PUROMYCIN-INDUCED NECROSIS OF CRYPT CELLS OF THE SMALL INTESTINE OF MOUSE

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ABSTRACT

Chemical and radioautographic analysis of the small intestine of mice injected intraperitoneally with puromycin revealed an immediate decrease of precursor incorporation into DNA and protein and a delayed decrease of precursor incorporation into RNA. In addition to this decrease of precursor incorporation, damage to the crypt cells, but not to the cells of the villus of the small intestine, was observed. Further examination of other dividing cells (spleen) and nondividing cells (liver and heart) of these mice showed again that only cells of actively dividing tissues were damaged. The metabolic inhibitors actinomycin D, cytosine arabinoside, actidione, and puromycin aminonucleoside were used in an attempt to clarify the mechanism of cell damage by puromycin. The results showed that there was no clear correlation between cell necrosis and the pattern of inhibition of synthesis of DNA, RNA, or protein.

INTRODUCTION

The antibiotic puromycin is known to inhibit protein synthesis by blocking the transfer of amino acids from aminoacyl-sRNA to the peptide chain (26). In addition, puromycin blocks the incorporation of precursors into both RNA and DNA (15, 24). The data of Lieberman and coworkers (24) indicate further that the decreased incorporation of precursors into both protein and DNA is almost complete within 1 hr after the addition of puromycin to primary cultures of rabbit kidney cells.

We have chosen the small intestine of mouse to assay in vivo the effect of puromycin on continuously dividing cells. Our results indicate a rapid and marked inhibition of precursor incorporation into DNA and protein and a more gradual decrease in precursor incorporation into RNA, thus confirming the results of Gottlieb et al. (15) and Lieberman et al. (24). Microscope examina-

tion of sections taken from the small intestine, spleen, heart, and liver of puromycin-treated mice revealed necrosis only among cells known to be actively dividing, namely crypt cells of the small intestine and cells of the germinal centers of the spleen and lymphoid tissue, whereas cells of the other tissues examined were not visibly damaged. Actidione, whose action is similar to that of puromycin, and other antibiotics and antimetabolites acting on a narrower range of metabolic processes were used in an attempt to correlate the production of necrosis in dividing cells with the pattern of inhibition of metabolic processes or with the phase of the cell cycle (29).

MATERIALS AND METHODS

Strain A male mice, 4 to 6 months old and weighing an average of 30 g each, were used. The following antibiotics or antimetabolites were injected intra-

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peritoneally: puromycin hydrochloride and the aminonucleoside of puromycin (Nutritional Biochemicals Corporation, Cleveland, Ohio) dissolved in saline-phosphate buffer (14); actinomycin D (a gift of Merck, Sharp and Dohme, West Point, Pennsylvania) dissolved in saline; actidione (cycloheximide, a gift of Dr. Savage of The Upjohn Co., Kalamazoo, Michigan) suspended in sterile water; and cytosine arabinoside (gift of Dr. A. E. Osterberg, NIH Cancer Chemotherapy Center, Bethesda, Maryland, and Dr. Savage of The Upjohn Co.) dissolved or suspended in sterile water. Controls were injected with appropriate solvents. All radioisotopes (New England Nuclear Corp., Boston, Massachusetts) were injected subcutaneously (28) and had the following specific activities: thymidine-methyl-H^3, 6.7 c/mmole; DL-leucine-4,5 hydrochloride-H^3, 5.0 c/mmole, L-leucine-C^{14}, uniformly labeled, 233 mc/mmole; uridine-H^3, 3.0 c/mmole; uridine-2-C^{14}, 30 mc/mmole, and cytidine-H^3, 2.0 c/mmole. All other chemicals were of reagent grade.

The animals were killed by cervical dislocation, and approximately 500 to 800 mg of small intestine, beginning 5 cm from the squamocolumnar junction of the stomach, were removed. A small segment of intestine from the most proximal portion was fixed for radioautography in calcium formalin or in Carnoy's solution. Intestinal contents were removed from the remaining portion which was weighed and homogenized with ice cold water in a Potter-Elvehjem homogenizer with a Teflon pestle.

NUCLEIC ACID DETERMINATION: Aliquots of homogenate were extracted by the method of Scott, Fraccastoro, and Taft (35), and three fractions, acid-soluble, RNA, and DNA, were obtained. The amounts of DNA and RNA were determined by ultraviolet spectrophotometry. The method of Scott, Fraccastoro, and Taft (35) was used for the determination of DNA, and the two-wavelength method of Tsanev and Markov (38) and Fleck and Munro (11) was used for determining RNA. The radioactivity of each fraction was determined by liquid scintillation counting (1). Specific activities were calculated as counts per minute per milligram of DNA or RNA.

DETERMINATION OF INCORPORATION INTO PROTEIN: The amount of leucine incorporated into proteins was determined by dissolving the residue, remaining after extracting nucleic acids with the above method (35), in 1 N KOH for 1 hr at 60°C. One-tenth ml of the digest was used for liquid scintillation counting. The limits of accuracy of this procedure were determined in previous studies (17). In tissues of animals injected with radioactive leucine, 80% of the acid-insoluble radioactivity was found in the 1 N KOH fraction, while the remaining radioactivity was found in the RNA (17%) and DNA (3%) fractions. Since leucine is incorporated only into proteins (1), the radioactivity in the 1 N KOH fraction represents 80% of the total radioactivity incorporated into proteins. In the present paper, the results of leucine incorporation are expressed as counts per minute per 100 mg (wet weight) of small intestine.

RADIOAUTOGRAPHY AND HISTOLOGY: The fixed tissues were embedded in paraffin, from which 3 to 5 μ sections were cut. Radioautographs were prepared by the dip coating method of Joftes and Warren (20), with Eastman Kodak NTB emulsion. Radioautographs, after exposure and photographic processing, and routine sections were stained with Mayer's hematoxylin and eosin. The percentage of labeled cells and the mean grain count were determined as previously described (1). The percentage of damaged cells was determined after 200 crypt cells were counted in at least four segments of the small intestine of each mouse.

RESULTS

Puromycin (2.5 mg per mouse) was injected intraperitoneally every hour for a total of four injections (10 mg per mouse) starting at zero time. Thymidine-H^3, leucine-H^3, and uridine-H^3 were injected subcutaneously, 30 min before killing, at times beginning immediately after the first injection of puromycin. Fig. 1 shows the effect of puromycin treatment on the incorporation of thymidine-H^3 into DNA, leucine-H^3 into protein, and uridine-H^3 into RNA, of small intestine.

Thymidine incorporation into DNA, expressed in counts per minute per milligram of DNA, was 46% of control values 30 min after the first injection of puromycin. Subsequently, incorporation dropped to 13% of control values at 90 min and remained slightly below this level for the remainder of the experiment. Incorporation of leucine-H^3 into protein of the small intestine (see Materials and Methods) decreased to 35% of control values at 30 min after the first injection of puromycin and remained at the same level at 120 min. The incorporation of uridine-H^3 into RNA, expressed in counts per minute per milligram of RNA, was actually increased 75 min after the first injection of puromycin, confirming the findings of Hamilton (16), but, by the second hour, it decreased to about 50% of control values. Radioautography of the small intestine confirmed the results of the chemical analyses, but in addition disclosed severe damage of crypt cells of animals receiving three and four injections of puromycin. The experiments were extended to
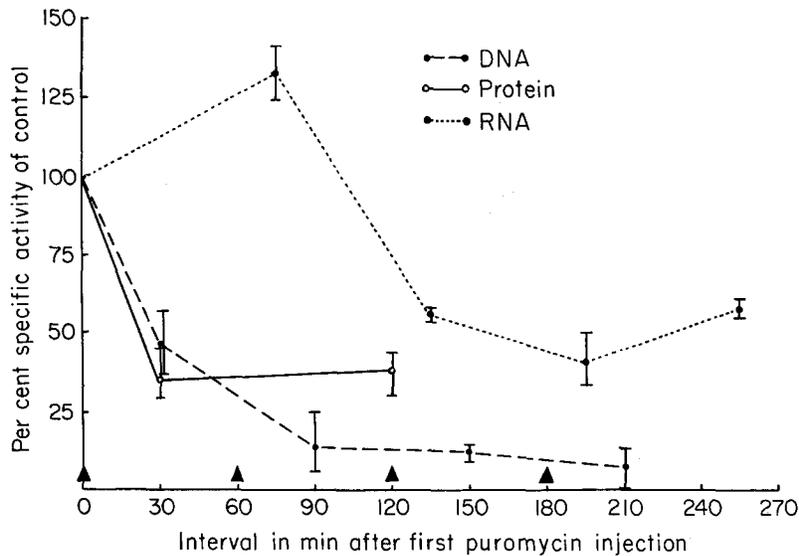
FIGURE 1 Effect of puromycin on incorporation of thymidine into DNA (●---●), leucine into protein (O—O), and uridine into RNA (●··●). Arrows on the abscissa indicate intraperitoneal puromycin injections (2.5 mg per mouse). All isotopes were injected subcutaneously 30 min before killing. Each point represents the mean of 3 to 5 animals. Vertical bars represent ranges.

determine: (a) whether the lesion could be reproduced with a similar dose of a different lot of puromycin, and (b) whether the crypt damage could be induced with fewer injections by leaving a longer time interval between the last injection of puromycin and killing. The results are shown in Table I. The upper half of the Table shows that crypt damage was produced by a different lot of puromycin. The lower group of values shows that two injections, which had produced fragmentation of occasional nuclei in the original experiment, produced extensive damage when a longer time interval was left between the last injection and killing. The data in this Table indicated that at least two injections and an interval of 3 to 4 hr were necessary for crypt damage to become visible.

Illustrations of crypt damage are shown in Figs. 2 to 5. Normally columnar cells became cuboidal in extensively damaged crypts. The cell borders became indistinct and the cytoplasm increasingly basophilic. Many of the cells lost definite nuclear membranes. Many nuclei were replaced by a thin rim of basophilic material surrounding a clear area in which were pyknotic nuclear fragments. There was no apparent damage to cells of the villi. In these experiments sections were also taken from liver, heart, and spleen. The

TABLE I

Effect of Puromycin on Histologic Appearance of Crypts of Small Intestine of Mice*

No. of puromycin injections	Time between first injection and death	Per cent necrosis of crypt cells
	hr	
1	0.5	0
2	1.5	<5
3	2.5	65–85
4	3.5	>85
1	4.0	<5
2	5.0	65–85
3	6.0	>85
4	7.0	>85

* Puromycin 2.5 mg per intraperitoneal injection every hour. The first four groups of animals were killed 30 min after the last puromycin injection, and the others, 4 hr after the last puromycin injection.

germinal centers of the spleen, in which the percentage of cells in DNA synthesis is relatively high (5), showed numerous necrotic cells. No damage was found in livers and hearts, tissues in which the number of cells in DNA synthesis is very small (25).
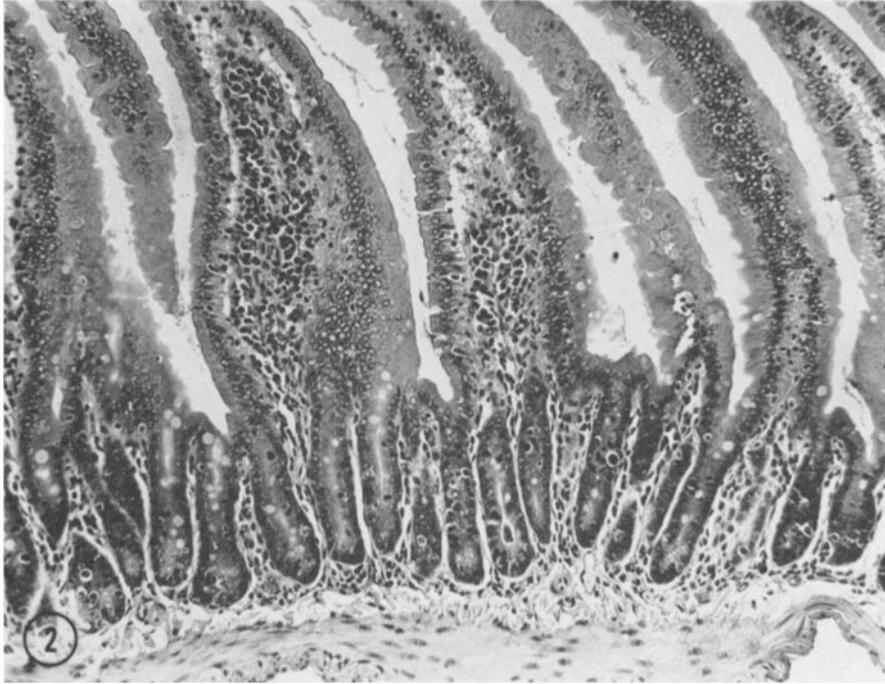
FIGURE 2   Slight to moderate crypt damage in small intestine of mouse given one injection of puromycin (5.0 mg) intraperitoneally, and killed 6 hr later. Hematoxylin and eosin. × 100.
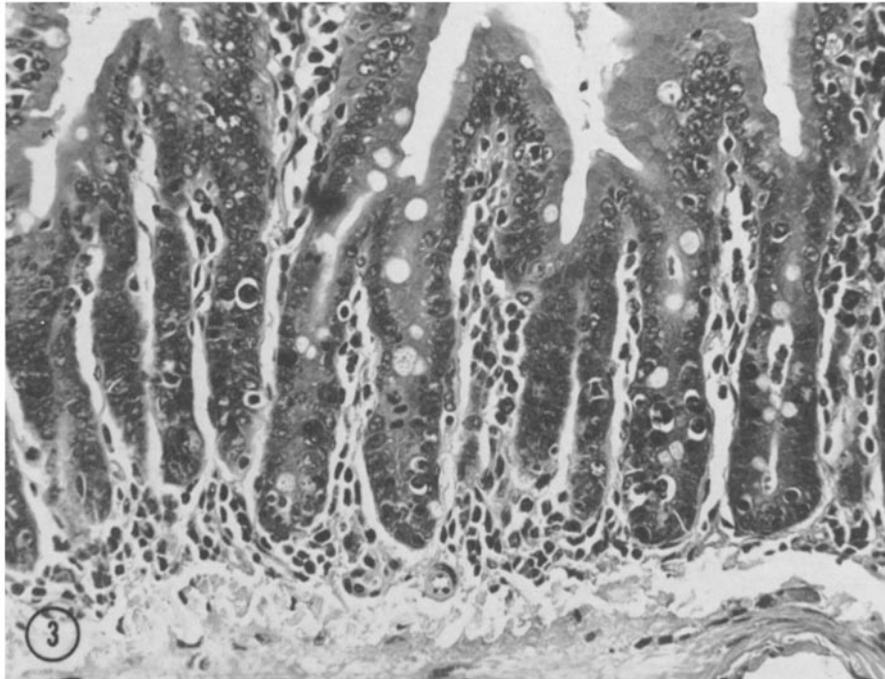

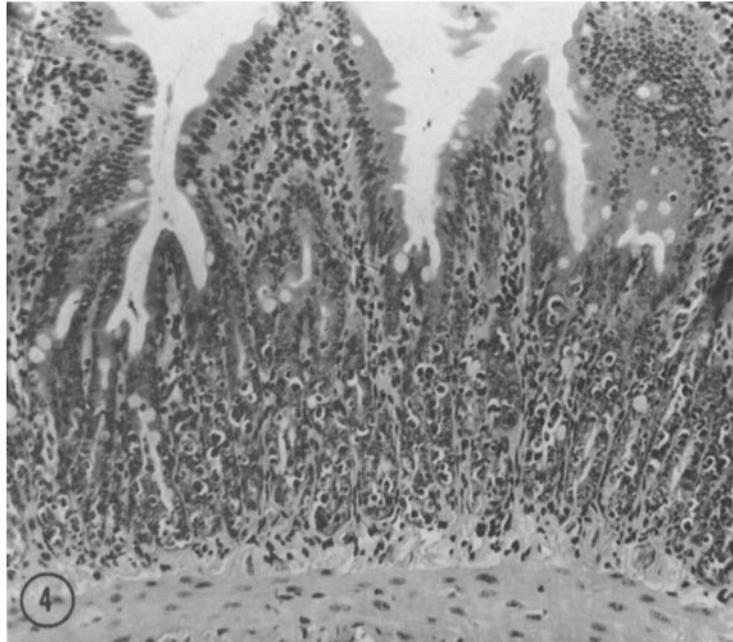FIGURE 3   Higher magnification of Fig. 2. Hematoxylin and eosin. × 250.

FIGURE 4 Extensive damage in crypts of small intestine of mouse given puromycin (2.5 mg) intraperitoneally hourly for 4 hr and killed 30 min after the final injection. Hematoxylin and eosin. × 100.

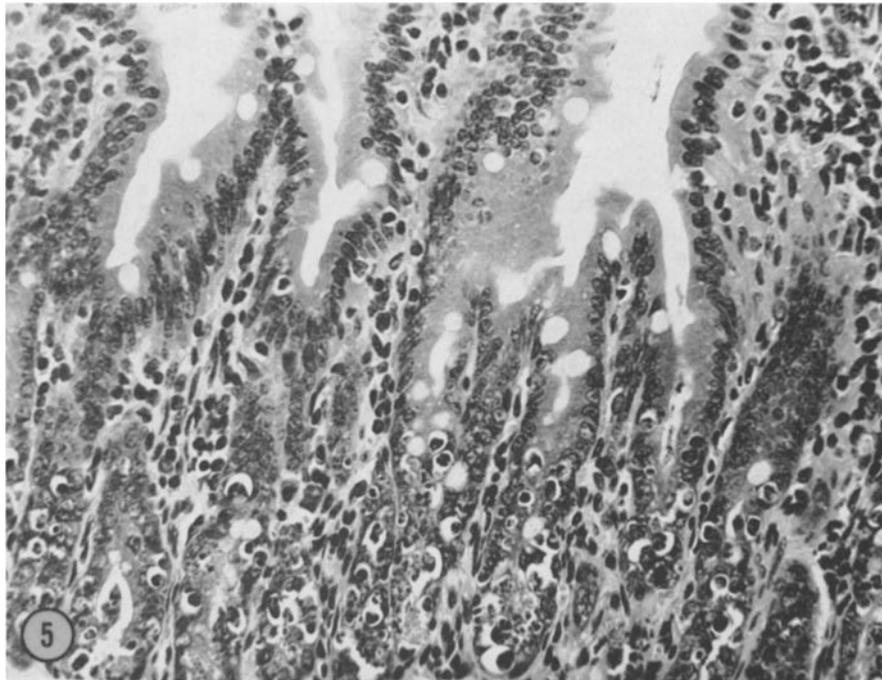FIGURE 5 Higher magnification of Fig. 4. Hematoxylin and eosin. × 250.

TABLE II

*Effect of Dose and Time of Injection of Puromycin on Incorporation of Leucine-H³ into Protein**

Tissue	No. of puromycin injections	Total dose	Interval between last injection of puromycin and death	Incorporation of leucine-H³ into protein: per cent of controls	Percentage of necrotic cells‡
		mg	*hr*		
Small intestine	1	2.5	1	33	0
	1	2.5	3	104	<5
	2	5.0	3	126	58–85
	3	7.5	3	130	>85
	4	10.0	3	46	>85
Small intestine	1	1.25	6	143	0
	1	2.5	6	134	0
	1	5.0	6	104	28–38
Liver	1	2.5	1	29	0
	1	2.5	3	73	0
	2	5.0	3	77	0
	3	7.5	3	61	0
	4	10.0	3	24	0
Heart	1	2.5	1	34	0
	1	2.5	3	83	0
	2	5.0	3	63	0
	3	7.5	3	75	0
	4	10.0	3	33	0

* Puromycin, 2.5 mg per mouse, was injected intraperitoneally at 1 hr intervals. Leucine-H³ was injected subcutaneously 1 hr prior to death.
‡ Cells in this column are crypt cells for small intestine; hepatocytes for liver; and myocardial cells for heart. There were at least 3 mice for each group.

Table II illustrates the effect of puromycin on the incorporation of leucine into protein of small intestine and other organs when the time interval between the last injection and killing is increased. Cell damage regularly appeared in the crypts of the small intestine. However, 3 hr after the last injection of puromycin, the incorporation of leucine was at control levels in the small intestine while remaining below control levels in both liver and heart. The Table also indicates that the lowest single dose which produced irreversible cell damage was 5 mg per mouse. It should be noted that, immediately after a single injection of puromycin, the incorporation of leucine into protein was equally inhibited in small intestine, liver, and heart. Apparently, there was a reversal in the inhibition of protein synthesis by puromycin when 3 or more hours were allowed to elapse between the last injection of puromycin and death. How- ever, inhibition of protein synthesis became seemingly irreversible after four injections of puromycin.

The results, thus far, may be summarized as follows: puromycin injected hourly in the amount of 2.5 mg per mouse produced a prompt inhibition of protein synthesis and DNA synthesis and a delayed inhibition of RNA synthesis in the small intestine. In addition, two injections, 1 hr apart, were sufficient to destroy the crypts of the small intestine and to produce necrosis in other tissues where cells in DNA synthesis are numerous. In the experiments to be described, we have attempted to answer two questions posed by these results:

1. In which phase of the cell cycle did this dose of puromycin act?
2. By what mechanism did puromycin produce

necrosis in tissues where cellular proliferation is prominent?

Effect of Puromycin on the Cell Cycle of Crypt Cells of the Small Intestine

The cell cycle (29) is defined as the time period between the midpoint of mitosis in the parent cell and the midpoint of mitosis in the daughter cell. This cycle is divided into four phases: G_1, the period between completion of mitosis and the beginning of DNA synthesis; the phase of DNA replication or S phase; G_2, the period between completion of DNA synthesis and mitosis; and mitosis or M. According to Lesher et al. (23) the cell cycle of the crypt cells of the small intestine of mice of this age can be subdivided

TABLE III

Effect of Puromycin on the G_2 Phase of the Cell Cycle of Crypt Cells of Small Intestine of Mice

Treatment	Time between thymidine-H[3] and killing	Labeled cells per 1000 cells	Per cent labeled mitoses, mean and range	Mitotic index
	min			
None	60	51.6	12 (3–16)	3.4
Puromycin*	60	52.6	7 (5–11)	3.4
None	105	50.0	69 (64–76)	4.5
Puromycin*	105	49.8	46 (40–54)	4.4

* 2.5 mg of puromycin per mouse given either 15 min or 15 and 75 min after a single injection of 25 µc of thymidine-H[3].

into phases of the following length: G_1, 4.5 to 5.5 hr; S, 5 hr; G_2 and mitosis, less than 2 hr.

If cells are labeled with thymidine-H[3] in the S phase and followed for various intervals thereafter, the length of the G_2 phase can be measured by the time of appearance of labeled mitoses. If cells are delayed in the G_2 phase, the appearance of labeled mitoses is also delayed. If cells are blocked in G_2, no labeled mitoses are seen. Similarly, the mitotic index can be used to indicate the presence of a block in mitosis or G_2. The mitotic index increases if cells are blocked in mitosis alone, or it decreases if the cells are blocked in G_2. Table III shows an experiment in which crypt cells prelabeled with thymidine-H[3] were treated with puromycin and examined radioautographically at suitable intervals. The interval between labeling and killing was sufficient to allow some labeled cells to pass from S through G_2 and into mitosis. The experimentally determined length of G_2 was 1 hr, which agreed with the figures given by other authors (23). The percentage of labeled mitoses decreased in puromycin-treated animals, indicating that a G_2 delay rather than a block was produced by this dose of antibiotic. The mitotic index remained the same, indicating that puromycin did not produce a mitotic block but that both G_2 and mitoses were delayed.

The data presented in Fig. 1 suggested that the cells were blocked in S phase, because the onset of inhibition of DNA precursor uptake was rapid and marked, but the possibility of a concomitant G_1 block cannot be ruled out in the present experimental conditions.

TABLE IV

*Effects of Some Antibiotics or Antimetabolites on Incorporation of Precursors by the Small Intestine of Mice**

Specific activity (Per cent of control)

Agent	DNA after thymidine-H[3]			RNA after cytidine-H[3]			Proteins after leucine-H[3]			Per cent necrosis of crypt cell
	30 min	2 hr	4 hr	1 hr	2 hr	4 hr	30 min	2 hr	4 hr	
Actinomycin D	100		53	65	14		100	100		0
Cytosine arabinoside	6		1		100		96	136		30
Actidione	19	14			10		7	11		0
Puromycin aminonucleoside			60			64			182	0

* Actinomycin D: one injection of 50 µg per mouse. Cytosine arabinoside: 4.5 mg per mouse. Actidione: 10 mg per mouse and puromycin aminonucleoside: 2.5 mg per mouse injected hourly starting at zero time. The total time of each experiment from initial injection to killing is indicated at the top of the Table for each isotope. Isotopes were injected subcutaneously 30 min prior to killing. Each figure represents at least 3 animals.

Effects of other Compounds on Crypt Cells

Other compounds were used to establish whether or not a pattern of inhibition of precursor uptake into DNA, RNA, or protein could be related to the production of crypt damage. Table IV shows the results of these experiments. A single injection of actinomycin D, 1.33 mg/kg, produced 90% inhibition of cytidine-H^3 incorporation into RNA in the small intestine. Thymidine-H^3 incorporation into DNA was inhibited 50% after 4 hr, but there was no inhibition of leucine-H^3 incorporation into protein. However, crypt cell damage did not appear in the small intestine of mice even 8 hr after a single injection of actinomycin D, although the dose used was greater than the 1.0 mg/kg which Schwartz et al. (34) found produced 94% inhibition of RNA synthesis and 50% inhibition of DNA synthesis, as well as cell damage, in regenerating rat liver.

Cytosine arabinoside promptly inhibited incorporation of precursors into DNA to 13% of control values, but neither leucine-H^3 incorporation into protein nor cytidine-H^3 incorporation into RNA was inhibited. In fact, leucine incorporation was above control levels at 2 hr. This drug produced crypt cell damage which was not qualitatively different from that seen with puromycin.

Actidione produced 93% inhibition of leucine incorporation into proteins, 85% inhibition of thymidine incorporation into DNA, and a 90% inhibition of cytidine incorporation into RNA. However, no crypt cell damage was seen in the small intestine of mice 5 hr after the first injection of actidione.

Puromycin aminonucleoside at a dose of 2.5 mg per mouse produced a 40% inhibition of both thymidine incorporation into DNA and uridine incorporation into RNA. However, leucine incorporation into protein was increased, as with cytosine arabinoside. No cell damage was seen in the crypts.

Radioautographs of the tissues, whose chemical analyses are given in Table IV, showed that the effect of the antibiotics and antimetabolites used in these experiments was roughly of the same magnitude in the several components of the small intestine. The only exception was the effect on the incorporation of thymidine-H^3, which was, of course, largely limited to the crypts. Inhibition of cytidine-H^3 incorporation by actinomycin D or

puromycin aminonucleoside affected every cell type. Radioautographs from actidione-treated animals gave evidence of so little radioactivity that no quantitative determination was attempted. However, this observation per se indicates that the inhibitory effect extended to every cell type.

DISCUSSION

Puromycin inhibits protein synthesis in vivo (14), and recent reports as well as our results show that it also inhibits incorporation of thymidine into DNA and incorporation of cytidine or uridine into RNA in vivo and in vitro (15, 18, 24). Our data further indicate that puromycin blocks the crypt cells of the small intestine in the S phase of the cell cycle, with a concomitant but slight G_2 and mitotic delay. Kishimoto and Lieberman (21) have demonstrated, in cultures of kidney cells, a G_2 delay or block, depending on the dose of puromycin used. Studzinski and Love (37) have demonstrated both a preprophase block, interpreted as a G_2 block, and mitotic arrest with varying doses of puromycin. The possibility of a G_1 block, concomitant with a block in the S phase, has not been ruled out. To our knowledge, the literature contains no reports of necrosis of cells after puromycin in vivo, although cell death in puromycin-treated tissue cultures has been described (22). No effort has been made in these experiments to ascertain whether the decreased incorporation of precursors reflected a true decrease in rate of macromolecular synthesis or was due to concomitant changes in other parameters, such as the size of the endogenous pool of precursors. However, it should be noted that the inhibitory action has already been studied in detail for most of the compounds used in these experiments. Thus: (a) the inhibitory effect of puromycin on protein synthesis has been determined in a variety of living systems (14–16), while Studzinski and Love (37) have shown that the amino acid and nucleotide precursor pools are not affected by puromycin; (b) the prompt inhibitory effect of actinomycin D on RNA synthesis and the delayed inhibitory effect on DNA synthesis without concomitant changes in precursor pools have also been described in several living systems (4, 27, 30); (c) the inhibition of DNA synthesis by cytosine arabinoside has also been demonstrated unequivocally (7); and (d) the generalized in-

hibitory effect of actidione on macromolecular synthesis has also been reported in the literature (6). In addition, unpublished experiments in our laboratory, with P^{32} and formate-C^{14}, confirm the inhibitory effects of puromycin and actinomycin D on nucleic acid synthesis in the small intestine. The only compound whose inhibitory effect has not been clearly ascertained is therefore the aminonucleoside of puromycin, although some evidence is available from bacterial studies (10). It therefore seems reasonable to assume that, with the exception of the aminonucleoside, decreased incorporation of precursors in these experiments can be taken to signify decreased macromolecular synthesis.

There is no clear-cut relationship between suppression of precursor incorporation into DNA, RNA, or protein and necrosis of cells. Actidione produced a pattern of suppression of precursor incorporation similar to that produced by puromycin and yet caused no cell necrosis. However, actidione inhibition of RNA synthesis is prompt and dramatic, whereas puromycin inhibition of RNA synthesis is delayed and does not go below 50% of control values. It is possible that puromycin-induced necrosis of cells engaged in DNA replication may be another instance of "unbalanced growth" (2, 32). It is interesting to note that hydroxyurea, a compound that was not available for these experiments, has been recently reported to produce lethal damage of tissue culture cells in DNA synthesis (36), and necrosis of intestinal epithelial cells (quoted by Schwartz et al., reference 33). Hydroxyurea has been found to be a potent inhibitor of DNA synthesis (39), although no effects have been observed on synthesis of RNA or protein (33, 39). However, if puromycin-induced necrosis is another example of "unbalanced growth," it remains to be explained why cytosine arabinoside is so much less effective than puromycin in producing necrosis of crypt cells.

In a more detailed analysis of the rapid fall in precursor incorporation into DNA, one should note that Rosenkranz and Bendich (31) have suggested that the continued synthesis of DNA is dependent upon the continued presence of a protein. However, there is also evidence that both puromycin and its aminonucleoside interfere directly with the synthesis of purines (8, 10, 13, 19).

It seems clear that dividing cells of puromycin-treated mice are severely injured. While requiring a lag period to show damage, these cells are irreversibly injured, although the inhibitory action of puromycin on protein synthesis is at least partially reversible (9, 12). It is also apparent that nondividing cells show no similar damage. The action of puromycin which results in damage to dividing cells remains unknown. The possibility of an increased uptake of puromycin by cells in DNA synthesis is not supported by the finding that protein synthesis is equally inhibited in proliferating and nonproliferating tissues. There is room for a reasonable doubt that the necrotizing effect of puromycin is not due to the effect on protein synthesis alone. Since there is evidence that puromycin directly affects DNA synthesis and since it causes necrosis only in dividing cells, one is tempted to speculate that this direct action is involved in the mechanism responsible for the necrosis of these cells. However, other factors, such as the continued synthesis of other macromolecules, must be part of the complex sequence of events leading to cell death.

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