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A COMPARISON OF THE EFFECTS OF BENZPYRENE ADMINISTRATION ON SOME HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASES OF RATS AND MICE*

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Abstract—There were marked differences in the responses of the hepatic microsomal enzymes of rats and mice after treatment of these animals with benzpyrene. Benzpyrene treatment caused qualitative as well as quantitative changes in the hepatic microsomal mixed-function oxidases of rats. Qualitative changes included: a decrease in the ratio of the peaks at 430 and 455 mμ in the pH-dependent ethyl isocyanide difference spectrum; an increase in the pH optimum of aniline *p*-hydroxylation; and changes in the apparent Michaelis–Menten kinetics of aniline, (+)-benzphetamine and benzpyrene metabolism. In addition, there were marked alterations in the substrate concentration-dependent "kinetics" of the difference spectra produced by the interaction of aniline and (+)-benzphetamine with rat liver microsomal P-450. Treatment of rats with benzpyrene increased both the apparent V_{max} and apparent K_m of aniline *p*-hydroxylation, and the degree of change was dependent upon substrate concentration and pH of the incubation systems. Benzpyrene treatment also increased the apparent ΔA_{max} of the aniline-induced microsomal difference spectrum, although the apparent spectral dissociation constant (K_s) was decreased. Both the rate of (+)-benzphetamine metabolism and the magnitude of the (+)-benzphetamine-induced microsomal difference spectrum were decreased after benzpyrene treatment. Treatment of rats with benzpyrene also increased the apparent V_{max} and decreased the apparent K_m of benzpyrene hydroxylation several-fold. In identical experiments performed concomitantly with mice, benzpyrene treatment produced no measurable effect on any of these characteristics of the mouse hepatic microsomal mixed-function oxidases, except for a decrease in the magnitude of the (+)-benzphetamine-induced microsomal difference spectrum. Although the administration of benzpyrene produced no stimulatory effects on the drug-metabolizing enzymes of the mouse, treatment with 3-methylcholanthrene did enhance the benzpyrene hydroxylase activity of mouse liver microsomes. Studies showed that the apparent V_{max} of this reaction was increased more than 2-fold.

A LARGE number of drugs and other chemicals foreign to the body can be biotransformed by the hepatic microsomal mixed-function oxidases. The activity of these "drug-metabolizing" enzymes can be altered greatly by exposure to a wide variety of substances found in the environment. One such substance is the carcinogenic aromatic polycyclic hydrocarbon, 3,4-benzpyrene.

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Although it is generally believed that polycyclic hydrocarbons increase the hepatic metabolism of certain drugs in the mouse,[1] Hansen and Fouts[2] were unable to detect significant changes in hepatic microsomal enzyme activity in this species after benzpyrene treatment, even when such treatment was prolonged and with high doses. Several widely accepted methods of detecting hepatic microsomal enzyme induction were employed in those studies[2] (viz. changes in drug toxicity and durations of action *in vivo*, rates of drug metabolism *in vitro*, and P-450 levels). Since, in experiments performed in our laboratory[3] and in many other laboratories,[1] benzpyrene did produce alterations in the characteristics of microsomal mixed-function oxidases in the rat, there seemed to be an animal species difference in response to this polycyclic hydrocarbon.

The main purpose of the present studies was to measure and compare more thoroughly various qualitative and quantitative effects of 3,4-benzpyrene treatment on the microsomal drug-metabolizing enzymes of the mouse and rat. Thus, by performing concomitant experiments with both rats and mice and by employing various types of test systems, we hoped to determine whether benzpyrene was an inducer of microsomal enzymes in the mouse, and whether the negative results obtained earlier could be attributed to a lack of either specificity or sensitivity in the methods used. Also, to determine whether the lack of effect seen after benzpyrene administration to mice was typical of all aromatic hydrocarbons, some studies were performed with 3-methylcholanthrene as the inducing agent. A variety of polycyclic hydrocarbons will induce increased microsomal mixed-function oxidase activity and, although these increases are thought to occur via the same mechanism,[4] the degree of induction produced varies among the compounds used.[5,6] Thus, comparative studies have clearly shown that, while 3,4-benzpyrene was a potent inducer of hepatic microsomal drug-metabolizing enzymes in rats, it was not the most potent of the hydrocarbons tested, and that 3-methylcholanthrene was one of the polycyclic hydrocarbons exhibiting an inducing potency greater than that of 3,4-benzpyrene in rats.[5,6] This difference in potency (or efficacy), although rather slight in rats, was thought to be of potentially greater significance in mice.

MATERIALS AND METHODS

Animals

Male, Swiss–Webster mice weighing between 25 and 35 g were used in these studies and were obtained from Sutter Farms, Springfield, Mo. Rats were males of the Sprague–Dawley strain from Simonsen Laboratories, Minneapolis, Minn., except in the studies with ethyl isocyanide, wherein male, Long–Evans strain from Simonsen Laboratories, Gilroy, Calif., were used. The weight range of the rats was 110–220 g. Both mice and rats were fed Wayne Lab Blox and tap water *ad libitum* up to the time of sacrifice. Animals were housed in suspended, stainless steel cages, in air conditioned rooms (70–72°F) with controlled lighting (10 hr of light/day).

The inducing agents were dissolved in corn oil by gentle heating and were administered by intraperitoneal injection in a volume of 0·5 ml/100 g of body weight. Benzpyrene was given as a single dose of 45 mg/kg of body weight once between 64 and 72 hr before sacrifice. Animals receiving 3-methylcholanthrene were injected with a dose of 20 mg/kg, once a day for 3 days, and were sacrificed approximately 24 hr after the last dose. In all cases, control animals received corn oil alone.

Preparation of microsomes

Animals were killed between 6 and 8 a.m. by decapitation, and were exsanguinated to aid in the removal of blood from the liver. Livers were excised (gall bladders were removed from mouse livers) and chilled immediately. All subsequent preparative procedures were performed at 0–4°. The livers were placed in 0·15 M KCl buffered with 0·005 M Tris or potassium phosphate, pH 7·4 (buffered KCl), minced with scissors, rinsed with additional buffered KCl, blotted and weighed. When microsomes were obtained for spectral studies, the tissue was minced and rinsed three times to aid in the removal of hemoglobin. Next, 2 vol. of buffered KCl was added to the minced livers, and the tissue was homogenized using two strokes of a motor-driven Potter–Elvehjem homogenizer (Teflon pestle). The homogenates were centrifuged at 9000 *g* for 20 min, and the resultant post-mitochondrial supernatant fractions were aspirated with care with a needle and syringe to avoid contamination by the sediment or the floating, fatty layer. Microsomal fractions of the livers were prepared by centrifuging each pool of 9000 *g* supernatant for approximately 100,000 *g*-hr on a preparative ultracentrifuge (IEC B-60 or Beckman L2 65-B), suspending the resultant pellets in buffered KCl, then resedimenting. The washed microsomes were resuspended in buffered KCl at a concentration of approximately 10 mg of microsomal protein/ml. The protein concentrations of the microsomal suspensions were determined by the method of Lowry *et al.*[7] using Pentex Fraction V bovine serum albumin in the standards. The protein concentrations were then adjusted so that assays comparing microsomes from different treatment groups were always performed with the same predetermined amount of protein in all systems.

Assays of drug-metabolizing enzyme activity

The incubation mixtures used to determine the rate of aniline *p*-hydroxylation contained 0·25 m-moles of Tris or Hepes buffer, 2·5 mg of microsomal protein, 0·25 ml of soluble fraction from control rats, 12·5 μmoles $MgSO_4$, 12·5 μmoles glucose 6-phosphate and 2·5 μmoles NADP together with sufficient water to produce a total volume of 2·5 ml. The incubations were carried out for 15 min in a Dubnoff metabolic shaker (100 oscillations/min) at 37° under an atmosphere of oxygen (flow rate 1000 ml/min). An ordinary glass marble was added to each 15-ml incubation beaker to ensure adequate mixing of the contents. Fouts[8] has shown that assays of microsomal mixed-function oxidase activity performed under these conditions were sensitive to the stimulation of drug metabolism produced by pretreatment of animals with phenobarbital.

The incubation conditions for the estimation of (+)-benzphetamine *N*-demethylation were identical to those described for the hydroxylation of aniline, except that 1·8 μmoles NADP was used and 12·5 μmoles semicarbazide was added to trap the formaldehyde formed. Systems used to measure the metabolism of benzpyrene *in vitro* were also identical to those used for aniline except that no $MgSO_4$ was added. Benzpyrene was added to the incubation media dissolved in acetone (0·1 ml acetone/2·5 ml incubation mixture).

In all experiments, the pH of the buffers was measured and adjusted at the temperature the buffers were to be used.

The analysis methods were as follows. The *p*-hydroxylation of aniline was estimated by the method of Kato and Gillette,[9] as modified by Gram *et al.*[10] The *N*-demethylation of benzphetamine was determined by measuring the production of formaldehyde using the method of Cochin and Axelrod,[11] as described by Hewick and Fouts.[12]

Benzpyrene metabolism was determined by spectrofluorometric measurement of products according to the method described by Wattenberg *et al.*[13] with the following modifications. The reaction was stopped by placing the incubation beakers in an ice bath and immediately adding 1·0 ml of cold acetone. Next, 7·0 ml hexane (Skelly Solve B) was added, and the incubation mixture plus solvents was transferred to 25-ml Erlenmeyer flasks. (In some experiments the enzyme–cofactor–substrate incubation was run directly in the Erlenmeyer flasks, thereby eliminating the transfer step.) The flasks were then capped with marbles and shaken in a Dubnoff incubator at 37° for 15 min. After shaking, the contents of the flasks were transferred to 45-ml glass-stoppered centrifuge tubes and stored in the dark at 4° for 24–72 hr. Next, the tubes were shaken vigorously for 10–15 min and centrifuged to separate the organic and aqueous phases, and 2·0-ml aliquots of the hexane phase were transferred to clean 45-ml centrifuge tubes. Finally, in subdued light, 7·0 ml of 1 N NaOH was added to each of the tubes; the samples were shaken for 20 min and centrifuged; and the fluorescence of aliquots of the NaOH solution was read on an Aminco–Bowman spectrophotofluorometer [excitation wavelength, 400 mμ; emission wavelength 525 mμ; and slits adjusted according to scheme No. 3 (p. 15, Aminco–Bowman operating manual)].

While this method seemed adequate when mouse liver microsomes were used, i.e. product formation was linear with time and proportional to microsomal protein concentration under the conditions used, preliminary studies showed that estimates of the initial velocities of benzpyrene hydroxylation *in vitro* by microsomes from benzpyrene-treated rats seemed grossly in error when low benzpyrene concentrations were employed. These errors could be attributed to changing rates of product formation during the 15-min incubation period. Therefore, the following additional modifications of the experimental method were devised to investigate more fully the time-course of benzpyrene metabolism *in vitro* by microsomes from benzpyrene-treated and control rats.

Volumes of 20–30 ml of buffer–cofactor incubation mixtures, identical in composition to those already described for benzpyrene metabolism, were warmed to 37° in 125-ml Erlenmeyer flasks. Appropriate aliquots of microsomal suspensions were then added and allowed to mix at 37° for 1 min. Finally, benzpyrene was added dissolved in acetone to start the reaction. The incubation flasks were shaken at 37° in air and, at various specified time intervals of 1·0 min or less, 2·5-ml aliquots of the reaction mixture were transferred to 45-ml glass-stoppered centrifuge tubes containing 7·0 ml hexane and 1·0 ml acetone. The remainder of the assay procedure was carried out as previously described, except that the step involving shaking the reaction mixture 10 min with acetone and hexane at 37° was carried out directly in the unstoppered centrifuge tubes. The apparent benzpyrene hydroxylase activity (relative fluorescence units per milligram of microsomal protein) was then plotted vs. the incubation time, and estimates of initial reaction velocities were obtained from the slopes of the linear portions of the lines fitted to the data points.

Spectral studies

All microsomal difference spectra were measured at room temperature on a Shimadzu MPS-50-L dual beam recording spectrophotometer.

The ethyl isocyanide difference spectra of dithionite-reduced microsomal suspensions (1·0 to 1·5 mg microsomal protein/ml) were determined according to the procedure of Sladek and Mannering,[14] modified so that the final concentration of the phosphate buffer was 1·0 M. The ethyl isocyanide was graciously provided by Dr. Don Shoeman of the Department of Pharmacology, University of Minnesota, Minneapolis, Minn.

Type I and type II difference spectra, attributed to the interaction of drug substrates with cytochrome P-450,[15–17] were determined by consecutive additions of (+)-benzphetamine or aniline, respectively, to microsomal suspensions containing 3·0 mg protein/ml in 0·1 M Hepes buffer, pH 7·3. With each addition to the sample cuvette, a corresponding volume of water was added to the reference cuvette (maximum volume added was 60 μl). Differences in extinction were measured between the peak at 385–390 mμ and the trough at 420 mμ for (+)-benzphetamine, and between the trough at 395 mμ and the peak at 430 mμ for aniline.

NADPH–cytochrome P-450 reductase

NADPH–cytochrome P-450 reductase activity was assayed essentially as described by Gigon *et al.*[18] The procedure incorporated the following modifications. The microsomal protein concentration was 3·0 mg/ml of cuvette contents, and the buffer used was 0·1 M Hepes (pH 7·4) which had been bubbled with oxygen-free nitrogen for at least 30 min at 37° prior to the addition of the microsomes. Before the assay, the microsomal suspension was bubbled with nitrogen and CO for an additional 10 min at room temperature. The source of reducing equivalents was either 1·2 μmoles NADPH (chemically reduced) or an NADPH-generating system which included 24 μmoles glucose 6-phosphate, 1–2 μmoles NADPH and 0·56 unit of bacterial glucose 6-phosphate dehydrogenase.

RESULTS

Ethyl isocyanide interacts with dithionite-reduced liver microsomes, producing a difference spectrum with peaks at 430 and 455 mμ.[19] At any given ionic strength, the relative intensities of these two peaks are dependent upon pH.[20] Sladek and Mannering[14] showed that the pH-dependent ratio of the peak heights is shifted in rats treated with 3-methylcholanthrene, and recently Parli and Mannering[5] demonstrated that measurement of this phenomenon provides a sensitive, quantitative index of alterations in microsomal cytochrome P-450 from rats treated with aromatic polycyclic hydrocarbons.

A useful method of ascertaining quantitatively the benzpyrene-induced change in the ethyl isocyanide difference spectrum is to measure the change in the pH at which the peaks at 430 and 455 mμ attain the same magnitude.

Results of a typical experiment of this kind are shown in Figs. 1 and 2. Points on these figures represent the heights of the peaks (at 430 and 455 mμ relative to 500 mμ) of the ethyl isocyanide difference spectrum at the indicated pH values. The data indicated that the ethyl isocyanide difference spectrum of rat liver microsomal cytochrome P-450 was substantially altered after the administration of benzpyrene to

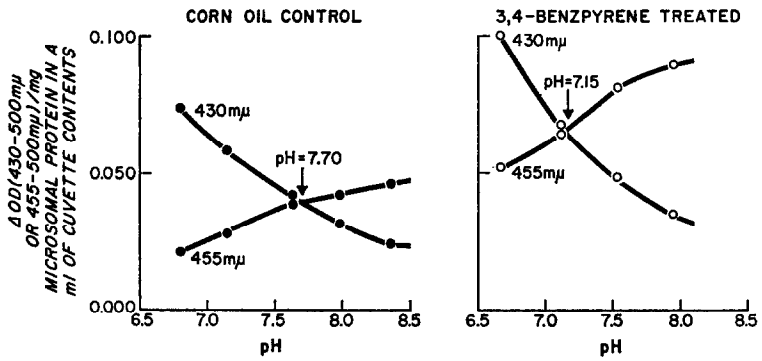

FIG. 1. Ethyl isocyanide-induced microsomal difference spectra in rats. Treatment procedures and assay methods are described in the text.

rats. However, benzpyrene treatment did not change the spectral characteristics of this hemoprotein–ethyl isocyanide interaction in mouse liver microsomes.

It has been shown[3] that the rate of aniline metabolism by rat liver microsomes is pH dependent, and that benzpyrene treatment shifts the pH optimum (in incubation systems employing Tris buffer) from pH 7·0 to about pH 8. Therefore, we investigated the effects of benzpyrene treatment on the pH optimum of hepatic microsomal aniline hydroxylase from both rats and mice.

The results of these experiments are shown in Figs. 3 and 4. Treatment of rats with benzpyrene produced a shift in the pH optimum of aniline hydroxylase from pH 7·0 or 7·1 to about pH 7·6. However, the data from concomitant experiments performed with mouse liver microsomes showed that benzpyrene treatment did not affect the pH optimum (about 7·2) of aniline hydroxylase in this species.

Michaelis–Menten analysis of reaction rates, which are often first order with respect to substrate concentration, has become a popular tool in the analysis of alterations in microsomal mixed-function oxidases. However, there are many theoretical and methodologic considerations which can limit the applicability of simple Michaelis–Menten kinetics to microsomal enzyme systems (cf. discussion by Smuckler *et al.*[21]).

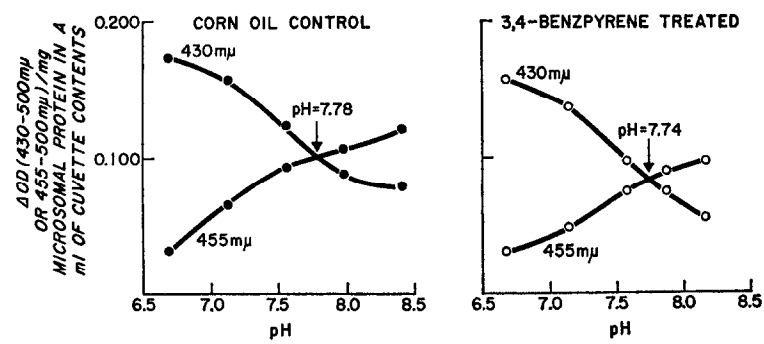

FIG. 2. Ethyl isocyanide-induced microsomal difference spectra in mice. Treatment procedures and assay methods are described in the text.

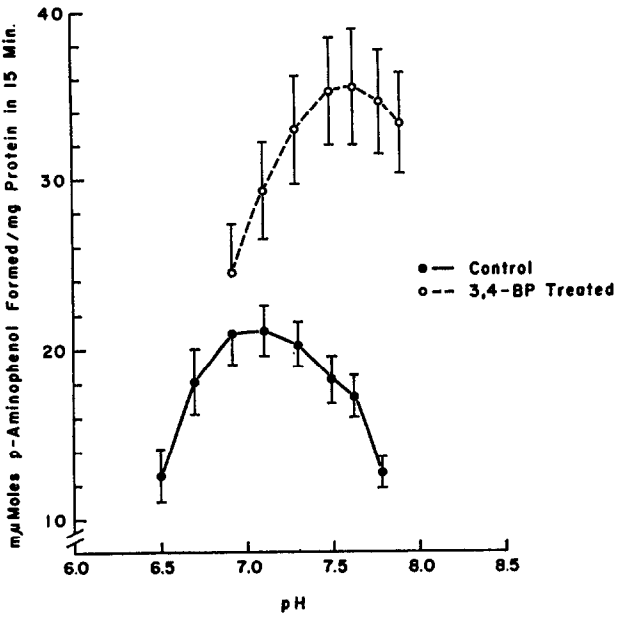

FIG. 3. The pH optima for the *p*-hydroxylation of aniline by microsomes from benzpyrene-treated and control rats. Treatment procedures and assay methods are described under Materials and Methods. The incubation systems contained 0·1 M Hepes buffer, 1·0 mg microsomal protein/ml and 4·0 mM aniline. The abscissa indicates the pH of the reaction mixtures (measured after approximately 5 min of incubation), and the rate of aniline metabolism is shown on the ordinate. Data points are means (± S.E.) of four experiments. The rate of aniline metabolism by microsomes from benzpyrene-treated rats is significantly greater ($P < 0·05$) than the rate by microsomes from control rats at all pH values higher than 7·0.

Therefore, in the studies in the present paper, analysis and interpretation of kinetic data will be essentially descriptive and limited to the relationship of substrate concentration to the rate of metabolism *in vitro*, or to the magnitude of substrate-induced microsomal difference spectra. All kinetic "constants" (e.g. K_m, K_s, V_{max}, ΔA_{max}) of membrane-bound, multi-component systems can serve only as indicators of change, not definers of change in the usual sense in which constants are employed (as in purified, soluble enzyme assays).

Castro and Gillette[22] have demonstrated one such practical ramification of kinetic "analysis" of metabolic data *in vitro*. They showed that both the appparent V_{max} and the apparent K_m for mouse liver microsomal ethylmorphine demethylase were higher in females than in males. Thus, the degree of difference in the rates of ethylmorphine metabolism between the two sexes was dependent upon the substrate concentration employed. We felt that similar differences in drug metabolism by microsomes from benzpyrene-treated as compared with control mice might be revealed by comparing rates of metabolism at several concentrations of substrate, even when such differences were not evident at "optimal" substrate concentrations.

Aniline and (+)-benzphetamine were chosen for these studies as representatives

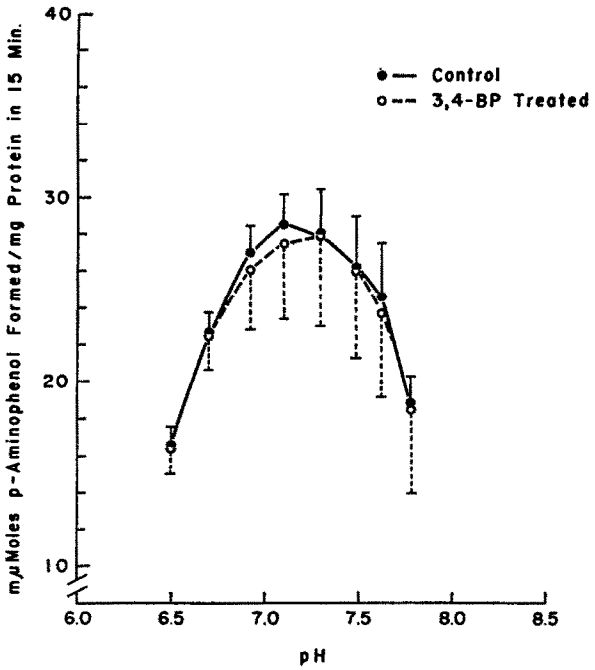

FIG. 4. The pH optima for the *p*-hydroxylation of aniline by microsomes from benzpyrene-treated and control mice. (See legend to Fig. 3.) There are no significant differences in the rates of aniline metabolism between treatment groups at any of the pH values shown.

of type II and type I substrates.[12,17] Figure 5 shows Lineweaver–Burk plots of aniline *p*-hydroxylase activity of liver microsomes from benzpyrene-treated and control rats and mice. The administration of benzpyrene to mice produced no noticeable effects on the rate of aniline hydroxylation at any substrate concentration employed (0·4–1·0 mM).

In contrast to the lack of effect on mice, treatment of rats with benzpyrene apparently produced both qualitative and quantitative changes in microsomal aniline *p*-hydroxylase activity. Thus, the Lineweaver–Burk plot of microsomal aniline *p*-hydroxylation by control rat liver microsomes was rectilinear. However, the plots obtained with liver microsomes from benzpyrene-treated rats were curved. Moreover, the degree of curvature seemed to depend upon the pH of the incubation system. When the incubations were performed at pH 7·2 (near optimal pH for control rat liver microsomes), there was little evidence of benzpyrene-induced increases in aniline metabolism until the substrate concentrations exceeded 0·25 mM. At higher concentrations of aniline, the Lineweaver–Burk plots curved downward, a phenomenon indicative of substrate activation. This effect was even more pronounced when the incubations were performed at pH 7·6 (optimal pH for liver microsomes from benzpyrene-treated rats). At aniline concentrations of 0·25 mM or less, the rate of aniline metabolism by microsomes from benzpyrene-treated rats was even lower than that obtained with control rat microsomes. As the substrate concentration was increased to 4 mM, the rate

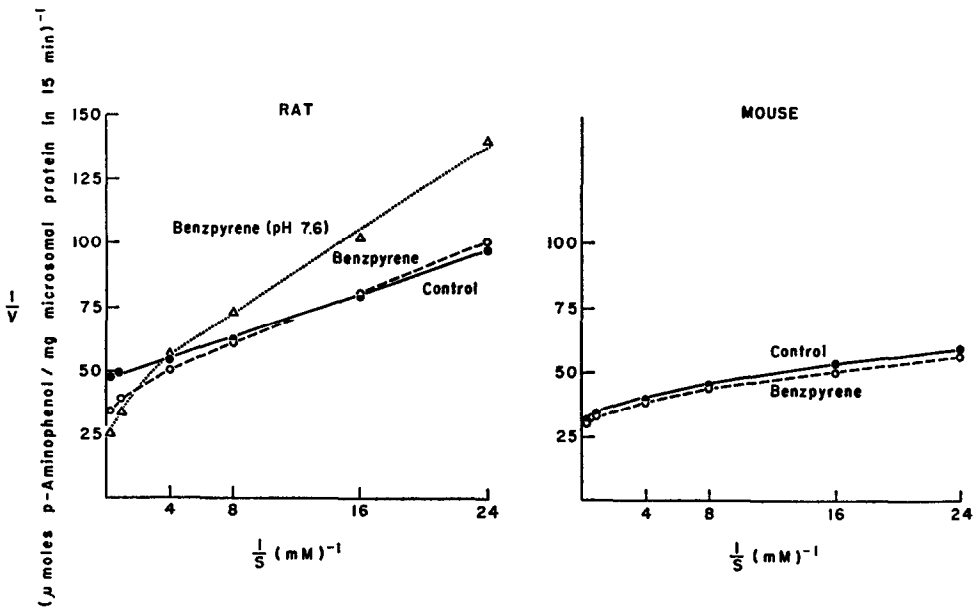

FIG. 5. Aniline *p*-hydroxylation by liver microsomes from benzpyrene-treated and control rats or mice. Treatment procedures and assay methods are described under Materials and Methods. The incubation systems contained 1·0 mg microsomal protein/ml and 0·1 M Hepes buffer. The pH of all the incubation mixtures was 7·2 ± 0·1, except for the systems employing microsomes from benzpyrene-treated rats at pH 7·6 ± 0·1 (indicated by open triangles). The reciprocal of the concentration of added aniline (range, 0·04–4 mM) is shown on the abscissa, and the reciprocal of the rate of aniline *p*-hydroxylation is shown on the ordinate. Data points are the means of values from four experiments.

of aniline metabolism increased until it substantially exceeded the control rate. Interestingly, these data indicate that the shift in pH optimum of aniline *p*-hydroxylation produced by treatment of rats with benzpyrene may also depend on the aniline concentration employed. If one were to measure the pH optimum of aniline *p*-hydroxylase from benzpyrene-treated and control rats using an aniline concentration of 0·1 mM, there would probably be no evidence of benzpyrene having caused either an increase in enzyme activity or a shift in the pH optimum. Further studies on this phenomenon have recently been published.[43]

Work from several laboratories[3,5,23,24] has demonstrated that the type II aniline-induced difference spectrum with rat liver microsomes is enhanced after treatment of the animals with 3-methylcholanthrene or benzpyrene, or other polycyclic hydrocarbons. Therefore, the aniline-induced difference spectra with microsomes from benzpyrene-treated and control rats or mice were determined in order to compare treatment-induced changes in rat liver microsomes with those in the mouse, and to correlate[24] these findings with changes in the rate of microsomal aniline metabolism *in vitro* by the two animal species. Aniline difference spectra were determined in both 0·1 M Tris and 0·1 M Hepes buffer (final pH 7·4). The results obtained with the two buffers were very similar; therefore, only the data from experiments utilizing Hepes buffer are shown (Fig. 6). Benzpyrene treatment appeared to have no marked effect on the type II difference spectrum produced by the addition of aniline to mouse liver

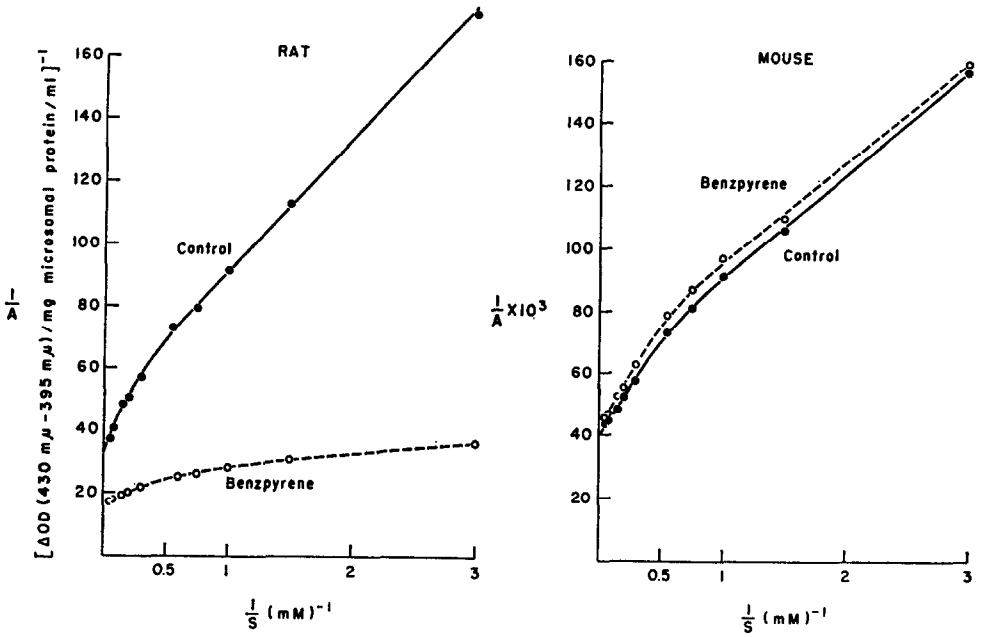

FIG. 6. Aniline-induced microsomal difference spectra in liver preparations from benzpyrene-treated and control rats or mice. Treatment procedures are described under Materials and Methods. The reciprocal of the concentration of added aniline (range, 0·33–20 mM) is shown on the abscissa, and the reciprocal of the magnitude of the absorbance change is shown on the ordinate. The cuvettes contained a suspension of 3·0 mg microsomal protein/ml of 0·1 M Hepes buffer (pH 7·4 ± 0·1). Data points are the means of values from three experiments.

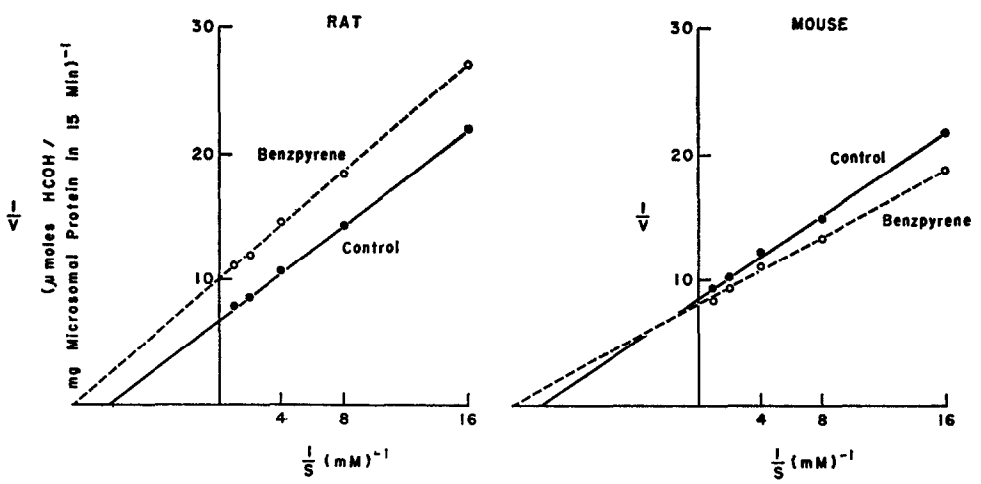

FIG. 7. *N*-demethylation of benzphetamine by liver microsomes from benzpyrene-treated and control rats or mice. Treatment procedures and assay methods are described under Materials and Methods. The incubation systems contained 0·1 M Hepes buffer (pH 7·35) and a microsomal protein concentration of 1·0 mg/ml. The reciprocal of the concentration of added (+)-benzphetamine (range, 0·06–1·0 mM) is shown on the abscissa, and the reciprocal of the rate of formaldehyde formation is shown on the ordinate. Data points are the means of values from six (rat) and four (mouse) experiments.

microsomes, and these results (with liver microsomes from control or benzpyrene-treated mice) closely resembled the data obtained with control rat liver microsomes. As expected, however, microsomes from benzpyrene-treated rats exhibited markedly enhanced type II substrate-induced difference spectra at all concentrations of aniline employed.

Regardless of the buffer used, the Lineweaver–Burk plots of the difference spectra were curves with the most pronounced bend occurring at aniline concentrations of approximately 1·5–4 mM. Similar curves have been reported previously by Hewick and Fouts[12] and by Schenkman.[25]

Lineweaver–Burk plots of (+)-benzphetamine metabolism by liver microsomes from benzpyrene-treated and control rats and mice are shown in Fig. 7. Contrary to the findings of Rickert and Fouts,[3] benzpyrene treatment of rats appeared to reduce the rate of formaldehyde formation *in vitro* at all benzphetamine concentrations used in these experiments. These results are consistent with the decrease in hexobarbital metabolism produced by 3-methylcholanthrene treatment of rats as reported by Shoeman *et al.*[24]

The administration of benzpyrene to mice seemed to have no inhibitory effect on benzphetamine metabolism *in vitro*. In all experiments, the rate of benzphetamine demethylation by liver microsomes from benzpyrene-treated animals was the same as or slightly higher than the rate obtained with control mouse liver microsomes incubated with the same concentration of substrate (Fig. 7).

Treatment of rats with polycyclic hydrocarbons has been shown to reduce the magnitude of the type I substrate-induced difference spectrum.[3,24] Experiments performed to compare the effects of benzpyrene treatment of animals on the benzphetamine-induced difference spectrum of liver microsomes from mice and rats (Fig. 8) showed that, in the rat, benzpyrene treatment decreased the magnitude of the type I substrate-induced difference spectrum at all concentrations of (+)-benzphetamine studied. The administration of benzpyrene to mice also decreased the (+)-benzphetamine-induced difference spectrum, although apparently not to the same extent as with rats. Moreover, with mouse liver microsomes, the reduction of the (+)-benzphetamine-induced difference spectrum that was caused by prior treatment of the animals with benzpyrene seemed to follow competitive-type inhibition kinetics; while with rat liver microsomes, the benzpyrene-caused inhibition of the type I substrate-induced difference spectrum appeared to be more noncompetitive in nature. With microsomes from either animal species, the Lineweaver–Burk plots of the substrate-induced difference spectra exhibited marked curvature.

Conney *et al.*[26] demonstrated that the administration of benzpyrene to rats stimulated benzpyrene metabolism *in vitro*. However, our preliminary experiments had indicated that prior treatment with benzpyrene *in vivo* had no significant effect on benzpyrene metabolism by mouse liver microsomes. These results were, in themselves, inconclusive because studies performed by several workers[3,27–29] demonstrated that treatment of rats with polycyclic hydrocarbons caused changes in the apparent Michaelis–Menten kinetics of hepatic microsomal benzpyrene hydroxylase. Although the specific changes which were reported varied among laboratories and probably depended upon the individual assay procedures used, clearly the induction of liver microsomal benzpyrene hydroxylase could be associated with changes in the apparent V_{max} and apparent K_m of the reaction, which might offset each other. Therefore, the

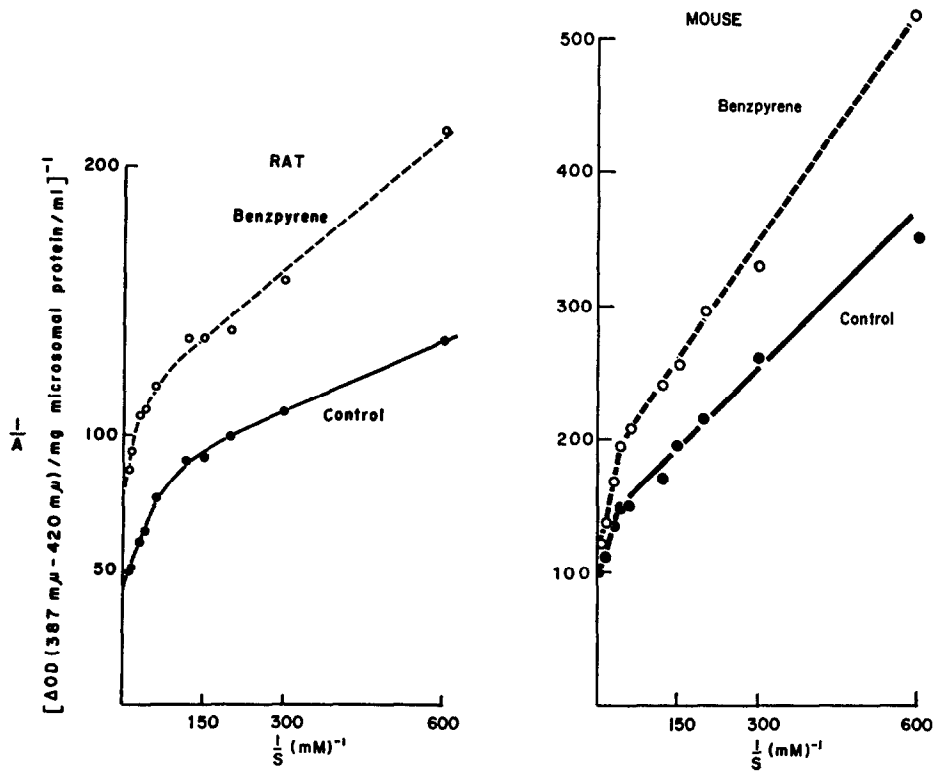

FIG. 8. Benzphetamine-induced microsomal difference spectra in liver preparations from benzpyrene-treated and control rats or mice. Treatment procedures are described under Materials and Methods. The reciprocal of the concentration of added (+)-benzphetamine (range, 0·0017–0·10 mM) is shown on the abscissa, and the reciprocal of the magnitude of the absorbance change is shown on the ordinate. The cuvettes contained a suspension of 3·0 mg microsomal protein/ml of 0·1 M Hepes buffer (pH 7·3 ± 0·1). Data points are the means of values from four experiments.

apparent Michaelis–Menten kinetics of liver microsomal benzpyrene hydroxylase from benzpyrene-treated and control rats and mice were studied.

The experiments performed with mouse liver microsomes showed that prior treatment of mice with benzpyrene did not affect the rate of benzpyrene hydroxylation *in vitro* at any of the substrate concentrations employed. When the data were expressed as Lineweaver–Burk plots (Fig. 9), straight lines were obtained, indicating that, like the liver microsomal enzyme system derived from control rats, benzpyrene hydroxylation by liver microsomes from both control and benzpyrene-treated mice appeared to conform to simple Michaelis–Menten kinetics. Moreover, the Lineweaver–Burk plots were virtually superimposable and were nearly identical in both slopes and intercept with those obtained using control rat liver microsomes (data not shown).

When liver microsomes from benzpyrene-treated rats were used as the source of benzpyrene hydroxylase, the metabolism *in vitro* of benzpyrene, added at concentrations less than 40 μM, proceeded so rapidly that significant substrate depletion occurred in much less than 15 min. Therefore, we used the sequential sampling technique (described under Materials and Methods) to obtain more accurate estimates of initial

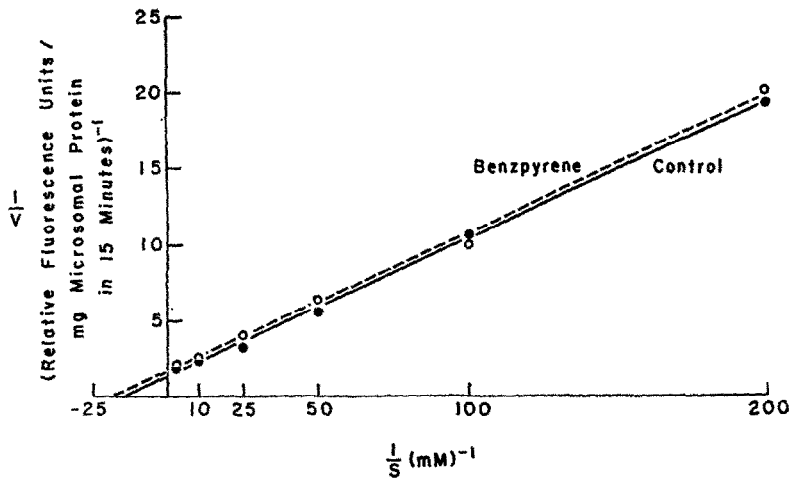

FIG. 9. Benzpyrene hydroxylation by liver microsomes from benzpyrene-treated and control mice. Treatment procedures and assay methods are described under Materials and Methods. The incubation systems employed 0·1 M Tris (pH 7·35) and a microsomal protein concentration of 1·0 mg/ml. The reciprocal of the concentration of benzpyrene (range, 5–400 μM) is shown on the abscissa. The reciprocal of the apparent reaction velocity is shown on the ordinate. Data points are the means of values from four experiments.

reaction velocities. Data obtained using the modified assay method (Fig. 10) clearly indicated that benzpyrene treatment both increased the apparent V_{max} of rat hepatic microsomal benzpyrene hydroxylation and decreased the apparent K_m (i.e. as the substrate concentration was reduced, the relative stimulation of benzpyrene hydroxylase activity increased).

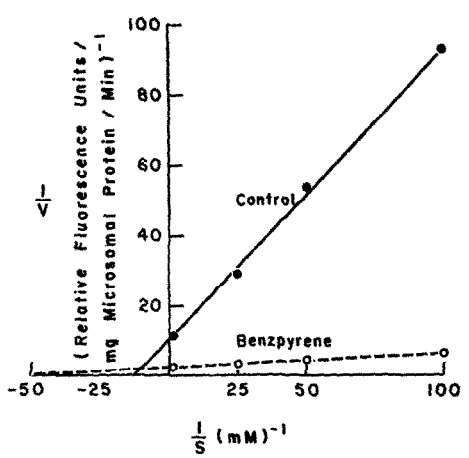

FIG. 10. Benzpyrene hydroxylation by liver microsomes from benzpyrene-treated and control rats. Treatment procedures and assay methods are described under Materials and Methods. The incubation systems employed 0·1 M Hepes (pH 7·35) and a microsomal protein concentration of 0·5 to 1·0 mg/ml. Apparent initial reaction velocities were determined from the slope of the linear portion of time-course experiments (assay times of 30 sec, 1, 2, 3, 4 and 5 min). The reciprocals of the apparent initial reaction velocities are shown on the ordinate. The reciprocals of the benzpyrene concentrations (range, 10–400 μM) are shown on the abscissa. Individual points are the means of four experiments.

Although the data presented thus far showed that benzpyrene treatment had no appreciable effect on the mouse liver microsomal mixed-function oxidases which were studied, other workers have documented effects attributed to liver microsomal enzyme induction in mice after treatment with 3-methylcholanthrene. Cramer *et al.*[30] showed that 3-methylcholanthrene treatment stimulated the metabolism of 2-acetylaminofluorene by mouse liver microsomes. Similarly, Alvares *et al.*[27] described a 2-fold increase in the rate of benzpyrene hydroxylation in mice treated with 3-methylcholanthrene, and a shift of the peak of the carbon monoxide-induced difference spectrum of dithionite-treated microsomes from 450 to 448 mμ. Therefore, benzpyrene hydroxylase activity of liver microsomes from 3-methylcholanthrene-treated and control rats and mice was measured and compared so as to establish whether the activity of mouse liver microsomal mixed-function oxidase could be stimulated by treatment with a polycyclic hydrocarbon other than 3,4-benzpyrene.

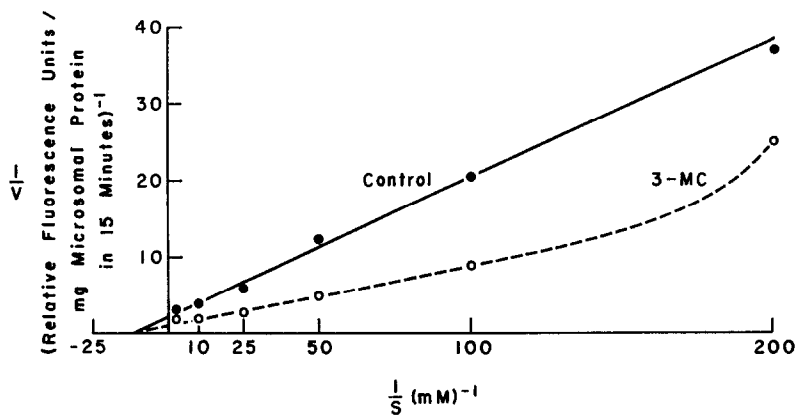

FIG. 11. Benzpyrene hydroxylation by liver microsomes from 3-methylcholanthrene-treated and control mice. See legend to Fig. 9 for other details.

Preliminary experiments indicated that injection of 20 mg 3-methylcholanthrene/kg, daily for 3 days, produced a 2-fold increase in mouse hepatic microsomal benzpyrene hydroxylase and a 6-fold increase in the activity of the rat liver microsomal enzyme.

Since this dose schedule produced a measurable response, experiments were performed to determine the effect of 3-methylcholanthrene treatment on the apparent Michaelis–Menten kinetics of benzpyrene hydroxylase in mouse liver microsomes. Lineweaver–Burk plots of the data (Fig. 11) show that prior treatment of mice with 3-methylcholanthrene produced an enhanced benzpyrene hydroxylase activity at all substrate concentrations employed.

As in previous work, control mouse liver microsomes appeared to metabolize benzpyrene according to a simple Michaelis–Menten relationship. The Lineweaver–Burk plot of benzpyrene hydroxylation by liver microsomes from 3-methylcholanthrene-treated mice also appeared rectilinear, with all points falling on a straight line except the one representing the apparent rate of metabolism at the lowest substrate concentration used (5 μM). Thus, although 3-methylcholanthrene altered the mouse hepatic microsomal mixed-function oxidase activity, the changes produced were

somewhat different from the effects observed after 3-methylcholanthrene[25] or benzpyrene treatment of rats (K_m appears to be altered in rats, but not in mice; Fig. 10 vs. Fig. 11).

Some experiments have been performed to determine whether treating mice with the polycyclic hydrocarbons, 3-methylcholanthrene or 1,2,3,4-dibenzanthracene, produces any marked effect on hepatic microsomal aniline hydroxylase activity. These experiments showed that neither 3-methylcholanthrene nor dibenzanthracene produced any such change in the apparent V_{max} of mouse liver microsomal aniline hydroxylase. Although these conclusions should be considered as tentative, it is reasonable to conclude that if these compounds do alter aniline metabolism by the mouse, these alterations are much less than those observed in the rat after treatment with 3,4-benzpyrene.

Gigon *et al.*[18] have proposed that the rate-limiting step in oxidation of drugs by liver microsomal enzymes is the reduction of the cytochrome P-450–substrate complex by NADPH. These workers found that complexes of cytochrome P-450 with type I substrates were reduced more rapidly, while type II substrate–cytochrome P-450 complexes were reduced more slowly, than the free cytochrome. These results have subsequently been confirmed in several laboratories including our own.[31] Schenkman *et al.*[32] showed that the absolute spectrum of liver microsomal P-450 from benzpyrene-treated rats resembles the spectrum of a type I substrate–microsome complex. Therefore, part of the enhanced drug metabolism resulting from benzpyrene treatment of animals might be due to the formation (via either enzyme synthesis or enzyme activation) of a microsomal cytochrome P-450 that is more readily reduced than the native form. Similarly, the lack of effect of benzpyrene in mice might be attributed to the absence of this phenomenon. To investigate these possibilities, NADPH–cytochrome P-450 reductase levels of microsomes from benzpyrene-treated and control rats and mice were measured.

The data from these experiments (Table 1) show that benzpyrene treatment slightly, but significantly, increased the rate of hepatic microsomal P-450 reduction by NADPH in rats, but not in mice. Note that these data are expressed only in terms of the change in absorbance (at 450 mμ) per milligram of protein per minute. No attempt was made to correct for possible benzpyrene-induced changes in the extinction coefficient of microsomal cytochrome P-450.[33]

DISCUSSION

The present study has compared the responses to benzpyrene injection of a number of hepatic microsomal enzymes and components in mice and rats. Most of the systems measured in rat liver microsomes were affected by the benzpyrene injection of these animals, whereas few if any changes could be detected in liver microsomes from mice pretreated with benzpyrene. The systems or components studied included: (a) the pH-dependent ratio of the peaks of the ethyl isocyanide-induced difference spectrum;[5] (b) the pH optimum of the aniline hydroxylation to *p*-aminophenol;[3] (c) the apparent K_m and apparent V_{max} for aniline *p*-hydroxylation and benzphetamine *N*-demethylation;[3] (d) the apparent K_s and apparent ΔA_{max} of the substrate-induced difference spectra produced when aniline and benzphetamine are added to microsomes;[3,34] (e) the apparent K_m and apparent V_{max} for benzpyrene hydroxylation;[3,27–29,35] and (f) the rate of reduction of cytochrome P-450 by NADPH in the presence of CO.[18,36] Of all

TABLE 1. EFFECT OF BENZPYRENE TREATMENT OF RATS OR MICE ON THE NADPH–CYTOCHROME P-450 REDUCTASE ACTIVITY OF LIVER MICROSOMES*

Species	Treatment group†	
	Corn oil controls	Benzpyrene-treated
Rat	0·11 ± 0·02‡	0·17 ± 0·01§
Mouse	0·11 ± 0·02	0·09 ± 0·01

* The assay procedures are described under Materials and Methods.
† Treatment procedures are described under Materials and Methods.
‡ Data shown are Δ O.D. (450 mμ) per milligram of microsomal protein per milliliter of cuvette contents per minute. Each value is the mean (± S. E.) of four experiments. Values from each experiment were the averages of two or three trials.
§ Treated group is significantly different from control group ($P < 0·05$).

these measurements, only the magnitude of the benzphetamine (type I)-induced microsomal difference spectrum was affected by pretreatment of mice with benzpyrene.

Some of the changes produced by treatment of rats with benzpyrene deserve comment. These include: the changes in aniline *p*-hydroxylation (kinetics and pH optimum) and aniline-induced microsomal difference spectrum; changes in the *N*-demethylation of benzphetamine (kinetics) and the benzphetamine-induced microsomal difference spectrum; changes in hydroxylation of benzpyrene (kinetics); and changes in NADPH–cytochrome P-450 reductase.

Aniline p-*hydroxylation*

Benzpyrene treatment of rats caused a shift upwards in pH optimum (Fig. 3) similar to that reported previously from this laboratory.[3] From further studies of the hydroxylation at both different pH and substrate concentrations, it was obvious that the measured reaction velocity and the dependence of this on pH were affected by substrate concentration in such a way that at certain (especially low) aniline concentrations, benzpyrene pretreatment of rats would apparently have been without effect. Reasons for the effects of benzpyrene on aniline hydroxylation might include: (1) Benzpyrene caused activation or synthesis of an enzyme (system) capable of hydroxylating (best) relatively high concentrations of aniline (high apparent V_{max}, high apparent K_m). Benzpyrene would then act somewhat like acetone[37] or ethyl isocyanide[38] added *in vitro*; Imai and Sato[38] showed that ethyl isocyanide activation of aniline hydroxylation involved a pH optimum shift and that the change in activity was also dependent on the aniline concentration added. (2) A second possibility is that microsomes obtained from benzpyrene-treated rats contained some substance (e.g. benzpyrene or metabolites) which competes with aniline for active sites on an enzyme with high V_{max}.[25] Interestingly, the addition *in vitro* of 1 mM prednisolone produced changes in apparent kinetics of aniline hydroxylation[39] which closely resembled those we have reported to occur after benzpyrene treatment of rats.

Benzpyrene treatment also changed the aniline-induced microsomal difference spectrum (type II), but the changes did not parallel those in the rate of hydroxylation

of aniline. The magnitude of the difference spectrum was increased, but this was more pronounced at low aniline concentrations (whereas increased hydroxylase activity was seen best at high substrate concentrations). The spectral dissociation constant (K_s) was reduced by benzpyrene treatment while the apparent K_m for aniline hydroxylation was increased. These results merely show again that difference spectra and hydroxylase activity do not run parallel.[12,31,34,40] Our results are similar in some respects to those obtained by Schenkman,[25] e.g. aniline K_s was dependent on substrate concentration.

Benzphetamine N-demethylation

Benzpyrene pretreatment apparently changed the kinetics of demethylation and substrate-induced microsomal difference spectra with benzphetamine. Both the rate of demethylation and the magnitude of the difference spectrum with benzphetamine were decreased by benzpyrene treatment of rats. Shoeman *et al.*[24] have reported a similar inhibitory effect of 3-methylcholanthrene treatment of rats on the substrate-induced microsomal difference spectrum and the rate of metabolism of a type I substrate, hexobarbital. However, Rickert and Fouts[3] have reported that benzpyrene treatment of rats did not affect either microsomal metabolism (apparent V_{max}, K_m) or substrate-induced difference spectrum (apparent K_s, ΔA_{max}) with benzphetamine as substrate (ligand). Such differences in results may be due to the fact that benzpyrene (or metabolites) can act as an inhibitor of microsomal mixed-function oxidases and of the substrate-induced (especially type I) microsomal difference spectra. Thus, any difference (between experiments) in microsomal contents of benzpyrene (or metabolites) might explain differences in effects of benzpyrene pretreatment on especially sensitive microsomal–substrate interactions. The presence (at the time of assay) of residual "inducer" or metabolite in microsomes from inducer-pretreated animals has been postulated to explain some of the effects of polycyclic hydrocarbon treatment on microsomal enzymes and components.[25]

Benzpyrene hydroxylation

The apparent kinetics of benzpyrene hydroxylation by rat liver microsomes were markedly affected by benzpyrene pretreatment of the rats. The results reported in this paper were obtained only after appreciably altering our usual assay methods, especially to measure metabolism at low substrate concentrations (short incubation times). The degree of benzpyrene-induced stimulation of its own metabolism was very dependent on the substrate concentration (and hence time of incubation at low substrate concentrations). The results reported here (Fig. 10) are in agreement with those of Alvares *et al.*[27,41] (increased apparent V_{max} and decreased apparent K_m). Our previously reported results[3] (increased apparent V_{max}, but increased apparent K_m may have been caused by substrate depletion at low substrate concentrations. The many differences which have appeared in the literature dealing with the effects of animal pretreatment with polycyclic hydrocarbons on hepatic microsomal benzpyrene hydroxylase[3,27–29,41] are probably of multiple causation. Assay method (fluorescence vs. radioactive[35]), substrate concentration range used, dose and frequency of inducer treatment plus time between inducer treatment and animal sacrifice, the amount of protein in the assay mixture,[41] and even the animal strain can all affect the results obtained—both qualitatively and quantitatively.

3-Methylcholanthrene pretreatment of rats causes changes in microsomal benzpyrene hydroxylation similar to those we saw here with benzpyrene pretreatment of rats. In contrast, in mice, there was no evidence for an effect of benzpyrene treatment on hepatic microsomal benzpyrene hydroxylation, whereas 3-methylcholanthrene did apparently produce effects on this hydroxylation in mice. These results indicated that benzpyrene hydroxylation can be an index of polycyclic hydrocarbon-induced effects in the mouse, and emphasized again the lack of effect of benzpyrene on the liver in this species.

NADPH-cytochrome P-450 reductase

Benzpyrene treatment of rats caused some increase in NADPH–cytochrome P-450 reductase activity. This increased activity may be important to the stimulation of microsomal mixed-function oxidases in this species and the converse applies to the mouse—a lack of effect on the rate of NADPH-linked reduction of cytochrome P-450 may be related to the lack of effect of benzpyrene treatment on microsomal enzymes in the mouse. Again though, it is important to recognize that changed enzyme activity may in part be due to the presence of the inducer (or metabolite). Thus, benzpyrene gives a type I substrate-induced difference spectrum with microsomes,[32] and type I substrates can produce increases in NADPH–cytochrome P-450 reductase (P-450 reductase) activity when added to the assay system *in vitro*.[18] Gnosspelius *et al.*[36] showed that benzpyrene added to microsomes (especially those from benzpyrene-treated rats) caused increased P-450 reductase activity. There is no way from our data to know how much of the increased P-450 reductase activity was due to a direct effect of residual inducer (benzpyrene or metabolite), and how much was caused by the presence of new enzymes (production of more enzyme or enzyme with an increased turnover number). Even experiments where antimetabolites (e.g. cycloheximide, actinomycin D, ethionine) are used to block the effects of benzpyrene as an inducer are not definitive,[29] until we know whether residual inducer (or metabolite) can be displaced from microsomal binding sites by the antimetabolite (or its metabolites).

It is interesting that benzpyrene treatment of mice did not affect P-450 reductase. Either benzpyrene (and perhaps other type I ligands) does not stimulate P-450 reductase in mouse liver, or this effect is masked by some other phenomenon. Sasame and Gillette[42] have also found that type I compounds did not produce the marked stimulation of P-450 reductase in mouse liver microsomes that they saw when these compounds were added to rat liver microsomes. They attributed this relative lack of effect of type I compounds on mouse liver microsomal P-450 reductase to the fact that the mouse liver system was so active already that the assay method was at its upper limit of response. The mice used by Sasame and Gillette[42] were pretreated with phenobarbital, and this treatment is known to stimulate P-450 reductase in the rat.[36] However, in the untreated animals, we found that the activity of P-450 reductase was the same in microsomes from rats and mice (Table 1), and that our assay could detect a stimulation of reduction rate if it occurred. We believe that benzpyrene treatment does not affect mouse liver P-450 reductase.

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