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CHARACTERIZATION OF THE BENZENE MONOOXYGENASE SYSTEM IN RABBIT BONE MARROW*

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Abstract—The microsomal fraction of bone marrow contains cytochrome P-450 (39 ± 11 pmoles/mg microsomal protein) and monooxygenase activity could be demonstrated by the *O*-dealkylation of 7-ethoxycoumarin (114 ± 65 pmoles/(min × mg microsomal protein)) and the hydroxylation of benzene to phenol (51 ± 8.6 pmol/45 min × mg microsomal protein). This monooxygenase system differs from that in liver in various aspects. The conversion of benzene to phenol calculated as molecular activity was about 4 times higher than in liver and no induction by phenobarbital could be observed. Aroclor 1254 induced the cytochrome P-450 content about twofold but lowered the *O*-dealkylation activity of 7-ethoxycoumarin in contrast to liver. Pretreatment with benzene did not change the *O*-dealkylation in bone marrow, but had a stimulating effect on benzene monooxygenation and covalent binding of ^{14}C-benzene metabolites.

From these results we conclude that the bone marrow monooxygenase system develops its own pattern of cytochrome P-450 isoenzymes. Especially after chronic exposure to benzene this system can convert this chemical to phenol and secondary metabolites. The similar behaviour of phenol formation and covalent binding strengthens the hypothesis of a common pathway for metabolism and toxicity but the active intermediate still remains unknown.

Benzene is a major industrial solvent and due to its antiknock properties has the prospects of being increasingly used to replace tetraethyl lead in gasoline. As a consequence, chronic exposure of man to this chemical will also increase and so may become a health risk factor in view of the long-known tendency of benzene to induce aplastic anemia in man [1]. Furthermore, a strong correlation between such exposure and the occurrence of leukemia is suggested.

In spite of many attempts to establish the biochemical basis for these toxic events, no convincing hypothesis on the underlying mechanism is currently available.

In 1953 Parke and Williams [2] reported on the oxidation of benzene to several different phenolic compounds in intact animals. This hydroxylation was later found to occur in liver microsomal preparations to be cytochrome P-450 mediated [3]. Experiments performed by Andrews *et al.* [4] and Tunek *et al.* [5, 6] strongly support the hypothesis that metabolic transformation and activation of this chemically inert compound is an absolute prerequisite to its adverse effects on bone marrow. Although liver is believed to be the major site of benzene metabolism, it still remains a subject of debate whether benzene metabolism by liver or by bone marrow is itself responsible for the toxicity observed. For benzo(*a*)pyrene mediated toxicity in bone marrow it was shown that this tissue contains covalently bound metabolites [7] which were not derived from liver or intestinal metabolism [8].

There are only few data available about bone marrow cytochrome P-450 [9, 10]. Although it shows only minor activities in benzo(*a*)pyrene and benzene hydroxylation it could play an important role in metabolizing lipophilic compounds due to organotropic actions. This has prompted us to study the cytochrome P-450 monooxygenase system in bone marrow in greater detail in order to find out in which way this enzyme participates in the generation of toxic metabolites.

MATERIALS AND METHODS

Animals used in the experiments were female chinchilla bastard rabbits (2–4 kg body weight). Induced animals received 500 mg Aroclor 1254/kg i.p. dissolved in sunflower oil 4 days before sacrifice or 80 mg phenobarbital/kg i.p. 5, 4, 2 and 1 day before sacrifice. Control animals received no treatment.

Rabbits were killed by exsanguination; humerus, femur and tibia were removed and placed on ice. Adhering tissue was scraped off and after opening the bones the marrow was completely removed and transferred into ice-cold pyrophosphate/HCl buffer (100 mM, pH 7.6 containing 250 mM sucrose and 10 mM EDTA). Then it was homogenized in a glass/Teflon homogenizer (B. Braun Melsungen AG) and centrifuged for 20 min at 12,000 *g*. The fatty layer on top was removed and the supernatant centrifuged for 60 min at 100,000 *g*. The resulting microsomal pellet was resuspended in Tris/HCl buffer (100 mM, pH 7.6 containing 1.15% KCl and 5 mM EDTA) and spun down again at 140,000 *g* for 40 min. The microsomal pellet was then suspended in Tris/HCl buffer (100 mM, pH 7.6 containing 20% glycerol and 1 mM EDTA) yielding a protein concentration of 30–40 mg/ml which was stored under liquid nitrogen until use.

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Liver microsomes were prepared from a 5 g aliquot using the same procedure as for bone marrow microsomes. The cytochrome P-450 content was determined with an Aminco DW-2 spectrophotometer according to the methods for hemoglobin contaminated samples as described by Estabrook *et al.* [11] using a molar extinction coefficient of 91 for E 450 minus 490 nm.

Benzene hydroxylation was assayed as follows: 10 mg of the microsomal suspension was incubated in 100 mM Tris/HCl buffer pH 7.6 containing radioactive labelled ^{14}C-benzene (125 μmoles/l, specific radioactivity 16 μCi/μmole) in the presence of glucose-6-phosphate dehydrogenase (5.6 U), 3.8 mmole/l $NADP^+$ and 10 mmole/l $MgCl_2$ in a final volume of 2 ml at 30°. Incubations were stopped by the addition of 0.5 ml $HClO_4$ (10% v/v) and 50 μl of a mixture of unlabelled phenol, catechol and hydroquinone (10 μmoles/ml, each). Protein was sedimented by centrifugation and benzene metabolites were extracted from the supernatant three times with 2 ml of ethylacetate. The combined extracts were evaporated to dryness and the remainder dissolved in 300 μl of methanol. A 50 μl aliquot of each sample was subjected to HPLC on a RP C8 column and eluted with water/methanol/acetic acid (800/200/1.6; w/w/w).

Fractions were collected and examined for radioactivity after the addition of 16 ml Unisolve 1® in a liquid scintillation counter. Recovery of benzene metabolites was based on peak heights of samples compared to the peak heights of standards.

The amount of benzene metabolites irreversibly bound to the microsomal protein was determined by extracting the denatured microsomes first with 5 ml ethanol, then with acetone/hexane (5/2, v/v) and twice with methanol. The last methanol extract was free of radioactivity. Thereafter the protein was solubilized in 1.5 ml of tissue solubilizer TS 1 and subjected to liquid scintillation counting in 18 ml Dimilume® after decay of chemiluminescence.

The 7-ethoxycoumarin *O*-dealkylation test as well as that of 7-methoxycoumarin and coumarin were performed as described by Ullrich *et al.* [12] using small cylindrical quartz cuvettes in a final volume of 0.1 ml. The substrate concentration was 0.9 mmole/l unless otherwise indicated.

The demethylation of 4-nitroanisole (1.5 mmoles/l) was assayed in Tris/HCl-buffer (100 mmoles/l, pH 7.8) in a final volume of 1 ml in the presence of an NADPH-generating system consisting of isocitrate (3 mmoles/l), 5'-AMP (1 mmoles/l), $MgCl_2$ (5 mmoles/l), NADP (0.5 mmoles/l) and isocitrate dehydrogenase (0.2 U). After preincubation for 2 min the incubations were started by the addition of the microsomes (1.5–2.0 mg) and the formation of nitrophenol was determined directly in a photometer at 436 nm and 30°.

NADPH-dependent cytochrome *c* reductase activity was determined in Tris/HCl buffer in a total volume of 1.0 ml at 30°. Cytochrome *c* (50 μmoles/l) was added and the reaction started in 1.0 ml cuvettes by the addition of NADPH to yield a final concentration of 0.12 mmole/l. An extinction coefficient of 21 at 550 nm was used.

Student's distribution was used as a test of the null hypothesis, using a level of significance of P equal to or less than 0.05.

RESULTS

The cytochromes b_5 and P-450 could be detected by difference spectroscopy in bone marrow microsomes from untreated rabbits. Figure 1 shows both a typical difference spectrum of cytochrome b_5 (reduced minus oxidized) and a typical difference spectrum of cytochrome P-450 (reduced plus CO minus oxidized). The average content was 75 (±20) pmol cytochrome b_5 and 39 (±11) pmoles cytochrome P-450 per mg microsomal protein. The NADPH-dependent cytochrome *c* reductase activity was measured as 11.2 nmoles cytochrome *c* reduced × (min × mg protein)$^{-1}$. The CO-complex of cytochrome P-450 was also formed in the presence of NADPH indicating that the electron transfer in the monooxygenase system was functioning.

This was further established when in the presence of NADPH bone marrow microsomes dealkylated 7-ethoxycoumarin to umbelliferone (Table 1). NADH being active only to about 20%. A small additive but no synergistic effect was observed with both pyridine nucleotides. As expected the reaction was blocked by about 75% in the presence of carbon monoxide. 7-Ethoxycoumarin as well as coumarin also yielded umbelliferone, but nitroanisole which is demethylated in liver microsomes was not converted to 4-nitrophenol in detectable amounts. In agreement with reports in literature, ^{14}C-benzene was converted to phenol, but with a low specific activity. Although the absolute rate of phenol formation in bone marrow microsomes was low, it was about four fold higher than in liver microsomes based on the total cytochrome P-450 content (Fig. 2 and Table 2). This was not due to the nonlinear time course of phenol formation over 45 min, but was observed at any time of incubation. The incubation time of 45 min was required to ensure the detection and measurement of the secondary metabolites hydroquinone and catechol, which were both found in liver but not in bone marrow microsomes.

Qualitative differences in the metabolite pattern of different organs are not unexpected since the pattern of cytochrome P-450 isozymes also may change within an individual. This can depend on

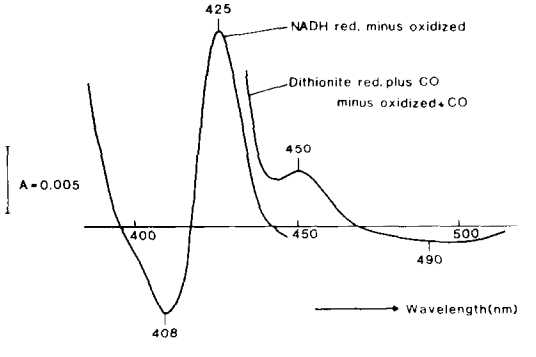

Fig. 1.

Table 1. Monooxygenation activities of bone marrow microsomes from untreated rabbits

System	Specific activity pmoles/(min × mg prot.)	Relative activity (%)
7-Ethoxycoumarin + NADPH	143 ± 32	100
+ NADH	27 ± 3.6	19
+ NADPH + NADH	163 ± 29.5*	114
+ NADPH + CO/O_2 (4:1 v/v)	36 ± 7.3	24
7-Methoxycoumarin + NADPH	107 ± 27	
Coumarin + NADPH	2.1 ± 0.5	
4-Nitroanisole + NADPH	n.d.†	
Benzene + NADPH	51 ± 8.6‡	100
+ NADPH + CO/O_2 (4:1 v/v)	24 ± 7.8‡	47

The assays were performed as described in Materials and Methods.
* Activity not significantly different from control values ($P > 0.05$).
† n.d. = no 4-nitrophenol detected.
‡ Reaction time: 45 min, phenol formed.

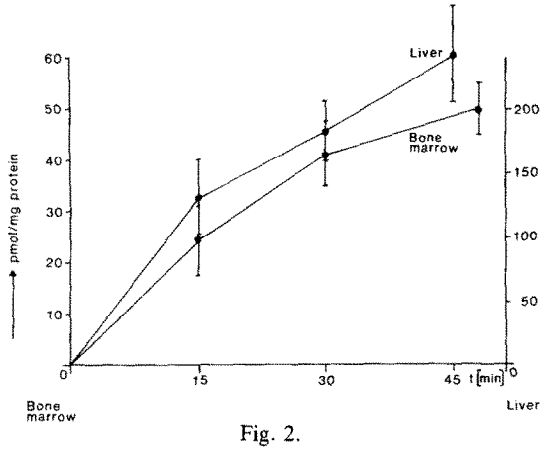

Fig. 2.

genetic factors but can also be influenced by the organotropic effect of endogenous or exogenous inducers of the monooxygenase system. Whether bone marrow was affected differently in this respect was investigated for the well-known inducers phenobarbital and Aroclor 1254 (Table 2). Phenobarbital pretreatment which in liver results in dramatic increases of all measured activities did not significantly affect the activities in bone marrow. In contrast Aroclor 1254 increased cytochrome P-450 by more than 100% but decreased the *O*-dealkylation activity towards 7-ethoxycoumarin. Benzene pretreatment had a moderate effect on liver but not on bone marrow.

The qualitative difference in the substrate specificity after Aroclor pretreatment is also reflected in a different inhibition pattern. Metyrapone blocked the dealkylation activity almost completely in controls but had little effect after Aroclor induction. α-naphtoflavone showed the opposite behaviour but benzene and tetrahydrofurane were hardly discriminating.

This dramatic change in the qualitative nature of the monooxygenase system after Aroclor pretreatment was expressed also in the protein band pattern of the cytochrome P-450 region of the SDS-gel electropherograms (Fig. 3).

A band representing a protein of about 58 kD was greatly increased after Aroclor pretreatment and

Table 2. Effects of inducers on the monooxygenase system in liver and bone marrow microsomes from rabbits

Parameter	Organ	Pretreatment: None	Phenobarbital	Aroclor 1254	Benzene
Cytochrome b_5	Liver	652 ± 199	1299 ± 217	929 ± 340	731 ± 56
[pmole × prot.$^{-1}$]	Marrow	74.8 ± 20	81.8 ± 29.5	57.9 ± 14.2	63.2 ± 13.4
Cytochrome P-450	Liver	809 ± 140	3388 ± 550	2877 ± 216	1292 ± 422
[pmole × prot.$^{-1}$]	Marrow	38.9 ± 11.4	31.8 ± 9.1	88.2 ± 9.7	30.5 ± 4.9
NADPH-cytochrome *c*-reductase	Liver	24.6 ± 10.6	49.3 ± 16.3	32.6 ± 15.4	29.5 ± 8.5
[nmole × (min × mg prot.)$^{-1}$]	Marrow	11.2 ± 4.6	5.3 ± 3.1	5.9 ± 3.8	6.5 ± 1.7
7-Ethoxycoumarin dealkylation	Liver	731 ± 79	3618 ± 612	1127 ± 167	1836 ± 670
[nmole × (min × mg prot.)$^{-1}$]	Marrow	114 ± 64.9	94.8 ± 27.4	30.4 ± 3.2	119 ± 31.4

Data given are means of 4 experiments except for untreated microsomes from liver and bone marrow (8 and 16, respectively).

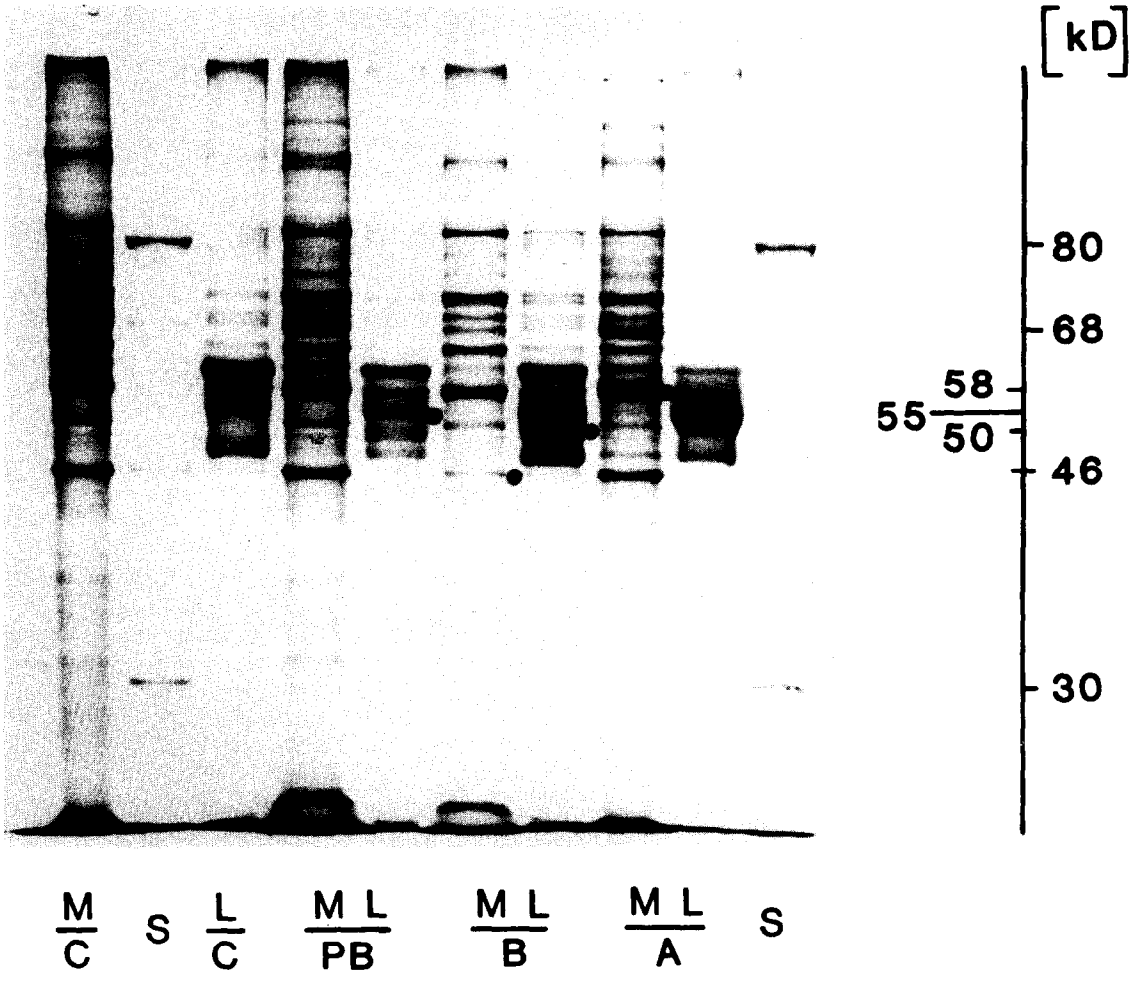

Fig. 3.

benzene caused a relative decrease of a 46 kD band in bone marrow microsomes.

In liver microsomes the pretreatment with phenobarbital increased a protein band of about 55 kD whereas benzene pretreatment increased a protein band of about 50 kD.

The change in the protein band pattern after benzene pretreatment led us to study its effect on benzene metabolism in these microsomes although the overall cytochrome P-450 content and the 7-ethoxycoumarin-*O*-dealkylation had revealed no changes (see Table 3). Indeed, phenol formation was almost doubled and covalent binding of ^{14}C-metabolites to microsomal protein, too. Liver showed a similar behaviour (Table 4).

Hydrochinone or catechol as further metabolites of phenol could also not be detected in bone marrow microsomes from induced animals. It is not clear whether this is due to a lack of formation or to an enhanced disappearance by oxidative processes. According to the latter hypothesis one would have expected a relative increase in covalent binding, which however was not the case. Table 5 provides some information on the mechanism of covalent protein binding. The close parallel inhibition of phenol formation and covalent binding suggests that a common intermediate is involved.

Table 3. Inhibition patterns for the *O*-dealkylation of 7-ethoxycoumarin in bone marrow microsomes from Aroclor 1254 pretreated and control rabbits

Inhibitor	Conc.	Relative activities (%)	
		Control	Aroclor 1254
None	–	100	100
CO/O_2	80/20; v/v	15 ± 4	8 ± 6
Metyrapone	0.1 mmol/l	8 ± 6	71 ± 13*
Benzene	11.3 mmol/l	12 ± 9	4 ± 1
Tetrahydrofurane	10.8 mmol/l	51 ± 6	31 ± 12
α-naphtoflavone	10 μmol/l	78 ± 13	0*

* Values significantly different from controls ($P < 0.05$).

DISCUSSION

In agreement with reports in the literature we established the presence of the unspecific monooxygenase system in bone marrow microsomes but

Table 4. Benzene metabolites in bone marrow and liver microsomes from controls, Aroclor 1254 and benzene pretreated rabbits

	Control		Aroclor 1254		Benzene	
	Marrow	Liver	Marrow	Liver	Marrow	Liver
Phenol	50 ± 14.4	238 ± 45.1	111 ± 6.4*	544 ± 23.8*	82 ± 14.0*	496 ± 79.2*
Hydroquinone	n.d.†	35 ± 12.8	n.d.	69 ± 16.1*	n.d.	153 ± 58.3*
Catechol	n.d.	6 ± 4.9	n.d.	23 ± 14.1	n.d.	25 ± 12.8*
Protein binding	1.6 ± 0.77	14 ± 3.5	1.7 ± 0.60	16 ± 1.9	4.2 ± 1.12	25 ± 7.6*
No of preparations	10	4	3	3	4	4

The concentration of products is expressed as pmole × (mg prot. × 45 min)$^{-1}$.
* Significantly different from control values ($P < 0.05$).
† n.d., not detected.

also noticed some important differences to the system in liver microsomes. 4-nitroanisole demethylation could not be detected although a comparable molecular activity to liver would have been within the sensitivity of the method. Benzene hydroxylation to phenol occurred at a higher rate than in liver, based on the overall cytochrome P-450 content. Phenobarbital was without an inducing effect which may be due to a lack of the transducing mechanism or, more likely, due to a low concentration of phenobarbital in the target tissue due to its effective removal by its hepatic metabolism. The high degree of inhibition by metyrapone and the low efficacy of α-naphtoflavone, however, suggest that most of the cytochrome P-450 isozymes present in bone marrow of untreated rabbits belong to the phenobarbital-responsible types and not to the isozymes inducible by polycyclic hydrocarbons.

Aroclor 1254 was found to change the isozyme pattern completely; α-naphtoflavone was now a powerful inhibitor and also the appearance of a band at about 55 kD in SDS-polyacrylamide electrophoresis pointed to the induction of a P-448 (LM4)-type of the monooxygenase. This isozyme must exhibit a very low or even no activity for 7-ethoxycoumarin and a moderate activity for benzene. Pretreatment with benzene did not change the overall P-450 content, but stimulated phenol formation from benzene. In the SDS-PAGE a band at 46 kD disappeared so that an increase in one or more other isozymes with higher activity towards benzene is a likely explanation.

The selective induction of one P-450 isozyme with even a decrease of other forms is a well-known phenomenon [13]. The proposed toxic action of a benzene metabolite leading to aplastic anemia or to leukemia in man can therefore well originate from a conversion of benzene to phenol in bone marrow itself and not from circulating metabolites. Due to their rapid metabolism by oxidation or conjugation, we also do not consider the hydrophilic phenol, catechol or hydroquinone as candidates for the observed toxicity. Hydroquinone was reported to cause some decrease in cell proliferation in bone marrow only at doses of more than 50 mg/kg daily over six days [6]. Benzene oxide as a possible reactive intermediate was disproved already by Tunek *et al.* [14] so that phenol or its secondary metabolites generated in bone marrow remain the most likely candidates. In this respect it is interesting that Tunek *et al.* [15] suggested an involvement of O_2^- in the process of covalent binding. Indeed, the O_2^- generation by neutrophils and macrophages but also by P-450 itself [16], could cause an oxidation of the phenols to radicals. Furthermore, these cells contain cyclooxygenase which is known to co-oxidize phenolic compounds [17]. Such radicals could cause covalent binding to macromolecules, but also an indirect action of oxygen radical formation leading to DNA-strand breakage may be a consequence.

One even has to consider the possibility that neither covalent nor strand breakage is involved but that according to a hypothesis of Harygara [18] a promoting rather than an initiating effect could play a role. In this respect it may be interesting that phenol which usually stimulates the cyclooxygenase-mediated prostaglandin formation from arachidonic acid is inhibitory in bone marrow microsomes (Castle

Table 5. Inhibitors of phenol formation and protein binding in bone marrow microsomes from untreated rabbits

		Relative activities (%)	
Inhibitor	Conc.	Phenol formation	Protein binding
None	—	100	100
CO/O_2	(80/20; v/v)	46	34
Metyrapone	0.5 mmole/l	50	55
Toluene	1.25 mmoles/l	42	54
Piperonylbutoxide	1.25 mmoles/l	82	97*

* Activity not significantly different from control value ($P > 0.05$).

and Ullrich, unpublished results). In view of the significance of prostaglandin regulators of cell growth, this observation may also be relevant for benzene toxicity.

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