of 30 mg of hydroxyurea, the specific activity of DNA was reduced to less than 2% of the NaCl solution-treated control in both normal (Chart 3) and croton oil-treated epidermis (Chart 4). By 3 hr the extent of inhibition was less in the croton oil-treated mice, and by 6 hr the rate of DNA synthesis had returned to the control level. In the normal epidermis, a 50% inhibition was still seen at that time. Thus, hydroxyurea produced a nearly complete inhibition of DNA synthesis in croton oil-treated mice, but the duration of the effect was shorter than in normal mice.

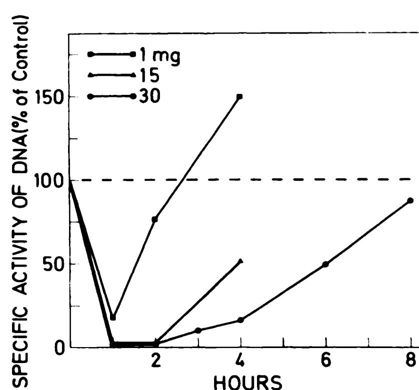

Chart 3. Inhibition of epidermal DNA synthesis by 1, 15, or 30 mg hydroxyurea. Groups of 4 mice were given i.p. injections of 0.154 M NaCl solution or 1, 15, or 30 mg hydroxyurea and killed at the times indicated. Thymidine-^{3}H, 30 μCi, was injected 30 min before sacrifice. At each time, the specific activity of epidermal DNA in the hydroxyurea-treated animals is plotted as a percentage of the specific activity in the controls given injections of NaCl solution.

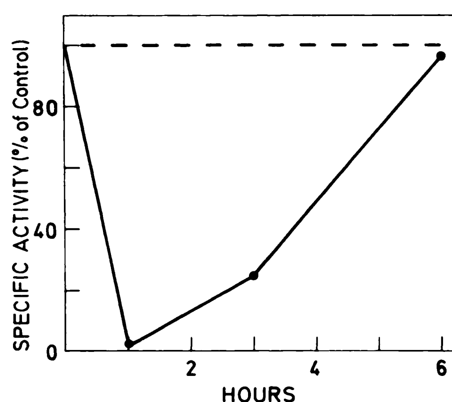

Chart 4. Inhibition of DNA synthesis by hydroxyurea in croton oil-treated mice. Groups of 4 mice were treated with 0.2 ml 0.5% croton oil in acetone 18 hr before i.p. injection of NaCl solution or 30 mg hydroxyurea. Mice were given i.p. injections of 30 μCi thymidine-^{3}H 30 min before killing at 1, 3, or 6 hr. At each time, the specific activity of DNA in the group treated with hydroxyurea is plotted as a percentage of that in the NaCl solution-treated control.

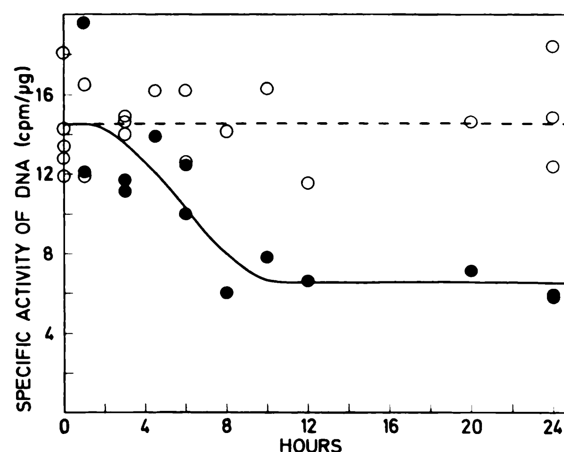

Chart 5. Kinetics of decrease in specific activity of prelabeled epidermal DNA after hydroxyurea injection. Groups of 4 mice were given i.p. injections of 30 μCi thymidine-^{3}H 30 min before zero time, when 0.2 ml NaCl solution or 30 mg hydroxyurea were injected i.p. Mice were killed at the times indicated, and hydrolysates of epidermal DNA were prepared. ○ and - - -, specific activity of DNA (cpm/μg) in the mice given NaCl injections; ● and —, specific activity of DNA in hydroxyurea-treated mice.

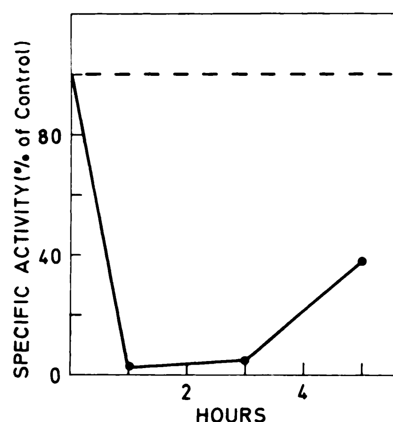

Chart 6. Inhibition of DNA synthesis in small intestine after injection of hydroxyurea. Groups of 4 mice were given i.p. injections of 0.154 M NaCl solution or 5 mg hydroxyurea. Thymidine-^{3}H, 30 μCi, was injected s.c. 30 min before sacrifice at 1, 3, or 5 hr. The specific activity of DNA (cpm/μg) in the hydroxyurea-treated mice is plotted as a percentage of the specific activity in the NaCl solution-treated controls.

In normal epidermis, injection of 1, 15, or 30 mg of hydroxyurea resulted in a prompt inhibition of DNA synthesis, with a more rapid recovery after the lower doses (Chart 3). After injection of 1 mg of hydroxyurea, a substantial inhibition of DNA synthesis lasted less than 2 hr.

The loss of tritium from prelabeled epidermis after hydroxyurea injections of 1 or 15 mg was compared with that found after 30 mg. The specific activity of DNA at 24 hr was reduced from the NaCl solution control value of 14.5 to 5.8 cpm/μg after injection of either 15 or 30 mg hydroxyurea. In contrast, injection of 1 mg hydroxyurea did not affect the specific activity of DNA (15.2 cpm/μg).

In intestine (12) and in cell culture (16), cells in S phase are

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Table 1

Number of prelabeled intestinal crypt cells after hydroxyurea injection

Samples of intestine for autoradiography were obtained from the mice used in the experiments shown in Chart 7. In mice prelabeled with 30 μCi thymidine-^{3}H, the number of labeled cells per crypt was estimated 1.5 and 26 hr after injection of 0.154 M NaCl solution or hydroxyurea.

Injection (zero time)	Time (hr)	Labeled cells/crypt ± S.D.	
		No X-irradiation	1000 R 3 days before
NaCl solution, 0.154 M	1.5	26 ± 6.2	43 ± 8.8
NaCl solution, 0.154 M	26	40 ± 5.5	67 ± 19
Hydroxyurea	1.5	27 ± 5.4	37 ± 8.2
Hydroxyurea	26	38 ± 6.3	36 ± 6.1

preferentially killed by hydroxyurea. In order to examine which cells are killed in the normal epidermis, mice were given thymidine-^{3}H, then given hydroxyurea at 1 of 3 times, and killed 20 hr after hydroxyurea. The times of hydroxyurea injection were chosen on the basis of the labeled mitosis curve in the interfollicular epidermis (2). Hydroxyurea was injected at either 0.5 hr, when labeled cells were in S or G_2 phase, or at 5 hr, when 80% of the mitoses were labeled, or at 10 hr, when only 20% of the mitoses were labeled and the rest of the cells were in G_1. Hydroxyurea injection reduced the specific activity of DNA only when labeled cells were in the S phase. No cytotoxic effect was observed when many labeled cells were in mitosis or G_1.

The time required for the hydroxyurea-induced loss of labeled S phase cells was investigated after injection of 30 mg hydroxyurea. No effect on the specific activity of DNA was seen at 1 hr, but by 3 to 6 hr, the specific activity was 20 to 25% lower than in NaCl solution-treated controls (Chart 5). At all times tested between 8 and 24 hr, the specific activity of DNA was reduced by 45 to 65%.

Experiments in Intestine. Experiments with the prelabeling technique were also performed with the intestine, in which the cytotoxic effects of hydroxyurea have been examined by histological methods (12).

An appropriate dose of hydroxyurea was chosen on the basis of its inhibition of DNA synthesis. As shown in Chart 6, the duration of the inhibition after injection of 5 mg hydroxyurea was similar to that found in the epidermis after injection of 30 mg. A large increase in the number of pycnotic cells in the crypts was seen 70 to 90 min after injection of 5 mg hydroxyurea. A peak in the number of pycnotic cells was found at 2 to 6 hr, after which the number decreased to normal by 24 hr.

Cytotoxicity was also examined by injecting thymidine-^{3}H, injecting hydroxyurea or NaCl solution 30 min later, and killing mice at either 1.5 or 26 hr. Autoradiographs were prepared from samples of small intestine taken midway between the duodenum and the cecum (3); DNA was isolated from intestine samples from the same mice.

In the controls given NaCl injections, the number of labeled cells per crypt increased from 26 to 40 between 1.5 and 26 hr (Table 1). The lack of doubling is probably due to division of some labeled cells by 1.5 hr, and migration of many labeled cells from the crypts to the villi within 26 hr (13). No

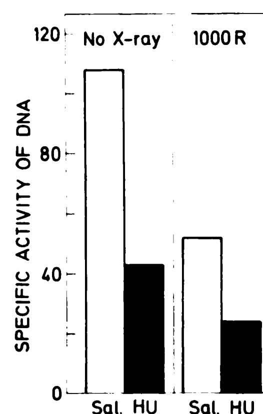

Chart 7. Effect of hydroxyurea on the specific acitivity of prelabeled DNA in the small intestine. Mice were given s.c. injections of 30 μCi thymidine-^{3}H 30 min before the i.p. injection of 0.154 M NaCl solution (*Sal.*) or 5 mg hydroxyurea (*HU*). In the experiment on the ***right***, mice also received whole-body X-irradiation of 1000 R. Groups of 4 mice were killed at 1.5 and 26 hr; for each treatment, the specific activity of DNA at 26 hr is expressed as the percentage of that found at 1.5 hr.

reduction in the number of labeled cells in the intestinal crypts was found after hydroxyurea injection (Table 1). However, 26 hr after hydroxyurea injection, the specific activity of DNA was reduced to less than 50% of that found in the NaCl solution-treated mice (Chart 7). In an attempt to explain this anomaly, the number of grains were counted over the cells labeled at 26 hr. In the NaCl solution-treated controls, the average number of grains per labeled cell ± S.D. was 11.6 ± 0.3, compared to a value of 8.9 ± 0.8 in the hydroxyurea-treated mice. On the average, the crypt cells in hydroxyurea-treated mice have apparently divided more times in 26 hr. However, this small difference in grain count can account for only part of the reduction in specific activity of DNA.

Since many labeled cells migrate to the villi within 26 hr, the distance traveled by the most distal-labeled cells was determined in the 2 groups. In the controls, labeled cells were found along 57% of the length of the villi. In contrast, labeled cells had traveled less than 40% of the length of the villi in hydroxyurea-treated mice. This result, along with the reduced grain count, may account for the decrease in specific activity of DNA.

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Three days after whole-body X-irradiation with 1000 R, the fraction of labeled cells in the intestinal crypts increased from 45 to 70%. When hydroxyurea was given at this time, the cytotoxic effect was slightly less than that found in the normal intestine. After prelabeling with thymidine-^{3}H, both the specific activity of DNA and the number of labeled cells per crypt were reduced by nearly 50% at 26 hr (Chart 7, Table 1). However, interpretation of the results is complicated by the decrease in specific activity of DNA in the control group. The reduction is due to a combination of a shortened maturation time (at 26 hr, labeled cells were seen at the tips of the villi) and an increase in the amount of DNA as a result of the very rapid regeneration between 3 and 4 days after 1000 R (3, 8).

DISCUSSION

The loss of labeled DNA from spleen, thymus, bone marrow, and small intestine has been reported after X-irradiation (10). Similarly, prelabeled DNA was lost from the small intestine within 24 hr after injection of mitomycin C (15). Hydroxyurea-induced loss of radioactivity from mouse duodenum and mammary tumor prelabeled with iododeoxyuridine-^{125}I or iododeoxyuridine-^{3}H has been reported (1).

Normal Epidermis. After thymidine-^{3}H labeling of the DNA of epidermal cells in S phase, the cytotoxicity of hydroxyurea to these cells has been shown by the loss of labeled cells and the reduction of the specific activity of DNA. No toxicity to epidermal cells in phases of the cell cycle other than S was demonstrated. The maximum loss of DNA-synthesizing cells, as indicated by a decrease in specific activity of DNA, was attained 8 to 12 hr after injection of 30 mg hydroxyurea. No further loss was observed within 24 hr.

After an injection of 30 mg hydroxyurea, an inhibition of epidermal DNA synthesis of more than 50% was maintained for 6 hr. At a dose of 15 mg, the cytotoxic effect was equivalent to that found after 30 mg, although DNA synthesis was inhibited more than 50% for only 4 hr. Injection of 1 mg hydroxyurea effectively inhibited DNA synthesis for at least 1 hr but produced no cytotoxic effect. In the normal epidermis, the killing of S phase cells by hydroxyurea is apparently not related to the degree of inhibition of DNA synthesis, but it may be related to the duration of the block.

Rapidly Proliferating Epidermis. In the rapidly proliferating epidermis 18 hr after treatment with croton oil, the apparent relationship between cytotoxicity and duration of inhibition of DNA synthesis found in normal epidermis has been altered. The cytotoxic effect of 30 mg hydroxyurea, which inhibited DNA synthesis by more than 75% for at least 3 hr, was much less pronounced than in normal epidermis. The effective dose of hydroxyurea reaching the epidermal cells may have been somewhat reduced since the duration of the inhibition of DNA synthesis was similar to that found in normal epidermis after injection of 15 mg hydroxyurea.

A prolonged inhibition of DNA synthesis does not necessarily imply that cells will be killed. Factors other than the duration of the block of DNA synthesis, perhaps associated with the proliferation rate, are apparently responsible for the difference in cytotoxicity of hydroxyurea in normal and croton oil-treated epidermis. The mechanism of the differential cytotoxic action of hydroxyurea on S phase cells with different rates of cell proliferation remains unclear.

Small Intestine. Loss of prelabeled DNA from the small intestine was found within 26 hr of injection of hydroxyurea. The cytotoxicity of hydroxyurea was only slightly reduced in the rapidly regenerating intestine 3 days after whole-body irradiation with 1000 R. If the decreased cytotoxicity of hydroxyurea found in croton oil-treated epidermis is indeed related to the greater than 10-fold increase in the rate of proliferation, then the intestine is not a favorable tissue to show a similar effect. Considering the rapid rate of proliferation in the normal small intestine, the labeling index can be increased by less than a factor of 2, even in conditions of maximal regeneration. Thus, the cytotoxicity of hydroxyurea in the stimulated epidermis and small intestine may be determined by the extent of increase in the rate of cell proliferation relative to the normal rate.

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