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UNIQUE BEHAVIOUR OF BENZENE MONO-OXYGENASE: ACTIVATION BY DETERGENT AND DIFFERENT PROPERTIES OF BENZENE- AND PHENOBARBITAL-INDUCED MONO-OXYGENASE ACTIVITIES

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Abstract—The benzene mono-oxygenase present in liver microsomes from untreated rats was activated 3.5-fold by the detergent Renex 690. This is the first report of an activation of a microsomal mono-oxygenase by detergent. Metyrapone also activated benzene mono-oxygenase, albeit not as strongly (1.5-fold). Treatment of the rats with benzene or phenobarbital induced the benzene mono-oxygenase activity about 3-fold. The benzene-induced form was also activated by the detergent and by metyrapone. However, the phenobarbital-induced activity was inhibited by both modulators.

When the mono-oxygenase activity was measured with 7-ethoxycoumarin as a model substrate, which is accepted by many microsomal mono-oxygenases, the phenobarbital-induced activity was inhibited strongly (~ 80 per cent) by metyrapone or Renex 690; however, both the benzene-induced and the control activity were inhibited only weakly (~ 30 per cent).

The results provide evidence for substantially different properties of the phenobarbital-induced mono-oxygenase(s) compared to control mono-oxygenase(s). Moreover, benzene appears to be a type of inducer different from the known types and of special interest since, whether assayed with benzene or 7-ethoxycoumarin as substrates, and Renex 690 or metyrapone as modulators, the benzene-induced mono-oxygenase activity possesses characteristics resembling the controls rather than that induced by phenobarbital.

Benzene is a component of most motor fuels and therefore a ubiquitous environmental pollutant [1]. The toxicity of benzene is specifically directed towards the bone marrow [2, 3]. Some evidence indicates that metabolism of benzene is required to produce the toxic effects [4]. It is thought that such metabolism-induced toxic effects originate from the formation of reactive intermediates, which are able to interact covalently with macromolecules, thereby disturbing the cellular functions. The formation of such reactive intermediates, especially epoxides, has been extensively studied for some aromatic polycyclic hydrocarbons [5–8].

Recently, we investigated the irreversible binding *in vitro* of benzene metabolites to microsomal proteins. We found that, although benzene oxide clearly is formed, it was not this epoxide but rather a secondary metabolite, namely a metabolite of phenol, which was mainly responsible for this binding [9].

In the present study we show some unusual properties of the rat liver benzene mono-oxygenase, the enzyme responsible for the first step in the metabolic activation of benzene. This enzyme has been shown to be a cytochrome P-450-dependent mono-oxygenase [10], and hydroxylation proceeds, at least to a large extent, via benzene oxide [9]. Furthermore, some properties of the benzene-induced mono-oxygenase activity are investigated and compared to those of PB† induced mono-oxygenase.

EXPERIMENTAL SECTION

7-Ethoxycoumarin was synthetized as described [11]. Metyrapone was a gift from Ciba-Geigy, Basel, Switzerland. All chemicals, obtained from commercial sources, were of analytical grade. [^{14}C] Benzene and [^{14}C] phenol from the Radiochemical Centre, Amersham, U.K., had specific activities of 107 and 35 mCi/mmole, respectively, and were > 99 per cent radiochemically pure. The detergent Renex 690 was purchased from Atlas-Chemie, Essen, West Germany, and 7-hydroxycoumarin from EGA- Chemie, Steinheim, West Germany.

Adult male Sprague–Dawley rats (180–280 g) were obtained from Versuchstier-Zuchtanstalt Wiga, Sulzfeld, West Germany, and kept in plastic cages with hardwood bedding under standardized conditions of light (light–dark cycle: 7 a.m. to 7 p.m.) and temperature (21–24°) for at least 4 days before treatment. They had free access to Aldromin pellets (Samen-Schmitt-Jakobi, Frankfurt/M., West Germany) and tap water. Care was taken to avoid any environmental effects such as cigarette smoke, insecticides, noise, etc. Animals were always treated

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† Abbreviations used: PB, phenobarbital.

between 8 and 10 a.m. The PB-induced animals received 80 mg PB per kg body weight, dissolved in 0.9% NaCl, intraperitoneally for three consecutive days and were sacrificed on the fourth day. The benzene-induced animals received 1.1 g benzene per kg body weight subcutaneously 24 and 18 hr before being sacrificed, as described previously [10]. Control animals received either intraperitoneal saline injections at the same time as the PB-treated, or no injection at all.

In this study mono-oxygenase activity with benzene and 7-ethoxycoumarin as substrates, respectively, is expressed as phenol and 7-hydroxycoumarin formed (often referred to as "benzene hydroxylase" and "ethoxycoumarin de-ethylase"). The preparation of rat liver microsomes, incubations and measurement of phenol formation were performed as has been described [9], except that the incubation time was reduced to 30 min and metyrapone, when added, was dissolved in 10 μl acetone (total incubation volume 3 ml). To all controls and incubations with detergent, 10 μl acetone was added. Renex 690 and unlabeled phenol were administered dissolved in 100 and 50 μl water, respectively. The protein concentrations of the microsomal suspensions were estimated using the method of Lowry *et al.* [12].

The mono-oxygenase activity with ethoxycoumarin as substrate was measured by determination of the fluorescence of the dealkylated product 7-hydroxycoumarin [11]. The cuvette contained: 0.8 ml 0.2 M phosphate buffer pH 7.6; 1.0 ml 2×10^{-4} M ethoxycoumarin; 0.2 ml NADPH-generating system (10^{-3} M NADPH), 3×10^{-3} M glucose-6-phosphate and glucose-6-phosphate dehydrogenase (10 μl/5 ml; Boehringer Mannheim grade II) and 0.35–0.40 mg microsomal protein. Metyrapone was added dissolved in 20 μl water to a final concentration of 2×10^{-5} M. The detergent Renex 690 was added dissolved in 10 μl water. The reaction was started by addition of the microsomes and was followed on a recorder for 3–4 min. During this time the rate was linear. The excitation and emission wave-lengths were 380 and 460 nm respectively. The instrument was calibrated by adding 1 μl of a solution of 16.2 mg 7-hydroxycoumarin/100 ml water to the cuvette (1 nmole).

RESULTS

Effects of Renex 690 and metyrapone on the recovery and metabolism of phenol

To evaluate the results of this study it was necessary to investigate how Renex 690 and metyrapone affected the recovery of phenol through the extraction procedure and the further metabolism of this compound. The method used to isolate phenol has been described elsewhere, and was shown to have a recovery of 64–65 per cent [9].

Table 1 shows that Renex 690 increased the recovery of phenol from 65 to 75 per cent (due to smaller loss during the evaporation of the ethyl acetate phase). The difference of the recovery of phenol in presence or absence of Renex 690 appeared slightly (7 per cent) greater when NADPH was added to the incubation system, indicating a (very weak) inhibition of the metabolism of phenol by Renex 690. However, the overall recovery of unmetabolized phenol in incubations containing a NADPH-generating system was increased from 52 per cent to only 64 per cent. Thus, the effects of Renex 690 on the metabolism of benzene, shown in Fig. 2 and described below can only to a very minor extent be explained by effects on recovery and metabolism of phenol.

Table 1. Effect of Renex 690 and metyrapone on the recovery and metabolism of phenol*

Modulator	Amount of phenol recovered (pmoles) Incubations without NADPH-generating system	Incubations with NADPH-generating system
None	387 (65%)†	312 (52%)
Renex 690‡	450 (75%)	383 (64%)
Metyrapone‡	390 (65%)	338 (56%)

* Standard incubations were preformed with 600 pmoles [^{14}C]phenol (35 mCi/mmole) as substrate, added dissolved in 10 μl acetone. After incubation unmetabolized phenol was isolated. The determinations were done in duplicates which differed less than 5 per cent from each other.

† Numbers in brackets indicate percentage of total amount of phenol added to the incubations.

‡ The ratio mg Renex 690/mg microsomal protein was 0.5 and the concentration of metyrapone was 10^{-4} M.

Table 1 also shows that metyrapone had no effect on extraction recovery, but a small effect on metabolism of phenol. Again the weak inhibition of phenol metabolism by metyrapone represents only a very minor contribution to the effects on benzene metabolism depicted in Fig. 1 and described below.

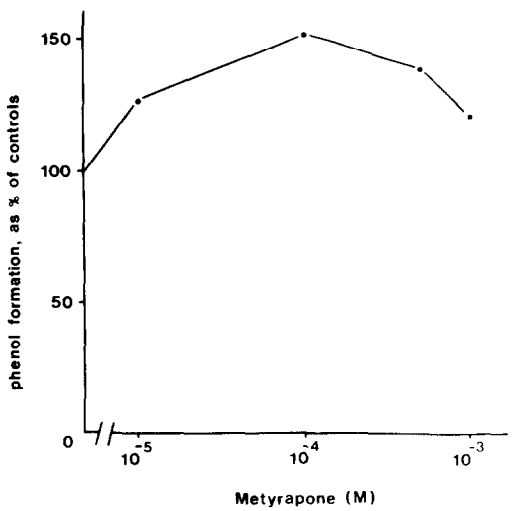

Fig. 1. The effect of metyrapone on the formation of phenol from [^{14}C]benzene in liver microsomes from untreated rats. The phenol formation in controls (incubations without metyrapone) was defined as 100 per cent. The specific activity in these controls was 79 pmoles phenol/mg microsomal protein/30 min. Indicated are means from two experiments, which differed by not more than 6 per cent

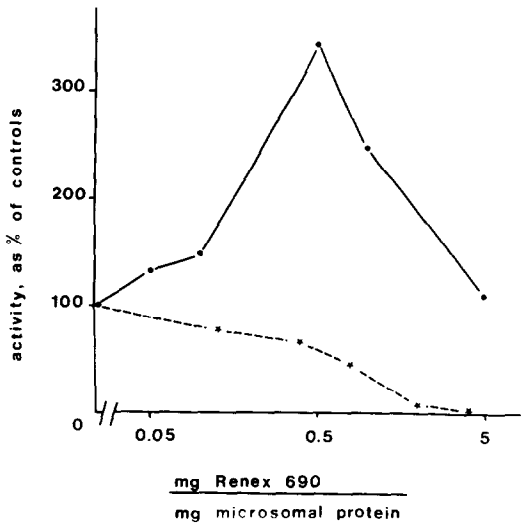

Fig. 2. The effect of the detergent Renex 690 on the formation of phenol from [^{14}C]benzene (●——●) and on the mono-oxygenase activity with ethoxycoumarin as substrate (*----*) in liver microsomes from untreated rats. The activities in controls (in the absence of Renex 690) are defined as 100 per cent. The specific activities in these controls were 72 pmol phenol/mg microsomal protein/30 min and 0.75 nmol 7-OH-coumarin/mg microsomal protein/min. Indicated are means from two experiments, which differed by not more than 5 per cent.

Effect of phenol on benzene metabolism

Table 2 shows the inhibition of benzene hydroxylation by phenol, the product of this hydroxylation. In presence of 50 μM or more phenol, transformation of benzene to phenol was markedly inhibited. However, the amount of [^{14}C] phenol formed from [^{14}C]benzene under the standard conditions used in this study, was around 250 pmoles, giving a phenol concentration of about 0.08 μM. In presence of 1.1 μM unlabelled phenol no measurable effect on transformation of [^{14}C]benzene to [^{14}C] phenol was observed. Thus, it seems unlikely that the amount of phenol formed under the standard conditions used would be limited by a feedback mechanism.

Table 2. Effect of unlabelled phenol on the microsomal formation of [^{14}C]phenol from [^{14}C]benzene*

Concentration of unlabelled phenol (μM)	% inhibition of [^{14}C] phenol formation
0	0
1.1	0
11	12
50	51
90	65

* Standard incubations with [^{14}C]benzene as substrate were performed. After incubation [^{14}C]phenol was isolated. In incubations without added unlabelled phenol 255 pmoles [^{14}C]phenol was formed. Unlabelled phenol was added dissolved in 50 μl of water. The determinations were made in duplicates which differed less than 8 per cent from each other.

Effects of metyrapone on phenol formation

The effects of different concentrations of metyrapone on the formation of phenol from [^{14}C]benzene in standard incubations are shown in Fig. 1. It is apparent that metyrapone, in the concentration range investigated, increased phenol formation.

Effect of detergent Renex 690 on phenol formation

In Fig. 2 the effect of the detergent Renex 690 on the phenol formation from [^{14}C]benzene in standard incubations and on the mono-oxygenase activity with ethoxycoumarin as substrate is shown. Surprisingly, the phenol formation greatly increased, while the mono-oxygenase activity measured with ethoxycoumarin was inhibited at all detergent/protein ratios investigated. At a detergent/protein ratio of 5, where mono-oxygenase activity with ethoxycoumarin as substrate was totally inhibited, the phenol formation was the same as in controls (incubations without detergent). In presence or absence of Renex 690 phenol was the only ethyl acetate-extractable metabolite of benzene formed in detectable amounts under the standard conditions of this study. This was checked using the TLC-systems described [9].

Induction of benzene and ethoxycoumarin mono-oxygenase by PB and benzene, and response of the induced activities to metyrapone and to the detergent Renex 690

Figure 3 shows that the benzene mono-oxygenase was induced about 3-fold after PB as well as after benzene treatment. As expected from the results presented in Figs. 1 and 2, both metyrapone and Renex 690 stimulated the benzene mono-oxygenase activity in control microsomes. However, the PB-induced activity was inhibited by metyrapone down to the level observed in metyrapone-treated controls. Also, in the presence of Renex 690 the activity was the same as in Renex 690-treated controls. The benzene-induced activity, on the other hand, was stimulated by metyrapone and Renex 690, as was the activity in controls.

In Fig. 4 data on mono-oxygenase activity with ethoxycoumarin as substrate are shown. Both PB and benzene induced the activity about 3-fold. The controls and benzene-induced activities were inhibited by 30–40 per cent by metyrapone as well as Renex 690. The PB-induced activity, on the other hand, was inhibited by about 80 per cent by the two modulators.

DISCUSSION

Metyrapone is known to be an inhibitor of several mono-oxygenase forms (see e.g. [13]). The activation of benzene mono-oxygenase by metyrapone in control and in benzene-induced microsomes reported in this paper stands in contrast to these inhibitory effects but is comparable to the activation [14, 15] of acetanilide hydroxylation by at least some metyrapone concentrations. Gonasun *et al.* [10] investigated the effect of metyrapone on phenol formation from benzene in mouse liver microsomes. At a concentration of 10^{-4} M, where we found 50 per cent activation in the rat, they reported a weak inhibition

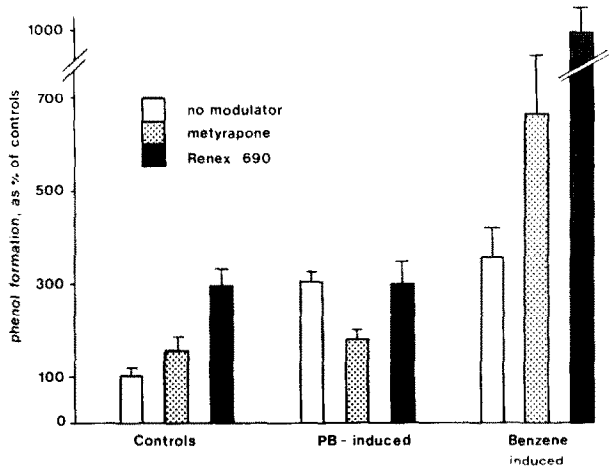

Fig. 3. Induction of benzene hydroxylase with PB and benzene and the effects on the induced and control activities of Renex 690 and metyrapone. Control: The activity in microsomes from untreated rats incubated without metyrapone or detergent. The specific activity in these controls was defined as 100 per cent and was 77 pmole phenol/mg microsomal protein/30 min. Metyrapone was used at a concentration of 5×10^{-4} M and the ratio mg Renex 690/mg microsomal protein was 0.5. Each bar represents the mean of independent measurements from three animals, and the standard deviations are indicated.

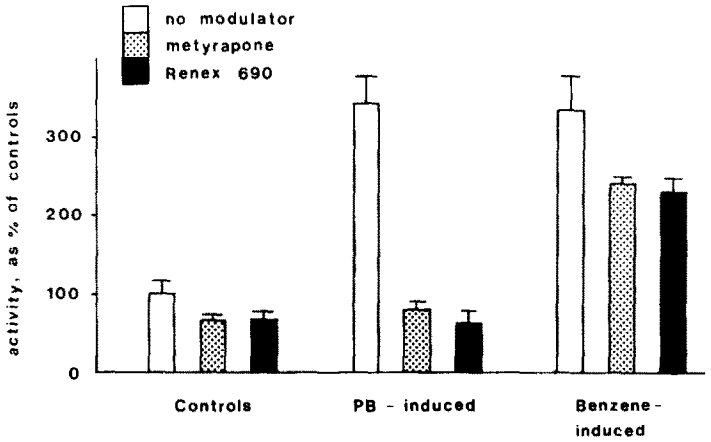

Fig. 4. Induction of mono-oxygenase activity, as measured with the ethoxycoumarin assay, with PB and benzene, and the effects, on the induced and control activities, of metyrapone and the detergent Renex 690. The control activity was 0.69 nmole/mg microsomal protein/min. Metyrapone was used at a concentration of 2×10^{-5} M. See also legend to Fig. 3.

(20 per cent). To decide if this discrepancy reflects different benzene mono-oxygenase enzymes in the two species, or if it is due to different experimental design, requires further investigation.

The activities of some non-oxidative microsomal enzymes are increased by detergent treatment [16–19]. If an ordered spatial arrangement between NADPH cytochrome P-450 reductase and cytochrome P-450 (cf. [20]) is important for mono-oxygenase activity, detergent treatment may decrease mono-oxygenase activity by loosening such an ordered structure. Moreover, it has been shown that detergents accelerate the denaturation of cytochrome P-450 to the metabolically inactive P-420 form [21]. Thus it was as expected when we found that addition of detergent inhibited the mono-oxygenase activity as measured with the ethoxycoumarin assay (Fig. 2). This assay is known to measure a very wide spectrum of mono-oxygenases [13]. To our surprise, however, we found a massive activation of the benzene mono-oxygenase activity on addition of the detergent (Fig. 2). At a detergent concentration, where the mono-oxygenase activity with ethoxycoumarin as substrate was totally inhibited, the benzene mono-oxygenase activity was the same as without the detergent. To the best of our knowledge this

represents the first demonstration of an activation of microsomal mono-oxygenase by detergent treatment.

Since the concentration of benzene in the incubation mixtures (7 μM) was far below the solubility limit (11 mM), we see two possible explanations for the activation by the detergent:

1. Although the results in Table 2 indicate that the amounts of phenol formed are much too low to inhibit benzene hydroxylation, it could still be that newly formed phenol reaches high local concentrations at its site of formation, i.e. at the cytochrome P-450, concentrations high enough to compete with unmetabolized benzene. In this case the detergent may increase the dissociation of the phenol from the cytochrome.
2. A special form of mono-oxygenase, with the unusual property of being activated by detergent, might be responsible for the benzene mono-oxygenase activity.

The results given in Fig. 3 favour the latter explanation. It is unlikely that some special property of the substrate benzene, or of the product phenol, would be responsible for the unusual effects of detergent and metyrapone on the benzene hydroxylation since these effects are not seen in PB-induced microsomes. The most obvious explanation of these findings would be instead that (1) there exists already in control microsomes an unknown number of mono-oxygenase forms which accept benzene as substrate and which are activated by metyrapone and by detergent; (2) PB induces a mono-oxygenase form or forms which can also accept benzene as substrate but which are basically different from the control benzene mono-oxygenase(s) in that they are not activated by either metyrapone or by detergent; (3) benzene induces the benzene mono-oxygenase form(s) which are already present in control microsomes.

The properties of induced mono-oxygenase activity, as measured with the ethoxycoumarin assay, are shown in Fig. 4. Benzene- and PB-treatment both induced the activity about 3-fold. The PB-induced activity was massively inhibited by metyrapone and detergent while the activity in controls and in microsomes from benzene-induced rats was inhibited by only 30–40 per cent.

Thus, whether assayed with benzene or 7-ethoxycoumarin as substrates, and Renex 690 or metyrapone as modulators, the benzene-induced mono-oxygenase activity possesses characteristics resembling the controls rather than that induced by phenobarbital. This observation is of interest since it is often assumed that PB induces mainly the control forms of cytochrome P-450 or forms having properties similar to the control forms. Thus it seems important for future studies to investigate the properties of the benzene-induced mono-oxygenase activity further, since the induction of a mono-oxygenase activity, in substrate specificity and cytochrome P-450 composition as much as possible resembling the control forms would be of great interest.

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