Molecular Mechanism of Liver Regeneration

THE EFFECT OF PUROMYCIN ON DEOXYRIBONUCLEIC ACID SYNTHESIS*

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The rate of synthesis of deoxyribonucleic acid in regenerating liver can be measured in vivo by following the incorporation of 14C-orotic acid (1) or H-thymidine (2) after intraperitoneal injection. The same process can also be studied in vitro by tracing the incorporation of labeled deoxynucleotides into boiled primer in the presence of a supernatant fraction from regenerating liver (3). Discrepancies between rates of synthesis in vivo and in vitro have been observed repeatedly (4, 5). Thus, although maximal incorporation occurs in vivo 24 hours after partial hepatectomy, the presence of a supernatant fraction from regenerating liver may provide information on the role played by proteins in this process, and may aid in explaining the differences observed in experiments in vivo and in vitro.

The present report is a comparative study of the effect of puromycin in DNA synthesis, in vivo and in vitro, in regenerating rat liver.

EXPERIMENTAL PROCEDURE

General—Male Sprague-Dawley rats, weighing 160 to 200 g, were used. Partial hepatectomies were performed according to the method of Higgins and Anderson (14). Food was withheld, but water was given ad libitum during the 24-hour period before the animals were killed. Puromycin dihydrochloride, obtained from the Nutritional Biochemicals Corporation, was suspended in an aqueous solution of 0.9% sodium chloride-0.04 M phosphate buffer, pH 7.4; 0.5 ml of this suspension, containing 12.5 mg of puromycin, was administered intraperitoneally. Control animals received intraperitoneal injections of 0.5 ml of the sodium chloride-phosphate buffer solution. All animals were killed by decapitation and thoroughly bled. The liver was excised and chilled in 0.25 M sucrose, and a 10% homogenate in 0.25 M sucrose was prepared with the aid of a Potter-Elvejem homogenizer, equipped with a Teflon pestle. The tissue suspension was maintained at the temperature of melting ice during this procedure.

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† Obtained from Sigma Chemical Company, St. Louis.


‡ The scintillation fluid contained, per 100 ml of solution, 38.5 ml of xylene, 38.5 ml of dioctane, 23 ml of absolute ethanol, 8.09 g of naphthalene, 0.504 g of 2,5-diphenyloxazole, and 0.00504 g of α-naphthylphenyloxazole.

The incorporation in vitro of 14C-dTMP in the presence of regenerating liver supernatant fluid—A high speed supernatant fraction from regenerating liver was prepared as previously described (3) to study the incorporation of 14C-thymidylic acid (9 mc per mmole) into DNA. In these experiments, highly polymerized calf thymus DNA, dissolved in distilled water and boiled for 10 minutes, was used as primer. After incubation of the system under conditions described previously (3), the reaction was stopped by addition of 5 ml of ice-cold 20% perchloric acid. The precipitate,
containing the protein and the nucleic acids, was washed with 5% cold perchloric acid (usually four times) until the acid-soluble fraction was free of radioactivity. The nucleic acids were extracted with 5% hot trichloroacetic acid, according to the method of Schneider (15), and a sample of the trichloroacetic acid extract was placed in the scintillation fluid for counting in a Packard Tri-Carb scintillation counter. Studies of DNA and RNA specific activities after extraction with hot 10% sodium chloride, as well as separation of DNA and RNA by the Schmidt-Thannhauser method (16), demonstrated that all the radioactivity present in the precipitate was associated with DNA.

Phosphorylation in Vitro of 3H-Thymidine in Presence of Regenerating Liversupernatant—3H-Thymidine

Phosphorylation to thymidine triphosphate was assayed according to the method of Weissman, Smellie, and Paul (17). After incubation of the precursor with the enzyme source at 37° for 15 minutes, the reaction was stopped with 20% perchloric acid. After neutralization with 5 N potassium hydroxide, the acid-soluble fraction was chromatographed on Celite cellulose. The columns were first washed with 100 ml of glacial-distilled water, and then eluted with 100-ml fractions of 0.01 N hydrochloric acid and 0.5 N hydrochloric acid. dTMP is eluted with the first hydrochloric acid fraction, and thymidine triphosphate with the second fraction. Attempts to detect thymidine diphosphate by gradient chromatography on Celite cellulose, or by paper chromatography, were unsuccessful under the conditions of the assay. The eluate, containing the thymidine triphosphate, was concentrated to dryness and redissolved with scintillation fluid for radioactive determination in a Packard Tri-Carb scintillation counter. Three concentrations of the enzyme source were assayed in each case. The plot of the counts per minute in the thymidine triphosphate fraction versus the concentration of the enzyme was linear, under the conditions of the assay, and of the first order.

Incorporation of 3H-dTMP into DNA in Presence of Regenerating Liver Acetone Powder—In these experiments, an acetone powder of the supernatant fluid from regenerating liver was used to catalyze the incorporation of 3H-dTMP into DNA. The composition of the incubation mixture was, in every other respect, identical with that described previously (3).

DNA, RNA, Nitrogen Determination, Enzyme Assays, and Units—DNA, RNA, nitrogen contents, and acid phosphatase, β-glucuronidase, and glucose 6-phosphatase activities were determined as described in previous studies (3). Enzyme activities are expressed in micromoles of phosphorus or phenolphthalein released per minute under the conditions of the assay.

### RESULTS

In the first series of experiments, changes in liver weight, nitrogen contents, enzyme activities (acid phosphatase, β-glucuronidase, glucose 6-phosphatase), and 14C-leucine incorporation were investigated after two, three, and four injections of puromycin administered as outlined in Table I. The animals that received two or three injections of puromycin appeared in good health until the moment of death. In contrast, those that received four injections were apathetic and presented respiratory difficulties, which often led to death 1 or 2 hours before scheduled. The results obtained after four injections of puromycin have therefore been omitted.

Three injections of puromycin have no significant effect on nitrogen, DNA and RNA contents, acid phosphatase, β-glucuronidase, or glucose 6-phosphatase activities of 24-hour regenerating liver (Table II). In contrast, the incorporation of 14C-leucine into regenerating liver protein is depressed, under these conditions, to 30% of the normal rate (Fig. 1). In view of the effect of puromycin on protein synthesis in vivo and in vitro (7-13), it would appear that the low protein radioactivities reflect interference with their biosynthesis.

The effect of puromycin on 14C-orotic acid and 3H-thymidine incorporation in vivo, and of 14C-dTMP incorporation in vitro,

### Table II

<table>
<thead>
<tr>
<th>Change in nucleic acid, nitrogen contents, and enzyme activities after puromycin injection</th>
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<tr>
<td>Nitrogen, DNA, and RNA contents are expressed in milligrams per gram of wet liver, and the acid phosphatase, glucose 6-phosphatase, and β-glucuronidase activities are expressed in units per gram of wet liver. The data presented refer to the mean and the standard deviation calculated according to the formula</td>
</tr>
<tr>
<td>[ \sigma = \sqrt{\frac{(x - \bar{x})^2}{n - 1}} ]</td>
</tr>
<tr>
<td>Each statistic was calculated from results obtained on individual livers.</td>
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</tbody>
</table>

### Table I

<table>
<thead>
<tr>
<th>Schedule of injections and death after partial hepatectomy</th>
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<tr>
<td>Incorporation studies</td>
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<tr>
<td>-----------------------</td>
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<tr>
<td>First</td>
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<tr>
<td>In vivo</td>
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<td></td>
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<tr>
<td>In vitro</td>
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In the first series of experiments, changes in liver weight, nitrogen contents, enzyme activities (acid phosphatase, β-glucuronidase, glucose 6-phosphatase), and 14C-leucine incorporation were investigated after two, three, and four injections of puromycin administered as outlined in Table I. The animals that received two or three injections of puromycin appeared in good health until the moment of death. In contrast, those that received four injections were apathetic and presented respiratory difficulties, which often led to death 1 or 2 hours before scheduled. The results obtained after four injections of puromycin have therefore been omitted.

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The effect of puromycin on 14C-orotic acid and 3H-thymidine incorporation in vivo, and of 14C-dTMP incorporation in vitro,
was investigated after administration of three injections of the antibiotic before death, according to the protocol outlined in Table I. The effect of puromycin on the incorporation of 14C-orotic acid and 3H-thymidine of rat regenerating liver is presented in Fig. 2. It is clear that the antibiotic blocks the incorporation of 14C-orotic acid into DNA and into nuclear and cytoplasmic RNA of regenerating liver 24 and 40 hours after hepatectomy. The effect of puromycin on the incorporation of 3H-thymidine into DNA of regenerating liver was investigated only at 40 hours after hepatectomy. Again, the incorporation of the isotope under these conditions was markedly inhibited.

Studies of the effect of puromycin on the ability of the supernatant fluids prepared from regenerating livers to catalyze the incorporation of 14C-dTMP into boiled DNA are presented in Fig. 3. In these experiments, rats were partially hepatectomized and were killed 24 and 40 hours after the operation. Animals that received three injections of puromycin during an 8-hour span preceding death were compared with animals that received three injections of buffered sodium chloride solution. The results demonstrate that the catalytic properties of the supernatant fluid prepared from livers obtained 24 hours after hepatectomy are reduced by puromycin. In contrast, such treatment is without effect on the supernatant fluid of liver of animals killed 40 hours after hepatectomy. Furthermore, three injections of puromycin do not interfere with the phosphorylation of 3H-thymidine to thymidine triphosphate by 40-hour regenerating liver supernatant fluid (Table III).

The effect of various concentrations of puromycin, added directly to the incubation mixture, was also investigated. In these experiments, an acetone powder of a regenerating liver supernatant was used as an enzyme source. The results demonstrate (Table IV) that the addition of puromycin to the incubation mixture does not alter 14C-dTMP incorporation into DNA, thus excluding a direct effect of the antibiotic on either primer or enzymes.

**DISCUSSION**

Our goal was to investigate the effect of puromycin on DNA synthesis in regenerating liver, in vivo and in vitro. The results obtained in vitro can be interpreted in light of existing information on the fate of the enzyme activities involved in the last steps of DNA synthesis. In animals that received three injections of puromycin, beginning 16 hours after hepatectomy, protein synthesis is blocked during an 8-hour span which coincides with the period during which thymidylic kinase and DNA polymerase appear (6, 17). Under these conditions, puromycin interferes with 14C-dTMP incorporation into DNA in vitro, and therefore it would appear that at least one of the two enzymes involved in the last steps of DNA formation is synthesized de novo. In contrast to what happens between 16 and 24 hours after hepatectomy, thymidylic kinase and DNA polymerase activities change very little between 32 and 40 hours after operation, suggesting that either little or no enzyme is synthesized during that period. It is not surprising, therefore, that the injections of puromycin during that 8-hour span do not affect the incorporation in vitro of 14C-dTMP into DNA.

If the effect of puromycin on the incorporation of 14C-dTMP
thymidine in vivo, would expect that three injections of puromycin would block DNA synthesis in vivo 24 hours after hepatectomy, but would be without effect on r4C-erotic acid incorporation 40 hours after operation. Nevertheless, the results obtained in two typical experiments with supernatant fluids prepared from 24- and 40-hour regenerating liver are presented.

**Table III**

**Phosphorylation of H-thymidine**

The supernatant of 40-hour regenerating rat liver was used in these experiments.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>H4-dTMP formed (μmoles/mg N)</th>
</tr>
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<tbody>
<tr>
<td>Control 1</td>
<td>3.728</td>
</tr>
<tr>
<td>Control 2</td>
<td>3.657</td>
</tr>
<tr>
<td>Puromycin 1</td>
<td>3.809</td>
</tr>
<tr>
<td>Puromycin 2</td>
<td>4.063</td>
</tr>
</tbody>
</table>

In vitro reflected the events in vivo, one would expect that three injections of puromycin would block DNA synthesis in vivo 24 hours after hepatectomy, but would be without effect on 14C-erotic acid or H-thymidine incorporation in regenerating livers obtained 40 hours after operation. Nevertheless, the results demonstrate that puromycin blocks the incorporation of DNA precursors in vivo in both 24- and 40-hour regenerating livers. Discrepancies between the utilization of 14C-erotic acid or H-thymidine in vivo, and of 14C-dTMP in vitro, may result from interferences with the availability of the precursors in vivo, or from the formation by the animal of factors modifying the activities of the enzymes involved in DNA formation. Puromycin could interfere with the absorption of the precursor by the cells of the peritoneal lining or by the hepatic cell. Although such possibilities require further investigation, observations made in other laboratories tend to eliminate this eventuality. Thus, puromycin interferes with the incorporation of H-thymidine into DNA of cells grown in tissue culture (13). Furthermore, the antibiotic is without effect on the incorporation of 32P-orthophosphate into RNA of various tissues, except the thymus, where it reduces 32P-orthophosphate incorporation into RNA (7). These results suggest that the effect of puromycin on nucleic acid synthesis is related to the metabolic activities of the cell, rather than to absorption of the precursor. In addition, the differences in the effect of puromycin on the incorporation of 14C-erotic acid into DNA and into nuclear and cytoplasmic RNA would seem difficult to explain on the basis of selective absorption.

The activities of the enzymes catalyzing the phosphorylation of H-thymidine or the polymerization of the deoxynucleotides could be altered by puromycin indirectly by (a) destruction of normal cellular compartmentalization, (b) interference with the availability of coenzymes, and (c) activation of inhibitors or catalytic pathways. These various possibilities cannot be excluded without further investigation of the morphological and biochemical alterations of the hepatic cell that result from the administration of puromycin.

Puromycin could also directly affect thymidylate kinase and DNA polymerase activities by altering the enzyme molecules or by interfering with their biosynthesis. In view of the well established effect of puromycin on protein synthesis in cells (7-13), it seems logical to postulate that the antibiotic interferes with DNA synthesis by blocking the biosynthesis of a protein involved in the conversion of 14C-erotic acid or H-thymidine to DNA.

A block of the pathway converting 14C-erotic acid to 14C-dTMP could explain the lack of incorporation of 14C-erotic acid into regenerating liver DNA, but not that of H-thymidine. Since the rate of the conversion of H-thymidine to thymidine triphosphate catalyzed by the supernatant fraction obtained from 40-hour regenerating liver is normal, interference of puromycin with the formation of the phosphorylating enzymes seems to be excluded also. Inasmuch as puromycin does not alter 14C-dTMP incorporation into boiled DNA when the supernatant fluid from 40-hour regenerating liver is used, it would appear that puromycin does not affect the formation of DNA polymerase. Definitive conclusions must, however, await experiments in which the DNA polymerase activity is tested, with the deoxynucleotide triphosphates as precursors. Thus, if puromycin interferes with the formation of DNA polymerase in vivo, it would appear that puromycin interferes with the phosphorylation of H-thymidine in vivo.

**Table IV**

**Effect of puromycin added in vitro**

The data refer to the incorporation of 14C-dTMP into DNA catalyzed by an acetone powder (5 mg per ml) supplemented with puromycin. The incorporation of 14C-dTMP is expressed in counts per minute incorporated under the conditions of the assay (see legend of Fig. 3). The puromycin is expressed in micrograms per ml of incubation mixture.

<table>
<thead>
<tr>
<th>Puromycin (μg/ml)</th>
<th>14C-dTMP incorporation (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2556</td>
</tr>
<tr>
<td>20</td>
<td>2676</td>
</tr>
<tr>
<td>40</td>
<td>2758</td>
</tr>
<tr>
<td>60</td>
<td>2822</td>
</tr>
<tr>
<td>80</td>
<td>2860</td>
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</tbody>
</table>
DNA synthesis of regenerating liver by blocking protein synthesis, it would appear that the protein involved might be different from thymidylic kinase and DNA polymerase. Such a conclusion would be in agreement with the effect of inhibitors of protein synthesis on DNA synthesis in tissue culture (13-17, 19). The present data do not, however, eliminate the possibility of a direct interaction, in vivo, between puromycin and DNA.

Puromycin does not alter acid phosphatase, β-glucuronidase, or glucose 6-phosphatase activities in 24-hour regenerating liver, probably because the turnover and restoration of these enzymes are too slow to allow the detection of significant interference with their biosynthesis.

The effect of puromycin on 14C-orotic acid incorporation in vivo into nuclear and cytoplasmic RNA is of interest. It is in agreement with observations made in virus (20). The mechanism by which this effect occurs cannot be explained, however, without further investigation.

**SUMMARY**

The effect of puromycin dihydrochloride injections on synthesis of deoxyribonucleic acid in regenerating rat liver was investigated 24 and 40 hours after partial hepatectomy. Synthesis in vivo was studied by measuring the incorporation of 14C-orotic acid into liver DNA. Synthesis in vitro was investigated by determining the incorporation of 14C-deoxythymidine 5'-phosphate into boiled DNA in the presence of a supernatant fraction from regenerating liver. Three puromycin injections, administered in the 8-hour span preceding death, inhibit DNA synthesis in regenerating liver obtained 24 hours after partial hepatectomy both in vivo and in vitro. In contrast, it affects DNA synthesis only in vivo in 40-hour regenerating liver.

**REFERENCES**
