Benzpyrene Hydroxylase Activity in the Gastrointestinal Tract

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SUMMARY

Quantitative and morphologic histochemical studies of the activity of the benzpyrene hydroxylase system in the gastrointestinal tract have been carried out. In normal rats activity is found only in the small intestine. In rats in which an increase in activity is induced by oral administration of 1,2-benzanthracene, activity is found throughout the entire gastrointestinal tract but varies in magnitude from one region to another. The effects of starvation and of a low-fat diet have been studied and were found to markedly lower the activity of the enzyme system. In addition to the rat, benzpyrene hydroxylase activity has been found to be present in the duodenum of mouse, guinea pig, rabbit, hamster, dog, monkey, baboon, and man. The possible role of this type of system as a protective mechanism against polycyclic hydrocarbons which might reach the gastrointestinal tract is discussed.

There has been described a group of closely related enzyme systems in liver which are capable of metabolizing compounds not normally present in the animal body (6–8). These enzymes occur in the microsomes and require reduced triphosphopyridine nucleotide (TPNH) and oxygen. An increase in the activities of many of these systems has been shown to be induced by administration of a number of polycyclic hydrocarbons (1, 6–8). Among the compounds acted upon by these systems are many potent carcinogens including those belonging to chemical groups such as the polycyclic hydrocarbons (8), the aminoazo dyes (7), the aromatic amines (5), and fluorenyl derivatives (10, 19). Studies of carcinogenesis induced by aminoazo dyes and 2-acetylaminofluorene in the rat have shown that an enhanced activity of the TPNH-dependent microsomal systems hydroxylating these compounds protects the animal against the carcinogen (17, 18).

In the present communication, studies of benzpyrene hydroxylase will be reported. This TPNH-dependent microsomal system oxidizes 3,4-benzpyrene (BP) to hydroxy derivatives and subsequently to quinones (8). In the earlier work its properties and behavior were studied only in the liver (6, 8). Subsequently a morphologic, histochemical procedure has been developed for demonstrating BP hydroxylase in tissue sections, and application of this procedure has revealed the presence of this system in a number of other tissues (81). In normal rats it has been found to be present in the kidney, adrenal, and small intestine as well as the liver. In rats in which a high activity of BP hydroxylase was induced by intraperitoneal injection of 3-methylcholanthrene, this enzyme system could also be found in the thyroid, lung, and testis. In rats in which the skin was painted with 3-methylcholanthrene, activity of this system could be demonstrated in the skin (20).

In the present investigation the distribution of BP hydroxylase activity has been studied in the gastrointestinal tract in normal animals and under several experimental conditions. This study seemed important to carry out in view of the increasing evidence of human exposure to environmental carcinogens and the possibility that polycyclic hydrocarbon-hydroxylating systems, of which BP hydroxylase can be considered a prototype, may represent a protective mechanism against ingested compounds of this type. Morphologic histochemical as well as conventional homogenate technics have been employed so as to give information as to the localization of the reaction as well as quantitative data on its activity.
MATERIALS AND METHODS

Histochemical procedure.—The morphologic histochemical procedure for the demonstration of BP hydroxylase activity in tissue sections has been described in detail elsewhere (31). In this procedure, frozen dried sections are stained with BP and then incubated in a buffered reaction mixture containing TPNH. The sections are fixed in formalin and then mounted in an aqueous medium containing 60 per cent glycerol and 1 n NaOH. By means of fluorescence microscopy, sites of BP hydroxylase activity are identified in tissue sections by the presence of the hydroxy derivatives, which have a bright green fluorescence in contrast to the unaltered BP which maintains a violet fluorescence under these conditions.

Quantitative procedure.—A quantitative adaptation of the histochemical procedure has been developed employing reaction conditions similar to those described by Conney, Miller, and Miller for quantitating this system by benzpyrene destruction as manifested by a decrease in the violet fluorescence of this compound (8). In the procedure employed in the present work mucosa was scraped from the bowel wall with a sharp scalpel blade. A 2.5 per cent homogenate of this material was made in cold (0°–8° C.) isotonic KCl with a Potter-Elvehjem type of homogenizer with a Teflon pestle. Two ml. of homogenate was pipetted into a cold (4° C.) 25-ml Erlenmeyer flask containing 50 μg of BP in 0.1 ml of acetone. One milliliter of reaction mixture was added last to initiate the reaction. It contained the following: TPNH (1 mg); DPNH (0.5 mg); 0.06 m nicotinamide; 0.05 m KCl; 0.06 m NaH2PO4·Na2HPO4 buffer at pH 7.4. The flasks were incubated aerobically at 37° C. in a covered water bath equipped with a mechanical shaker. The reaction was stopped by the rapid addition of 3.0 ml of cold acetone and the flasks placed in an iced bath. To this mixture an aliquot (0.5–1.0 ml) of the solvent was reemulsified by homogenization with a Potter-Elvehjem type of homogenizer with a Teflon pestle. Two ml. of homogenate was pipetted into a cold (4° C.) 25-ml Erlenmeyer flask containing 50 μg of BP in 0.1 ml of acetone. One milliliter of reaction mixture was added last to initiate the reaction. It contained the following: TPNH (1 mg); DPNH (0.5 mg); 0.06 m nicotinamide; 0.05 m KCl; 0.06 m NaH2PO4·Na2HPO4 buffer at pH 7.4. The flasks were incubated aerobically at 37° C. in a covered water bath equipped with a mechanical shaker. The reaction was stopped by the rapid addition of 3.0 ml of cold acetone and the flasks placed in an iced bath. To this mixture was then added 9.0 ml of petroleum ether (Skelto solve B, b.p. 66°–68° C.). The flasks were placed in a covered water bath at 37° C. for 10 minutes, which facilitates quantitative extraction of the metabolite into the 10.0-ml organic solvent phase. After storage in the dark at 4° C. for 24–48 hours, an aliquot (0.5–1.0 ml) of the solvent was removed and extracted with 10.0 ml of 1 n NaOH. The fluorescence of the aqueous extract was determined in a Farrand photoelectric fluorometer, model A, equipped with a primary filter which transmits light maximally at 400 μm and a secondary interference filter with a peak wavelength at 522 μm and a half band width of 14 μm. A quinine sulfate solution (0.5 μg/ml of 0.1 N H2SO4) was used as a fluorescence standard. A specimen of pure 8-hydroxy-3,4-benzpyrene (8-OH-BP) has been used to standardize the procedure. The fluorescence of this compound is linear in the range assayed (0.001 μg–0.02 μg/ml of 1 n NaOH). All samples were read within 1–5 minutes after the alkaline aqueous extracts were obtained. This is necessary, since a slow decrease in intensity of the fluorescence occurs with time. The fluorescence spectra of the pure sample of 8-OH-BP in 1 n NaOH and the alkaline aqueous extract from our procedure applied to the mucosa of the small intestine and liver were recorded in an Aminco-Bowman spectrofluorometer. The fluorescence spectra were almost identical, all showing a single maximum at 520 μm. This was true for highly reactive rat duodenum from animals fed 1,2-benzanthracene and liver from rats injected intraperitoneally with methylcholanthrene as well as less reactive duodenal and hepatic specimens from control rats. No effort has been made to identify the specific hydroxy derivatives produced by the BP-hydroxylase reaction of the duodenum, but instead they have been quantitated as a group. Previously reported studies of this reaction in rat liver homogenates have shown that the reaction products consist almost entirely of a mixture of hydroxy derivatives of BP of which the most abundant is 8-OH-BP. A small amount of quinone is also produced, but the quinone derivatives of BP do not fluoresce (3, 4). Since the fluorescence spectra of the reaction products so closely resemble that of 8-OH-BP, a unit of BP-hydroxylase activity has been defined as the activity which will result in an increase in fluorescence at 522 μm equivalent to that of 1 μg of 8-hydroxy-BP per mg. wet weight of tissue per minute. No correction is required for unmodified BP, since under the conditions employed the final alkaline extract shows no fluorescence at 522 μm from this material.

The activity obtained at 5 minutes was routinely employed in making calculations except in the case of weakly reactive tissues in which 10 and in some instances 20 minutes were used. Tissues reported as negative did not show demonstrable activity after a 1-hour incubation. Under the conditions used there was a linear relationship between tissue concentration and enzyme activity over the range of tissue concentrations tested, which was between 12.5 and 50 mg. wet weight of tissue in the reaction mixture.

Animals.—Most of the animal experimentation was done on female Sprague-Dawley rats of 10 weeks of age. The source of supply of rodents was as follows: Sprague-Dawley rats, Holtzman Company, Madison, Wisconsin; Syrian hamsters, Lakeview Hamster Colony, Newfield, New Jersey;
white Swiss mice, Taconic Farms, Germantown, New York; guinea pigs, Gopher State Caviary, St. Paul, Minnesota; albino rabbits, Oak Crest Rabbitry, Glen Lake, Minnesota. A specimen of baboon duodenum was obtained from Dr. Claude Hitchcock during an operative procedure on this animal; a specimen of duodenum from a 1-year-old female rhesus monkey was obtained immediately after sacrifice of this animal by cerebral trauma. The dogs employed were female mongrel animals obtained from the Animal Rescue League; the duodenum was removed immediately following exsanguination. Specimens of human duodenum DPNH (Pabst), and sesame oil U.S.P. grade (Magnus, Maybee and Reynard) were used without further purification. 1,2-Benzanthracene (Terra Chemicals, Inc.) was decolorized with activated charcoal, then recrystallized from ethanol. A highly purified preparation of 8-hydroxybenzopyrene was generously made available to us by Dr. Kenneth H. Harper of the Huntington Research Center, Huntington, England. The fat-free diet employed was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. All inorganic salts and organic solvents were Mallinckrodt, AR grade.

![Chart 1](chart.png)

Chart 1.—Distribution of benzpyrene hydroxylase activity along the gastrointestinal tract of untreated rats and rats given 20 mg. of 1,2-benzanthracene by mouth 36 hours prior to being sacrificed. The small intestine has been divided into seven segments so that differences in activity in sequential portions of the structure may be depicted.

RESULTS

Quantitative studies: BP-hydroxylase activity in the small intestine of the rat.—Quantitative studies of the BP-hydroxylase activity in the small intestine of the female rat showed that a moderate activity was present throughout much of the length of this structure except for the distal portion, in which it was extremely low (Chart 1). Activity has not been demonstrated elsewhere in the gastrointestinal tract. In rats given 4 ml. of sesame oil containing 1,2-benzanthracene (5 mg/ml by stomach tube) and sacrificed 36 hours later, a profound increase in activity was found throughout the small intestine. Activity could also be demonstrated in the stomach and the large intestine.
(Chart 1). In the stomach, in the small intestine, and in the large intestine, activity was notably lower in the distal portion of these structures than in the proximal portion.

Effects of various experimental regimens on the BP-hydroxylase activity of the rat duodenum.—Starvation for 72 hours markedly reduced the BP-hydroxylase activity of the duodenum, as can be seen in Table 1. A second procedure which has been found to markedly decrease the BP-hydroxylase activity of the duodenum is feeding of a fat-free diet. After 8 days on such a diet very little BP-hydroxylase activity remained (Table 1). The enzyme system in the duodenum seems particularly sensitive to these effects, since studies of other organs in which BP-hydroxylase activity occurs, such as liver, kidney, and adrenal, show no significant alteration under these conditions. Data on the magnitude of the increase in BP-hydroxylase activity following administration of 1,2-benzanthracene are also presented in Table 1, along with those for control animals fed sesame oil, which was used as the vehicle for this polycyclic hydrocarbon. The level of activity in the duodenum of the normal male rat was similar to that of the female, Table 1.

BP-hydroxylase activity in species other than the rat.—Studies have been carried out to determine whether the duodenum of species other than the rat have BP-hydroxylase activity. All the species included showed activity, but the magnitude varied quite widely (Table 2). Of all the specimens studied, those for man had the lowest activity. Information as to the extent to which the preoperative and operative procedures as well as the diseased states of these individuals result in an alteration of activity from a normal level is not available at present. In man, the stomach and colon have also been tested for BP-hydroxylase activity, and none has been found.

Morphologic histochemical studies of the distribution of BP-hydroxylase in the gastrointestinal tract of the rat.—In the normal rat a positive reaction can be observed in the small intestine, where it is found in the epithelial cells covering the villi. Cells in the glandular crypts, the stroma, and muscle are negative. The intensity of the activity is not uniform throughout the length of the small intestine, but can be perceived only in approximately the proximal half of this structure. Even in the proximal portion of the small intestine, the reaction is not intense, and in some specimens it cannot be seen at all. Activity has not been observed in the esophagus, stomach, cecum, or the large intestine.

In rats given 1,2-benzanthracene by mouth and sacrificed 36 hours later, a high activity of the BP-hydroxylase activity in the proximal portion of the small intestine was seen. In addition, activity became perceptible in portions of the gastrointestinal tract in which a positive reaction could not be found in normal animals. A positive reaction could be observed in the forestomach. The activity was limited to the squamous epithelium and was uniform throughout the forestomach except in its most distal portion, where the reaction became weaker. In the squamous epithelium immediately adjacent to the glandular portion of the stomach no activity was apparent. The entire glandular portion of the stomach gave a negative reaction. The duodenal mucosa exhibited an intense reaction (Fig. 1). As in the normal animal, the positive reaction was confined to the epithelium covering the villi. This intense activity persisted throughout most of the length of the small intestine but diminished progressively in the distal portion of the ileum. In the cecum and right half of the colon weak activity could be seen in the surface epithelium. No activity was observed in the glandular crypts. In the more distal portions of the large intestine a positive reaction has not been observed.

A comparison of the results of the morphologic histochemical procedure for demonstrating BP-hydroxylase activity with the quantitative one clearly showed that the quantitative method was more sensitive. This was evident from the fact that structures such as the glandular stomach and left half of the colon of rats fed 1,2-benzanthracene gave a negative reaction with the histochemical procedure but did have definite activity which could be quantitated.
Histochemical procedures in which perylene was used as a substrate (21) gave very similar results to those with BP. With both BP and perylene as substrates, enhanced activity in the small intestine and stomach has been obtained in experiments in which 3-methylcholanthrene, 3,4-benzpyrene, or 1,2,5,6-dibenzanthracene have been administered by stomach tube as well as those in which 1,2-benzanthracene was given. 1,2-Benzanthracene has been employed to induce increases in BP-hydroxylase activity in most of the work, since this compound is effective and also presents a minimum carcinogenic hazard.

### TABLE 2
**BENZPYRENE HYDROXYLASE ACTIVITY IN THE DUODENAL MUCOSA OF VARIOUS ANIMAL SPECIES**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Benzpyrene hydroxylase activity (unit*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>2.0, 5.0</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1.5, 2.5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.0, 1.3</td>
</tr>
<tr>
<td>Hamster</td>
<td>1.7, 1.3</td>
</tr>
<tr>
<td>Dog</td>
<td>2.3, 1.3</td>
</tr>
<tr>
<td>Monkey</td>
<td>13.4</td>
</tr>
<tr>
<td>Baboon</td>
<td>3.0</td>
</tr>
<tr>
<td>Man</td>
<td>0.3, 0.3, 0.2</td>
</tr>
</tbody>
</table>

* Each value represents an individual animal.

### DISCUSSION

The results of the present study have shown that in normal rats BP-hydroxylase activity is present in the small intestine and is either absent or of extremely low magnitude in other segments of the gastrointestinal tract. In animals which have received 1,2-benzanthracene by stomach tube, activity can be demonstrated throughout the entire gastrointestinal tract but shows considerable variation from one region to another. In addition, in each of the anatomic subdivisions of the gastrointestinal tract the activity is markedly higher in the proximal part than in the distal one. Thus the squamous portion of the stomach shows a moderate activity, whereas in the glandular portion it is barely detectable; most of the small intestine has a very high activity except for its distal segment, and the cecum and proximal half of the large intestine has a moderate activity while the left half of this structure including the rectum has a low activity.

The presence of polycyclic hydrocarbon-hydroxylating systems in the gastrointestinal tract may represent a mechanism whereby the animal is protected against the carcinogenic effects of ingested polycyclic hydrocarbons, since it has been shown for BP that its hydroxy derivatives are considerably less carcinogenic than the parent compound (4, 9). Such a mechanism would protect not only the gastrointestinal tract, but along with liver and other organs which have BP-hydroxylase activity would serve in having a more general protective effect, since it has been shown that ingested polycyclic hydrocarbon can produce malignant neoplasms at distant sites such as the breast (13). A protective mechanism of this type in the gastrointestinal tract might be particularly important for man, since there is substantial evidence that the human may ingest benzpyrene as well as other polycyclic hydrocarbons by virtue of their formation during the cooking or smoking of foods (2, 11, 12, 14, 15). In addition to ingested material, some polycyclic hydrocarbon which is inhaled probably reaches the gastrointestinal tract in swallowed respiratory secretions which contain these compounds.

The results of the present study indicate that the level of BP-hydroxylase activity in gastrointestinal mucosa may be varied by diet. Starvation or a low-fat diet decreases the activity, whereas ingestion of polycyclic hydrocarbons induces an increase in activity. If the excess of unaltered carcinogenic polycyclic hydrocarbon over that which is hydroxylated represents the main carcinogenic hazard, the amount of unaltered material would depend not only on the dose of these compounds taken into the gastrointestinal tract but also the hydroxylase activities of the sequential segments of the gastrointestinal tract. This latter in turn would depend on the intrinsic ability of the particular segment to form this enzyme and the various dietary stimuli which modify this intrinsic activity.
level. In considering the types of hazard from polycyclic hydrocarbons in terms of the ability of the gastrointestinal tract to detoxify these compounds, the maximum one would be sporadic high doses of carcinogen with a dietary regimen giving minimum stimulation to the induction of polycyclic hydrocarbon hydroxylases. In contrast, a considerably more favorable situation would exist where frequent lower doses are taken and where the diet stimulates a high hydroxylase formation. Under these conditions there would be present not only a relatively high level of activity to begin with, but this would be further enhanced by the additional inducing effect of the low doses of polycyclic hydrocarbon.

REFERENCES


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