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REDUCTION OF BENZYL HALIDES BY LIVER MICROSOMES

FORMATION OF 478 NM-ABSORBING σ-ALKYL–FERRIC CYTOCHROME P-450 COMPLEXES

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Abstract—The benzyl halides benzyl bromide and 4-nitrobenzyl chloride are reduced anaerobically by NADPH and rat liver microsomes to yield toluene and 4-nitrotoluene, respectively. These reductions are cytochrome P-450-dependent since they are inhibited by CO and metyrapone, and are increased after pretreatment of rats by phenobarbital and 3-methylcholanthrene. During benzyl halide reduction, cytochrome P-450 complexes, which are very unstable to O_2 and characterized by a Soret peak at 478 nm, are formed in steady-state concentrations. These concentrations are very dependent on pretreatment of rats and on the nature of the reducing agent (NADPH or dithionite) and the benzyl halide: 4-methylbenzyl bromide and benzyl bromide lead to 478 nm absorbing complexes in the presence of NADPH whereas 4-nitrobenzyl chloride and benzyl chloride lead to such complexes only in the presence of dithionite. Microsomal reductions of 4-nitrobenzyl chloride and benzyl bromide in D_2O lead to partially deuterated 4-nitrotoluene and toluene. From these results, we propose a mechanism for anaerobic microsomal reduction of benzyl halides involving the intermediate formation of σ-alkyl cytochrome P-450–Fe(III)–CH_2Ar complexes which exhibit red-shifted Soret peaks around 478 nm. Toluenes, $ArCH_3$, are formed either by protonation of the σ-alkyl complexes or by hydrogen abstraction by the intermediate free radical $ArCH_2$.

The main physiological role of liver microsomal cytochrome P-450 is to activate dioxygen by a two-electron reduction [1–3]. However, there is now ample evidence that several compounds, such as nitroarenes [4], epoxides [5, 6], amine oxides [7] and halogenated alkanes [8–13], can compete with dioxygen at cytochrome P-450–iron(II) and can be reduced by NADPH and liver microsomes, especially under anaerobic conditions. It has been shown that cytochrome P-450–iron–metabolite complexes are formed upon microsomal reduction of several polyhalogenated compounds [8, 9, 11–13]. Recent studies on reactions between iron–porphyrins and polyhalogenated compounds, in the presence of excess reducing agent, have shown that two types of iron complexes can be formed from compounds containing at least two halogen (Cl, Br or I) substituents at the same carbon: σ-alkyl–Fe(III) and carbene–Fe(II) complexes, [equation (1)] [14].

$$R_1R_2CX_2 + (P)Fe(II) \xrightarrow[-X^-]{+e^-} (P)Fe^{III}\text{—}CXR_1R_2$$

σ-Alkyl complex

$$\xrightarrow[-X]{+e^-} (P)Fe^{II} \leftarrow CR_1R_2$$

Carbene complex (1)

X = Cl, Br, I
P = Porphyrin dianion

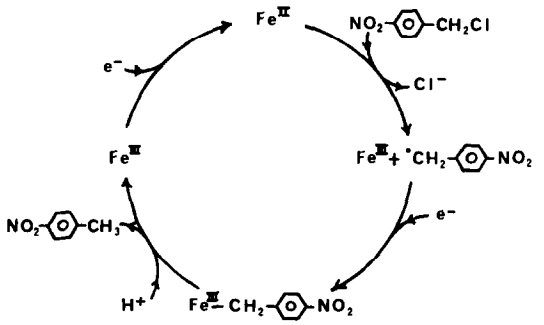
Generally, in the presence of excess reducing agent and with polyhalogenated compounds as different as CCl_4, CCl_3F, CBr_4, $CFBr_3$ [14–16], CCl_3CN, CCl_3COOEt [17], CCl_3R (R = CH_3, $CHOHCH_3$, $CHOHC_6H_5$) [18] and CCl_3SR [19], one observed the formation of (porphyrin) Fe(II)–carbene complexes, the structure of which has been fully established. However, under identical conditions, the reduction of halothane $CF_3CHClBr$ stopped at the stage of the σ-alkyl Fe^{III}—$CHClCF_3$ complex allowing its isolation in the crystalline state [20].

As far as cytochrome P-450 is concerned, it is naturally more difficult to determine the structures of its iron complexes with polyhalogenated compounds metabolites. However, evidence has been provided in favour of a carbenic structure for the complexes derived from reduction of CCl_4, CCl_3F and other polyhalomethanes, which are characterized by Soret bands around 460 nm [9].

On the contrary, a σ-alkyl Fe^{III}—$CHClCF_3$ structure has been recently proposed for the 470 nm absorbing complex formed upon microsomal reduction of halothane, on the basis of its reactivity [11] and spectral characteristics which are very similar to that of a thiolate heme Fe^{III}—$CHClCF_3$ model complex [21].

One could expect that the reduction of haloalkanes containing only one carbon–halogen bond would lead to a simpler situation: the formation of a carbene complex from equation (1) cannot occur in this case whereas that of a σ-alkyl complex remains possible. We have recently described a heme model system, using ascorbate as a reducing agent in water and Fe(III)(TPP = tetraphenylporphyrin dianion)(Cl) in catalytic amounts in a hydrophobic solvent, able to

perform most of the microsomal NADPH- and cytochrome P-450-dependent reductions of organic substrates [22]. This system is able to reduce benzyl halides, such as 4-nitrobenzyl chloride or benzyl bromide, with the intermediate formation of a σ-benzyl complex (TPP)Fe^{III}—CH_2Ar [23] and evidence has been provided for the following mechanism for 4-nitrobenzyl chloride reduction (Scheme 1).



Scheme 1. Proposed mechanism for the reduction of 4-nitrobenzyl chloride by a heme model system. Intermediate formation of a σ-alkyl-iron(III) complex [23].

This result prompted us to study the reduction of benzyl halides by microsomal cytochrome P-450, and this paper reports that benzyl halides, $ArCH_2X$, are reduced by rat liver microsomes and NADPH to the corresponding toluene $ArCH_3$, with the intermediate formation of cytochrome P-450 complexes characterized by a Soret peak around 478 nm.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (150–200 g) were given three daily doses of sodium phenobarbitone (PB) (80 mg/kg body weight i.p.) or 3-methylcholanthrene (3-MC) (20 mg/kg body weight i.p.). Liver microsomes were prepared [24] and protein [25] and cytochrome P-450 [26] contents were determined as previously described.

Chemical compounds were obtained from regular commercial sources.

Anaerobic NADPH oxidation. The benzyl halide-induced NADPH oxidation was measured in rat liver microsomal preparations (1 mg protein/ml) suspended in 0.1 M phosphate buffer, pH 7.4, in stoppered 1 cm glass cuvettes. Dioxygen was removed by bubbling the buffer 1 hr with argon prior to the addition of microsomes, followed by bubbling another 15 min with argon. After addition of NADPH (final concentration 100 μM) and $ArCH_2X$ (final concentration 1 mM) to the test cuvette, the absorption change at 340 nm was recorded in an AMINCO DW 2 spectrophotometer at 30°. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate the rate of NADPH oxidation [11].

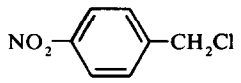
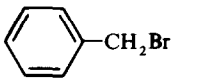
Formation of metabolites. The assay for benzyl halide metabolites was performed in 10 ml rubber septum-capped tubes containing 6 mg of microsomal protein in 3 ml of 0.1 M phosphate buffer, pH 7.4, and a NADPH-generating system (glucose-6-phosphate 16 mM, $MgCl_2$ 12 mM, glucose-6-phosphate dehydrogenase 3 U, NADP 0.5 mM). Dioxygen was removed by bubbling argon through the buffer for 1 hr, then adding the microsomes and bubbling argon for another 15 min. The reaction was started by adding $ArCH_2X$ (1 mM). After incubation at 30°, 0.3 ml of 10% perchloric acid was added to precipitate the protein and stop the reaction. Metabolites were extracted with CH_2Cl_2 (3 ml) and analyzed by GLC and by gas chromatography–mass spectrometry (GC–MS).

For GLC, an INTERSMAT IG 120 FL equipped with a flame ionization detector and a 3% SE30 glass column were used. The injector temperature was 210° and the column temperature 150° for 4-nitrobenzyl chloride, and 75° for benzyl bromide. The carrier gas was N_2 at a pressure of 1.1 and 0.7 bar, respectively. For combined GC–MS, a RIBER R10-10 mass spectrometer and a PDP8 computer were coupled with a GIRDEL chromatograph. The temperature of the SE52 glass column increased from 100 to 250° with a rate of 5°/min. The carrier gas was helium at a pressure of 1 bar.

Incubations with deuterated water. The 0.1 M phosphate buffer, pD 7.4, was prepared by incubating KH_2PO_4 and K_2HPO_4 in D_2O and by lyophilizing the solution. This incubation–lyophilization procedure was repeated three times. The liver microsomal pellet was suspended in this buffer for 30 min. The suspension was centrifuged (105,000 *g*) and the pellet resuspended in the same deuterated buffer. This procedure was repeated three times to obtain a microsomal suspension in D_2O containing 2 mg protein/ml. Anaerobic incubations were performed in 10 ml rubber septum-capped tubes containing the microsomal suspension (3 ml) and NADPH (1 mM). The reaction was started by injection of $ArCH_2X$ (1 mM final concentration). After 10 min incubation at 30°, CH_2Cl_2 (3 ml) was introduced to extract the products. Aliquots of the extract were injected into a gas chromatograph linked to a mass spectrometer. The relative abundance of the ions M and M + 1 was used to determine the relative amounts of $ArCH_3$ and $ArCH_2D$ formed in the incubates. By ^1H NMR it was established that the aqueous buffer contained 99.3% D_2O and 0.7% H_2O.

Formation of the 478 nm absorbing complexes. To follow the reaction by UV–visible spectroscopy and for the measurement of the metabolic intermediate complex from cytochrome P-450 and benzyl halide, microsomes were suspended in 6 ml of 0.1 M phosphate buffer, pH 7.4, made anaerobic by argon-bubbling and equally separated between two stoppered 1 cm glass cuvettes containing microsomal proteins (1 mg/ml) and NADPH (0.2–2 mM) or sodium dithionite (5 mM) at 30°. The reaction was started by addition of $ArCH_2X$ (final concentration 1 mM) to the test cuvette and the formation of the complex was monitored by difference visible spectroscopy in an AMINCO DW 2 spectrophotometer for 20 min. By adding known amounts of aerated buffer, the inhibition of complex formation by dioxygen was studied. The stability of the complexes towards CO and O_2 was studied by adding CO or O_2 to the test cuvette (20 sec bubbling).

Table 1. Effects of cytochrome P-450 inhibitors on the benzyl halide-induced oxidation of NADPH by liver microsomes of PB-pretreated rats* in anaerobic conditions

Conditions	*Vo*†, nmole NADPH consumed/nmole P-450 per min (% inhibition)	
		
100 μM NADPH	1.45 ± 0.20	1.30 ± 0.20
100 μM NADPH	0.05	0.15
+ CO saturated	(97%)	(88%)
100 μM NADPH	0.70 ± 0.10	0.45 ± 0.10
+ 10^{-4} M metyrapone	(48%)	(65%)
100 μM NADPH	0.05	0.05
+ boiled microsomes	(97%)	(97%)

The assays were performed as described in Materials and Methods. Metyrapone was present in the incubation medium before starting the reaction. CO was bubbled for 30 sec in the anaerobic suspension.

* Cytochrome P-450 content was 2.2 nmole/mg protein for benzyl bromide and 2.8 nmole/mg protein for 4-nitrobenzyl chloride.

† *Vo* is the initial rate of the reaction. The results are given as mean values from three or four experiments (± S.D.).

RESULTS

NADPH-dependent reduction of benzyl bromide and 4-nitrobenzyl chloride by rat liver microsomes

Under strictly anaerobic conditions, no NADPH consumption by liver microsomes from phenobarbital (PB)-treated rats was observed. Addition of 4-nitrobenzyl chloride or benzyl bromide led to an immediate anaerobic NADPH oxidation. The results of Table 1 show that this is an enzymatic reaction, since it does not occur with boiled microsomes, and that cytochrome P-450 is involved since known inhibitors of this hemoprotein, such as CO and metyrapone, inhibit the reaction.

The products of the NADPH- and liver microsomes-dependent anaerobic reduction of benzyl bromide and 4-nitrobenzyl chloride were analyzed by GLC and MS of CH_2Cl_2 extracts of the reaction mixture. The only observed metabolites of benzyl bromide and 4-nitrobenzyl chloride were, respectively, toluene and 4-nitrotoluene. It is noteworthy that no 4-nitrosotoluene or 4-methyl aniline could be detected from microsomal reduction of 4-nitrobenzyl chloride. The results of Table 2 provide further evidence that the reduction of these benzyl halides is a cytochrome P-450-dependent reaction. Accordingly, the reduction rate decreased markedly in the presence of metyrapone and CO, and increased significantly with microsomes from rats treated by classical inducers of cytochrome P-450, such as phenobarbital and 3-methylcholanthrene. It is noteworthy that dioxygen greatly inhibits the reactions, but that 15–20% of the reducing activity remains in aerated microsomes.

In order to know the origin of the hydrogen that replaces the halogen atom in these reductions,

Table 2. Effects of various inducers and inhibitors of cytochrome P-450 on the formation of 4-nitrobenzyl chloride and benzyl bromide metabolites by anaerobic NADPH-reduced rat liver microsomes

Conditions	Pretreatment of rats	*Vo**, nmole/mg protein per min [nmol/nmol P-450 per min] (% inhibition)			
		4-Nitrotoluene		Toluene	
Anaerobic	PB	3.7 ± 0.6	[1.3]	5.3 ± 0.5	[2.4]
Anaerobic	3-MC	2.8 ± 0.3	[1.1]	6.1 ± 0.6	[2.4]
Anaerobic	Control	1.2 ± 0.2	[1.0]	3.6 ± 0.5	[3.0]
CO-Saturated	PB	0.1 (97)	[0.05]	0.3 ± 0.1 (94)	[0.15]
10^{-4} M metyrapone	PB	2.2 ± 0.4 (40)	[0.8]	2.1 ± 0.3 (60)	[0.95]
Air-saturated	PB	0.5 ± 0.1 (84)	[0.2]	1.0 ± 0.2 (81)	[0.45]
Boiled microsomes	PB	0 (100)		0.1 (98)	[0.05]
NADPH omitted	PB	0 (100)		0 (100)	

The assays were performed as described in Materials and Methods.

* *Vo* is the initial rate of formation of the metabolites. The results are given as mean values from three or four experiments (± S.D.)

Table 3. Formation of deuterated 4-nitrotoluene and toluene from 4-nitrobenzyl chloride and benzyl bromide reduction by anaerobic NADPH-reduced microsomes

Incubation conditions	% $ArCH_2D$	
	Ar = 4-$NO_2C_6H_5$	Ar = C_6H_5
Microsomes, H_2O, NADPH	0	0
Microsomes, D_2O, NADPH		
after two centrifugations*	31 ± 2	—
after three centrifugations*	43 ± 2	15 ± 1
after four centrifugations*	43 ± 2	—

The assays were performed as described in Materials and Methods.
* Successive incubations of microsomes in D_2O, pD 7.4, followed by centrifugation were performed to achieve complete exchange of acidic protons of microsomes by deuterons (see Results).

anaerobic incubations of benzylhalides with NADPH and rat liver microsomes in D_2O, pD 7.4, were performed. In order to ensure the exchange of the acidic hydrogens present in microsomes by deuteriums, microsomes were suspended in D_2O phosphate buffer for 30 min and then centrifuged, this operation being repeated several times. Deuterium incorporation in the products (Table 3) was measured after each suspension–centrifugation cycle, and found to be almost constant after three cycles. It is noteworthy that deuterium incorporation is much higher in 4-nitrotoluene than in toluene.

Formation of cytochrome P-450–metabolite complexes during anaerobic microsomal reduction of benzyl halides

Addition of benzyl bromide to rat liver microsomes reduced by NADPH in anaerobic conditions produced the appearance of a difference spectrum characterized by an unusually red-shifted Soret peak at 478 nm. With NADPH and benzyl bromide (1 mM), the difference spectrum increased within time with isobestic points at 446 and 526 nm, and reached its maximum intensity after about 20 min (Fig. 1). The 478 nm absorbing complex only exists in a steady-state concentration, since when low concentrations of NADPH are used, it rapidly disappears as soon as NADPH is consumed (Fig. 2). Upon new addition of NADPH, the formation of the complex started again at the same rate (Fig. 2).

Moreover, upon addition of CO to the microsomal suspension, after maximum formation of the 478 nm band, two bands at 450 and 478 nm were first observed, then a decrease (≃ 2 min) of the 478 nm peak with a concomitant increase of the 450 nm peak, indicating decomposition of the intermediate com-

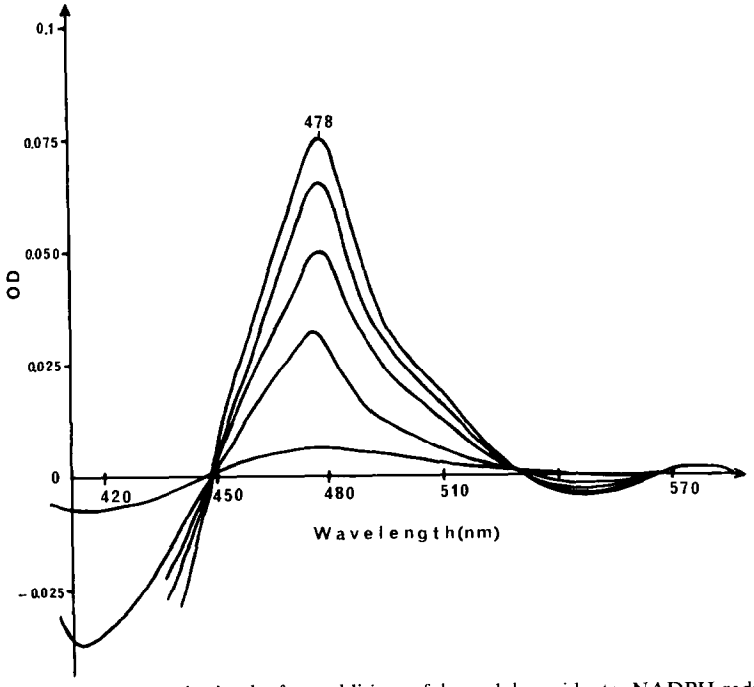


Fig. 1. Difference spectrum obtained after addition of benzyl bromide to NADPH-reduced liver microsomal preparations from PB-pretreated rats. The two cuvettes contained 3 ml of a microsomal suspension (1 mg/ml) and NADPH (1 mM). Benzyl bromide (1 mM) was added to the sample cuvette. Scans were done at 0.5, 2, 5, 10 and 20 min.

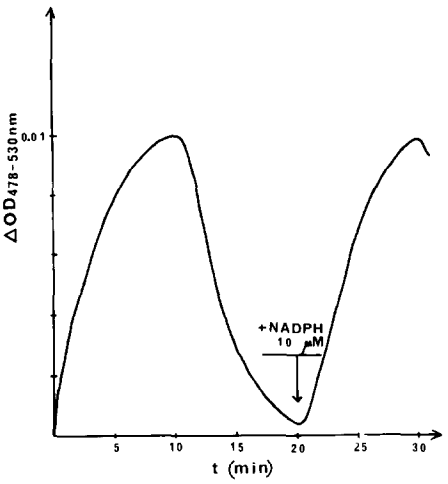


Fig. 2. Evolution of the benzyl bromide-derived 478 nm absorbing complex in the presence of limited amounts of NADPH. The cuvette contained 3 ml of anaerobic microsomal suspension (1 mg/ml) and NADPH (10 μM). Benzyl bromide (1 mM) was added to the sample cuvette and $\Delta OD_{478-530\ nm}$ recorded as a function of time. After complete decomposition of the complex, 10 μM NADPH was added.

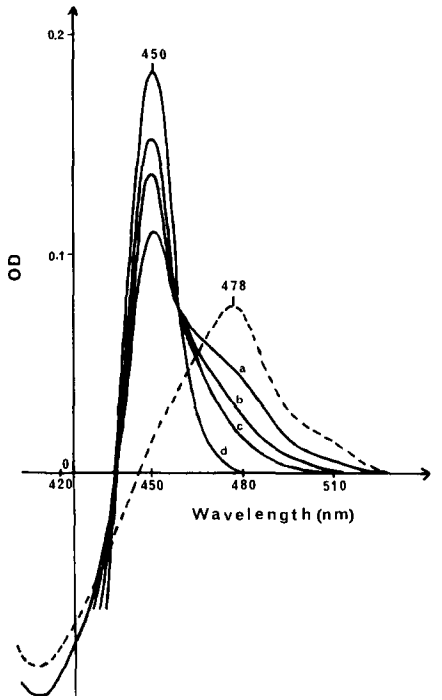


Fig. 3. Decomposition of the benzyl bromide–metabolite complex in the presence of CO. The two cuvettes contained 3 ml of an anaerobic suspension of rat liver microsomes (1 mg protein/ml and 2.2 nmole cytochrome P-450/mg protein) previously incubated with 1 mM NADPH. (---) Difference spectrum obtained after addition of 1 mM benzyl bromide to the sample cuvette and 20 min incubation. (———) CO was then bubbled through the suspension and scans were done at 0.5 (a), 1 (b), 2 (c) and 5 (d) min.

plex and formation of cytochrome P-450–Fe(II)–CO (Fig. 3). This experiment allows an estimation of the millimolar extinction coefficient of the 478 nm absorbing complex, $E(478–530\ nm) = 75 \pm 5\ mM^{-1}\ cm^{-1}$. After complete disappearance of the 478 nm peak, final determination of the remaining cytochrome P-450 able to bind CO indicated that about 20% of the starting cytochrome P-450 had been destroyed over a 20 min incubation period with NADPH and benzyl bromide in anaerobic conditions. This loss of cytochrome P-450 does not correlate with the formation of the 478 nm absorbing complex, since the same 20% loss was observed after identical incubations but in the absence of NADPH, i.e. under conditions where no 478 nm absorbing complex is formed. This cytochrome P-450 destruction is certainly related to the alkylating properties of benzyl bromide. Accordingly, 4-nitrobenzyl chloride, which is a much less alkylating agent [27], formed a similar complex with a 477 nm Soret peak with dithionite-reduced liver microsomes (Table 4) without loss of cytochrome P-450 after CO addition at the end of a 20 min incubation period with NADPH- or $Na_2S_2O_4$-reduced microsomes.

In microsomes from PB-treated or untreated rats, the steady-state level of the 478 nm absorbing complex was higher than in microsomes from 3 MC-treated animals (Fig. 4). This level increased when the powerful reducing agent dithionite was used instead of NADPH, up to 80% of total cytochrome P-450 being engaged in a 478 nm absorbing complex. In that respect, it is noteworthy that in the case of 4-nitrobenzyl chloride the cytochrome P-450 iron–metabolite complex is only detectable when dithionite is used as a reducing agent (Table 4).

The 478 nm absorbing complex derived from benzylbromide reduction is very unstable to dioxygen: bubbling O_2 through a microsomal suspension after

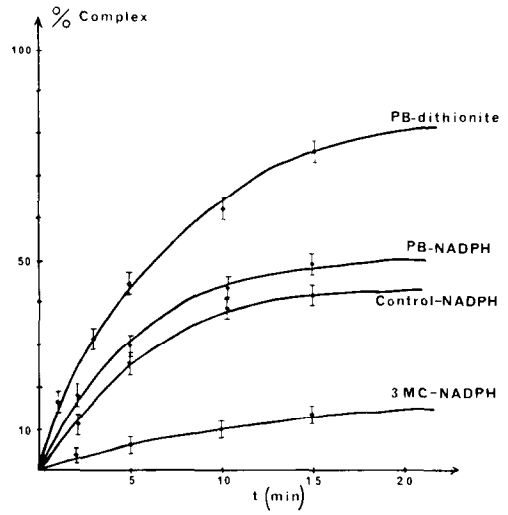


Fig. 4. Kinetics of formation of the 478 nm absorbing complex during the reduction of benzyl bromide by rat liver microsomes: influence of the nature of the reducing agent and of rat pretreatment. The procedure is described in Materials and Methods. % Complex yield is based on the total amount of cytochrome P-450 not destroyed, taking into account that 1% cytochrome P-450 per min is destroyed in the conditions used. An extinction coefficient of 75 mM ^{1}cm ^{1} was used for calculating the % complex yield.

Table 4. Cytochrome P-450–metabolite complex formation during anaerobic microsomal reduction of various benzyl halides

Substrate	Steady-state level of complex (%)* with			λ_{max} (nm)
	NADPH	$Na_2S_2O_4$ Conditions A	$Na_2S_2O_4$ Conditions B	
4-Nitrobenzyl chloride	0	15	20	477
4-Nitrobenzyl bromide	0	20	25	477
Benzyl bromide	40	70	80	478
Benzyl chloride	0	0	10	478
4-Methylbenzyl bromide	35	70	80	478
4-Cyanobenzyl bromide	0	0	0	

The assays were performed as described in Materials and Methods.
* In experiments with NADPH, 1 mM NADPH and 1 mM substrate concentrations were used. In experiments with sodium dithionite, 5 mM $Na_2S_2O_4$ and 1 mM substrate concentrations (conditions A) or 500 mM $Na_2S_2O_4$ and 10 mM substrate concentrations (conditions B) were used. % Complex at its maximum level relative to starting cytochrome P-450.

maximum development of the complex in anaerobic conditions and in the presence of NADPH resulted in an immediate disappearance of the 478 nm peak. However, the formation of the 478 nm absorbing complex in NADPH-reduced microsomes in the presence of low dioxygen concentrations could be detected: with 20 μM O_2 and after 10 min incubation, 12% of starting cytochrome P-450 was in the form of an iron–metabolite complex, instead of 50% in anaerobic conditions. No complex formation was detected with O_2 concentrations above 60 μM.

There is a clear dependence of the formation of the 478 nm absorbing complexes on the kinetics of microsomal reduction of benzyl halides into toluenes. During microsomal NADPH-dependent reduction of 4-nitrobenzyl chloride in conditions where one cannot detect 478 nm absorbing complex formation, 4-nitrotoluene formation and NADPH oxidation were linear with time, at least for 15 min. On the contrary, during reduction of benzyl bromide by PB-pretreated rat liver microsomes, a situation where high levels of cytochrome P-450–metabolite complex (Fig. 4) are observed, toluene formation was only linear during the first 2 min and then decreased markedly (Fig. 5). With 3-MC treated rat microsomes, lower levels of the 478 nm absorbing complex were obtained as well as a lower deviation of toluene formation from linearity.

As indicated in Table 4, benzyl bromide and 4-methylbenzyl bromide are able to form 478 nm absorbing complexes with NADPH-reduced anaerobic microsomes, whereas 4-nitrobenzyl bromide and 4-nitrobenzyl chloride lead only to cytochrome P-450–metabolite complexes in the presence of $Na_2S_2O_4$. The appearance of such complexes should depend critically upon their relative rates of formation and destruction, since a similar benzyl halide, 4-CN-benzyl chloride, was unable to produce a similar complex either with NADPH or dithionite as reducing agents.

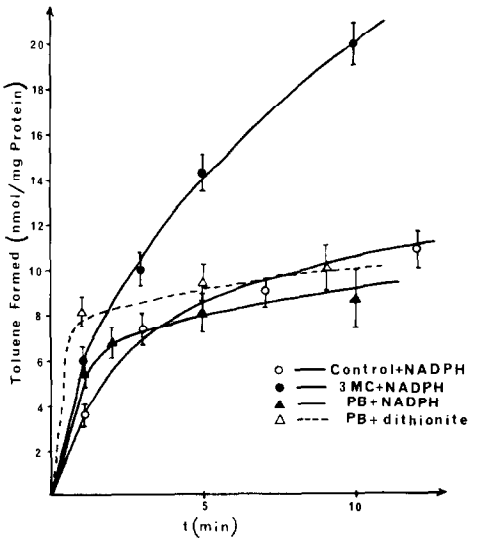


Fig. 5. Kinetics of toluene formation upon benzyl bromide reduction by rat liver microsomes: influence of the nature of the reducing agent and of rat pretreatment. The assays were performed as described in Materials and Methods (NADPH-generating system from 0.5 mM NADP, 1 mM benzyl bromide and 6 mg of microsomal protein in 3 ml phosphate buffer, pH 7.4). The results are given as mean values from three or four experiments (±S.D.).

DISCUSSION

It is now well known that iron(II)–porphyrins [28] or iron(II)–hemoproteins [29] are able to transfer one electron to certain reducible organic compounds. For compounds containing a carbon–halogen bond, this electron transfer leads to the formation of a free radical derived from the substrate and release of a halide anion, equation (2) [28].

$$Fe^{II} + RX \longrightarrow Fe^{III} + R^{\cdot} + X^{-} \qquad (2)$$

Similar reactions have been shown to occur during reduction of CCl_4 [12], CCl_3–CCl_3 [10] or halothane $CF_3CHClBr$ [11] by cytochrome P-450. The intermediate free radical may be released from the active site and react with microsomal proteins and unsaturated lipids, producing irreversible modifications of these macromolecules as shown for $^{\cdot}CCl_3$ derived from CCl_4 for instance [30, 31]. It might also bind to cytochrome P-450–Fe(III) leading to a reactive intermediate Fe(IV)–R σ-alkyl complex [equation (3)]; in that respect, the formation of such a complex upon reaction of porphyrins–Fe(III) with $^{\cdot}CH_3$ radicals has been indicated using pulse radiolysis techniques [32].

$$[Fe^{III} + R^{\cdot}] \leftrightarrows Fe^{IV}—R \qquad (3)$$

At this stage, the [Fe^{III} + R·] pair or the Fe^{IV}—R complex can receive one electron from NADPH leading to a σ-alkyl–Fe(III)–cytochrome P-450 complex which can be viewed as deriving from the binding of the R^- anion to cytochrome P-450–Fe(III) by a simple Fe–C bond [equation (4)].

$$\begin{array}{ccc} [Fe^{III} + R^{\cdot}] & & [Fe^{III} + R^-] \\ \downarrow\uparrow & \xrightarrow{+e^-} & \downarrow\uparrow \\ Fe^{IV}—R & & Fe^{III}—R \end{array} \qquad (4)$$

A very similar scheme has been recently proposed for the cytochrome P-450-dependent reductions of CCl_3–CCl_3 [10] and halothane [11]. Depending on the nature of R, there are several possible evolutions of the Fe^{III}—R complex: (i) a simple protonation of the Fe–R bond leading to RH, as previously found for σ-alkyl–Fe(III)–porphyrin complexes [23]; (ii) the elimination, when possible, of a halogen substituent in the β-position, as previously found for CCl_3–CCl_3 and halothane [10, 11]; (iii) the elimination of a halogen substituent in the α-position, as previously found for CCl_4 for instance, leading to the formation of a carbene complex [9] [equation (1)]. In the case of halothane $CF_3CHClBr$, these three possible evolutions are theoretically possible, and the second one has been shown to occur with NADPH-reduced microsomes, leading to CF_2=CHCl [11].

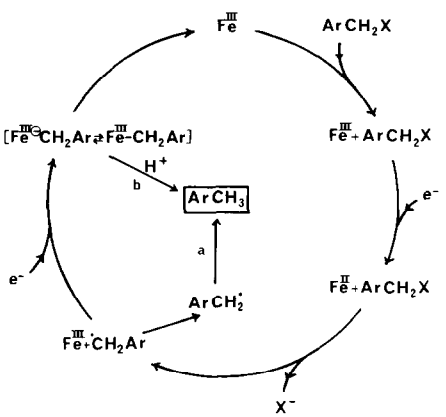
The case of benzyl halides is simpler and our results show that the NADPH-dependent reduction of benzyl bromide or 4-nitrobenzyl chloride by anaerobic microsomes is a cytochrome P-450-dependent process which gives toluene or 4-nitrotoluene as the only metabolite. By analogy to the mechanism previously indicated for the iron(II)–porphyrins-dependent reduction of 4-nitrobenzyl chloride into 4-nitrotoluene (Scheme 1) [23], and for the cytochrome P-450-dependent reductions of halothane and CCl_3–CCl_3 [10, 11], we propose the mechanism indicated in Scheme 2 for benzyl halides reduction by NADPH and microsomes. From the aforementioned results, the 478 nm absorbing complex observed during these reductions is formed in steady-state concentration and this formation does not cause any irreversible modification of cytochrome P-450. This complex exhibits several properties very similar to those of the σ-alkyl–Fe(III)(TPP) (4—NO_2—C_6H_4—CH_2) complex observed during reduction of 4-nitrobenzyl chloride by a heme model system [22], such as its formation in steady-state concentration from reduction of 4-nitrobenzyl chloride in the presence of a reducing agent in excess or the fast destruction of its iron–carbon bond in the presence of O_2. We thus propose a σ-alkyl–Fe(III)–CH_2Ar structure for these 478 nm absorbing complexes. In agreement with this structure and with the proposed Scheme 2, we noticed that the rate of decomposition of the σ-alkyl–Fe(III)–CH_2Ph complex obtained from benzyl bromide by using limited amounts of NADPH (conditions of Fig. 2) increases as the pH decreases from 9 to 7.4 and 6 (data not shown). So far, only very few cytochrome P-450 complexes are known to exhibit Soret peaks above 470 nm; this is the case for the halothane-derived complex [11] or for cytochrome P-450–Fe(III)–L complexes, with L being negatively charged and very good electron-donor ligands such as thiolates [33]. In the Fe^{III}—R structure proposed for the complexes derived from $ArCH_2X$ (this paper) or $CF_3CHClBr$ [11], the carbanions $ArCH_2^-$ and CF_3CHCl^- are also very good electron-donor ligands. Accordingly, we recently found that the σ-Fe(III)(porphyrin)-($nBuS^-$)(R^-) complex with R = CF_3CHCl exhibits, as Fe(III)(porphyrin)(RS^-)$_2$ complexes [34], a hyperporphyrin spectrum with a red-shifted Soret peak around 470 nm [21].

Two routes are possible for toluene, $ArCH_3$, formation from microsomal reduction of benzyl halides: either the abstraction of a hydrogen atom by the intermediate free radical $ArCH_2^{\cdot}$ (Scheme 2, route a), or the protonation of the carbanion $ArCH_2^-$ ligand of the Fe^{III}—CH_2Ar complex (Scheme 2, route b). From the results of experiments using D_2O, it seems that both routes are involved, route a being more predominant in the NADPH-dependent reduction of benzyl bromide than in the reduction of 4-nitrobenzyl chloride. The $ArCH_3$/$ArCH_2D$ ratio would give a good estimation of the relative importance of routes a and b, if $ArCH_2D$ comes only from route b and $ArCH_3$ from route a. Actually, $ArCH_2D$ could also come from route a by abstraction by $ArCH_2$ of some deuterium atoms incorporated into microsomal components during microsomes equilibration with D_2O; however, the isotopic effect (k_H/k_D) for a free radical hydrogen abstraction is generally high, favouring very much $ArCH_3$. Minor amounts of $ArCH_3$ could also come from route b upon protonation of the σ-alkyl complex by traces of remaining H^+, but the isotopic effects for protonation reactions are generally low and do not favour $ArCH_3$ formation. So $ArCH_3$/$ArCH_2D$ should give a good idea of the relative importance of routes a and b. By comparison, it has been reported that route a is largely predominant in the microsomal reduction of CCl_4 [12] and $CF_3CHClBr$ [35] into $CHCl_3$ and CF_3CH_2Cl, respectively. The relative importance of routes a and b should depend on the rate of the second electron transfer necessary to get the σ-alkyl–Fe(III) complex vs that of the dissociation of the elements of the [Fe^{III} ·CH_2Ar] pair (or the Fe^{IV}—CH_2Ar bond), which releases free $ArCH_2^{\cdot}$. It is likely that the [Fe^{III} ·CH_2Ar] pair, or the Fe^{IV}—CH_2Ar complex, is more reducible with the electron-withdrawing group Ar = 4-$NO_2C_6H_4$ than with Ar = C_6H_5. Formation of $ArCH_3$ by route a implies the transfer of one electron from NADPH whereas its formation from route b needs two electrons. Accordingly, about 2 moles of toluene are formed from benzyl bromide reduction per mole of NADPH consumed, whereas about 1 mole of 4-nitrotoluene is formed from 4-nitrobenzyl chloride reduction per mole of NADPH used (Tables 1 and 2).* The abstraction of a hydrogen atom by $ArCH_2^{\cdot}$ (route a) that could occur on an amino acid residue close to the catalytic site of cytochrome P-450 could lead to some degradation of this cytochrome. However, since we observed that the

* In fact, from the results of Table 3, it is expected that about 50% of 4-nitrotoluene would be formed by route a (one-electron pathway) and 50% by route b, implying the consumption of 0.75 mole of NADPH per mole of 4-nitrotoluene.

NADPH-dependent reduction of benzyl halides did not cause any decrease of the amount of cytochrome P-450 (measured as its CO complex), such a degradation should not lead to a loss of the ability of cytochrome P-450 to bind CO and produce a 450 nm peak.

The steady-state concentration of the 478 nm absorbing complexes formed in these reductions should critically depend, if one takes into account Scheme 2, on the relative rates of complex proton-



Scheme 2. Proposed mechanism for the anaerobic reduction of benzyl halides by microsomal cytochrome P-450.

ation (step b) and formation from two-electron reduction of $ArCH_2X$. Heme model studies showed us that the (porphyrin)Fe^{III}—CH_2Ar (Ar = 4-$NO_2C_6H_4$) complex is more rapidly protonated than its analogue Fe^{III}—$CH_2C_6H_5$ [23, 36]. Faster protonation of the cytochrome P-450–Fe^{III}—CH_2Ar complex when Ar = 4-$NO_2C_6H_4$ than when Ar = C_6H_5 would lead to lower steady-state concentrations of the former, explaining why we could not detect it, in contrast to the latter (Ar = C_6H_5). By using the stronger reducing agent, dithionite, its formation rate is increased, explaining why it becomes easily detectable when Ar = 4-$NO_2C_6H_4$ and why its level increases from 50 to 80% (Fig. 4) when Ar = C_6H_5. Moreover, with benzyl chloride contrary to benzyl bromide, the level of the possible σ-benzyl–iron complex is not high enough to be detected with NADPH or 5 mM $Na_2S_2O_4$. However, by using a larger excess of $Na_2S_2O_4$ (500 mM), it becomes detectable (10% of total cytochrome P-450) (Table 4). This last result shows that, at least with $PhCH_2X$ (X = Cl or Br), the rate of formation and steady-state concentration of the σ-alkyl complex depend upon the nature of X (Table 4). This indicates that the different steps of the catalytic cycle (Scheme 2) that are independent of X [after Fe(III) + $^{\cdot}CH_2Ar$] are not the only ones involved in the kinetics of the σ-alkyl complex formation. This is easily understandable if one considers that the electron transfer from cytochrome P-450–Fe(II) to $ArCH_2X$ leading to cytochrome P-450–Fe(III) and $ArCH_2^{\cdot}$ is also involved as a relatively slow step.

In a more general manner, the absence of a 478 nm absorbing complex upon microsomal reduction of a benzyl halide does not indicate that this complex is not formed and does not play an important role in the reduction process, but only means that its steady-state concentration is too low for its detection by difference visible spectroscopy. Accordingly, in the case of 4-nitrobenzyl chloride reduction by NADPH-reduced microsomes, the σ-alkyl–iron complex could not be detected by visible spectroscopy, although experiments using D_2O indicated that this complex is involved to an important extent in the formation of 4-nitrotoluene.

REFERENCES

1. R. Sato and T. Omura, in *Cytochrome P-450*. Kodansha, Tokyo (1978).
2. V. Ullrich, *Topics Curr Chem.* **83**, 68 (1979).
3. I. C. Gunsalus, J. R. Meeks, J. D. Lipscomb, P. Debrunner and E. Munck, in *Molecular Mechanisms of Oxygen Activation* (Ed. O. Hayaishi), p. 559. Academic Press, New York (1974).
4. J. R. Gillette, J. J. Kamm and H. A. Sasame, *Molec. Pharmac.* **4**, 541 (1968).
5. J. Booth, A. Hewer, G. R. Keysall and R. Sims, *Xenobiotica* **5**, 197 (1975).
6. R. Kato, K. Iwasaki, T. Shiraga and N. Noguchi, *Biochem. biophys. Res. Commun.* **70**, 681 (1976).
7. M. Sugiura, K. Iwasaki and R. Kato, *Molec. Pharmac.* **12**, 322 (1976).
8. H. Uehleke, K. H. Hellmer and S. Tabarelli, *Xenobiotica* **3**, 1 (1973).
9. C. R. Wolf, D. Mansuy, W. Nastainczyk, G. Deutschmann and V. Ullrich, *Molec. Pharmac.* **13**, 698 (1977).
10. W. Nastainczyk, H. J. Ahr and V. Ullrich, *Biochem. Pharmac.* **31**, 391 (1982).
11. H. J. Ahr, L. J. King, W. Nastainczyk and V. Ullrich, *Biochem. Pharmac.* **31**, 383 (1982).
12. H. J. Ahr, L. J. King, W. Nastainczyk and V. Ullrich, *Biochem. Pharmac.* **29**, 2855 (1980).
13. C. R. Wolf, L. J. King and D. V. Parke, *Chem.-biol. Interact.* **21**, 277 (1978).
14. (a) D. Mansuy, *Pure appl. Chem.* **52**, 681 (1980). (b) D. Mansuy, *Rev. Biochem. Toxic.* **3**, 283 (1981).
15. D. Mansuy, M. Lange, J. C. Chottard, P. Guérin, P. Morlière, D. Brault and M. Rougee, *J. chem. Soc. chem. Commun.* 648 (1977).
16. D. Mansuy, M. Lange, J. C. Chottard, J. F. Bartoli, B. Chevrier and R. Weiss, *Angew. Chem. int. Edit.* **17**, 781 (1978).
17. D. Mansuy, P. Guérin and J. C. Chottard, *J. organometall. Chem.* **171**, 195 (1979).
18. P. Guérin, J. P. Battioni, J. C. Chottard and D. Mansuy, *J. organometall. Chem.* **218**, 201 (1981).
19. J. P. Battioni, J. C. Chottard and D. Mansuy, *Inorg. Chem.* **21**, 2056 (1982).
20. D. Mansuy and J. P. Battioni, *J. chem. Soc. chem. Commun.* 638 (1982).
21. H. J. Ahr, H. H. Ruf, W. Nastainczyk, V. Ullrich, J. P. Battioni and D. Mansuy, to be published.
22. D. Mansuy and M. Fontecave, *Biochem. biophys. Res. Commun.* **104**, 1651 (1982).
23. D. Mansuy, M. Fontecave and J. P. Battioni, *J. chem. Soc. chem. Commun.* 317 (1982).
24. P. Beaune, P. Dansette, J. P. Flinois, S. Columelli, D. Mansuy and J. P. Leroux, *Biochem. biophys. Res. Commun.* **88**, 826 (1979).
25. O. H. Lowry, N. J. Roseborough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
26. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
27. N. Kornblum and R. A. Brown, *J. Am. chem. Soc.* **86**, 2681 (1964).
28. R. S. Wade and C. E. Castro, *J. Am. chem. Soc.* **95**, 226 (1973).

29. R. S. Wade and C. E. Castro, *J. Am. chem. Soc.* **95**, 231 (1973).
30. M. C. Villaruel, M. I. Diaz Gomez and J. A. Castro, *Toxic. appl. Pharmac.* **33**, 106 (1975).
31. R. O. Recknagel, E. A. Glende and A. M. Hruszkewycz in *Free Radicals in Biology* (Ed. W. A. Pryor), Vol. III, p. 97. Academic Press, New York (1977).
32. D. Brault and P. Neta, *J. Am. chem. Soc.* **103**, 2705 (1981).
33. W. Nastainczyk, H. H. Ruf and V. Ullrich, *Chem.-biol. Interact.* **14**, 251 (1976).
34. H. H. Ruf and P. Wende, *J. Am. chem. Soc.* **99**, 5499 (1977).
35. V. L. Kubic and M. W. Anders, *Chem.-biol. Interact.* **34**, 201 (1981).
36. D. Mansuy and M. Fontecave, unpublished results.