Characterization of Mutagenic Glucuronide Formation from Benzo(a)pyrene in the Nonrecirculating Perfused Rat Liver

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ABSTRACT

Excretion of mutagenic metabolites of benzo(a)pyrene into bile from livers of corn oil- or 3-methylcholanthrene-treated Sprague-Dawley rats perfused with a nonrecirculating perfusion system was quantitated. Mutagenic benzo(a)pyrene metabolites were detected using Salmonella typhimurium (strain TA 98) grown in the presence of limiting amounts of histidine. Microsomes were not included in the bacterial assay since metabolic activation was carried out by the perfused liver. Mutagenic activity was detected only if β-glucuronidase was added to the assay mixture or if bile was treated with acid to hydrolyze glucuronides prior to assay. When livers were perfused with 20 μM benzo(a)pyrene, stable, mutagenic glucuronides were exported from corn oil-treated livers at maximal rates of 149 ± 24 (S.E.) revertants/g/hr and at rates of 225 ± 22 revertants/g/hr in livers from 3-methylcholanthrene-treated rats.

Chromatography of bile by high-performance liquid chromatography demonstrated that two peak areas contained phenolic glucuronides which were hydrolyzed by β-glucuronidase. These two peaks, one of which cochromatographed with authentic 3-benzo(a)pyrenyl-β-D-glucuronide, accounted for all of the mutagenic activity in bile from livers perfused with benzo(a)pyrene. A good correlation (r = 0.86) between rates of mutagen production and rates of formation of phenolic glucuronides was observed under a variety of experimental conditions. The mutagenic activity observed with pure 3-benzo(a)pyrenyl-β-D-glucuronide exposed to β-glucuronidase was 4 revertants/nmol. When the rate of mutagen production was divided by the rate of production of 3-benzo(a)pyrenyl-β-D-glucuronide by the perfused liver, a value of 4 revertants/nmol was also obtained. Therefore, it is concluded that mutagens exported in bile from livers perfused with benzo(a)pyrene can be accounted for predominantly by hydrolysis products of phenolic glucuronides.

INTRODUCTION

Short-term bacterial bioassays for mutagenesis, as described originally by Ames et al. (2), are used widely to predict the potential carcinogenic activity of a variety of chemicals. In the presence of added microsomes, the production of activated electrophiles causes dose-dependent mutations in bacteria that can be quantitated using the Ames test. In an effort to define the relationships between various physiological and environmental factors (i.e., nutrition, age, sex, exposure to inducing agents, etc.) and the metabolism of procarcinogens to mutagenic intermediates, investigators have used microsomes isolated from animals in different physiological states (3); however, results obtained with subcellular fractions do not necessarily reflect events in vivo. For example, procarcinogen entry into the cell, subsequent transport to and from the endoplasmic reticulum, and rates of production of cofactors for mixed-function oxidation and conjugation reactions are all involved in the production of reactive mutagens and carcinogens by the liver. To study the role of these factors in the metabolism of chemical carcinogens, whole-cell preparations are required.

An earlier study by Forti and Trieff (10) demonstrated that benzo(a)pyrene was converted to conjugated mutagens which appeared in bile collected from rat livers perfused using a recirculating system. The conjugated metabolite was, however, not identified. Furthermore, interpretation of data from the recirculating perfusion system is complicated because products of intermediary metabolism accumulate in the perfusate, alterations in pH occur, and concentrations of substrates diminish constantly. Therefore, we have chosen to evaluate the nonrecirculating perfusion system as a model for mutagen production. This system allows precise control of substrate concentration and composition of the perfusion medium during the course of experiments.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (Zivic-Miller) weighing between 200 and 300 g were used in all experiments. 3-Methylcholanthrene (80 mg/kg) in corn oil or corn oil vehicle (10 ml/kg) was injected 3 days prior to perfusion experiments. Tap water and laboratory chow were fed ad libitum; fasted animals were deprived of food 24 hr prior to perfusion experiments.

Liver Perfusion. Details of the nonrecirculating liver perfusion technique have been described elsewhere (24). The common bile duct was cannulated with polyethylene tubing (PE-10; Clay-Adams), and sterile aliquots of bile were collected in tared vials over a period of 4 min. Livers were perfused with Krebs-Henseleit bicarbonate buffer (37°, pH 7.4) saturated with oxygen:carbon dioxide (95:5) (17). The fluid was pumped via a cannula placed in the vena cava past a Teflon-shielded, Clark-type oxygen electrode before being discarded or analyzed for metabolites. Rates of oxygen uptake were calculated from the influent minus effluent concentration differences, the flow rate, and the liver wet weight.

Preparation of Benzo(a)pyrene. A stock solution of 30 mm benzo(a)pyrene (Sigma Chemical Co.) was prepared in HPLC grade acetone and stored in the dark at −20°. Prior to perfusion, it was diluted with 15% (w/v) bovine serum albumin (Fraction V; Sigma) in Krebs-Henseleit buffer and stirred under nitrogen for 1 hr to evaporate the acetone. This suspension was infused into the liver at final albumin concentrations of 0.15% and benzo(a)pyrene concentrations ranging from 0.2 to 80 μM. Vehicle controls were prepared in an identical manner except that benzo(a)pyrene was omitted.

Mutagen Assay. Bile and effluent perfusate were assayed for muta-
genic activity by the method of Ames et al. (2) using Salmonella typhi-
murium, strain TA 98. Aliquots of bile (10 to 20 µl) effluent perfusate (up
to 1 ml), or concentrations of effluent perfusate were added to 2 ml of top
agar (0.9% agar, 0.5% NaCl, 0.055 mm L-histidine, and 0.055 mm biotin)
containing 0.1 ml (approximately 1.0 × 10^6 bacteria) of a resuspended
nutrient broth culture of bacteria with or without 500 Fishman units of β-
gluconoridase activity and 25 units of sulfatase activity (Sigma G-0876).
Hepatic microsomes and cofactors were not added. The agar was mixed
and poured onto Petri plates (15 x 100 mm) with 30 ml of solidified
Vogel-Bonner agar containing 2% glucose as the carbon source. Bacte-
rial colonies were counted after 72 hr of incubation at 37°C. For assays
of bacterial viability, 0.2 ml of a 10^8-fold dilution of the bacterial suspen-
sion was plated as described above except that 125 µm histidine was
included.

Concentration of Mutagenic Activity in Effluent Perfusate. Effluent
perfusate (120 ml) was lyophilized for 24 hr. The resultant residue was
extracted 3 times with 8 ml of methanol. The combined methanol extracts
were separated from the salt precipitate by centrifugation and evaporated
under a stream of N2 at 50°C. This extraction procedure was repeated
twice, and the final residue was dissolved in 1.0 ml of dimethyl sulfoxide.
Recovery of fluorescent phenols of benzo(a)pyrene with this procedure
was from 55 to 60%.

Analysis of Perfusate for Benzo(a)pyrene Phenols. Phenols of
benzo(a)pyrene in perfusate were measured by a modification (23) of
the Dehnen assay (8). Aliquots of bile (2.5 µl) or effluent perfusate (1.0 ml)
were incubated with 0.1 ml of 0.5 M Tris buffer, pH 7.4, in the presence
or absence of β-glucuronidase (500 Fishman units β-glucuronidase and
25 units aryl sulfatase activities) in a final volume of 1.1 ml. After a 3-h
incubation period (23°), 1.0 ml was added to 0.3 ml of triethylamine:Triton
X-100 (9:1) and 2.0 ml aqueous EDTA (0.5 mg EDTA/ml H2O), and
fluorescence was determined (435 to 522 nm) using an Aminco DW2A
spectrophotometer equipped with a fluorescence attachment. Phenols of
benzo(a)pyrene were quantitated using chromatographically pure 3-
hydroxybenzo(a)pyrene (IT Research Institute, Chicago, IL) as a stand-
ard. Rates of phenol production were calculated from concentrations in
effluent perfusate or bile, the flow rate, and the liver wet weight.

HPLC Analysis of Mutagenic Glucuronides in Bile. [3H]-
benzo(a)pyrene (40 Ci/mmol; Amersham/Searle Corp., Arlington Heights,
IL) was diluted with nonradioactive benzo(a)pyrene to a specific activity
of 1.0 µCi/mmol and infused into the liver at final concentrations of 20 µm.
Radioactive bile (30 µl) was incubated overnight in sealed test tubes
flushed with nitrogen in a final volume of 600 µl of 0.5 M Tris buffer (pH
7.4) containing o-saccharic acid 1,4-lactone (6 mg/ml) to inhibit any β-
gluconoridase present in bile. In some incubations, saccharic lactone was
omitted and replaced with 1000 units of β-glucuronidase.

Chromatography of bile samples was performed with Waters Associ-
ates, Inc. (Milford, MA) equipment using a Waters Associates Bondapak/Porasil 300-
x 4-mm column. Metabolites were eluted with 5 ml of water at a flow
rate of 1 ml/min followed by a 0 to 75% linear methanol:water gradient
for 40 min. Every 30 seconds, 0.5-ml fractions were collected, and
 aliquots (100 µl) were mixed with 13 ml Aquasol (New England Nuclear).
Radioactivity, which was recovered completely from the column,
determined in a Packard scintillation spectrophotometer.

Samples representing peaks were pooled and concentrated by evap-
oration of solvent at 37°C under a stream of N2. The resultant residues
were redissolved in dimethyl sulfoxide for analysis of phenols and mu-
tagens. Authentic 3- and 7-benzo(a)pyrenyl-β-D-glucuronide (National
Cancer Institute Chemical Repository) and a racemic mixture of 4,5-
benzo(a)pyrene dione-β-D-glucuronide were chromatographed under
identical conditions.

RESULTS

A nonrecirculating perfusion system allows simultaneous
quantitation of oxygen uptake, rates of bile flow, and rates of
production of mutagenic activity in bile and effluent fluids. Follow-
ing a brief (10 min) equilibration period after surgery, the hemo-
globin-free perfused rat liver consumed oxygen at rates ranging
from 80 to 135 µmol/g/hr (Chart 1, top). During this basal period,
bile flow (Chart 1, middle) was relatively constant and was devoid of
mutagenic activity (Chart 1, bottom). Infusion of albumin at
concentrations used to deliver benzo(a)pyrene caused a small increase in
oxygen uptake of about 10 µmol/g/hr, most probably due
to oxidation of bound fatty acids. The albumin vehicle did
not affect rates of bile production, and bile collected during
albumin infusion did not contain mutagenic activity.

Infusion of benzo(a)pyrene (20 µm) into livers from 3-methyl-
cholanthrene-treated rats did not affect significantly rates of
oxygen uptake. Rates of bile flow decreased steadily throughout
the perfusion period (Chart 1) but were the same in the presence
and absence of benzo(a)pyrene. Within 8 min after infusion of
benzo(a)pyrene, mutagenic activity in the bile increased steadily
above background until steady rates of production were reached
with mean values of 225 ± 22 (S.E.) revertant colonies/g/hr (n =
9). The time necessary to reach half-maximal rates of mutagen
production was 17 ± 1.3 min (Chart 2). Maximal rates of mutagen
production were significantly lower (p < 0.05) in livers of corn
oil-treated rats (149 ± 24 revertants/g/hr; n = 6); half-maximal

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rates of mutagen production were reached in 30 ± 2.6 min.

Rates of bile production were unaffected by termination of the benzo(a)pyrene infusion; however, rates of mutagen export into bile remained elevated for at least 40 min (Chart 1).

Upon infusion of benzo(a)pyrene, the color of the bile changed from yellow to dark green coincident with the production of mutagenic products in livers from control and 3-methylcholanthrene-treated rats. Hepatic "green" pigments have been isolated from microsomes incubated with a variety of chemicals, and it has been postulated that this color is due to the release of porphyrins from destruction of cytochrome P-450 (9).

The production of mutagenic glucuronides was investigated as a function of benzo(a)pyrene concentration using livers from 3-methylcholanthrene-treated rats. Maximal rates of mutagen production were observed with concentrations of benzo(a)pyrene ranging between 2 and 80 μM (Chart 3).

Mutagenic activity in bile could not be detected if purified β-glucuronidase (EC 3.2.1.31) was omitted from the assay (Table 1); however, the reversion frequency of the bacteria increased in a linear fashion when enzyme activity was increased up to a maximum value of 500 Fishman units. Addition of 100 units (1 unit = 1.0 μmol p-nitrocatechol hydrolyzed per hr at pH 5.0 and 37°C) of purified arylsulfatase (EC 3.1.6.1) without β-glucuronidase did not alter the spontaneous reversion frequency (Table 1). Thus, it is concluded that mutagenic activity is due to hydrolysis of glucuronides.

The stability of the conjugated mutagen was assessed under conditions of cold storage, heat treatment, and low pH. Mutagenic activity in the bile was unaffected by cold storage (4°C) for periods of up to 72 hr (data not shown). Similarly, when bile was boiled for 30 min, conjugated mutagens numbered 79 ± 10 colonies/plate/10 μl of bile, a value indistinguishable from non-boiled controls (77 ± 12). Incubation of the glucuronide in acid (pH 1.0) for 1 hr at 23°C resulted in the nonenzymatic cleavage of the glucuronide bond and liberation of mutagenic activity (Table 2). About one-half of this activity could be recovered after storage for up to 27 days in acid. Thus, mutagenic glucuronides are heat stable but acid labile.

When varying amounts of bile were assayed in the presence of excess β-glucuronidase (Chart 4), the number of revertant colonies increased in a curvilinear manner until a plateau was reached with volumes greater than 20 μl. To examine if benzo(a)pyrene or its metabolites were toxic to bacteria, we examined bacterial viability. Pure 3-benzo(a)pyrenyl-β-D-glucuronide (IIT Institute), a major metabolite of benzo(a)pyrene, was added to the bioassay system in the absence of β-glucuronidase.

Concentrations of up to 195 nmol/plate of 3-benzo(a)pyrenyl-β-

![Chart 3. Effect of benzo(a)pyrene concentration on rates of mutagen export into bile. Livers from 3-methylcholanthrene-treated rats were perfused with the concentrations of benzo(a)pyrene indicated. Values are average maximal rates of mutagen production; bars, S.E. (0.2 μM, n = 2; 2.0 μM, n = 5; 20 μM, n = 9; 80 μM, n = 2).](chart3)

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Table 1

Effect of β-o-glucuronidase and aryl sulfatase on reversion frequency in bile from livers perfused with benzo(a)pyrene

Aliquots (20 μl) of a mixed sample of mutagen-containing bile were collected from a liver infused with benzo(a)pyrene. Bioassays were carried out as described in "Materials and Methods" with varying amounts of purified β-glucuronidase or sulfatase. β-o-Glucuronidase activity is expressed as Fishman units; sulfatase is expressed in IU. The spontaneous reversion frequency was 27 colonies/plate and was subtracted from total colonies to yield net revertants per plate. These experiments were performed on a pooled-bile sample, and rates are not directly comparable with data in the Charts. The data are the mean of 2 replications.

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<tr>
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<td>20 μl mutagen-containing bile</td>
<td>30 ± 1</td>
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<tr>
<td>+</td>
<td>236 ± 22</td>
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Table 2

Effect of pH on conjugated mutagens from bile

Where indicated, the pH of the assay was adjusted to 1.0 with perchloric acid. After 1 hr of incubation, the pH was brought to 7.6 with potassium carbonate (1.6 m). Following centrifugation to remove precipitated salts, the supernatant was plated with or without 500 units β-glucuronidase, as described in "Materials and Methods."

![Chart 4. Relationship between volume of bile plated and reversion frequency. Bile was collected from a liver infused with 20 μM benzo(a)pyrene as described in Chart 1. Various volumes of bile were incubated with 1000 Fishman units of β-glucuronidase. Values represent total uncorrected colonies per plate.](chart4)
Chart 5. Effect of purified 3-benzo[a]pyrenyl-β-D-glucuronide on toxicity (A) and mutagenicity (B) in Salmonella typhimurium. Zero to 229 nmol of 3-benzo[a]pyrenyl-β-D-glucuronide was dissolved in 100 μl of dimethyl sulfoxide and added to bacterial assays as described in "Materials and Methods." Values are total uncorrected colonies per plate and the mean of duplicate assays.

DISCUSSION

Using oxygen consumption as a criterion, the nonrecirculating perfused liver was viable and metabolized benzo(a)pyrene to mutagenic products for at least 2 hr when the concentration of infused benzo(a)pyrene was varied from 0.2 to 80 μM (Charts 1 and 3). Thus, concentrations of benzo(a)pyrene up to 80 μM can be studied in the perfused liver without toxicity to the liver. Moreover, the concentrations of benzo(a)pyrene used in this study are similar to those used by other groups studying microsomal fractions (7), highly purified, reconstituted monooxygenase systems (27), whole cells isolated from a variety of human and nonhuman tissue types (11–13, 15), livers perfused with recirculating systems (4, 10), and in vivo preparations (5).

Our results extend the studies of Forti and Trieft (10) which demonstrated that unidentified conjugated metabolites, mutagenic towards S. typhimurium, appear in bile from rat livers perfused with benzo(a)pyrene. In our study, a good correlation (r = 0.86) between rates of phenolic glucuronide production and rates of mutagen release in bile was observed (Chart 6), suggesting that the stable conjugated mutagens released are phenolic glucuronides. This hypothesis is supported by the observation that pure 3-benzo[a]pyrenyl-β-D-glucuronide produced 4 net revertants/nmol (Chart 5B). When the rate of mutagen production was divided by the rate of production of phenolic glucuronides, a value of 4 revertants/nmol was also obtained. Furthermore, about one-half of all fluorescent metabolites had retention times on HPLC identical with pure 3- and 7-benzo(a)pyrenyl-β-D-glucuronide (Peaks I, Chart 7A). Peaks I and II (Chart 7) accounted for all of the mutagenic activity recovered from the column. Therefore, we conclude that mutagenic activity in bile results predominantly from hydrolysis of 3-benzo(a)pyrenyl-β-D-glucuronide and other phenolic glucuronides sharing similar fluorescence characteristics. The glucuronides of 6- and 9-hydroxybenzo(a)pyrene occur in bile (5) and their respective phenols are mutagenic toward S. typhimurium (strain TA98) (27). Thus, they may also contribute to the mutagenic activity observed in bile.

mutagenic activity present in bile. Similarly, only Peaks I and II contained fluorescent phenols. When bile was incubated with β-glucuronidase prior to HPLC analysis (Chart 7B), 2 new radioactive metabolites not present in untreated bile appeared between Fractions 110 and 120.

ensure that toxicity to bacteria is avoided.

Since pure 3-hydroxybenzo(a)pyrene glucuronide is both mutagenic in our assay and is a major metabolite of benzo(a)pyrene by the liver, we correlated observed rates of mutagenesis with rates of glucuronide production. A good correlation (r = 0.86) between rates of benzo(a)pyrene glucuronide formation and rates of mutagen production in bile was observed (Chart 6). About 4 revertant colonies were produced per nmol of phenolic glucuronides formed by the liver.

Export of mutagens from the perfused liver via the effluent perfusate was also determined in the same livers from which bile was collected. Mutagens in the perfusate were concentrated as described in "Materials and Methods." The extract also produced about 4 revertants/nmol of 3-hydroxybenzo(a)pyrene (data not shown).

Chromatographic analysis of bile from livers with benzo(a)pyrene demonstrated 4 major peak areas (Chart 7A) which were diminished by treatment with β-glucuronidase (Chart 7A). Metabolites labeled as Peaks I and II in Chart 7 contained all of the
Formation of Mutagenic Glucuronides by PERFUSED LIVERS

Because phenolic products of benz[a]pyrene are weakly mutagenic, considerably more attention has been paid to the extremely mutagenic diol-epoxides. However, phenols represent a major fraction of benz[a]pyrene metabolites (13). (±)-7ß,8α-Dihydroxy-9ß,10ß-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene is about 1500 times more mutagenic than is 3-hydroxybenzo[a]pyrene (25-28). Since diol-epoxides are produced by hepatocytes (13, 20) and are apparently sufficiently stable to reach the lung when injected i.p. into newborn mice at high doses (6), an obvious question that arises from our work is why most of the biliary mutagenic activity can be attributed to hydrolysis products of phenolic glucuronides. One possibility is that diol-epoxides are excreted into the bile in quantities below the detection limits of our bioassay when they are generated in the cell (i.e., they may bind intracellularly).

Work presented here shows that phenolic glucuronides of benz[a]pyrene metabolites represent a major source of potential mutagenicity to other organs. This conclusion is supported by the observation that DNA adducts resulting from further metabolism of benz[a]pyrene phenols have been detected in extrahepatic tissues of rats given an i.v. dose of benz[a]pyrene (5). To be carcinogenic, however, phenolic glucuronides must be further metabolized to mutagenic products, since they are not mutagenic per se (Chart 5; Table 1). The enzymatic hydrolysis of benz[a]pyrene-3-glucuronide by β-glucuronidase results in liberation of metabolites which bind to DNA to a greater extent than does 3-hydroxybenzo[a]pyrene, the theoretical hydrolysis product (16). This may result from rearrangement during hydrolysis to yield a complex mixture of metabolites with yet undefined structures and biological activities (19, 21). In our system, the glucuronide was about 5 times more mutagenic on a molar basis than was 3-hydroxybenzo[a]pyrene itself (data not shown). Since β-glucuronidase activity is widely distributed in mammalian organs (18), the liberation of mutagenic phenols from systemically circulating glucuronides is probable. Surprisingly, little attention has been paid to this possible mechanism of initiation.

Glucuronides of benz[a]pyrene phenols can also be cleaved by acid (Table 3). This opens the possibility that stable molecules produced in the liver may be transported to other organs followed by subsequent nonenzymatic production of electrophiles. For example, N-hydroxy-N-2-naphthylamine is oxidized and glucuronidated by the liver. The subsequent binding of arylnitrenium residues to nucleic acids in the bladder results from the acidic cleavage of the glucuronide and produces tumors in the bladder (14). Moreover, human colon cancer has been correlated with β-glucuronidase activity (22). Thus, glucuronides of chemical carcinogens could be exported in the bile via the pathways described in this study. In general, transport of stable glucuronides into urine, feces, or the blood may represent a common mechanism involved in chemical carcinogenesis in the body.

The nonrecirculating, hemoglobin-free, perfused liver provides a convenient system which can be used to study the effect of acute and chronic nutritional manipulation on the export of mutagenic glucuronides of benz[a]pyrene into the bile under near-physiological conditions. This preparation has advantages over whole-cell suspensions because it allows identification of metabolites in bile and has advantages over recirculating perfusion systems since first-pass kinetics can be studied. For example, treatment of rats with 3-methylcholanthrene produced more biliary mutagens faster than did livers from control rats (Chart 2). Since this system is quantitative, rates of mutagen export can be correlated with metabolite production under a variety of dynamic conditions (Chart 6). Moreover, the production of stable, phenolic mutagenic products from polycyclic aromatic hydrocarbons can be studied in a model that allows parameters such as bile production, flow rate, and exposure to various nutrients and hormones to be varied experimentally. Diet plays an extremely important role in carcinogenesis, possibly equaling that of smoking (1). However, mechanisms concerning effects of various nutrients on biological processes to produce neoplasms remain largely unknown. It is possible that the relatively simple, physiologic model described here could be useful in further defining interactions between cancer and nutrition.

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