The Effect of Urethan on the Incorporation of Thymidine-\(^{3}\)H into DNA and the Activities of Some Enzymes Required for DNA Biosynthesis in Rat Regenerating Liver

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SUMMARY

Urethan, a hepatocarcinogen in adult regenerating liver, was administered to rats at various intervals after partial hepatectomy to determine the effect of this drug on (a) the pattern of DNA replication and (b) the induction by partial hepatectomy of some enzymes required for DNA synthesis. A single i.p. dose of urethan, 1 mg/g, was injected into rats at either 0.5, 12, or 24 hr after surgery. When urethan was given 0.5 hr after partial hepatectomy, the initiation of DNA synthesis was delayed by 6 hr, followed by greater uptake of thymidine-\(^{3}\)H into nuclear DNA than into that of untreated regenerating liver. When urethan was given 12 hr after partial hepatectomy, the first wave of DNA synthesis was suppressed by 50 to 60%; the second cycle of DNA replication was essentially unaffected. Administration of urethan 24 hr postsurgery resulted in maximum inhibition at 14 hr after treatment; the incorporation of thymidine-\(^{3}\)H during the second wave of DNA replication occurred within a shorter period of time than that of the control. The induction of certain enzymes linked to DNA replication during liver regeneration was affected by the urethan in a similar manner. Ribonucleoside diphosphate reductase and thymidine kinase were sensitive to urethan treatment, while DNA polymerase activity was insensitive. Other enzymes not tightly coupled to DNA synthesis in regenerating liver, such as adenine and hypoxanthine-guanine phosphoribosyltransferases, were not affected by the carcinogen. The results indicate that urethan selectively inhibits the induction by partial hepatectomy of enzymes necessary for DNA synthesis.

INTRODUCTION

Urethan (ethyl carbamate) is a multipotential carcinogen which appears to be a particularly useful tool to investigate the mechanism of hepatocarcinogenesis by chemical agents. The utility of urethan to studies of the process of liver carcinogenesis derives in particular from the findings that only minimal death of hepatic cells occurs following exposure to this agent (11) and that the susceptibility of hepatocytes to the carcinogenic action of urethan depends upon the metabolic state of the cells (11, 13, 27, 29, 33, 44, 45).

A number of investigations have implicated nucleic acid metabolism in the action of urethan. For example, Gelboin et al. (18) have shown that topical administration of actinomycin D immediately after administration of urethan prevents the appearance of tumors promoted by croton oil, while other investigations have demonstrated inhibition of the synthesis of DNA and RNA in regenerating liver, as well as in the livers of adult and newborn animals (23, 32). In contrast, the synthesis of protein on both free and membrane-bound polyribosomes of the livers of sham-operated and partially hepatectomized rats was shown to be stimulated by exposure to urethan in vivo (19).

The binding of urethan to DNA, RNA, and nuclear proteins of normal liver was shown by Prodi et al. (39), who also indicated that the reaction products were stable to alkaline and enzymatic hydrolysis. The ethyl ester of cytosine-5-carboxylic acid was reported to be formed from the interaction in vivo of urethan and RNA of mouse liver (5). The findings that urethan interacted with nuclear macromolecules were supported by the report that this carcinogen had a toxic action on cell nuclei causing pycnosis and chromatid breaks (4, 9, 10, 34). In contrast to other studies with labeled urethan, no significant incorporation of radioactivity from urethan-carbonyl-\(^{14}\)C was reported to be present in the DNA or RNA of liver from intact or partially hepatectomized mice, and no difference was observed in the retention and metabolism of urethan by both normal and partially hepatectomized animals (20). Lawson and Pound (32), however, reported binding of urethan to DNA of regenerating liver; the degree of labeling of the regenerating tissue was no greater than that of normal liver.

The present report describes the effects of urethan, administered at various times after partial hepatectomy, on the replication of DNA in the regenerating liver of the rat. Significant interference with the biosynthesis of DNA was produced; this inhibition appeared to be due to prevention of the synthesis of certain enzymes required for the biosynthesis of the deoxyribonucleotide precursors of DNA of the regenerating liver.

MATERIALS AND METHODS

Urethan and thymidine were obtained from Sigma Chemical Co., St. Louis, Mo.; dATP, dCMP, dCTP, dGTP, and dTTP were purchased from P-L Biochemicals, Inc., Milwaukee, Wis.; dATP-\(^{14}\)C, thymidine-\(^{3}\)H, CDP-\(^{3}\)H,
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Ether anesthesia by the same investigator between 8:30 and libitum. Partial hepatectomies were performed under light ether anesthesia by the same investigator between 8:30 and 10:30 a.m. according to the method of Higgins and Anderson (21). Urethan was dissolved in 0.9% NaCl solution and injected i.p. either 0.5, 12, or 24 hr after partial hepatectomy at a level of 1 mg/g of body weight; control animals received an equal volume of 0.9% NaCl solution.

The nuclei were isolated by the method of Widnell and Tata (14). Urethan was dissolved in 0.9% NaCl solution and injected i.p. either 0.5, 12, or 24 hr after partial hepatectomy at a level of 1 mg/g of body weight; control animals received an equal volume of 0.9% NaCl solution.

The rate of synthesis of DNA was determined by the degree of incorporation of thymidine-3H into nuclear DNA. At various times after partial hepatectomy and urethan treatment, each rat received an i.p. injection of 70 μCi of thymidine-3H (8.47 μCi/μmol) and was killed 1 hr later. The nuclei were isolated by the method of Widnell and Tata (48). Aliquots of nuclei were washed with 4.5 volumes of 0.5 N cold perchloric acid and the precipitates that formed were vigorously dispersed with a vortex mixer and collected by centrifugation. Further washings showed no significant cold acid-soluble radioactivity. DNA was extracted from this pellet, and the concentration was determined by the method of Burton (8).

Cell-free supernatant fractions of regenerating liver were prepared from 25% homogenates in 0.14 M KCl-10 mm Tris-HCl (pH 7.4) by centrifugation at 100,000 × g for 1 hr in a Spincno Model L centrifuge. Specific activities of enzymes in most cases were defined as the number of nmols of substrate converted to product at 37° per mg of protein for the period of time of incubation used in each experiment. Under the conditions of assay, the enzyme activities were linear with respect to the amount of enzyme added during the incubation time. The protein concentration was determined by the method of Lowry et al. (35). No diurnal variation in these enzyme activities was observed under the conditions used.

Thymidine kinase was assayed according to the procedure of Labow et al. (28). The reaction mixture, incubated for 30 min at 37°, contained thymidine-3H, 1 μmole (3900 cpm/μmole); ATP, 5.0 μmoles; MgCl₂, 5.0 μmoles; Tris-HCl (pH 8.0), 30 μmoles; NaF, 100 μmoles; and 0.1 ml of a 100,000 × g liver supernatant in a total volume of 0.35 ml. The reaction was terminated by heating the mixture in a boiling water bath for 2 min. A 50-μl aliquot of the deproteinized reaction mixture was applied to a 1-inch-square DEAE-cellulose DE 81 paper disc (Whatman, Maidstone, Kent, England), treated as described by Voytek et al. (47), and radioactivity was determined in Aquasol (New England Nuclear) using a Packard liquid scintillation spectrometer.

Total DNA polymerase activity was determined by a modification of the procedure of Bollum (2, 3). The reaction mixture, incubated at 37° for 30 min, contained a mixture of Tris-deoxyribonucleotides (25 nmols of each) and dATP-14C (7 mCi/mmmole); MgCl₂, 1 μmole; phosphate buffer (pH 7.0), 0.5 μmole; mercaptoethanol, 0.25 μmole; Tris-ATP, 1 μmole; calf thymus DNA, 75 μg (denatured by heating at 95° for 15 min); and 75 μl of liver supernatant in a total volume of 0.25 ml. A 50-μl aliquot of the reaction mixture was applied to a Whatman No. 1 paper disc and then dropped into cold 5% trichloroacetic acid containing 1% Na₂P₂O₇ to terminate the reaction. The disc was washed twice with cold 5% trichloroacetic acid, twice with 95% ethanol, and twice with anhydrous ethyl ether; dried under infrared light; and counted in Aquasol using a Packard liquid scintillation spectrometer.

Ribonucleoside diphosphate reductase was assayed according to the procedures of Moore (37) and Elford et al. (14). The incubation mixture contained 115 nmols of substrate, either CDP-14C (1 to 3 × 10⁴ cpm/μmole) or CDP-14C (2 × 10⁴ cpm/μmole); FeCl₃, 10 nmols; ATP, 1.0 μmole; MgCl₂, 1.0 μmole; dithiothreitol, 5.0 μmoles; phosphate buffer (pH 7.0), 3.0 μmoles; and 100 μl of liver homogenate in a total volume of 0.35 ml. After incubation at 37° for 1 hr, the reaction was terminated by the addition of 1 ml of 1 N perchloric acid and carrier dCMP was added. Samples were centrifuged and supernatant solutions hydrolyzed in a boiling water bath for 15 min, cooled, and neutralized with 4 N KOH. After centrifugation the supernatant solutions were applied to Dowex 50-H⁺ columns (13 × 0.8 cm) and dCMP was eluted with 0.2 m acetic acid (40). The eluates were dried and dissolved in water; the recovery was determined spectrophotometrically and counted for radioactivity.

The phosphoribosyltransferase assay contained 50 mm Tris-HCl (pH 8.0); 5 mm MgCl₂; 0.56 mm phosphoribosyl pyrophosphate tetrasodium; and radioactive purine substrate in a total volume of 100 μl. The concentrations of the purine bases were 0.163 mm hypoxanthine-14C (30 μCi/mg) and 0.168 mm adenine-14C (30 μCi/mg). The reactions were initiated by addition of enzyme (usually 40 to 80 μg protein). Appropriate control tubes in which phosphoribosyl pyrophosphate was omitted were included routinely. After incubation for 15 min at 37°, reactions were terminated by the addition of 50 μl of 0.2 M EDTA and kept at 4° until chromatographed. Separation of the substrate from the nucleotide product was achieved using a modification of the technique of Adye and Gots (1). Columns of DEAE-cellulose (50 × 7 mm; Cellex D, exchange capacity, 0.72 mEq/g) were washed with 0.01 M Tris-HCl (pH 8.0). An aliquot of the reaction mixture (usually 100 μl) was applied to the column and washed with 8 ml of 0.01 M Tris-HCl (pH 8.0) which eluted unreacted purine bases. The nucleotide product was then eluted with 3 ml of 1.0 N HCl and the radioactivity was determined in Aquasol. The results obtained using this procedure were in agreement with values obtained by high-voltage electrophoresis separation (24).

RESULTS

The effects of urethan on the incorporation of thymidine-3H into nuclear DNA of regenerating liver was measured at
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various times after exposure to the carcinogen; the data obtained are shown in Charts 1 to 3. The pattern and rate of DNA synthesis in untreated partially hepatectomized animals was rigorously controlled; over a 50-hr period 2 cycles of DNA synthesis occurred, with the 2nd wave being less tightly synchronized. This pattern of DNA synthesis is similar to that described for weanling rats by Bucher et al. (6, 7); however, with the older rats used in the present study the biphasic peaks of replicative activity occurred slightly later in time than those of the weanling. The 1st wave of DNA synthesis took place at approximately 21 to 23 hr in 170- to 175-g animals (20 to 22 hr in weanlings) and the 2nd broader peak at approximately 38 to 43 hr (33 to 35 hr in weanlings) after partial hepatectomy. Urethan appeared to affect the replication of DNA in different ways depending upon the time of administration after surgery. When urethan was injected i.p. at 0.5 hr after partial hepatectomy, the initiation of DNA synthesis was delayed about 6 hr (Chart 1); thus, the entire 1st wave of DNA replication was shifted to a later period and the specific activity of DNA was increased about 60% by treatment with the carcinogen at the peak of DNA biosynthesis, as compared to peak synthesis in the control. The 2nd cycle of DNA replication appeared to be relatively unaffected. When urethan was given to rats at 12 hr after partial hepatectomy, the initial wave of DNA replication was suppressed by about 50 to 60% and the 2nd period of DNA synthesis appeared to be relatively normal (Chart 2). The i.p. injection of urethan at 24 hr after partial hepatectomy (Chart 3), a period following the 1st replicative cycle, caused progressive inhibition of the incorporation of thymidine-3H for about 14 hr. This effect was followed by a recovery in which the DNA was synthesized in a shorter period of time than normal. This result implied a greater degree of synchronization of the system.

It has been well documented that several enzymes involved in the pathways of DNA biosynthesis, such as TTP-forming enzymes, ribonucleoside diphosphate reductase, and DNA polymerase (15, 28, 30, 38), increase to the relatively high levels of activity required to initiate and support DNA replication following partial hepatectomy. The effect of urethan on the partial hepatectomy-induced appearance of enzymes involved in the biosynthesis of DNA at various stages of regeneration are shown in Tables 1 to 3. The data in Table 1 indicate that urethan inhibited the induction of enzymes required for replication following partial hepatectomy. Such inhibition appears to be specific for certain enzymes. Ribonucleotide reductase and thymidine kinase were the most sensitive enzymes measured to urethan inhibition, while DNA polymerase was inhibited to
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The results shown in Table 2 indicate that the continual suppression of reductase activity from the 1st phase (0.5 hr after partial hepatectomy) is due to the inhibitory activity of urethan, whereas adult animals are virtually resistant (12, 25-27, 41, 45, 46). The surgical removal of 70% of the liver, however, initiates cell replication and liver regeneration; under these conditions the carcinogenic potential of urethan has been shown to be expressed in adult male mice (11, 22, 29, 31). Since hepatocarcinogenesis appears to require active replication of cells, the effects of urethan on the biosynthesis of DNA were studied. Lawson and Pound (32) demonstrated that the formation of both RNA and DNA of regenerating mouse liver was susceptible to the inhibitory activity of urethan; however, this study was not designed to compare the action of this agent at early times after partial hepatectomy and during DNA replication, conditions under which urethan is essentially noncarcinogenic and carcinogenic to liver, respectively (11). Our experiments have demonstrated that urethan interferes not only with the incorporation of thymidine-3H into nuclear DNA, an event equated with the replicative phase (22 hr). In contrast, after urethan treatment at either 0.5 or 12 hr, thymidine kinase activity was inhibited at 22 hr (Table 1) but recovered by 32 hr after partial hepatectomy and then increased to levels greater than that of the control (Table 2). In addition, urethan had essentially no inhibitory effect on DNA polymerase, HGPRT, and APRT activities under all conditions tested.

The data in Table 3 show that the activities of ribonucleoside diphosphate reductase and thymidine kinase were greater at 42 hr after partial hepatectomy than at 32 hr indicating a biphasic elevation of these activities in regenerating liver. When urethan was administered early in the regeneration process, such as at 0.5 or 12 hr after partial hepatectomy, enzyme activity at 42 hr was either increased or not affected; no inhibition was observed. It appears that, after early inhibition by urethan, as observed at 22 and 32 hr after partial hepatectomy, enzyme activities recover to control levels by 42 hr. The inhibition of thymidine kinase appears to be reversed more rapidly, while the recovery of ribonucleoside diphosphate reductase was delayed for a longer period of time and no large increase over control activity was seen as in the case of thymidine kinase. However, when urethan was given 24 hr after partial hepatectomy, enzyme activity was inhibited in a manner similar to that observed in the 1st replicative phase (Table 1).

To determine whether urethan caused inhibition of enzymic activities by direct interaction with enzymes, crude cell-free extracts were prepared from the livers of rats 42 hr after partial hepatectomy and were used to assay enzyme activities in the presence of urethan in vitro. Urethan in the range of concentrations tested (10^-3 to 10^-6 M) had essentially no effect on the activities of ribonucleoside diphosphate reductase, thymidine kinase, and DNA polymerase.

**DISCUSSION**

Newborn mice and rats are relatively susceptible to the hepatocarcinogenic actions of urethan, whereas adult animals are virtually resistant (12, 25-27, 41, 45, 46). The surgical removal of 70% of the liver, however, initiates cell replication and liver regeneration; under these conditions the carcinogenic potential of urethan has been shown to be expressed in adult male mice (11, 22, 29, 31). Since hepatocarcinogenesis appears to require active replication of cells, the effects of urethan on the biosynthesis of DNA were studied. Lawson and Pound (32) demonstrated that the formation of both RNA and DNA of regenerating mouse liver was susceptible to the inhibitory activity of urethan; however, this study was not designed to compare the action of this agent at early times after partial hepatectomy and during DNA replication, conditions under which urethan is essentially noncarcinogenic and carcinogenic to liver, respectively (11). Our experiments have demonstrated that urethan interferes not only with the incorporation of thymidine-3H into nuclear DNA, an event equated with the synthesis of DNA, but also with the partial hepatectomy-induced formation of certain enzymes necessary for DNA biosynthesis in the regenerating liver. Interference by ure-
than with the incorporation of thymidine into DNA occurred when the carcinogen was given at either 0.5, 12, or 24 hr after partial hepatectomy. Measurements of the effects of urethan through 2 cycles of DNA biosynthesis indicated that the inhibition was transient and reversible and a normal pattern of synthesis of these macromolecules was rapidly reestablished. In this manner the action is similar to that described for dimethylbenzanthracene (36).

The precise mechanism of the inhibitory action of urethan on DNA synthesis is presently unknown; however, our data...
clearly show that this agent selectively inhibits the development of certain enzymic activities closely linked to the biosynthesis of DNA (i.e., ribonucleoside diphosphate reductase and thymidine kinase), while DNA polymerase and enzymes not essential to the formation of DNA (i.e., HGPRT and APRT) and, therefore, not elevated in activities following partial hepatectomy were relatively insensitive to the action of urethan. The resistance of HGPRT and APRT activities to inhibition may relate to the stability of these proteins or their mRNA in situ; however, the insensitivity to urethan of the partial hepatectomy-induced increase in DNA polymerase activity cannot be explained by this mechanism. Thus, the carcinogen seems to interfere preferentially with the expression of certain gene functions. Of the enzymes measured, which increase in activity with partial hepatectomy, the most sensitive to urethan was ribonucleoside diphosphate reductase. Inhibition of reductase activity by the carcinogen correlated with the sensitivity of DNA synthesis to this agent, indicating that this enzyme is a candidate for the rate-limiting step in the biosynthesis of DNA, a concept in keeping with the findings of Elford et al. (14).

That the interference by urethan with certain enzyme-forming systems was a pretranslational event was suggested by the findings (a) that the rate of formation of protein on both membrane-bound and free polyribosomes was stimulated by urethan (19) and (b) that the inhibition of the synthesis of DNA was greater when urethan was given at 0.5 hr after partial hepatectomy than when the carcinogen was injected at 12 hr postsurgery. Under the latter conditions, RNA has been extensively synthesized and is entering the cytoplasm at a maximal rate (15-17, 42, 43). The precise site of action of urethan on the enzyme-forming system is presently under investigation.

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REFERENCES

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