Pergamon

Biochemical Pharmacology, Vol. 47, No. 12, pp. 2233–2242, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0066-T

CYTOCHROME P450 DESTRUCTION AND RADICAL SCAVENGING BY BENZENE AND ITS METABOLITES

EVIDENCE FOR THE KEY ROLE OF QUINONES

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(*Received* 3 *November* 1993; *accepted* 25 *January* 1994)

Abstract—Exposure to benzene was reported to lower the cytochrome P450 (CYP; EC 1.14.14.1) content in phenobarbital-pretreated (PB) rats *in vivo* (Gut I, *Zbl Pharm* **122**: 1139–1161, 1983). In this paper we followed the ability of benzene and its metabolites, phenol, catechol, hydroquinone and benzoquinone to destroy CYP in liver microsomes from PB rats *in vitro*. The spectrophotometric determinations of the total CYP content, 7-pentoxyresorufin *O*-depentylase and aniline hydroxylase activities, electrophoresis and western blot analysis after incubation of PB-microsomes with benzene or its metabolites revealed that: (1) benzene is metabolically activated to intermediates causing CYP destruction; phenol is not responsible for this effect. (2) Quinonic metabolites of benzene cause CYP destruction with different potency (30% CYP was destroyed by 3 mM catechol, 0.3 mM hydroquinone and 0.03 mM benzoquinone). (3) Low concentrations of quinones are capable of protecting CYP against reactive oxygen species produced in the CYP futile cycle. (4) Ascorbate effectively protects CYP against quinones, apparently by maintaining them in the reduced state. (5) Quinones attack both heme and protein of CYP. (6) CYP activities differ in the sensitivity to quinone-mediated destruction. In conclusion, we suggest that quinones may be responsible for CYP destruction by benzene *in vivo*.

Key words: cytochrome P450; destruction; benzene; quinones; 7-pentoxyresorufin *O*-depentylase; aniline hydroxylase

CYP† (EC 1.14.14.1) plays an important role in the metabolism of various drugs, steroids, mutagens and carcinogens [1]. It can metabolize chemicals to the reactive intermediates binding spontaneously to proteins, nucleic acids, lipids and to CYP itself [2].

Suicide substrates can cause CYP destruction by irreversible binding of their metabolites to the CYP heme like terminal olefins and acetylenes, to the apoprotein like chloramphenicol or to both like CCl_4 [3–5]. A strong, but reversible inhibition of CYP activity may arise from the binding of the inducer, inhibitor or its metabolite to the CYP heme (SKF 525-A, isosafrole, polyhalogenated aromatic hydrocarbons, triacetyloleandomycin) [6–8]. It was postulated that CYP can be destroyed by superoxide anion radical, H_2O_2 or other ROS formed during the futile cycle without substrate [9] or via ROS-initiated lipid peroxidation [10, 11]. Some CYPs appear to be more sensitive to the effects of certain substrates: PB-inducible forms seem to be particularly sensitive to ethinylcyclopentanol, 17-propadienyl-19-nortestosterone, aminobenzotriazole, allylisopropylacetamide, cumene hydroperoxide and *m*-xylene which all only slightly affect 3-methylcholanthrene-inducible CYP forms [12–14].

Inhibition or destruction of CYP may cause prolongation of xenobiotic action, increase or decrease xenobiotic toxicity and could even affect some physiological functions, e.g. steroid biosynthesis [15]. Selective destruction of various CYP forms (e.g. pulmonary CYP 2B1 activity by toluene, *m*- or *p*-xylene) alters the activation/detoxication ratio of procarcinogens (e.g. benzo[*a*]pyrene) and thus may increase the cancer risk in conditions of combined exposure [14, 16, 17].

Benzene is a human and animal carcinogen [18] acting through an epigenetic mechanism. Benzene-induced myelotoxic and carcinogenic effects are supposed to be caused by covalent binding of its metabolites quinones and muconaldehyde to biomacromolecules [19, 20]. Benzene and its derivatives toluene and xylenes affect the PB-inducible CYP enzyme(s) [13, 16, 17, 21]. On the other hand, benzene was found to induce CYP 2E1 and is preferentially metabolized by this CYP form in rats [22, 23] and even in humans [24]. It is suggested that CYP 2E1 catalyses benzene oxidation at least partly via producing ROS [25, 26], while benzene metabolite hydroquinone reacts with dioxygen forming superoxide anion radical [27].

Our aim was to explore *in vitro* the role of benzene and its metabolites in the previously reported decrease of CYP content and its activity in PB-pretreated rats subsequently exposed to benzene

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† Abbreviations: CYP, cytochrome P450; PB, phenobarbital; ROS, reactive oxygen species; PROD, 7-pentoxyresorufin *O*-depentylase.

[21]. The determination of PROD activity a sensitive marker for measuring of CYP 2B1 activity [13, 28] and aniline hydroxylation for estimation of overall CYP activity, particularly of 2E1, 2B1 and 1A2 [29, 30] was used. The experiments were also intended to show the mechanism of destruction and methods of protecting CYP.

MATERIALS AND METHODS

Chemicals. All chemicals used were of A.R. grade. NADPH, 7-pentoxyresorufin, resorufin, EDTA, superoxide dismutase, EC 1.15.1.1 (3900 U/mg), catalase EC 1.11.1.6 (9740 U/mg), α-tocopherol acetate and ascorbate were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Desferal was a product of CIBA-GEIGY (U.S.A.). Benzene and its metabolites were purchased from Aldrich (Milwaukee, WI, U.S.A.). Electrophoresis and immunoblotting reagents were supplied by Bio-Rad (Hercules, CA, U.S.A.). Chemicals used as substrates were purified before use; benzene and phenol by distillation; catechol and hydroquinone by crystallization; benzoquinone by sublimation [31]. Specific polyclonal antibodies against CYP 2B1 were a generous gift of Dr Magnus Ingelman-Sundberg, Karolinska Institutet, Stockholm, Sweden.

Animals. Adult male, Wistar rats, weighing 200–250 g (VELAZ, Praha, Czech Republic), pretreated with sodium phenobarbital (0.1% in drinking water for 6 days and one i.p. injection, 80 mg/kg, 24 hr before killing) were used.

Microsomes. Microsomes were prepared according to van der Hoeven and Coon [32], protein was estimated according to Lowry *et al.* [33] with bovine serum albumin as a standard.

Incubations All incubations were carried out in glass-stoppered test tubes using a shaking water bath at 37° in the dark for 60 min (except experiments where the periods of incubation were 5, 10, 15, 30, 60, 90 and 120 min). The incubation mixture contained final concentrations of microsomal protein, 1 mg/mL, NADPH-generating system (10 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.5 mM, NADP, glucose-6-phosphate dehydrogenase, EC 1.1.1.49; 0.5 U per mL), 1 mM substrate (except experiments where 0.01, 0.03, 0.1, 0.3, 1 or 5 mM concentrations were used). A 150 mM KCl/50 mM Tris–HCl, pH 7.4 buffer was added to make 2 mL final volume. Final concentrations of the reagents anticipated to protect P450 were: 5 mM EDTA, 0.1, 1 or 5 mM desferal, superoxide dismutase (40, 200 or 400 U/mL), catalase (100, 500 or 1000 U/mL), 0.1, 1 or 5 mM α-tocopherol acetate (diluted in methanol) and 0.1, 1 or 5 mM ascorbate. After incubation, the microsomes were centrifuged for 15 min for 22,000 *g* at 4° and the pellet resuspended in KCl–Tris buffer prior to determination of aniline hydroxylase and electrophoretic procedures to exclude interference of water-soluble quinones. The protein loss due to centrifugation was <25%.

Spectrophotometric determination. The total CYP content in microsomes before and after the incubations was estimated according to Omura and Sato [34].

Catalytic activities. PROD was assayed according to Lubet *et al.* [35]. Aniline-hydroxylation was assayed according to Kato and Gillette [36] as modified by Gram *et al.* [37].

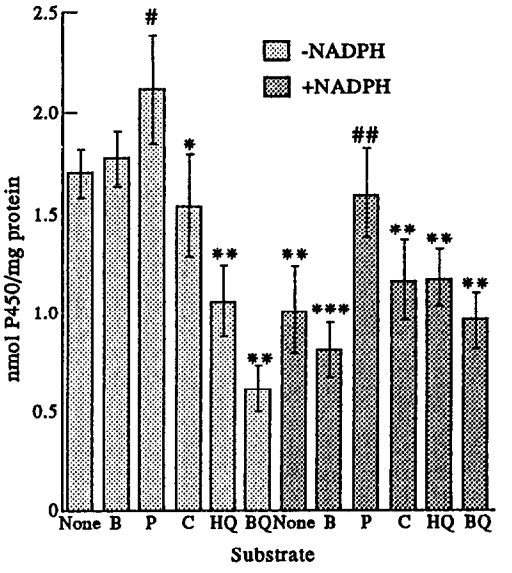

Fig. 1. CYP destruction by benzene and its metabolites—spectrophotometric evaluations. PB-microsomes were incubated for 60 min at 37° in the absence or presence of NADPH, without (NONE) or with 1 mM substrate (B, benzene; P, phenol; C, catechol; HQ, hydroquinone; BQ, benzoqunoine). Results are expressed as total CYP content at the end of incubation (nmol/mg protein), mean ± SD of eight observations. The initial CYP content ranged between 1.60 and 1.82 nmol/mg microsomal protein. *Protective effects*: #significantly different ($P < 0.003$) from sample incubated without substrate in the absence of NADPH, and ##($P < 0.001$) in the presence of NADPH. *Destructive effects*: significantly different from sample incubated without substrate in the absence of NADPH; *$P < 0.03$, **$P < 0.001$, and ***($P < 0.03$) in the presence of NADPH.

Electrophoresis. Electrophoresis in polyacrylamide gel was performed according to Laemmli [38] using the modification of O'Farrell [39]. Gels were stained for heme by 3,3′,5,5′-tetramethylbenzidine according to Thomas *et al.* [40] and for protein by Coomassie Brillant Blue R250 according to Fairbanks *et al.* [41]. Western blotting of proteins was performed according to Towbin *et al.* [42] using immunostaining with horseradish peroxidase system.

Statistical significance. This was analysed by the Student's *t*-test.

RESULTS

Destruction of CYP by benzene and its metabolites evaluated by CO-binding spectra

Microsomes were incubated for 60 min at 37° without substrate or with 1 mM concentration of substrate. In order to distinguish between the direct effect of substrates and the influence of their metabolism, the incubations were carried out in the absence or presence of NADPH-generating mixture

(subsequently called NADPH). The CYP content in different batches of microsomes at the beginning of incubation ranged from 1.60 to 1.82 nmol CYP/mg of protein. In the absence of NADPH, benzene did not change CYP content; phenol significantly ($P < 0.003$) enhanced CYP content; catechol significantly ($P < 0.003$) lowered CYP level; hydroquinone and benzoquinone showed a more significant ($P < 0.001$) destruction (40 and 65% decrease) of total CYP content, respectively (see Fig. 1). In the presence of NADPH, a significant ($P < 0.001$) decrease of total CYP content (35–45%) occurred in samples incubated both with and without substrates, except the sample incubated with phenol, when compared with the CYP content in a sample incubated without substrate and NADPH. On the other hand, in comparison with the sample incubated without substrate but with NADPH, benzene lowered the CYP content significantly ($P < 0.03$); but quinones did not cause a significant decrease of the CYP level and phenol significantly ($P < 0.001$) protected CYP.

The effect of quinone concentration of CYP destruction. Microsomes were incubated for 60 min at 37° with various concentrations of quinones (0.01, 0.03, 0.1, 0.3, 1 and 5 mM) in the absence or presence of NADPH. In the absence of NADPH (Fig. 2A), a significant decrease of CYP content was reached by 1 mM catechol, 0.3 mM hydroquinone and 0.01 mM benzoquinone (30% decrease was produced by 3 mM catechol, 0.3 mM hydroquinone and 0.03 mM benzoquinone). In contrast in the presence of NADPH (Fig. 2B), low concentrations of quinones protected CYP against the NADPH-mediated destruction. The effective protective concentration ranged between 0.03 and 0.3 mM for hydroquinone and 0.03 and 0.1 mM for benzoquinone.

Time course of CYP destruction. Microsomes were incubated with or without 1 mM quinones at 37° for various periods of time (5, 10, 15, 30, 60, 90, 120 min) in the absence or presence of NADPH. The results showed that in the absence of NADPH (Fig. 3A), catechol lowered the CYP level slowly with the constant rate during 0–120 min. In contrast hydroquinone and particularly benzoquinone exerted a very rapid effect within the first 5 min but after 30 min the hydroquinone effect ceased. In the presence of NADPH (Fig. 3B), CYP destruction caused by NADPH without substrate was faster than that caused by simultaneously added NADPH and catechol or hydroquinone, but slower than the effect of NADPH plus benzoquinone. On the other hand catechol and hydroquinone protected CYP against NADPH-mediated destruction from the start and benzoquinone from the 30th minute of incubation. The destructive action of benzoquinone seemed to be completely stopped at minute 10. Both the effect of quinone concentration and time courses confirmed benzoquinone to be the most potent quinone, hydroquinone being less effective and catechol the least effective in CYP destruction.

Possible mechanisms of CYP destruction: agents protecting CYP against the NADPH-and/or quinone-mediated destruction. Anticipated protective agents were included in the usual incubation mixture. A possible role of ROS or organic radicals was estimated by the addition of 1 or 5 mM concentration of chelators (EDTA, desferal); enzymes (40, 200 or 400 U/mL of superoxide dismutase used to dismutate the superoxide anion radical; 100, 500 or 1000 U/mL of catalase to eliminate H_2O_2); 0.1, 1 or 5 mM α-tocopherol which traps ROS and organic radicals and 0.1, 1 or 5 mM ascorbate (maintaining the reducing environment). The chelators produced a significant protection of CYP against NADPH-mediated destruction (data for EDTA are not presented), desferal being the more effective ($P < 0.005$), but both were unable to protect CYP against quinones (Table 1). Ascorbate was the only

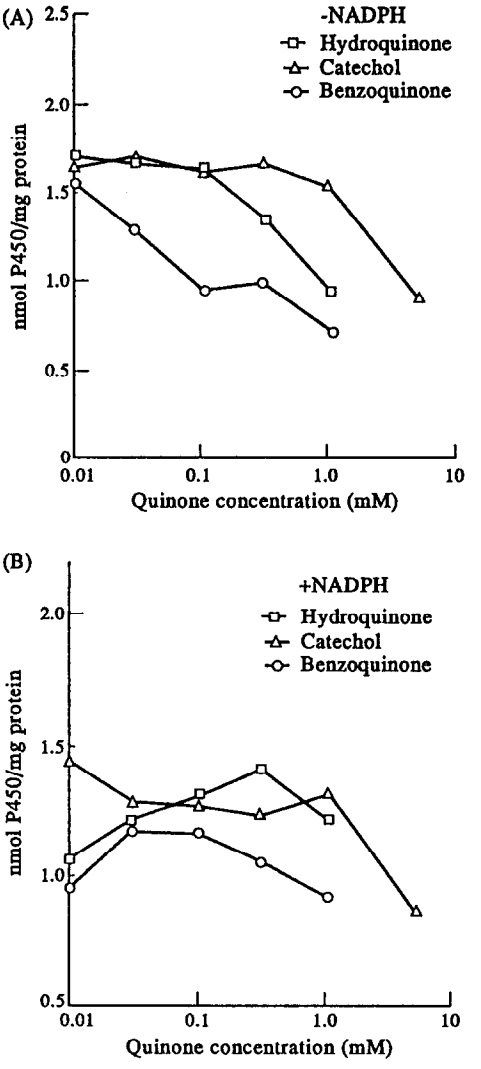

Fig. 2. Concentration course of the CYP destruction by quinones—spectrophotometric evaluations. PB-microsomes were incubated for 60 min at 37° in the absence (A) or presence (B) of NADPH with various concentrations of quinones (0.01, 0.03, 0.1, 0.3, 1 or 5 mM). Each point represents the CYP content (nmol/mg protein) in duplicate sample.

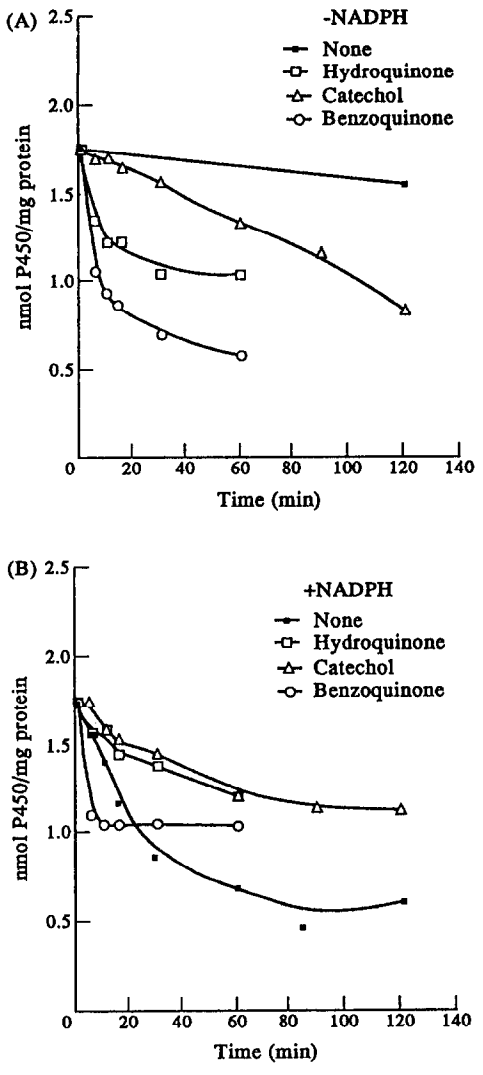

Fig. 3. Time course of the CYP destruction by quinones—spectrophotometric evaluations. PB-microsomes were incubated at 37° in the absence (A) or presence (B) of NADPH, without quinone (NONE) or with 1 mM quinones for various periods of time (5, 10, 15, 30, 60, 90, 120 min). Each point represents the CYP content (nmol/mg protein) in duplicate sample.

agent able to protect CYP against all quinones significantly (for level of significance see Table 1). The effective concentrations of ascorbate were: 1 mM (protecting against hydroquinone and benzoquinone in the absence of NADPH) and 5 mM (protecting against all quinones). A 0.1 mM concentration of ascorbate was without effect (data not presented). At 5 mM concentration, ascorbate protected CYP against NADPH-mediated destruction ($P < 0.005$) as well. Surprisingly, 1 mM concentration of ascorbate produced a significant ($P < 0.001$) decrease of CYP level in the sample incubated without quinones in the absence of NADPH, while 5 mM ascorbate was without destructive effect. Presently there is no explanation for this phenomenon. Superoxide dismutase and catalase did not appear to protect CYP in any concentrations used (data not presented). α-Tocopherol protected CYP against NADPH-mediated destruction with lower potency than chelators (decrease to 70%) but did not provide any significant CYP protection against quinones (data not presented).

CYP destruction evaluated by measuring the CYP enzyme activity.

Microsomes were preincubated for 60 min at 37°, with or without 1 mM quinones in the absence or presence of NADPH. For the aniline hydroxylase activity assay it was necessary to remove the excess of interfering quinones by centrifugation of the sample (15 min at 22,000 *g* and 4°) and resuspending the pellet in 150 mM KCl/50 mM Tris, pH 7.4 buffer. Aliquots of microsomal protein (200 μg for PROD and 1 mg for aniline hydroxylase) were used to assay CYP activity. The initial activity was 1170 ± 230 pmol of resorufin formed/min/mg protein for PROD and 675 ± 113 pmol of *p*-aminophenol formed/min/mg protein for aniline hydroxylase. In the absence of NADPH, catechol did not affect PROD (Fig. 4) but it significantly ($P < 0.03$) diminished aniline hydroxylase activity (Fig. 5); hydroquinone and benzoquinone significantly lowered both PROD ($P < 0.001$) and aniline hydroxylation ($P < 0.03$). In the presence of NADPH, catechol did not change PROD but produced a significant depression of aniline hydroxylation ($P < 0.03$); hydroquinone did not influence either PROD or aniline hydroxylation; benzoquinone significantly depressed PROD ($P < 0.001$) but did not affect aniline hydroxylation. There was a conspicuous difference in NADPH-mediated CYP destruction: it significantly reduced PROD ($P < 0.001$) but did not decrease aniline hydroxylation.

CYP destruction evaluated by electrophoresis and western blot analysis of microsomal proteins

Microsomes were preincubated for 60 min at 37°, with or without 0.1 or 1 mM hydroquinone, 0.01, 0.1 or 1 mM benzoquinone in the absence of NADPH. After this preincubation the sample was centrifuged (15 min at 22,000 *g* and 4°) and the pellet resuspended in lysis buffer, boiled for 5 min and 45 μg (for heme staining), 30 μg (for staining of total protein) or 2 μg (for immunoblotting) of microsomal protein were subjected to electrophoresis. For western blot analysis, proteins from the gels were transferred onto nitrocellulose paper by electroblotting and probed with the polyclonal CYP2B1 antibodies. Nitrocellulose was then incubated with horseradish peroxidase-conjugated second antibody and stained using aminoethylcarbazole as a substrate. Staining of the gels for the heme peroxidase activity (Fig. 6A) showed that CYP-heme was inactivated. This occurred by preincubation with: 0.1 mM hydroquinone and 0.01 benzoquinone (17% decrease of CYP-heme determined by densitometry, $P < 0.03$). Greater effects were seen with 0.1 mM benzoquinone (33%, $P < 0.005$), 1 mM hydroquinone (55%, $P < 0.005$) and 1 mM benzoquinone

Table 1. Protection against CYP destruction—spectrophotometric evaluations

		Protection				
			Desferal		Ascorbate	
Quinone	NADPH	—	1 mM	5 mM	1 mM	5 mM
None	−	97.1 ± 7.0	103.4 ± 1.9	104.1 ± 0.9	17.9 ± 1.2*	95.0 ± 1.4
	+	58.0 ± 12.8	77.5 ± 3.3†	75.8 ± 2.4†	48.2 ± 2.6	97.0 ± 3.6†
Catechol	−	87.7 ± 15.3	72.1 ± 2.3	81.2 ± 1.8	85.0 ± 2.4	85.6 ± 5.2
	+	66.4 ± 12.6	74.5 ± 1.0	59.7 ± 7.6	78.4 ± 11.1	88.0 ± 6.6‡
Hydroquinone	−	60.8 ± 9.9	56.0 ± 8.1	58.1 ± 3.3	86.4 ± 2.3§	98.8 ± 2.1§
	+	67.3 ± 9.0	71.5 ± 1.4	71.5 ± 3.3	77.4 ± 6.6‖	86.3 ± 4.2‖
Benzoquinone	−	35.5 ± 6.7	35.2 ± 2.4	35.6 ± 10.2	70.1 ± 1.1¶	88.7 ± 7.7¶
	+	56.2 ± 8.1	66.4 ± 3.0	56.1 ± 2.3	50.4 ± 6.1	79.4 ± 1.9**
N		8	4	4	4	4

Incubations were made in the same manner as in Fig. 1 and the protective agents (1 or 5 mM desferal or ascorbate) were included.

Results are presented as percentage of initial CYP content (1.71 ± 0.11 nmol/mg protein), mean ± SD, N = number of observations.

* Significantly different ($P < 0.001$) from microsomes incubated without substrate in the absence of NADPH (NONE−), or †($P < 0.005$) in the presence of NADPH (NONE+).

‡ Significantly different ($P < 0.005$) from microsomes incubated with catechol in the presence of NADPH.

§ Significantly different ($P < 0.001$) from microsomes incubated with hydroquinone in the absence of NADPH, or ‖($P < 0.05$) in the presence of NADPH.

¶ Significantly different ($P < 0.001$) from microsomes incubated with benzoquinone in the absence of NADPH, or **($P < 0.001$) in the presence of NADPH.

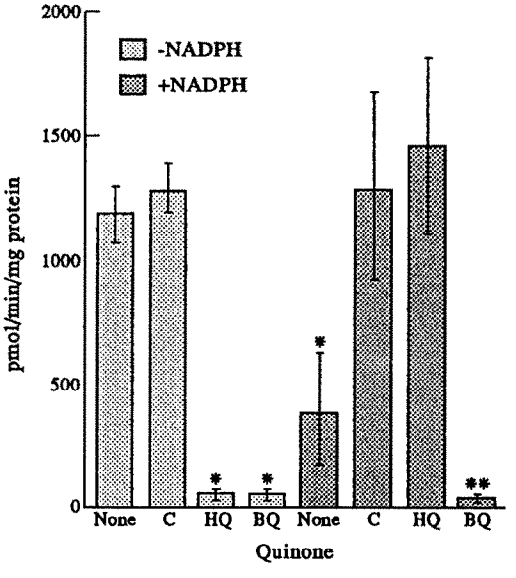

Fig. 4. PROD activity destruction by quinones. After the preincubation (see Fig. 1), 200 μg of microsomal protein were taken and PROD activity assayed. Each value represents the mean ± SD of eight assays. PROD activity in non-incubated PB-microsomes ranged between 940 and 1400 pmol of resorufin formed/min/mg protein. *Significantly different ($P < 0.001$) from microsomes preincubated without substrate in the absence of NADPH, and **($P < 0.001$) in the presence of NADPH.

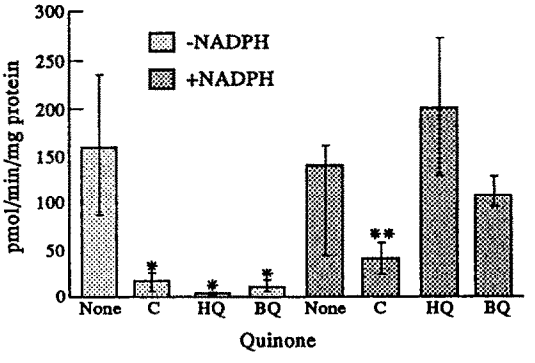

Fig. 5. Aniline hydroxylase activity destruction by quinones. After the preincubation (see Fig. 1), microsomes were centrifuged for 15 min at 22,000 *g* and 4°, the pellet resuspended in KCl–Tris buffer. An aliquot of 1 mg of microsomal protein was taken and aniline hydroxylase activity assayed. Each value represents mean ± SD of four assays. Aniline hydroxylase activity in non-incubated microsomes ranged between 562 and 788 pmol of *p*-aminophenol formed/min/mg protein. *Significantly different ($P < 0.03$) from microsomes preincubated without substrate in the absence of NADPH, and **($P < 0.03$) in the presence of NADPH.

(82%, $P < 0.001$; for densitometry see Table 2). A very similar trend was seen when the gels were stained for protein by Coomassie blue (Fig. 6B). The number of microsomal proteins in the 36.5–58 kDa range was substantially lowered after preincubation with 0.1 mM benzoquinone or 1 mM

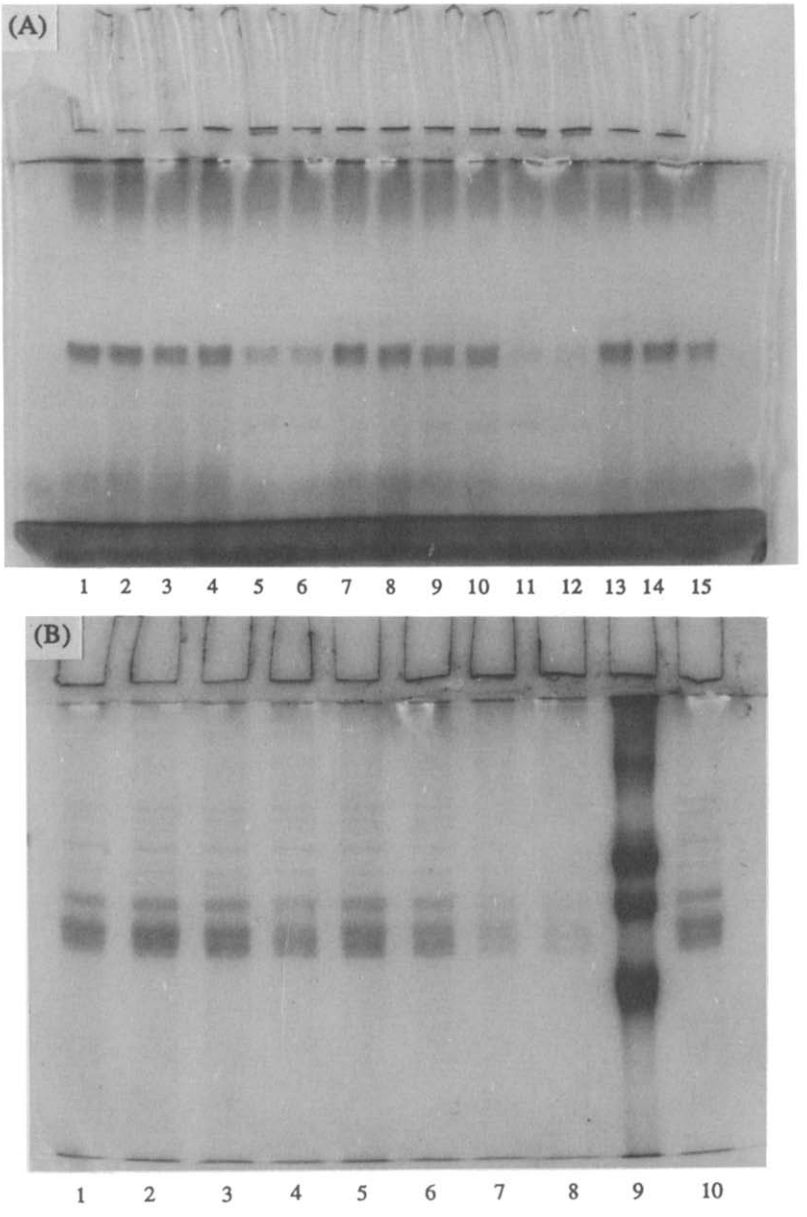

Fig. 6. CYP destruction by quinones in the absence of NADPH evaluated by electrophoresis. After the preincubation (60 min, 37° in the absence of NADPH without or with 0.01, 0.1 or 1 mM quinones), microsomes were centrifuged for 15 min at 22,000 *g* and 4°, the pellet resuspended in sample lysis buffer. Aliquots of 45 μg (for heme staining) or 30 μg (for protein staining) of microsomal protein were subjected to electrophoresis and gels stained for heme (A) or protein (B). (A) Heme staining: lanes 1, 2—PB-microsomes preincubated without quinone; lanes 3, 4—PB-microsomes preincubated with 0.1 mM hydroquinone; lanes 5, 6—PB-microsomes preincubated with 1 mM hydroquinone; lanes 7, 8—PB-microsomes preincubated with 0.01 mM benzoquinone; lanes 9, 10—PB-microsomes preincubated with 0.1 mM benzoquinone; lanes 11, 12—PB-microsomes preincubated with 1 mM benzoquinone; lanes 13, 14—microsomes from untreated rats not preincubated; lane 15—PB-microsomes preincubated without quinone (22.5 μg of protein per lane). (B) Protein staining: lanes 1, 10—PB-microsomes preincubated without quinone; lanes 2, 3—PB-microsomes preincubated with 0.1 mM hydroquinone; lane 4—PB-microsomes preincubated with 1 mM hydroquinone; lane 5—PB-microsomes preincubated with 0.01 mM benzoquinone; lane 6—PB-microsomes preincubated with 0.1 mM benzoquinone; lanes 7, 8—PB-microsomes preincubated with 1 mM benzoquinone; lane 9—prestained molecular weight standards, Sigma [lactic dehydrogenase (EC 1.1.1.27) 36.5 kDa, fumarase (EC 4.2.1.2) 48.5 kDa, pyruvate kinase (EC 2.7.1.40) 58 kDa, 10 μg of each standard per lane].

Table 2. CYP destruction by quinones evaluated by densitometry

Quinone	mM	Heme	N	Immunostaining for 2B		N
				Band 1	Band 2	
—		100 ± 3.0	2	100	0	8
Hydroquinone	0.1	78.6; 88.1*	2	103.4 ± 18.5	0	8
Hydroquinone	1.0	44.3; 45.6†	2	27.3 ± 14.6†	39.0 ± 7.2	8
Benzoquinone	0.01	87.3; 79.8*	2	74.7 ± 20.1‡	0	8
Benzoquinone	0.1	70.3; 65.4‡	2	13.8 ± 11.0†	35.4 ± 15.0	12
Benzoquinone	1.0	18.6; 17.7†	2	13.1 ± 14.5†	28.0 ± 18.3	16

Gels and blots presented in Figs 6 and 7 were scanned with a densitometer.

Values represent the percentage of staining intensity compared with the sample preincubated without quinone, N = number of scanned samples.

Significantly different from sample preincubated without quinone; *$P < 0.03$, †$P < 0.001$, ‡$P < 0.005$.

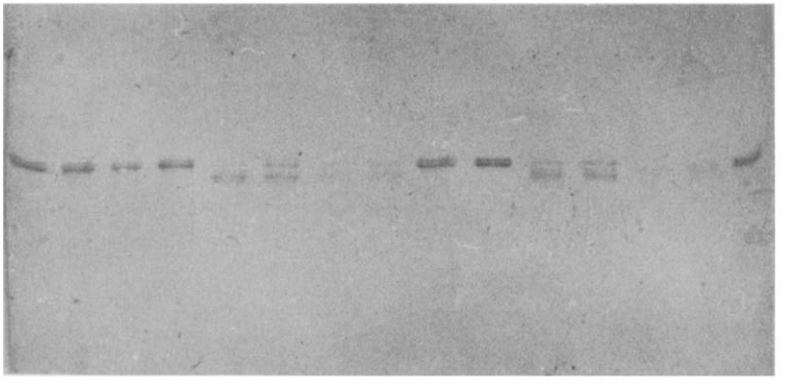

Fig. 7. CYP protein destruction by quinones in the absence of NADPH evaluated by western blot analysis. After the preincubation and centrifugation (see Fig. 6), 2 μg of microsomal protein were subjected to electrophoresis, transferred to nitrocellulose paper and probed with the polyclonal CYP2B1 antibody using horseradish peroxidase conjugated second antibody. Lanes 1, 2, 15—PB-microsomes preincubated without quinone; lanes 3, 4—PB-microsomes preincubated with 0.01 mM benzoquinone; lanes 5, 6—PB-microsomes preincubated with 0.1 mM benzoquinone; lanes 7, 8—PB-microsomes preincubated with 1 mM benzoquinone; lanes 9, 10—PB-microsomes preincubated with 0.1 mM hydroquinone; lanes 11, 12—PB-microsomes preincubated with 1 mM hydroquinone; lanes 13, 14—microsomes from untreated rats not preincubated.

hydroquinone or 1 mM benzoquinone. The preincubation with 0.01 mM benzoquinone or 0.1 mM hydroquinone failed to cause disappearance of microsomal proteins. The immunoblotting analysis (Fig. 7) showed that preincubation with 0.1 mM hydroquinone did not lead to a significant destruction of CYP 2B1, while preincubation with 0.01 mM benzoquinone lowered CYP 2B1 content significantly ($P < 0.03$; see Table 2). The preincubation with 1 mM hydroquinone or 0.1 mM benzoquinone produced two bands with a highly reduced band corresponding to CYP 2B1 ($P < 0.001$). At 1 mM concentration, benzoquinone also caused a strong reduction of both bands ($P < 0.001$).

Correlation analysis of CYP destruction by quinones

This revealed the highest level of correlation between the values for the total CYP content and CYP 2B1 destruction ($r = 0.997$; see Table 3). A high level of correlation between the values for the total CYP content and CYP-heme destruction was also observed ($r = 0.883$). The correlation between the data for CYP-heme and CYP 2B1 destruction ($r = 0.753$) and between CYP total content and PROD activity destruction ($r = 0.707$) was also good, but on the other side the correlation between aniline hydroxylase and PROD activity ($r = 0.425$) and that between aniline hydroxylase activity and total CYP content destruction ($r = 0.383$) was weak.

Table 3. Correlation between data for CYP destruction evaluated by various methods

	P450	PROD	Aniline hydroxylase	Heme	2B1
P450	—	0.707	0.383	0.883	0.977
PROD		—	0.4255	ND	ND
Aniline hydroxylase			—	ND	ND
Heme				—	0.7535
2B1					—

Correlation coefficient r presented, ND = not determined.

Thus, these data show that effects of quinones were specific for the CYP 2B1 form. Taken together, deleterious effects of quinones involve both the CYP heme as well as the CYP protein.

DISCUSSION

Benzene inhalation was previously shown to lower the total CYP content in PB-pretreated rats *in vivo* [21]. In the experiments presented here we revealed that *in vitro* incubation of microsomes from PB-treated rats with benzene in the presence of NADPH significantly diminished the total CYP content (evaluated by the CO-binding spectra—Fig. 1), whereas in the absence of NADPH benzene did not decrease the CYP content. This finding suggests that benzene is metabolically activated to reactive intermediates which can attack either CYP heme, protein or both. The covalent binding of benzene metabolites to biomacromolecules has been shown previously [19, 20, 43].

Phenol the main metabolite of benzene in microsomes from rats treated by PB significantly elevated the CYP content during incubation without NADPH. This effect may occur through a disruption of a complex between PB or its metabolite and CYP. Phenol also protected CYP against NADPH-mediated loss apparently by scavenging ROS (produced in the futile cycle).

In order to identify the destructive intermediates and the mechanism of their action we focused our attention to the other benzene metabolites—quinones: catechol, hydroquinone and benzoquinone. Quinones damaged CYP directly (in the absence of NADPH) with an increasing potency in the series of catechol < hydroquinone < benzoquinone. This trend was confirmed by the time- and concentration-effects (Figs 2A and 3A). In the presence of NADPH, all quinones caused almost the same CYP loss as that mediated by NADPH alone. Low concentrations of quinones (<0.3 mM) were able to protect CYP against the NADPH-mediated destruction (Figs 2B and 3B) thus documenting the reported ROS-scavenging capacity of quinonic substances [44, 45].

The time and concentration effects show that the destructive effect of benzoquinone is very rapid and significant at very low concentrations (0.03 mM). On the other hand, the effect of hydroquinone is slower, requires a higher concentration (>0.3 mM) and can be mitigated by a reducing environment (ascorbate). These observations suggest that hydroquinone must be oxidized to benzoquinone (via semiquinone) to cause CYP destruction. Our results agree with the data of Lunte and Kissinger [46] who found that hydroquinone incubated with microsomes was oxidized to benzoquinone which resulted in an increased rate of covalent binding. The immunoblots stained for CYP 2B1, showing that the destruction pattern produced by 1 mM hydroquinone is very similar to that of 0.1 mM benzoquinone (Fig. 7), support these observations.

Thus, it appears that ROS do not play a significant role in the quinone-mediated CYP destruction as superoxide dismutase and catalase failed to protect CYP against quinones. In addition another ROS and organic radical scavenger—α-tocopherol [47] was also without effect.

Ascorbate maintains hydroquinone and catechol in the reduced state [24] and it was used to inhibit covalent binding of quinones to microsomal proteins and DNA during metabolism of halogenated benzenes [48–51]. This was apparently the way ascorbate protected CYP against all quinones in our experiments (Table 1). The effect of ascorbate indicated that CYP destruction was caused by covalent binding of quinones to heme and/or protein moiety. Electrophoresis of microsomes stained for heme and total protein supported this view (Fig. 6A and B). The attack of quinones was directed to both heme and protein moiety (see Table 3). A similar mode of action was reported for CCl_4 [4].

The substantial decrease of CYP content incubated with 1 mM ascorbate without quinone and NADPH is apparently caused by ROS generated from Fe^{2+}-ascorbate redox system which is formed in conditions of aerobic incubation [52].

It is of interest that the sensitivity of PROD a marker for CYP 2B1 in rat liver microsomes and aniline hydroxylase represented preferentially by CYP 2E1 to preincubation of microsomes with catechol which show aniline hydroxylation damaged and benzoquinone with NADPH (PROD activity damaged) differed (Figs 4 and 5). Moreover, PROD was strongly affected by the CYP futile cycle after preincubation with NADPH, whereas aniline hydroxylation was not. These results strongly suggest different sensitivities of the particular CYP forms (2B1 versus 2E1 both present in our PB-microsomes at significant levels [23]) to quinones. In addition there is a possibility that CYP 2E1 is considerably less sensitive to ROS than CYP 2B1. Various reports have shown a high sensitivity of CYP 2B1 activity to xenobiotics. A significantly higher inhibition of

2B1 activity than 1A activity by avarol—sesquiterpenoid hydroquinone and its quinone avarone was observed [53]. A selective inhibition of PROD activity (in contrast with 7-ethoxyresorufin *O*-deethylase for 1A1) by benzene derivative *m*-xylene was also evidenced [14].

The apparent split of CYP 2B1 protein into two bands detected by immunostaining (Fig. 7) seems to be a rather unusual way of CYP inactivation.

Results similar to ours on the effects of benzene and its metabolites have not been reported so far. Our data presented here enable us to draw the following conclusions. (1) Benzene when metabolized may destroy CYP through its metabolites—quinones, in low concentrations (0.03–0.3 mM). (2) The action of benzoquinone is direct whereas catechol and hydroquinone must undergo oxidative reactions yielding corresponding benzoquinones. Ascorbate inhibits these reactions and thus protects CYP against quinones. The role of reactive intermediate of quinone redox cycling—semiquinone radical remains to be evaluated. (3) Both heme and protein moiety of CYP serve as the target for the benzoquinone action. (4) CYP2B1 seems to be very sensitive to both ROS- and quinone-mediated destruction. This fact is of great importance because the detoxication/activation ratios of many procarcinogens may be altered [14].

Acknowledgements—The specific anti-CYP2B1 antibodies were prepared at the Berzelius Laboratory, Karolinska Institutet, Stockholm. The authors wish to express their sincere thanks to Dr Magnus Ingelman-Sundberg for supporting this work.

REFERENCES

1. Soucek P and Gut I, Cytochromes P450 in rats: structures, functions, properties and relevant human forms. *Xenobiotica* **22**: 83–103, 1992.
2. Parke DV, Activation mechanisms to chemical toxicity. *Arch Toxicol* **60**: 5–15, 1987.
3. Ortiz de Montellano PR and Correia MA, Suicidal destruction of cytochrome P-450 during oxidative drug metabolism. *Annu Rev Pharmacol Toxicol* **23**: 481–503, 1983.
4. Halpert J, Covalent modification of lysine during the suicide inactivation of rat liver cytochrome P-450 by chloramphenicol. *Biochem Pharmacol* **30**: 875–881, 1981.
5. Davies HW, Britt SG and Pohl LR, Carbon tetrachloride and 2-isopropyl-4-pentenamide-induced inactivation of cytochrome P-450 leads to heme-derived protein adducts. *Arch Biochem Biophys* **244**: 387–392, 1986.
6. Buening MK and Franklin MR, SKF 525-A inhibition, induction and 452 nm complex formation. *Drug Metab Dispos* **4**: 244–255, 1976.
7. Voorman R and Aust SD, Inducers of cytochrome P450d: influence on microsomal catalytic activities and differential regulation by enzyme stabilization. *Arch Biochem Biophys* **262**: 76–84, 1988.
8. Watkins PR, Wrighton SA, Scheutz EG., Maurel P and Guzelian PS, Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P-450p in rat hepatocytes *in vivo* and in primary monolayer culture. *J Biol Chem* **261**: 6264–6271, 1986.
9. Guengerich FP and Strickland TW, Metabolism of vinyl chloride: destruction of the heme of highly purified liver microsomal cytochrome P-450 by a metabolite. *Mol Pharmacol* **13**: 993–1004, 1977.
10. Levin W, Lu AYH, Jacobson M, Kuntzman R, Poyer JL and McCay PB, Lipid peroxidation and the degradation of cytochrome P450 heme. *Arch Biochem Biophys* **158**: 842–852, 1973.
11. Schaefer WH, Harris TM and Guengerich FP, Characterization of enzymatic and nonenzymatic peroxidative degradation of iron porphyrins and cytochrome P450 heme. *Biochemistry* **24**: 3254–3263, 1985.
12. Ortiz de Montellano PR, Mico BA, Mathews JM, Kunze KL, Miwa GT and Lu AYH, Selective inactivation of cytochrome P450 isozymes by suicide substrates. *Arch Biochem Biophys* **210**: 717–728, 1981.
13. Balvers WG, Boersma MG, Veeger C and Rietjens IMCM, Differential cumene hydroperoxide sensitivity of cytochrome P-450 enzymes IA1 and IIB1 determined by their way of membrane incorporation. *Biochim Biophys Acta* **1117**: 179–187, 1992.
14. Stickney JA, Silverman DM and Schatz RA, Role of isozyme-specific inhibition of cytochrome P450IIB1 activity in *m*-xylene-induced alterations in rat pulmonary benzo(*a*)pyrene metabolism. *Xenobiotica* **21**: 641–649, 1991.
15. Osawa Y, Yarborough C and Osawa Y, Norethisterone a major ingredient of contraceptive pills is a suicide inhibitor of estrogen biosynthesis. *Science* **215**: 1249–1251, 1982.
16. Furman GM, Silverman DM and Schatz RA, The effect of toluene on rat lung benzo[*a*]pyrene metabolism and microsomal membrane lipids. *Toxicology* **68**: 75–87, 1991.
17. Roberts AE, LeBel CP, Stickney JA, Silverman D, Brown DR and Schatz RA, Changes in rat lung microsomal lipids after *p*-xylene: Relationship to inhibition of benzo[*a*]pyrene metabolism. *J Toxicol Environ Health* **25**: 479–494, 1988.
18. IARC Monographs on the Evaluation of Carcinogenic Risk to Humans. An Updating of *IARC Monographs*, Vol. 1–42, Suppl 7, Lyon, France, 1987.
19. Smith MT, Yager JW, Steinmetz KL and Eastmond DA, Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. *Environ Health Perspect* **82**: 23–29, 1989.
20. Witz G, Latriano L and Goldstein BD, Metabolism and toxicity of *trans,trans*-muconaldehyde, an open-ring microsomal metabolite of benzene. *Environ Health Perspect* **82**: 19–22, 1989.
21. Gut I, Influence of frequently used industrial solvents and monomers of plastics on xenobiotic metabolism. *Zbl Pharm* **122**: 1139–1161, 1983.
22. Nakajima T, Elovaara E, Park SS, Gelboin HV, Hietanen E and Vainio M, Immunological characterization of cytochrome P450 isozymes responsible for benzene oxidation in the rat liver. *Carcinogenesis* **10**: 1713–1717, 1989.
23. Gut I, Terelius Y, Frantik E, Linhart I, Soucek P, Filipcova B and Kluckova H, Exposure to various benzene derivatives differently induces cytochrome P450 2B1 and 2E1 in rat liver. *Arch. Toxicol* **67**: 237–243, 1993.
24. Guengerich FP, Kim DH and Iwasaki M, Role of human cytochrome P450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4**: 168–179, 1991.
25. Ingelman-Sundberg M and Hagbjoerk AL, On the significance of the cytochrome P-450-dependent hydroxyl radical-mediated oxygenation mechanism. *Xenobiotica* **12**: 673–686, 1982.
26. Karam LR and Simic MG, Mechanisms of free radical chemistry and biochemistry of benzene. *Environ Health Perspect* **82**: 37–41, 1989.
27. Powis G, Free radical formation by antitumour quinones. *Free Radical Biol Med* **6**: 63–101, 1989.

28. Liu PT, Ioannides C, Shavila J, Symons AM and Parke DV, Effects of ether anaesthesia and fasting on various cytochromes P450 of rat liver and kidney. *Biochem Pharmacol* **45**: 871–877, 1993.
29. Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes P450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or beta-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
30. Ryan DE, Ramanathan L, Iida S, Thomas PE, Haniu M, Shively JE, Lieber CS and Levin W, Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. *J Biol Chem* **260**: 6385–6393, 1985.
31. Perrin DD, Armarego WLF and Perrin DR, *Purification of Laboratory Chemicals*. Pergamon Press Ltd, Oxford, 1966.
32. van der Hoeven TA and Coon MJ, Preparation and properties of partially purified cytochrome P450 and reduced NADPH-cytochrome P450 reductase from rabbit liver microsomes. *J Biol Chem* **249**: 6302–6310, 1974.
33. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
34. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
35. Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T and Guengerich FP, Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P450 by phenobarbital and other xenobiotics. *Arch Biochem Biophys* **238**: 43–48, 1985.
36. Kato R and Gillette JR, Effect of starvation on NADPH-dependent enzymes in rat liver microsomes of male and female rats. *J Pharmacol Exp Ther* **150**: 279–284, 1965.
37. Gram TE, Rogers LA and Fouts JR, Further studies on the metabolism of drugs by subfractions of hepatic microsomes. *J Pharmacol Exp Ther* **155**: 479–493, 1967.
38. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**: 680–685, 1970.
39. O'Farrell PH, High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**: 4007–4021, 1975.
40. Thomas PE, Ryan DE and Levin W, An improved staining procedure for the detection of the peroxidase activity of cytochrome P450 on sodium dodecyl sulfate polyacrylamide gels. *Anal Biochem* **75**: 168–176, 1976.
41. Fairbanks G, Steck TL and Wallach DFH, Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**: 2606–2617, 1971.
42. Towbin, HT, Staehelin, T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
43. Gut I and Soucek P, Covalent binding of benzene and its metabolites to biomacromolecules *in vitro*. Investigation on the role of cytochromes P450 2B1 and 2E1. *Plzen Lek Sborn* **67** (Suppl.): 89–94, 1993.
44. Prasanna HR, Lotlikar PD, Hacobian N, Ho LL and Magee PN, Effect of (+)-catechin, dimethyl-sulfoxide and ethanol on the microsome-mediated metabolism of two hepatocarcinogens, *N*-nitrosodimethyl-amine and aflatoxin B_1. *IARC Sci Publ* **84**: 175–177, 1987.
45. Melikian AA, Bagheri K and Hoffman D, Oxidation and DNA binding of (+)-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene in mouse epidermis *in vivo* and effects of coadministration of catechol. *Cancer Res* **50**: 1795–1799, 1990.
46. Lunte SM and Kissinger PT, Detection and identification of sulfhydryl conjugates of *p*-benzoquinone in microsomal incubations of benzene and phenol. *Chem Biol Interact* **47**: 195–212, 1983.
47. Battioni JP, Fontecave M, Jaonen M and Mansuy D, Vitamin E derivatives as new potent inhibitors of microsomal lipid peroxidation. *Biochem Biophys Res Commun* **174**: 1103–1108, 1991.
48. van Ommen B, Adang A, Muller F and van Bladeren PJ, The microsomal metabolism of pentachlorophenol and its covalent binding to protein and DNA. *Chem Biol Interact* **60**: 1–11, 1986.
49. den Besten C, Peters MM and van Bladeren PJ, The metabolism of pentachlorobenzene by rat liver microsomes: the nature of the reactive intermediates formed. *Biochem Biophys Res Commun* **163**: 1275–1281, 1989.
50. den Besten C, Smink MC, de Vries EJ and van Bladeren PJ, Metabolic activation of 1,2,4-trichlorobenzene and pentachlorobenzene by rat liver microsomes: a major role for quinone metabolites. *Toxicol Appl Pharmacol* **108**: 223–233, 1991.
51. Buben JA, Narasimhan N and Hanzlik RP, Effects of chemical and enzymic probes on microsomal covalent binding of bromobenzene and derivatives. Evidence for quinones as reactive metabolites. *Xenobiotica* **18**: 501–510, 1988.
52. Johnson BC, The vitamin K-dependent reaction. *J Chromatogr* **440**: 499–508, 1988.
53. Belisario MA, Pecce R, Arena AR, De Giulio A, Strazzullo G and De Rosa S, Effect of avarol, avarone and nine of their natural and synthetic derivatives on microsomal drug-metabolizing enzymes. *Toxicol Lett* **57**: 183–193, 1991.