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Microsomal Metabolism of Benzene to Species Irreversibly Binding to Microsomal Protein and Effects of Modifications of This Metabolism

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SUMMARY

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It has been shown that when [^{14}C]benzene is incubated with rat liver microsomes in the presence of a NADPH-generating system, metabolites which irreversibly bind to biomacromolecules are formed. This binding occurs mainly to microsomal protein rather than ribonucleic acids. The addition of 2 mM reduced glutathione or cysteine to the incubation mixture prevented 90–95% of the binding but had only a small effect on the formation of phenol, the main metabolite of benzene and rearrangement product of the putative reactive intermediate benzene oxide. The rate of phenol formation was approximately the same when fully deuterated benzene and normal benzene were used as substrates. This is compatible with phenol formation via benzene oxide and not possible if the C-H cleaving of a direct insertion reaction were the rate-limiting step. The decrease in binding in presence of reduced glutathione was accompanied by a corresponding increase in the amount of water soluble metabolites. In contrast to the situation with metabolically activated benzene, the putative reactive metabolite benzene oxide led to negligible binding and reduced glutathione had no effect on the formation of water soluble metabolites, i.e., the spontaneous conjugation of benzene oxide and reduced glutathione was practically zero at the physiological pH of the experiments. However, when [^{3}H]-phenol was incubated with rat liver microsomes and a NADPH-generating system, effects similar to those with benzene were observed in that marked irreversible binding occurred and this binding was prevented by reduced glutathione. The extent of binding was much greater when using phenol. These results are compatible with the assumption that an immediate metabolite of phenol rather than of benzene is responsible for the binding. This assumption is supported by the fact that addition of uridine-diphosphate-glucuronic acid to incubations with [^{14}C]benzene decreased the amount of both phenol and the irreversibly bound metabolites. Metabolism of benzene to 1,2-dihydro-1,2-dihydroxybenzene was observable, but only when pure epoxide hydratase was added in amounts greatly exceeding those already present in the microsomes. Increased formation of this diol upon addition of homogeneous epoxide hydratase is unequivocal proof for the metabolic

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formation of benzene oxide, which has so far eluded isolation. The observations (i) prove that reactive metabolite(s) which irreversibly bind to microsomal protein are formed during microsomal metabolism of benzene and that this binding is prevented by low molecular weight nucleophiles such as glutathione or cysteine; (ii) exclude benzene oxide as the metabolite predominantly responsible for this binding; and (iii) strongly indicate that a secondary metabolite of benzene, namely a metabolite of phenol, is mainly responsible for this binding.

INTRODUCTION

Benzene is one of the most widely distributed environmental pollutants, mainly because it is a constituent of most motor fuels. For many decades, it has been known that chronic exposure to benzene may lead to bone marrow damage. The current concepts of benzene toxicology and metabolism have recently been thoroughly reviewed (1).

It has been shown that benzene is oxidized by a microsomal cytochrome P-450-dependent monooxygenase (2). The principal metabolite is phenol, which is excreted via the urine either in the free form or as sulfate and/or glucuronic acid conjugates (3, 4). *In vivo* a number of other metabolites have been detected in small quantities: CO_2, hydroquinone, catechol, hydroxyhydroquinone, *trans-trans*-muconic acid, L-phenyl mercapturic acid (3, 5) and benzene dihydrodiol[2] (6). In experiments with isolated microsomes, only phenol (2, 7) and to a small extent benzyl alcohol (8) have been reported as metabolites of benzene.

Some evidence exists to support the idea that a metabolite of benzene is responsible for the adverse effects of benzene on bone marrow (9). Polycyclic aromatic hydrocarbons have been shown to be metabolically oxidized via epoxides, and epoxides are believed to be the ultimate toxic and carcinogenic derivatives of these compounds (10–14). In analogy, benzene oxide has been suspected to be the ultimate toxic agent of benzene. This concept was supported by the finding that *in vitro* metabolism of benzene oxide gave the same main groups of metabolites as the *in vivo* metabolism of benzene (15). If benzene were metabolized via an electrophilic intermediate, e. g., an epoxide, it would be expected to bind covalently to nucleophilic centers in biomacromolecules. Protection against this binding by competing nucleophiles such as GSH[2] would be expected. If so, it would also be expected that changes in the concentration of GSH would lead to changes in the pattern of benzene metabolites. Reaction of GSH with a number of electrophilic agents (16) has been reported. Of special interest is that it has been shown to react with benzene oxide enzymatically and also to a certain extent spontaneously (15). However, attempts to prove metabolism of benzene to benzene oxide using radiotracer trapping technique were unsuccessful (15).

In this study we have investigated the possibility that benzene metabolites become irreversibly bound to microsomal macromolecules during metabolism of benzene by rat liver microsomes and the influence of modulators of metabolism on the distribution of benzene metabolites between irreversibly bound, water soluble and ethyl acetate-soluble metabolites. Our findings show that benzene metabolites do bind irreversibly to microsomal proteins during incubation in the presence of a NADPH-generating system, exclude benzene oxide as the metabolite predominantly responsible for this binding and indicate that the metabolite that causes the main portion of the binding is a secondary metabolite of benzene, namely a metabolite of phenol.

MATERIALS AND METHODS

Compounds. All chemicals, obtained from commercial sources, were of analytical grade. [^{14}C]Benzene from the Radiochemical Centre, Amersham, had a specific activity of 107 mCi/mmol, and was >99% radiochemically pure. The standards needed for the recovery study were obtained from benzene oxide-[3,6-^{3}H] which was synthesized

[2] Abbreviations used are: benzene dihydrodiol, *trans*-1,2-dihydro-1,2-dihydroxy benzene; GSH, reduced glutathione; TCA, trichloroacetic acid; UDPGA, uridine-diphosphate-glucuronic acid.

as described (17) and had a specific radioactivity of 0.08 mCi/mmol.

[^{3}H]phenol was prepared by adding about 2×10^6 dpm [^{3}H]benzene oxide dissolved in 25 μl acetonitrile, to 1 ml 5 M HCl. After two extractions with 4 ml ethyl acetate, the pooled organic phases were dried over Na_2SO_4 and evaporated. The residue was dissolved in 200 μl ethyl acetate and applied as a band to a thin layer plate which was developed in system 1 (see below) together with unlabeled commercial phenol as a reference. The phenol band was scraped off and eluted with ethyl acetate. After filtration and evaporation the remainder was dissolved in acetone to give 1.44×10^5 dpm/10 μl. The identity of the [^{3}H]-phenol was confirmed by thin layer chromatography in system 2 and 3 (see below) and the purity was found to be >99%.

[^{3}H]benzene dihydrodiol was prepared by incubating overnight at 5° C approximately 6×10^6 dpm [^{3}H]benzene oxide with rat liver microsomes (3 mg protein) in 2 ml 0.5 M tris buffer pH 9.0 containing 1.25% acetonitrile. After incubation the [^{3}H]benzene dihydrodiol was isolated by thin layer chromatography as described above for [^{3}H]-phenol but using ethyl acetate as solvent. Unlabeled benzene dihydrodiol used as reference was synthesized as described (18). The product was dissolved in acetone to give 27,000 dpm/10 μl. The identity of the diol was confirmed by thin layer chromatography in system 3 (see below) and the purity was found to be >97%.

Thin layer chromatography. Thin layer chromatography was carried out on precoated glass plates, silica gel, 60 F-254, Merck. The plates were developed with benzene:chloroform:ethyl acetate (1:1:1) (system 1), ethyl acetate (system 2) or benzene:dioxane:acetic acid (19:5:1) (system 3). Phenol had R_f values of 0.48, 0.65, and 0.60, respectively and benzene-dihydrodiol 0, 0.27 and 0.21, respectively, in the three systems.

Preparation of microsomes. Male Sprague-Dawley rats, weighing about 250 g, were killed by decapitation. The livers were removed, washed in ice cold 50 mM tris buffer, pH 7.5, containing 0.25 M sucrose and homogenized in a Potter-Elvehjem homogenizer in four volumes of the same buffer. This homogenate was centrifuged for 10 min at 10,000 *g*, and the supernatant fraction centrifuged for 60 min at 105,000 *g*. These pellets were suspended in 0.15 M KCl, 4 ml/g liver wet weight, and centrifuged for 30 min at 105,000 *g*. The pellets were then suspended in 50 mM tris buffer, pH 7.5 containing 0.25 M sucrose, to yield a protein concentration of 15 mg/ml, estimated using the method of Lowry *et al.* (19) with bovine serum albumin as standard.

Incubations. The standard incubations contained: 1.5 ml of buffer A (100 mM tris pH 7.5, 10 mM $MgCl_2$ and 10 μM $MnCl_2$), a NADPH-generating system (3 mg glucose-6-phosphate, 2 mg NADP and 4 μg (0.56 U) glucose-6-phosphate dehydrogenase (Boehringer, Mannheim)) and 0.2 ml microsomal suspension (3 mg protein) in a total volume of 3 ml. The substrates, 21.5 nmoles [^{14}C]benzene, 812 nmoles [^{3}H]-phenol or 14.1 μmoles [^{3}H]benzene oxide were added dissolved in 10 μl acetone. Incubations were started by adding the substrate to incubation mixtures which had been preincubated for 2 min. All incubations were performed at 37°C in stoppered glass tubes. Incubation time was 1 hr, unless otherwise stated.

In two series of experiments the standard incubations were modified: When epoxide hydratase, purified as described (20), was added to the incubation mixtures, these were reduced to one sixth the volume described above in order to reduce the amount of enzyme needed. The epoxide hydratase was added in 100 μl of buffer A diluted with one volume of water. One hundred microliters of enzyme-free diluted buffer A was added to the controls. Substrate [^{14}C]benzene (4.0 nmoles) was added in 2 μl acetone. After stopping the incubations as described below, 0.2 ml heat-denatured microsomal suspension and 2.0 ml buffer A diluted with one volume of water was added in order to reduce the relative loss of protein during the wash and extraction procedure described below.

The second modification of the incubation mixtures was in the determination of isotope effect on the phenol formation, where the amounts and volumes of the standard incubations were doubled and the

incubation time was reduced to 30 min. One μl of normal or of fully deuteriated benzene dissolved in 9 μl acetone was added as substrate. Thus the substrate concentration in this experiment was 2.0 mM.

Separation of metabolites into different groups. The incubations were stopped by addition of 5 ml ethyl acetate and shaking on a rotoshake (Kühner AG, Basel) for 5 min at 40 rpm. The samples were then centrifuged at 1800 *g* for 10 min, and the organic phases transferred to other tubes. The ethyl acetate extraction was repeated twice more and the organic phases from each sample were combined and treated as described below. Three ml ethanol were added to the water phases to precipitate the proteins and the samples were vigorously shaken and centrifuged for 15 min at 1800 *g*. One ml of the resulting supernatant fraction was then added to 10 ml Unisolve (Koch-Light Laboratories, Colnbrooks Bucks, England) followed by determination of radioactivity by scintillation spectrometry. This fraction is termed water-soluble metabolites.

The remainder of the water-ethanol supernatant fraction was decanted off, and the precipitated microsomal pellets were washed with 5 ml acetone/hexane (25:10) and 5 ml methanol, by shaking on a Vortex shaker with a stainless-steel spatula inserted in the tube to facilitate mixing as described by Jollow *et al.* (21). The pellets were then dissolved in 2 ml 1 M NaOH at 80°C for 30 min. After neutralization with 2 ml 1 M HCl, an aliquot of 1 ml was added to 10 ml Unisolve and the radioactivity estimated by scintillation spectrometry. This fraction is called irreversibly bound metabolites.

The irreversibly bound nature of these metabolites was checked by further washing the pellets with hexane, chloroform, ethyl ether, acetone, ethanol, methanol and 10% TCA[2]. Our method of preparing irreversibly bound metabolites was also compared with that described by Jollow *et al.* (21).

The ethyl acetate phases were evaporated to dryness on a rotation evaporator. This process was carefully observed and stopped as soon as all the solvent had evaporated. The remainder was dissolved in 1 ml ethyl acetate. An aliquot of 200 μl was then applied as a band on thin layer chromatography plates using unlabeled phenol and/or benzene dihydrodiol as reference. The plates were developed in one of the three solvent systems described above, and in each experiment the identity of the metabolites in one incubation mixture was confirmed by using also the other two systems. The phenol and benzene-dihydrodiol bands were scraped off and the silica gel added to 6 ml 0.6% Butyl-PBD (Koch Light Laboratories, Colnbrooks Bucks, England) and counted for radioactivity after standing 15 hr in a refrigerator.

In the study of the isotope effect on phenol formation, a different method was used to quantitate phenol. The incubation mixtures, modified as described above, were extracted three times with 10 ml ethyl acetate and the organic phases thereafter evaporated to dryness. The residue was dissolved in 100 μl acetone and 2 μl was injected into a gas chromatograph (Packard model 427) using a column of 2.5% SE 52 on 80–120 mesh Chromosorb W and a flame ionization detector. The carrier gas was nitrogen and the temperatures of the column, the injector and the detector were 70°, 170° and 250° C, respectively. The area of the phenol peak was determined using an integrator (Packard model 603).

RNase and protease treatment. Standard incubations were performed as described. The incubations were stopped by boiling for 5 min. After cooling, the microsomes were rehomogenized and treated for 24 hr at 37°C with 6000 units Ribonuclease T_1 (Boehringer Mannheim) or 80 units Protease Type VI(Sigma), dissolved in 100 μl buffer A diluted with one volume of water. Irreversibly bound metabolites were then separated from radioactivity associated with low molecular weight material as described above.

Recovery studies. Recovery studies were performed for [^{3}H]phenol and [^{3}H]benzene dihydrodiol in the above described extraction scheme. Standard incubations lacking the NADPH-generating system were performed with 10 μl of the acetone solutions described under Compounds.

Calibration. The results from the scintillation spectrometry were transformed to

dpm by the use of the external standard of the instrument. This procedure was occasionally checked by the use of [^{14}C]toluene, [^{3}H]toluene or [^{3}H]water as an internal standard. In all experiments zero time incubations were run, and the results from these were subtracted from all other results, except in Figs. 2 and 4 where results are compared to such blanks.

Since the ortho-tritium of the 2,5-ditritiophenol used may be exchanged, the tritium loss during the standard incubation was monitored. This loss was only 5–9% and the results are not corrected for this loss. Molarities and specific radioactivities given refer to amount of phenol determined spectrophotometrically and the radioactivity determined by scintillation spectrometry.

RESULTS

Recovery study. The recovery of phenol and benzene dihydrodiol was estimated by extracting the [^{3}H]-labeled compounds in the normal manner from incubation mixtures lacking a NADPH generating system. The recovery of phenol from the thin-layer chromatography plates was 64 ± 5% (n = 5). Phenol contamination of the water phase and the irreversibly bound metabolites were estimated to be 3 ± 1% and 2 ± 1%, respectively. The recovery of benzene dihydrodiol was 50% ± 5% (n = 3). Benzene dihydrodiol did not contaminate the irreversibly bound fraction, but 15% of the compound was found in the water phase. However, as shown below, benzene dihydrodiol was not formed under standard conditions and thus this possible contamination of the water phase was unimportant for the purposes of this study.

Time course studies. The results of the time course studies are shown in Fig. 1. The formation of water soluble and irreversibly bound metabolites was practically linear for at least 30 min, while phenol formation was only linear for 10 min. GSH inhibited the formation of irreversibly bound metabolites, and increased the production of water soluble compounds about fourfold. The phenol formation was less affected. The sum of recovered metabolites in the presence of GSH was 81% of the sum in the absence of this cofactor, reflecting either a slight inhibition of the metabolism or a slight deviation to more volatile metabolites.

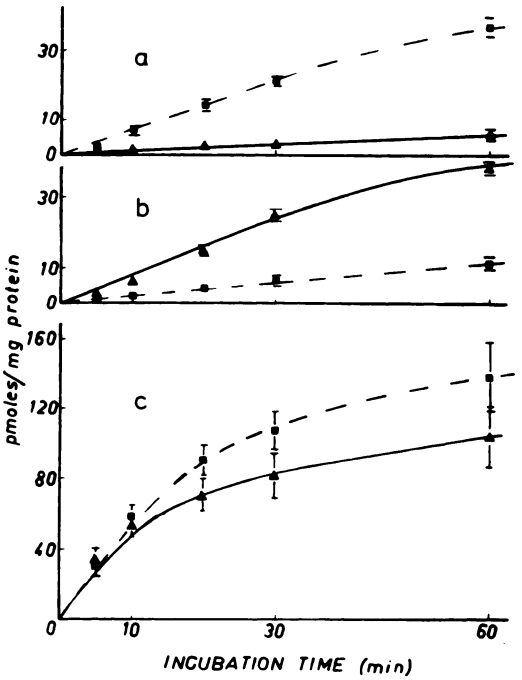

FIG. 1. *Formation of microsomal metabolites derived from benzene as a function of the incubation time*

a. Metabolites irreversibly bound to microsomes, b. water soluble metabolites, c. phenol. Incubations were performed in the absence (■- - -■) or presence (▲——▲) of GSH (2 mM). All values are based on three determinations, and zero time results have been subtracted. Except for the irreversibly bound metabolites in the presence of GSH and the water soluble metabolites without GSH, the radioactivity recovered in the different phases from zero time incubations, as well as from 1 hr-incubations lacking a NADPH-generating system, was always less than 5% of the radioactivity recovered from the standard 1 hr-incubations.

After 1 hr incubation in the absence of GSH 555 pmole metabolites were formed per incubation mixture (3 mg protein), i.e., 2.6% of the [^{14}C]benzene had been metabolized. Of these metabolites 20% became irreversibly bound to the macromolecules. This time point was chosen for the further studies described in this paper.

We were unable to show the formation of benzene dihydrodiol. If at all formed under normal conditions, the diol amounts to less than 1% of the amount of phenol, i.e., less than 0.03% of the incubated benzene. Phenol always accounted for at least 95%

of the radioactivity above background recovered from the thin layer plates.

Further studies on the irreversible binding. In order to gain some insight into the characteristics of the binding of benzene metabolites the experiments summarized in Fig. 2 were performed.

The method of washing the microsomes to remove free and reversibly bound metabolites described by Jollow *et al.* (21) gave 20% higher values of the binding (II) than our method (I). Further washing after our standard washing procedure did not decrease the binding to any significant degree (V). The radioactivity in the fraction of irreversibly bound metabolites was not significantly higher than zero time incubations (III) after incubation with boiled microsomes (XI) or with active microsomes without NADPH (IV). After protease treatment only 10% of the binding remained (VI), while RNase treatment had little effect (VII). Both GSH (VIII) and cysteine (IX) inhibited the binding by about 90%. GSH added 5 min before the end of 1 hr incubation had no detectable effect (X), indicating that GSH did not cleave the bound material but prevented the binding.

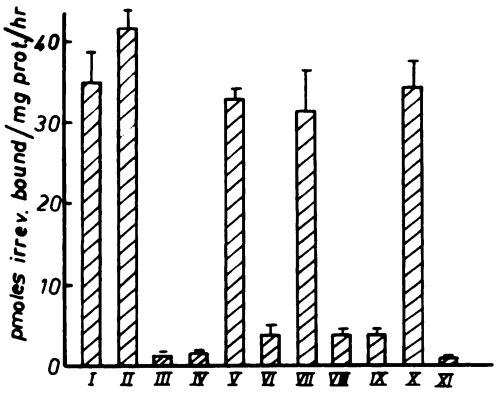

FIG. 2. *Some studies on the irreversibly bound metabolites*

Standard incubations with [^{14}C]benzene as described under MATERIALS AND METHODS. I. Irreversibly bound fraction when washed as described in MATERIALS AND METHODS; II. irreversibly bound fraction when washed as described by Jollow *et al.* (21); III. zero-time incubations; IV. 1-hr-incubations lacking a NADPH-generating system; V. effect of further washes with hexane, chloroform, ethyl ether, acetone, ethanol, methanol and 10% TCA as described; VI. effect of protease treatment; VII. effect of RNase treatment; VIII. effect of 2 mM GSH; IX. effect of 2 mM cysteine; X. effect of 2 mM GSH added 5 min before the end of incubation; and XI. incubation with boiled microsomes. I and II were determined in 5 experiments and standard deviations are indicated. III–XI were performed duplicates and the ranges are indicated. Zero-time incubations (III) have been subtracted as blank values from all values reported in this paper except those of this figure and figure 4.

Effects of different concentrations of GSH on the irreversible binding and phenol formation. The effect of different concentrations of GSH on phenol formation and on the irreversible binding in standard incubations is shown in Fig. 3. While the inhibition of the binding is dose dependent reaching 95% inhibition, the effect on phenol formation is weak, the ranges reaching maximally 30% inhibition. Between 500 and 5000 μM GSH, where inhibition of binding is still strictly dose-dependent, no further inhibition of phenol formation was visible. The increase in the amount of water soluble metabolites approximately corresponded to the decrease in binding (data from 0–1 mM not shown; for a concentration of 2 mM GSH see Fig. 1).

Determination of the isotope effect on the rate of phenol formation. Incubations were performed as described and phenol was isolated and analyzed by gas chromatography. Using normal benzene the rate of phenol formation was 13.2 ± 0.84 nmole/30 min (n = 5) while from fully deuterated benzene the rate of phenol formation was 12.0 ± 1.16 nmole/30 min (n = 5). Thus $K_H/K_D = 1.10 \pm 0.13$, which shows that the rate of hydroxylation of benzene to phenol is not subject to a significant isotope effect.

Determination of irreversible binding and spontaneous GSH conjugation of [^{3}H]benzene oxide. When standard incubations lacking a NADPH generating system were performed using [^{3}H]benzene oxide as substrate very little radioactivity was recovered in the irreversibly bound fraction (0.3–0.4%). Addition of 2 mM GSH to the incubation mixtures had no effect on this binding. The amount of radioactivity recovered in the water phase was 2–3%. This was also unaffected by addition of GSH.

Incubations with [^{3}H]phenol. Figure 4 shows that metabolites which could bind irreversibly to macromolecules were formed from [^{3}H]phenol and that this bind-

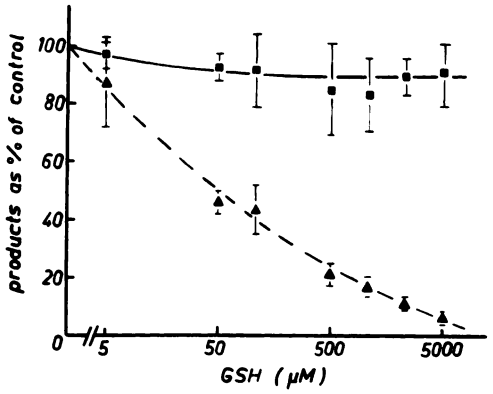

FIG. 3. *Formation of phenol* (■—■) *and irreversible binding* (▲- - -▲) *at different concentrations of GSH*

Standard incubations with [^{14}C]benzene and isolation of metabolites were performed as described in MATERIALS AND METHODS. As controls standard incubations without GSH were used. The amount of products formed in these was defined as 100%. Means and standard deviations of four experiments are shown.

ing was prevented by GSH. However, while only 0.6% of the administered dose of benzene bound as shown above, almost 10% of the phenol dose administered in this experiment bound, although about 40-fold more phenol than benzene was given.

Incubations with microsomes and purified epoxide hydratase. Incubations were performed in the presence of different amounts of epoxide hydratase. As stated above we could not show the formation of benzene-dihydrodiol during standard incubation conditions. Fig. 5 shows that the diol could be detected if relatively large amounts of purified epoxide hydratase were added.[3] At the highest amount of epoxide hydratase the formation of diol was about 20% of the phenol formation. This large amount of purified epoxide hydratase (about 40-fold that present in the microsomes used for this experiment) inhibited the irreversible binding during benzene metabolism by about 40%. This does not reflect epoxide hydratase activity, however, since the same degree of inhibition was observed using the same amount of boiled epoxide hydratase. Destruction of enzyme activity during boiling was proven by absence of diol formation. It is unlikely that free sulphhydryl groups of the denatured

[3] The point of the graph at 0 units epoxide hydratase added (i. e. in presence of the 3-4 units present in the microsomes used) represents the duplicate sample of the individual experiment shown. However, many (>10) similar experiment showed no or similar trace amounts of diol.

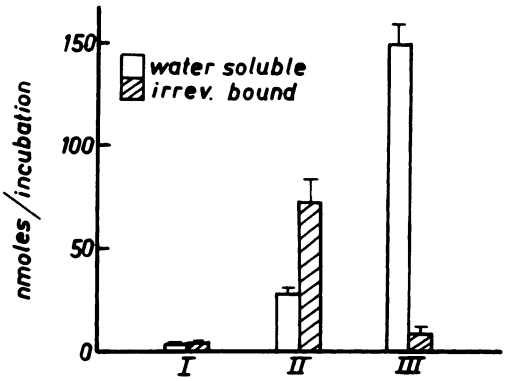

FIG. 4. *Formation of water soluble and irreversibly bound metabolites from [^{3}H]phenol and the dependence of this formation on the NADPH-generating system and GSH*

I. Standard incubations lacking a NADPH-generating system, II. standard incubations and III. standard incubations with 2 mM GSH. As substrate 812 nmoles [^{3}H]phenol was used. Two experiments were performed and means and ranges are indicated.

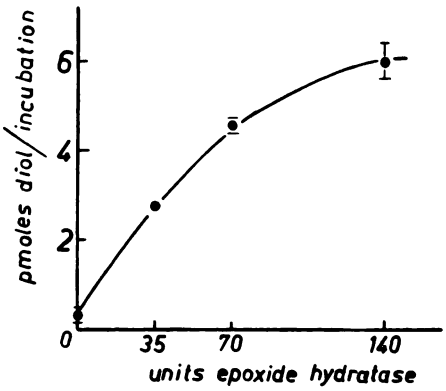

FIG. 5. *Formation of benzene-dihydrodiol on addition of purified epoxide hydratase*

Isolation and identification of the diol and modification of incubation mixtures are described in MATERIALS AND METHODS. In incubations containing the largest amount of hydratase, the amount of diol measured by scintillation spectrometry, was 6-fold higher than in blank incubations lacking the NADPH-generating system or zero-time incubation. Incubations were performed in duplicate, and means and ranges are indicated.

enzyme are reponsible for the inhibition of binding, since bovine serum albumin (up to twice the amount of epoxide hydratase used) did not show this effect.

Effect of UDPGA[2]. Standard incubations using [^{14}C]benzene as substrate and with concentrations of UDPGA ranging between 0 and 30 mM, were performed. The results for phenol and irreversibly bound metabolites are shown in Fig. 6. As expected, UDPGA lowered the amount of phenol recovered. However, the effect on the irreversible binding was even stronger. The formation of water soluble metabolites increased on addition of UDPGA in such a way that the total metabolism up to 10 mM UDPGA concentration was constant or slightly (~10%) elevated. At 30 mM UDPGA, however, the total metabolism was only 80% of that observed without the cofactor.

DISCUSSION

The method of detecting irreversibly bound metabolites, developed for the purpose of this study, enabled us to measure the formation of phenol, benzene dihydrodiol, water soluble metabolites and irreversibly bound metabolites in the same incubation mixture. Although we have not isolated and identified any water soluble metabolites in this study, we felt it was essential to determine their total amount, in order to see whether the different modifications to the incubation conditions simply inhibited (or activated) benzene metabolism. The results of the recovery study show that the possible contaminating compounds phenol and benzene dihydrodiol do not significantly contaminate the irreversibly bound fraction. From Fig. 2 it is clear that the contamination by unmetabolized benzene is quite small (III, IV and XI), that the irreversible binding occurs to proteins rather than to ribosomal RNA (VI and VII) and that nucleophilic agents such as GSH and cysteine effectively inhibit the binding (VIII and IX).

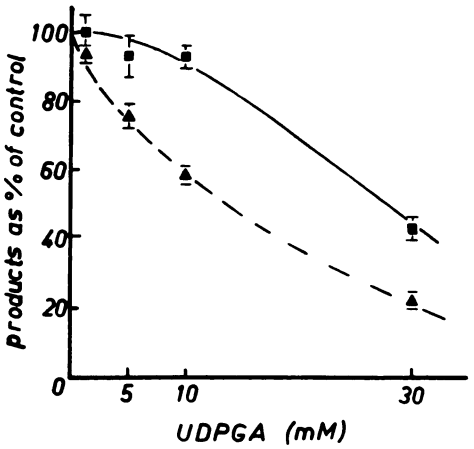

FIG. 6. *The effect of UDPGA on the amount of phenol (■——■) and irreversibly bound metabolites (▲- - -▲)*

Standard incubations with [^{14}C]benzene as substrate were performed in duplicate and means and ranges are indicated. As controls standard incubations without UDPGA were used. The amount of products formed in these was defined as 100%.

These findings are consistent with the hypothesis that benzene is hydroxylated via an epoxide intermediate and that this intermediate is responsible for the binding. The results presented in Fig. 3, however, are difficult to explain on the basis of this hypothesis. The effect of GSH on phenol formation is very weak (<20% inhibition, the ranges of the effects reaching maximally 30%) and the effect does not increase significantly between 50 μM and 5 mM. The effect of GSH on the binding at these concentrations is, however, clearly dose-dependent and reaches 95% inhibition at 5 mM.

We therefore decided to investigate whether phenol was formed via a route which did not involve any electrophilic intermediates, e.g., by direct insertion. The rate of hydroxylations that proceed via direct insertion show an isotope effect (K_H/K_D) of 1.3–1.7 (except where a step other than C-H cleaving is rate limiting, e.g., binding of the substrate to the enzyme), while the rate of hydroxylation via epoxide formation followed by NIH-shift shows no isotope effect (22). Thus, since no isotope effect was found, phenol is most probably formed (at least predominately) via the arene oxide route (23). This is further supported by the findings presented in Fig. 5. It is shown that benzene dihydrodiol is formed upon addition of epoxide hydratase to incubation mixtures with [^{14}C]benzene as substrate. The activity of epoxide hydratase occurring in rat liver microsomes

is 7-9 units[4]/mg protein (20). Thus 3-4 units were present in the microsomes used in these modified incubations, while 35-104 units purified enzyme were added in the experiment depicted in Fig. 5. This need for high epoxide hydratase addition agrees with the instability of benzene oxide and its high apparent K_m (about 30 mM) in the hydration reaction.[5]

Traces of benzene dihydrodiol have been reported to occur in rabbit urine after a heavy dose of benzene (6). It has also been suggested that the catechol excreted by rabbits after dosage with benzene possibly originates from a dihydrodiol (3, 5), although in these studies the diol itself could not be detected. However, since these were studies in whole animals, a contribution from intestinal bacteria is always possible and multistep reactions leading to dihydrodiol not via an epoxide can less readily be excluded than in the presence of liver microsomal fraction. The findings presented in this study constitute the first evidence that benzene dihydrodiol is formed from benzene *in vitro*. This in combination with the fact that benzene was converted to observable quantities of benzene dihydrodiol only after addition of homogeneous epoxide hydratase constitutes unequivocal proof of the metabolic formation of benzene oxide. Moreover, the fact that the diol formation, in the presence of large amounts of epoxide hydratase, is about 20% of the phenol formation shows that a considerable portion of benzene hydroxylation proceeds via the epoxide intermediate.

Benzene oxide, when incubated with microsomes under standard conditions, did not (or at most minimally) bind irreversibly to macromolecules, nor did it form water soluble conjugates with GSH. These observations are in contrast to those when benzene was used as substrate. Experiments with benzene oxide should always be interpreted with caution, due to the instability of the compound. However, the half-life of benzene oxide in water pH 7.0 is estimated to be about 2 min (11), and since the binding of an electrophilically reactive metabolite can be expected to proceed non-enzymically, a proportionality with respect to the concentration of the reactive species may be assumed. Thus, these observations exclude benzene oxide as the agent predominantly responsible for the binding observed.

We further showed that 10% of the dose of phenol added bound irreversibly upon incubation and that this binding, as in incubations with benzene, was prevented by GSH (Fig. 4). In incubations with benzene only 0.6% of the dose bound. Thus, although the dose of phenol was 40-fold higher than that of benzene, a 20-fold higher portion of the phenol dose bound. This is consistent with the interpretation of phenol being a metabolite more proximate to the binding species than benzene. Addition of UDPGA to incubation mixtures with [^{14}C]benzene as substrate, decreased the amount of irreversibly bound metabolites as well as the amount of phenol (Fig.6). An enzyme, UDP-glucuronyl transferase, is present in the microsomal fraction, which catalyzes the conjugation of UDPGA with acceptors of different sorts (24) including phenolic hydroxyl groups. Recently extensive conjugation was also found with benzo(a)pyrene-4,5-oxide and 7,8-oxide (25). The conjugation of the 4,5-oxide is thought to proceed via the diol and the conjugation with 7,8-oxide mainly by isomerization to 7-OH-benzpyrene before conjugation. Thus, to our present knowledge, the addition of UDPGA should have no effect on the irreversible binding if benzene oxide were the binding agent, while such an effect should be seen if a further metabolite of phenol bound. In Fig. 6 it appears as if UDPGA at low concentrations decreased the binding without significantly affecting the amount of phenol. A possible explanation for this is that at low UDPGA-concentrations the conjugation capacity is only enough to reduce the local concentration of phenol in the membranes, the critical compartment for the further enzymatic activation of phenol. At higher concentrations of UDPGA, the conjugation is so fast that an effect on the overall amount of phenol also is clearly seen.

On the basis of the findings described in this paper it can be concluded that benzene

[4] One unit epoxide hydratase is defined as the amount of enzyme producing 1 nmole styrene glycol per min from styrene oxide (26).

[5] M. Golan, P. Bentley, K. Platt and F. Oesch, publication in progress.

oxide is metabolically formed from benzene, since addition of homogeneous epoxide hydratase led to easily quantifiable amounts of benzene dihydrodiol. The data are compatible with the concept that phenol formation occurs by isomerization of benzene oxide and not by direct insertion of oxygen. On the other hand the data show that benzene oxide is not trapped by glutathione under the conditions used (pH 7.5; absence of cytoplasmic fraction which contains glutathione S-transferases) and is not the metabolite responsible for the majority of the irreversible binding to macromolecules. What binds is instead an unknown product of the further oxidation of phenol. Whether or not these findings are of significance for the toxicity of benzene must be investigated in further studies, where the metabolism of benzene in the bone marrow and levels of different enzyme activities in this organ will be determined. If a metabolite of phenol is also the binding agent in bone marrow it could be that a deficient conjugating system in this organ is responsible for the peculiar organ specificity of benzene toxicity.

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