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Effects of Hydroxyurea and Benzo(a)pyrene on DNA Synthesis in the Isolated Perfused Rat Lung

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A perfused rat lung model is described for the measurement of DNA synthesis by [^{3}H]-thymidine incorporation in terms of its response to inhibitors of DNA synthesis and carcinogens. Hydroxyurea (5-50 mM) inhibited DNA synthesis by 95% yet did not disrupt lung energy metabolism as indicated by cytosolic NADH/NAD$^+$ measured by lactate/pyruvate ratios. Perfused lung DNA synthesis was also inhibited 60% by in vivo administration of benzo(a)pyrene (6 mg/kg, i.p.) for 3 days. The application of this system as a sensitive indicator of lung damage caused by pneumotoxic environmental compounds is discussed.

A wide variety of compounds, including chemotherapeutic and other pharmacologic agents, herbicides and insecticides, industrial solvents, and various naturally occurring toxicants, produce acute pneumotoxic effects when administered to animals by oral, subcutaneous, intravenous, or intraperitoneal routes (COLLIS 1980; BOYD 1980). A potentially sensitive indicator of lung damage is alteration of lung DNA synthesis, a parameter not readily measured in intact animals.

We have, therefore, investigated the feasibility of utilizing a perfused rat lung model to: (1) determine whether DNA synthesis can be measured ex vivo by [^{3}H]-thymidine incorporation, (2) determine the response of DNA synthesis to hydroxyurea with the eventual aim of characterizing chemically-induced DNA damage by measuring unscheduled DNA synthesis, and (3) to examine the effect of in vivo pretreatment of the known pneumotoxic compound benzo(a)pyrene on DNA synthesis in the rat lung.

MATERIALS AND METHODS

Animals. Weanling male Fischer rats were obtained from the Charles River Breeding Laboratories, Wilmington, MA. The animals were housed singly and fed a Purina rat chow diet and water ad libitum until they weighed 150-175 g. In carcinogen treatment experiments, benzo(a)pyrene (Aldrich Chemical Co., Milwaukee, WI; 99+ %, "Gold Label") was administered i.p. on 3 consecutive days at a dose of 6 mg/kg per mL of corn oil.

Lung Perfusion. The lungs were isolated under pentobarbital anaesthesia with minor modification of the technique previously described by BASSETT et al. (1976) and placed in a perfusion chamber

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maintained at 37°C. The lungs were perfused for 60 min via the cannulated pulmonary artery at 10 mL/min with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 4% dialyzed bovine serum albumin (Fraction V, fatty acid poor; Miles Biochemicals, Elkhart, IN), 5 mM glucose and 10 mU of insulin per mL. The lungs were ventilated at 60 cycles/min with 95% O_2/5% CO_2 using a Harvard rodent respirator. The tidal volume was 2-2.5 mL and end-expiratory pressure was 2-3 cm H_2O.

The lungs were allowed to equilibrate for 10 min before any addition was made to the perfusate. Where indicated, hydroxyurea (Sigma Chemical Co., St. Louis, MO) was added at 10 min. In all cases measurement of DNA synthesis commenced at 40 min by the addition of 200 µCi [^{3}H]-thymidine (New England Nuclear Corp., Boston, MA) and perfusion continued for another 30 min. Perfusate samples were removed every 15 min and subsequently analyzed for pyruvate and lactate (CZOK & LAMPRECHT 1974; GUTMAN & WAHLEFELD, 1974). After perfusion the lungs were freeze-clamped between aluminum blocks precooled in liquid N_2 and stored at -70°C (WOLLENBERGER et al. 1960).

DNA Isolation. The frozen lung tissue was homogenized in 10 mL of ice-cold HKM-sucrose buffer (0.05 M Hepes, pH 7.0; 25 mM KCl; 5 mM $MgCl_2$; 0.25 M sucrose) with a Polytron (Brinkmann Instruments, Westbury, NY) at low speed for 1 min. The extract was filtered through 2 layers of cheesecloth and 1 layer of Nitex nylon mesh (160 mesh). After centrifuging the suspension for 10 min at 800 x g the crude nuclear pellet was resuspended in 10 mL HKM-sucrose buffer containing 0.5% Triton X-100 (V/V) and recentrifuged. After repeating the washing step the nuclear pellet was dispersed in 1.7 mL lysing buffer (0.05 M Hepes, pH 7.0; 5 mM Na_2EDTA; 3% Sarkosyl). The suspension was gently homogenized by hand with 5 up-and-down strokes in a Teflon-glass homogenizer with 10.3 mL medium (1.0 mL 3M Cs_2SO_4 - 0.05 M Hepes, pH 7.0 - 5 mM Na_2EDTA, pH 7.0; 9.3 mL 6 M CsCl - 0.05 M Hepes, pH 7.0 - 5 mM Na_2EDTA, pH 7.0) to produce a density of 1.66 and centrifuged (16 h, 18°, 217,000 x g) in a Beckman VTi 50 rotor. After fractionation of the gradient, fractions corresponding to the DNA peak were pooled and aliquots taken for specific activity determination by measurement of radioactivity and absorbance.

Data were submitted to statistical analysis by a one-way analysis of variance followed by Duncan's multiple range test (STEEL & TORRIE 1960). The 0.05 level of probability was used as the criterion of significance.

RESULTS AND DISCUSSION

DNA synthesis was determined in perfused rat lungs by measuring the incorporation of [^{3}H]-thymidine into lung DNA isolated by neutral density gradient centrifugation. The use of a vertical rotor (WELLS & BRUNK 1979) permitted the overnight separation of the DNA as opposed to the 24-48 h spins customarily employed with

swinging bucket rotors.

A representative density gradient profile of [^{3}H]-thymidine-labeled DNA is shown in Fig. 1. The radioactivity peak is closely associated with the major A_{260} peak. On the average 0.75 mg DNA was obtained from approximately 750 mg lung tissue with an average A_{260}/-A_{280} ratio of 1.85. [^{3}H]-Thymidine incorporation was reduced by over 95% by treatment with 10 mM hydroxyurea, a potent inhibitor of DNA synthesis (BAUGNET-MAHIEU et al. 1971). Lactate/pyruvate ratios were within the normal physiological range (10-15) at all hydroxyurea concentrations used.

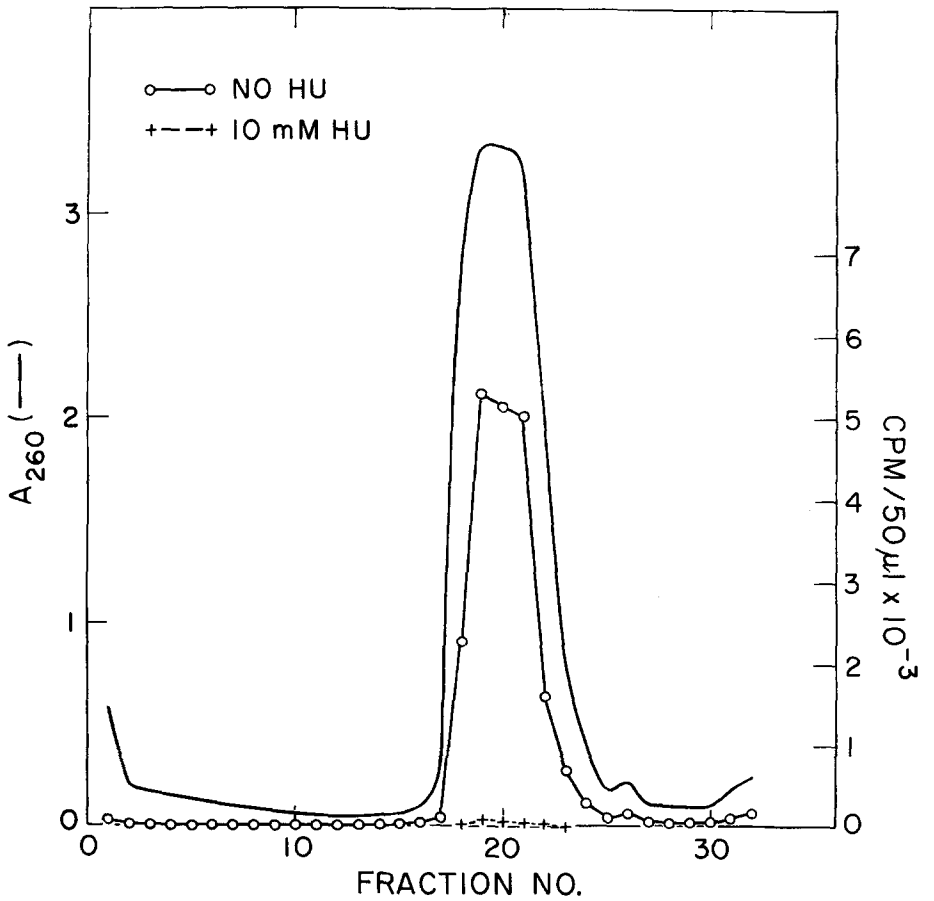

Fig. 1. Neutral CsCl gradient fractionation of [^{3}H]-thymidine-labeled DNA from perfused rat lung. A_{260} (————); radioactivity with (-+--+-) or without (0——0) 10 mM hydroxyurea.

Perfusion of rat lungs with 5-50 mM hydroxyurea was equally effective in rapidly inhibiting lung DNA synthesis with values ranging from 94-97% (Fig. 2). Considerable variation was noted in lung DNA synthesis from control preparations; this was not the case in hydroxyurea-treated tissues. Partial inhibition of DNA synthesis was obtained in preliminary experiments at lower (1-5 mM) hydroxy-urea concentrations (data not shown). On the basis of these results 10 mM hydroxyurea was used in later studies.

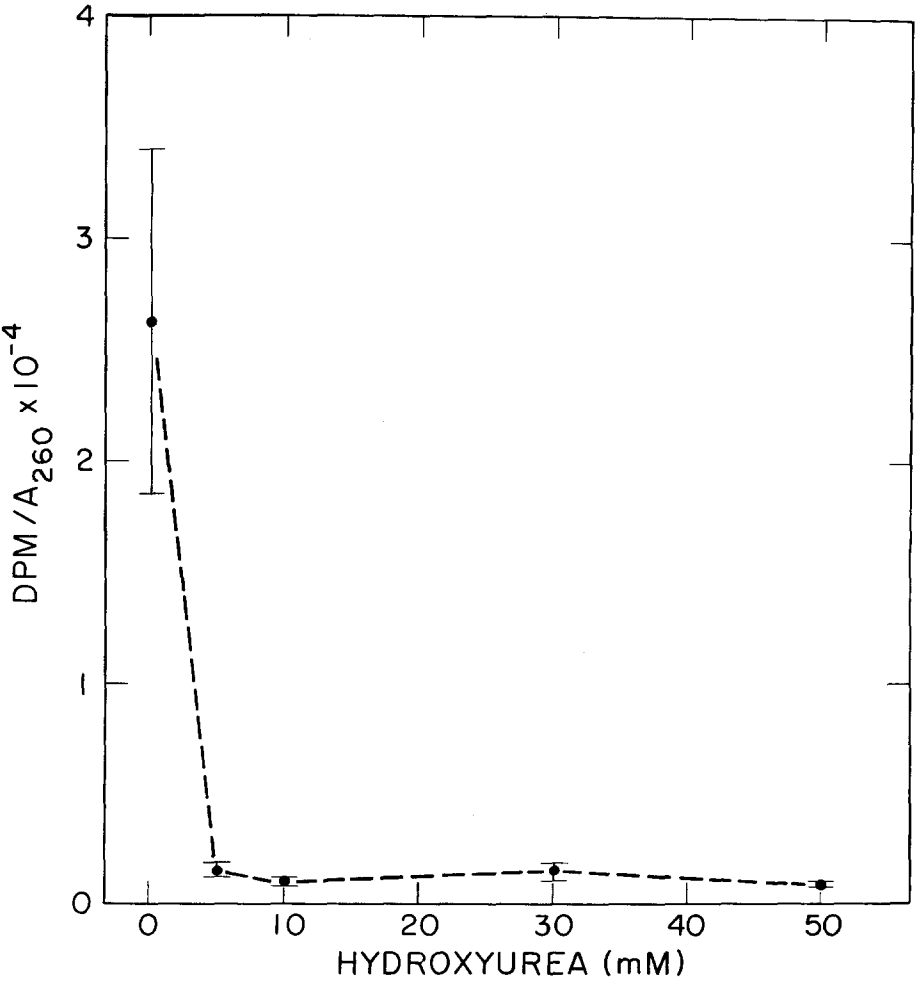

Fig. 2. Inhibitory effect of hydroxyurea on perfused rat lung DNA synthesis measured by [^{3}H]-thymidine incorporation. The data are expressed as mean ± S.E. using six animals per experiment.

In the next set of experiments, rats were pretreated *in vivo* with the pneumotoxic carcinogen benzo(*a*)pyrene (6 mg/kg in corn oil, i.p. for 3 days) or given corn oil alone to assess the effects of this compound on DNA synthesis in the perfused lung. [^{3}H]-Thymidine incorporation was measured in control and benzo(*a*)-pyrene-treated rat lungs in the presence and absence of 10 mM hydroxyurea. Benzo(*a*)pyrene treatment did not produce any gross indication of lung damage but significantly inhibited DNA synthesis (60%) in the perfused lung. DNA synthesis was inhibited by 90-95% with hydroxyurea both in the benzo(*a*)pyrene-treated and the corn oil control group. There was no statistically significant difference between the two groups.

TABLE 1

EFFECT OF BENZO(A)PYRENE ON DNA SYNTHESIS IN THE PERFUSED RAT LUNG

Conditions			DNA Synthesis	
Experiment[a]	Treatment *in vivo*	Hydroxyurea in perfusate (mM)	DPM [^{3}H]-Thymidine incorporation/A_{260} DNA[b]	% inhibition
A	Vehicle (1 mL corn oil/kg, i.p., 3 days)	0	29,898 ± 9,085	0
B	"	10	1,983 ± 384	93.4
C	B(*a*)P (6 mg/kg, i.p., 3 days)	0	11,623 ± 1,492	61.1
D	"	10	2,518 ± 312	91.6

[a]Groups underscored by the same line were not significantly different ($p \geq 0.05$) from each other: A, C, BD.

[b]Each value represents the mean ± SEM determined in six rats.

These studies demonstrated that the perfused rat lung model may be applicable to the study of the effects of pneumoactive agents on lung DNA synthesis, since DNA synthesis, as measured by [^{3}H]-thymidine incorporation, is detected with difficulty and at low sensitivity in the intact rat. Furthermore it was shown that DNA synthesis in the perfused lung is sensitive to pretreatment with benzo(*a*)pyrene *in vivo*. Benzo(*a*)pyrene is metabolized by rat lung microsomes and perfused rat lungs and is activated and covalently bound to lung DNA both in perfused lungs and *in vivo* (VAHAKANGAS et al. 1979; BOROUJERDI et al. 1981).

With further development it may be possible to extend the studies on hydroxyurea inhibition of DNA synthesis with these gradient techniques to investigate the induction of unscheduled DNA synthesis by appropriate compounds, using bromodeoxyuridine as a density label to separate parental from replicated DNA populations (SMITH & HANAWALT 1976).

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