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Oxidation of xenobiotics by plant microsomes, a reconstituted cytochrome P450 system and peroxidase: a comparative study

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Abstract

The microsomal fraction from tulip bulbs (*Tulipa fosteriana*, L.) contains cytochrome P450 (CYP[3], EC 1.14.14.1) and peroxidase (EC 1.11.1.7.) enzymes catalyzing the NADPH — and hydrogen peroxide — dependent oxidation of the xenobiotic substrates, *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylaniline (NMA), aminopyrine and 1-phenylazo 2-hydroxynaphthalene (Sudan I), respectively. Oxidation of these model xenobiotics has also been assessed in a reconstituted electron-transport chain with a partially purified CYP fraction, phospholipid and isolated tulip NADPH:CYP reductase (EC 1.6.2.4.). Peroxidase isolated from tulip bulbs (isoenzyme C) oxidizes these xenobiotics, too. Values of kinetic parameters (K_m, V_{max}), requirements for cofactors (NADPH, hydrogen peroxide), the effect of inhibitors and identification of products formed from the xenobiotics by the microsomal fraction, partially purified CYP and peroxidase C were determined. These data were used to estimate the participation of the CYP preparation and peroxidase C in oxidation of two out of the four studied xenobiotics (NMA, Sudan I) in tulip microsomes. Using such detailed study, we found that the CYP-dependent enzyme system is responsible for the oxidation of these xenobiotics in the microsomal fraction of tulip bulbs. The results demonstrate the progress in resolving the role of plant CYP and peroxidase enzymes in oxidation of xenobiotics.

Keywords: *Tulipa fosteriana*; Cytochrome P450; Peroxidase; Xenobiotics

1. Introduction

The oxidation of xenobiotics in animals is thought to be catalyzed mainly by mixed function oxidases, usually with a cytochrome P450 (CYP, EC 1.14.14.1.) as the terminal oxidase. However, it is not exactly known which plant enzymes are the most important for the oxidation of xenobiotics in plants. Several types of oxidative enzymes such as CYPs, peroxidases (EC 1.11.1.7.) and lipoxygenases (EC 1.13.11.12.) have been suggested to be implicated in the oxidation of xenobiotics in plants (Sandermann, 1988, 1992; Fonné-Pfister et al., 1988; Higashi, 1988; Durst and Benveniste, 1993; Pflugmacher and Sandermann, 1998).

An economically significant function of plant CYPs, and one which is only partially understood, is the role of CYPs in the detoxication of herbicides (Moreland et al., 1993a, 1993b; Bolwell et al., 1994; Frear, 1995; Thies et al., 1996). This function is one which provides the first committed step in the detoxification process responsible for herbicide selectivity in a variety of plant-herbicide combinations. Other plant CYP

Abbreviations: BDI, benzenediazonium ion; CYP, cytochrome P450; HPLC, high-performance liquid chromatography; K_m, Michaelis constant; NDMA, *N*-nitrosodimethylamine; NMA, *N*-nitroso-*N*-methylaniline; 4′-OH-Sudan I, 1-(4-hydroxyphenylazo)-2-hydroxynaphthalene; 6-OH-Sudan I, 1-phenylazo-2,6-dihydroxynaphthalene; 4′,6-di(OH)-Sudan I, 1-(4-hydroxyphenylazo)-2,6-dihydroxynaphthalene; R_f, relative mobility; TLC, thin layer chromatography; V_{max}, maximal velocity.

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enzymes serve critical functions in numerous biosynthetic pathways, especially those involved in the production of a diverse array of secondary metabolites such as terpenoids, alkaloids, phenylpropanoids, phytosterols, pigments, phytoalexins, giberellins, etc. (Durst and Benveniste, 1993; Durst and O'Keefe, 1995; Hallahan and West, 1995; Schuler 1996; Schalk et al., 1997; Chapple, 1998; Tijet et al., 1998) as well as in several reactions related to metabolism of xenobiotics (Riviére and Cabanne, 1987; Higashi, 1988; Sandermann, 1988, 1992; Frear et al., 1991; Durst and Benveniste, 1993; Frear et al., 1993; Thies et al., 1996; Pflugmacher and Sandermann, 1998; Robineau et al. 1998).

The metabolism of xenobiotics in many plant species (Dohn and Krieger, 1981; Sandermann, 1987, 1988, 1992) or plant microsomal enzymes has been previously shown (Fonné-Pfister et al., 1988; Sandermann, 1992, 1994; Moreland et al., 1993a, 1993b; Durst and O'Keefe, 1995; Schalk et al., 1997; Batard et al., 1998). Microsomal localization of the activity, O_2 and NADPH requirements, inhibition by CO and its reversal by light provided conclusive evidence for the involvement of CYPs in the oxidative metabolism. However, the metabolism of xenobiotics by purified plant CYPs reconstituted with NADPH:CYP reductase (EC 1.6.2.4.) was studied only in a few cases. CYP isolated from avocado was found to metabolize xenobiotics (e.g. 4-chloro-*N*-methylaniline) (O'Keefe and Leto, 1989). Demethylation of this substrate was also measured in yeast transformed with recombinant CYP71A1 from avocado (Bozak et al., 1992), demonstrating that CYP71A1 product oxidizes this xenobiotic. A recombinant plant cinnamate 4-hydroxylase CYP73A (EC 1.14.13.11.) (from *Helianthus tuberosus* tubers) produced in yeast was highly specific for its natural substrate (Pierrel et al., 1994; Urban et al., 1994; Schalk et al., 1997). However, it also demethylated herniarin (Urban et al., 1994; Pierrel et al., 1994), hydroxylated 2-naphthoic acid (Durst et al., 1996; Schalk et al., 1997) and oxygenated five xenobiotics including the herbicide chlorotoluron (Urban et al., 1994; Pierrel et al., 1994). An inducible CYP76B1 enzyme isolated from *Helianthus tuberosus* dealkylated a model xenobiotic compound 7-ethoxycoumarin and metabolized also a wide range of xenobiotics, including alkoxycoumarins, alkoxyresorufins, and several herbicides of the class of phenylureas (Robineau et al. 1998). Another CYP oxidizing xenobiotics such as phenylurea herbicides was a gene product of heterologously expressed CYP cDNA (CYP71A10) (Siminszky et al., 1999). We have shown that a protein fraction highly enriched for CYPs, isolated from tulip (*Tulipa fosteriana*, L.) bulbs, is active in the in vitro reductase-mediated oxidation of *N*-nitrosamines and aminopyrine (Hansíková et al., 1994, 1995).

Table 1
Inhibition of xenobiotics oxidation catalyzed by microsomes, a reconstituted CYP system and peroxidase from tulip bulbs[a]

Incubation conditions	Relative activity											
	Crude microsomal system				CYP reconstituted system				Peroxidase			
	NDMA	NMA	Aminopyrine	Sudan I	NMDA	NMA	Aminopyrine	Sudan I	NMDA	NMA	Aminopyrine	Sudan I
Complete (with NADPH)	100 ± 5.3[a]	100 ± 5.1[b]	100 ± 9.8[c]	100 ± 8.3[d]	100 ± 7.3[e]	100 ± 2.4[f]	100 ± 8.3[g]	100 ± 7.5[h]	0	0	0	0
–NADPH	6.1 ± 0.6	5.8 ± 0.6	8.9 ± 0.5	5.1 ± 0.5	0	0	0	0	0	0	0	0
+ NADH (0.5 mM) instead of NADPH	25.0 ± 2.9	20.8 ± 2.1	13.5 ± 1.3	22.5 ± 7.2	0	0	0	0	0	0	0	0
+ H_2O_2(0.5 mM) instead of NADPH	12.5 ± 0.1	10.5 ± 0.1	30.0 ± 2.3	4.8 ± 0.5	0	0	0	0	100 ± 4.8[i]	100 ± 5.7[j]	100 ± 4.4[k]	100 ± 5.9[l]
+ CO-buffer (50 μl) darkness	47.8 ± 2.8	45.3 ± 2.8	71.3 ± 5.6	41.3 ± 4.4	35.3 ± 3.4	38.5 ± 3.2	36.5 ± 3.4	38.8 ± 3.9	n.m.	n.m.	n.m.	n.m.
+ CO-buffer (50 μl) light	90.0 ± 9.1	92.1 ± 8.5	79.8 ± 6.3	72.5 ± 7.6	68.1 ± 6.2	65.1 ± 9.8	66.5 ± 6.9	67.3 ± 7.8	n.m.	n.m.	n.m.	n.m.
+ metyrapone (0.1 mM)	25.2 ± 2.2	21.5 ± 2.2	51.3 ± 6.3	21.0 ± 2.5	32.3 ± 3.3	35.6 ± 3.8	35.3 ± 6.3	25.3 ± 2.8	99.3 ± 9.8	99.9 ± 8.3	100.2 ± 8.7	100.2 ± 9.3

[a] The oxidation of xenobiotics was measured as described in Section 3. The 100% activity was 1.20[a], 1.24[b], 0.70[c], 0.51[d], 2.49[e], 2.41[f], 0.10[g] and 0.50[h] nmole HCHO or Sudan I/min per nmol CYP and 0.14[i], 0.24[j], 1.81[k]and 0.30[l] nmole HCHO or Sudan I/min per nmol peroxidase. Means and standard deviations of 3 experiments are presented. n.m.: not measured.

Care should be taken into account of any interpretation of data from crude microsomal fractions. Plant microsomes contain not only CYPs as do microsomes of animals, but peroxidases are also present in relatively high levels in this plant subcellular system (Chiapella et al., 1995). These enzymes were assumed to be even more effective in metabolism of several xenobiotics in plants than CYPs (Sandermann, 1988; Stiborová and Anzenbacher, 1991; Stiborová et al., 1988a, 1990, 1992; Plewa and Wagner, 1993; Gichner et al., 1994, 1995; Plewa et al., 1996; Stavreva et al., 1997).

The aim of the present work is to elucidate the participation of CYPs and peroxidases in oxidation of xenobiotics by plant microsomes. Here, we complete the in vitro comparative study of oxidation of *N*-nitrosodimethylamine (NDMA), *N*-nitroso-*N*-methylaniline (NMA), aminopyrine and 1-phenylazo-2-naphthol (Sudan I) using enzyme systems (microsomes, CYP and peroxidase) prepared from tulip bulbs.

2. Results and discussion

2.1. Oxidation of NDMA, NMA, aminopyrine and Sudan I by tulip microsomes

Microsomes isolated from tulip bulbs (20.9 ± 1.8 mg protein/g fresh weight of tulip bulbs) contained CYP (45.0 ± 3.2 pmol/mg protein), cytochrome P420 (0.05 ± 0.01 pmol/mg protein), cytochrome b_5 (0.10 ± 0.01nmol/mg protein) and exhibited the activity of NADPH:CYP reductase with respect to cytochrome *c* as a substrate (2.71 ± 1.91 nmol/min per mg protein) and the activity of peroxidase with guaiacol and benzidine as substrates (13.2 ± 1.4 nmol/min per mg and 14.1 ± 1.4 nmol/min per mg protein, respectively). Hence, we have confirmed that tulip microsomes contain not only CYPs, but also the peroxidase activity.

Four structurally diverse chemicals (NDMA, NMA, aminopyrine, and Sudan I) are oxidized by this microsomal fraction. Their oxidation by microsomes had an absolute requirement for NADPH. The reaction was negligible when the NADPH regenerating system was omitted (Table 1). NADH is less efficient cofactor than NADPH. Hydrogen peroxide (the peroxidase cofactor) is even less efficient cofactor for oxidation of NDMA, NMA and Sudan I than NADH. Only demethylation of aminopyrine, in the presence of hydrogen peroxide, was greater than that in the presence of NADH (Table 1). The oxidation of NDMA, NMA and Sudan I was significantly inhibited by CO and this inhibition was reversible by light (Table 1). The inhibition of the aminopyrine demethylation was less effective and it was only partially reversible by light. Likewise, metyrapone (2-methyl-1,2-di-pyridyl-1-propanone), which acts as a heterocyclic ligand, binding to both the oxidized and reduced form of CYP heme, competing with substrate and oxygen, inhibited more efficiently the oxidation of NDMA, NMA and Sudan I than the oxidation of aminopyrine (Table 1). Unfortunately, inhibitors of peroxidase, which are often used in the in vitro experiments (Gichner et al., 1994, 1995; Chiapella et al., 1995), could not be utilized in the study. We found that most of such well known peroxidase inhibitors (i.e. CN^-, diethyldithiocarbamate, pyrogallol) also inhibit CYP (results not shown).

Oxidation of the compounds was measured in the reaction mixture, which contained microsomal enzymes, NADPH as cofactor and various concentrations of the xenobiotic compounds. The reactions followed the Michaelis-Menten kinetics. Among the studied xenobiotics, NMA is the best substrate for plant microsomal enzymes. Under the conditions used, the highest value of the maximal velocity (V_{max}) and the lowest value of the apparent Michaelis constant (K_m) for NMA were observed (Table 2). The values of kinetic parameters for the reactions catalyzed by the microsomal system, in which hydrogen peroxide was used, were not determined because of the low efficiency of microsomes to oxidize the studied xenobiotics under the presence of this cofactor (see Table 1).

2.2. Oxidation of NDMA, NMA, aminopyrine and Sudan I by a CYP reconstitution system and peroxidase

All studied xenobiotics are also oxidized by partially purified CYP (a protein fraction highly enriched for CYPs) reconstituted with NADPH:CYP reductase and by peroxidase (one of the most abundant isoenzyme of tulip bulbs – isoenzyme C) (Tables 1 and 2). The reactions catalyzed by the CYP reconstituted system were not detectable when the NADPH-regenerating system or the reductase was omitted. Likewise, the presence of hydrogen peroxide was absolutely required for the activity of isolated peroxidase (Table 1). A molar ratio of 0.65 of CYP to the reductase, which was shown to be the most appropriate for the catalysis of the metabolism of xenobiotics (Hansíková et al., 1995), was used to determine the kinetic parameters of the oxidation of all studied xenobiotics. The reactions followed Michaelis-Menten kinetics. The apparent K_m values and maximal velocities (V_{max}) for the oxidation of xenobiotics by this system are shown in Table 2. Similarly to the crude microsomal fraction, NMA was also the best substrate for the reconstituted system containing partially purified CYP.

The reaction of NDMA, aminopyrine and Sudan I oxidation catalyzed by isolated tulip peroxidase followed the Michaelis-Menten kinetics, too. Among the studied xenobiotics, aminopyrine is the best substrate for tulip peroxidase (Table 2). We had described pre-

viously that non-Michaelian saturation curves were observed when the initial velocity of NMA oxidation catalyzed by isoenzymes of tulip peroxidase was plotted as a function of NMA concentrations (Stiborová and Hansíková, 1997). Therefore, we cannot characterize efficiency of NMA oxidation by plant peroxidases using the values of V_{max} and apparent K_m.

The values of apparent K_m obtained for NDMA, NMA and Sudan I as the substrates of CYP reconstituted system in vitro are similar to those obtained for these substrates in microsomes (Table 2). A different K_m value was, however, determined for aminopyrine. Other CYP enzymes different from the isolated enzyme fraction or other enzymes, which oxidize aminopyrine, but have lower affinities for aminopyrine, are present in the tulip microsomes.

On the basis of found effects of inhibitors, the requirement for cofactors and values of kinetic parameters, we can speculate that CYP isolated from tulip bulbs should be responsible for the NDMA, NMA and Sudan I oxidation in microsomes of tulip bulbs. In order to confirm this speculation, special emphasis was laid on the comparison of metabolites formed by these three used plant enzyme systems. NMA and Sudan I were used for such a detailed comparative study.

2.3. Metabolites generated from NMA and Sudan I by tulip microsomes, a CYP reconstitution system and peroxidase

Formaldehyde, methylaniline, aniline, *p*-methylaminophenol and *p*-aminophenol were found to be metabolites formed from NMA by the tulip CYP reconstituted with NADPH:CYP reductase, and also by the NADPH-dependent reactions catalyzed by the crude microsomal system (Table 3, Fig. 1). Phenol was not detected in these systems under the experimental conditions used (Table 3 and Hansíková et al., 1995). This metabolite was produced by peroxidase and, in a very low amount, also by microsomes with H_2O_2 as cofactor (Table 3). Methylaminophenol was not detected in the peroxidase system.

Major metabolites produced from Sudan I by tulip microsomes with the NADPH-regenerating system [4′-OH-Sudan I,6-OH-Sudan I and the colourless product, which was identified previously as compound derived from the benzenediazonium ion (BDI) (Stiborová et al., 1988b; Stiborová et al., 1995)] are the same as the major products formed from this compound by partially purified CYP (Table 3). A minor product is 4′,6-di(OH)-Sudan I (Table 3, Fig. 2). The formation of Sudan I oxidative products by microsomes were negligible when hydrogen peroxide was used as cofactor (Table 3). Tulip peroxidase oxidized Sudan I to form only a small amount of 6-OH-Sudan I, BDI and two other products with R_f 0.35 and 0.7 (Table 3, Fig. 2). These two products decomposed during mass spectroscopy. The fragmentation peaks at m/z 77 and 105 in the mass spectra of both products indicate the presence of phenyl and phenyldiazonium ion fragments, respectively,whereas the most prominent peak at m/z 247 in the mass spectrum of the products with R_f 0.7 apparently belongs to the Sudan I ion. We compared the fragmentation patterns of the two products with those of Sudan I. The most prominent peaks in the mass spectrum of Sudan I arise from the phenyl ion (m/z 77), the phenyldiazonium ion (m/z 105), and the Sudan I ion (m/z 247). Therefore, we suggest that the two products may be dimers and/or oligomers of Sudan I, being formed from Sudan I radicals. Their complete structure elucidation by NMR spectroscopy was precluded by their instability. These metabolites are formed neither by microsomes nor by CYP. Tulip peroxidase is less efficient with respect to oxidation of Sudan I than CYP (Table 3).

The identification of metabolites formed from NMA and Sudan I confirmed our suggestion (see above) that the CYP-dependent enzyme system is mainly responsible for the oxidation of both xenobiotics in the microsomal fraction of tulip bulbs.

The biological significance of both CYP- and peroxi-

Table 2
Kinetics of metabolism of different xenobiotic substrate by microsomes, a reconstituted CYP system and peroxidase from tulip bulbs[a]

Substrate	Crude microsomal system		CYP reconstituted system		Peroxidase	
	K_m μM	V_{max}[b]	K_m, μM	V_{max}[b]	K_m, μM	V_{max}[c]
NDMA	151 ± 32	1.23 ± 0.210	142 ± 27	2.51 ± 0.230	420 ± 60	0.150 ± 0.003
NMA	45 ± 3	1.25 ± 0.230	44 ± 1	2.42 ± 0.030	n.d.[d]	n.d.[d]
Aminopyrine	3300 ± 280	0.71 ± 0.072	117 ± 29	0.11 ± 0.016	80 ± 9	1.950 ± 0.090
Sudan I	682 ± 63	0.53 ± 0.062	635 ± 60	0.53 ± 0.030	893 ± 53	0.320 ± 0.030

[a] Means and standard deviations from 3 parallel experiments are presented.
[b] nmoles HCHO or Sudan I/min per nmol CYP.
[c] nmoles HCHO or Sudan I/min per nmol peroxidase.
[d] n.d.: not detectable.

dase-mediated oxidation of NMA and Sudan I metabolism in intact plants in vivo is still unknown and cannot be derived from the results as shown here. The determination of metabolites of both studied xenobiotics formed in tulip plants in vivo would resolve this question. Such study is planned for the future work. From this point of view, metabolites which are formed as unique products of oxidation by CYP or peroxidase (e.g. phenol and methylaminophenol formed from NMA by peroxidase and CYP, respectively, C-hydroxy derivatives and oligomeric products exclusively formed from Sudan I by CYP and peroxidase, respectively) will serve as *marker metabolites* for a CYP- or peroxidase-mediated metabolism of both compounds in the intact tulip plant.

3. Experimental

3.1. Chemicals

Chemicals were obtained from the following sources: cytochrome *c*, $NADP^+$, NADPH and dilauroylphosphatidylcholine from Fluka (Switzerland), Sudan I from British Drug Houses (UK), NADH from Boehringer (Germany), dithiothreitol from Koch-Light (UK), Emulgen 911 from Atlas Co. (Japan), benzenediazonium hexafluorophospate from Aldrich (USA) and all other chemicals were reagent grade or better. NMA and NDMA were synthesized as described previously (Stiborová et al., 1992). The derivatives 1-(4-hydroxyphenylazo)-2-hydroxynaphthalene (4′-OH-Sudan I),1-phenylazo-2,6-dihydroxynaphthalene (6-OH-Sudan I) and 1-(4-hydroxyphenylazo)-2,6-dihydroxynaphthalene [4′,6-di(OH)-Sudan I] were synthesized (Stiborová et al., 1988b) and purified by column chromatography on basic alumina and by thin layer chromatography (TLC) on silica gel (Woelm). [^{14}C]-Labelled Sudan I (20 MBq/mmol) was synthesized as shown earlier (Stiborová et al., 1988b) and stored in methanol at −17°C.

3.2. Preparation of microsomes

Tulip bulbs (*Tulipa fosteriana* L., var. Red Emperor) were extracted without any inducing pretreatment. Microsomes were prepared as described (Hansíková et al., 1994, 1995) and resuspended in 0.1 M sodium phosphate (pH 7.4), containing 30% glycerol (v/v) and stored at −80°C.

3.3. Purification of CYP

The CYP enzyme used for the reconstitution experiments was isolated by the procedure we described previously (Hansíková et al., 1994, 1995). The specific content of CYP in the final preparation (a protein fraction highly enriched for CYPs, see Hansíková et al., 1995) was 5.2 nmol/mg protein. The CYP preparation was stored in 10 mM potassium phosphate (pH 7.4) at −80°C.

3.4. Purification of NADPH CYP reductase

A homogeneoues preparation of NADPH:CYP reductase was isolated from solubilized tulip bulb micro-

Table 3
In vitro metabolism of NMA and Sudan I by microsomes, a CYP reconstituted system and peroxidase from tulip bulbs[a]

Metabolite	Yields (%)[c]			
	Crude microsomal system		CYP reconstituted system	Peroxidase
	with NADPH	with H_2O_2		
N-Methylaniline	3.54 ± 0.40	n.d.[b]	3.60 ± 0.39	0.32 ± 0.02
Phenol	n.d.	0.10 ± 0.03	n.d.	5.20[d] ± 0.43
Aniline	3.84 ± 0.40	0.02 ± 0.01	4.00 ± 0.41	0.82 ± 0.09
p-Methylaminophenol	5.06 ± 0.51	n.d.	5.20 ± 0.49	n.d.
p-Aminophenol	7.54 ± 0.80	n.d.	8.25 ± 0.80	1.40 ± 0.11
BDI[e]	3.10 ± 0.30	0.08 ± 0.01	2.52 ± 0.27	0.51 ± 0.05
4′,6-di(OH)-Sudan I	0.80 ± 0.08	n.d.	0.75 ± 0.08	n.d.
4′-OH-Sudan I	6.67 ± 0.63	n.d.	6.99 ± 0.71	n.d.
6-OH-Sudan I	1.69 ± 0.16	n.d.	1.95 ± 0.18	0.73 ± 0.08
Unknown	n.d.	n.d.	n.d.	1.24 ± 0.13
Unknown	n.d.	n.d.	n.d.	1.42 ± 0.14

[a] Values represent the means and standard deviations of three experiments. Experimental conditions are described in Section 3.
[b] n.d.: not detectable.
[c] The values of 100% were 300 nmoles NMA/min and 5 nmoles Sudan I/min.
[d] Corrected for 20% recovery of phenol from peroxidase.
[e] Other products formed from BDI could also be present (Stiborová et al., 1988a, 1988b).

somes by chromatography on DEAE-Sepharose CL 6B and affinity chromatography on a 2′,5′-ADP Sepharose 4B column (Hansíková et al., 1994). The final preparation of NADPH:CYP reductase had a specific activity of 77.5 nmol/min per mg protein. The reductase was stored in 10 mM potassium phosphate (pH 7.4) containing 0.02% Emulgen 911 after addition of 2 μM flavinmononucleotide at −80°C.

3.5. Isolation of peroxidases

A slight modification of the procedure for isolation of peroxidases from horseradish roots described by Shannon et al. (1966) was used. Chromatography on CM-Sephadex C-25 was used for isolation of cationic peroxidases B, C, D and E. Three anionic peroxidases (A_1, A_2, A_3) were isolated by chromatography on DEAE-cellulose DE 52 (Shannon et al., 1996; Stiborová and Hansíková, 1997). Fraction C (one of the most abundant peroxidase isoenzyme of tulip bulbs), which was used for detailed kinetic measurements with xenobiotics, was then purified by repeated chromatography on CM-Sephadex until elution patterns indicated that tulip peroxidase C was free from other peroxidase fractions or other protein contaminants.

3.6. Analytical methods

Quantitative determination of CYP, cytochrome P420 and cytochrome b_5 was carried out according to Omura and Sato (1964). The concentration of NADPH:CYP reductase was estimated as described (Vermilion and Coon, 1978). The activity of NADPH: CYP reductase was measured according to Sottocasa

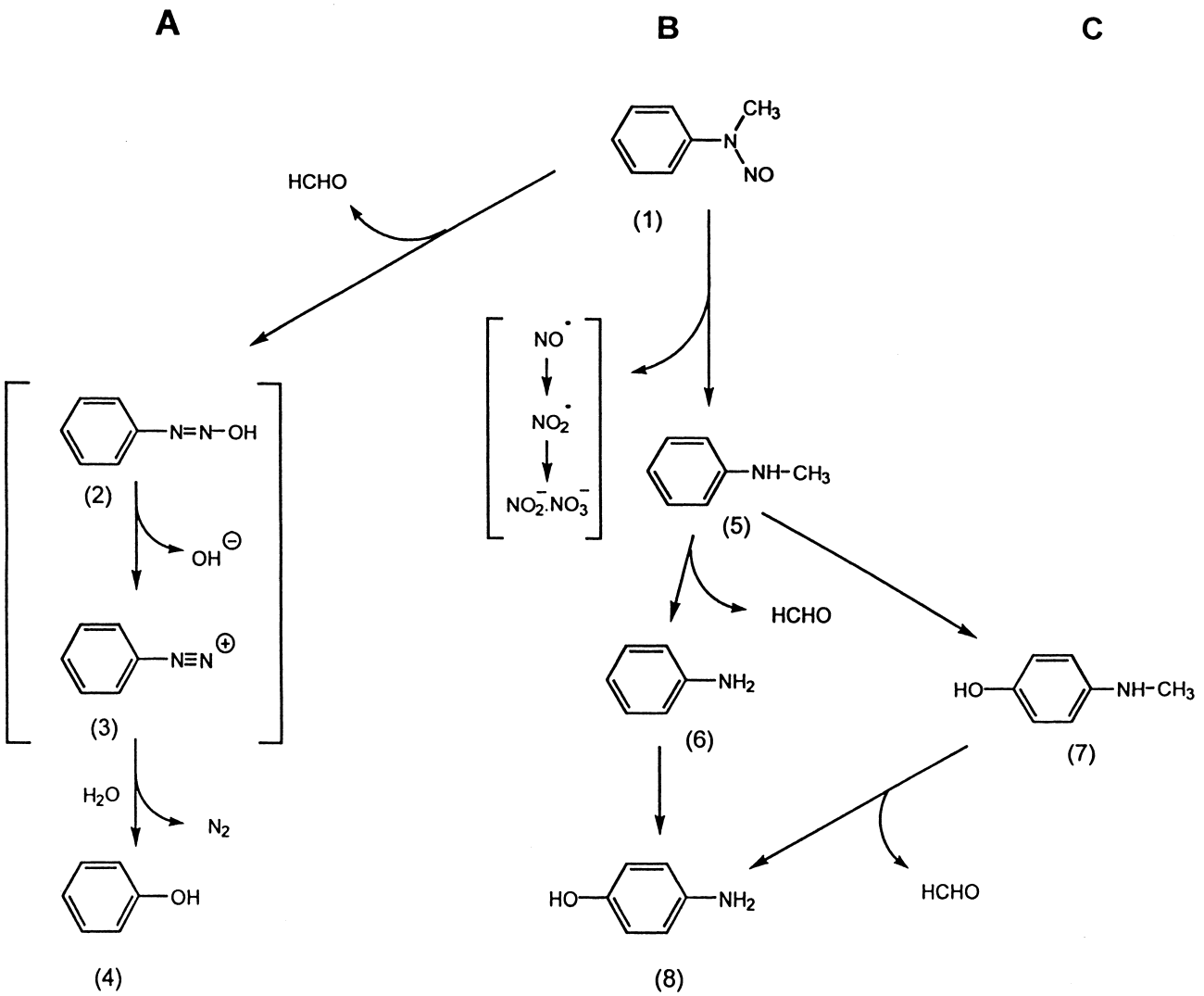

Fig. 1. Proposed metabolic scheme of NMA in the tulip peroxidase system (A,B) and the tulip CYP reconstituted system (B,C). (1) *N*-nitrosomethylaniline; (2) benzenediazoniumhydroxide; (3) benzenediazonium ion; (4) phenol; (5) methylaniline; (6) aniline; (7) *p*-methylaminophenol; (8) *p*-aminophenol. The metabolites in parentheses are unstable and therefore not detectable.

et al. (1967) using cytochrome *c* as the substrate (i.e. as NADPH:cytochrome *c* reductase). The peroxidase activity was measured according to Chiapella et al. (1995) using guaiacol and benzidine as substrates. Protein concentrations were estimated according to Bradford (1976) with bovine serum albumin as a standard. Kinetic analyses were carried out by using the non-linear square methods as described by Cleland (1983).

The composition of the assay mixtures for *N*-demethylation reactions of NMA, NDMA and aminopyrine was described earlier (Hansíková et al., 1995). Briefly, 60 pmol CYP from tulip bulbs, 90 pmol of the tulip NADPH:CYP reductase, 50 mM potassium phosphate pH 7.4, 7.5 mM $MgCl_2$, 8 mM semicarbazide, NADPH regenerating system, 60 μg of D,L-dilauroylphosphatidylcholine and 0.1–50 mM NMA, NDMA or aminopyrine. The amount of formaldehyde formed was measured as described by Nash (1953). The same reaction mixtures were used for demethylation of the compounds by peroxidase except that 200 μg tulip peroxidase was used instead of the CYP-dependent enzyme system. These mixtures contained 1 mM H_2O_2 as a second substrate (cofactor) instead of the NADPH regenerating system.

The composition of the assay mixture and the procedure for demethylation reactions of NMA, NDMA and aminopyrine catalyzed by microsomes were the same as that described for the reconstitution experiments except that 0.2 nmol of CYP (measured in microsomal preparations diluted to contain 2–3 mg/ml protein) were used instead of the reconstituted system. The incubation mixtures containing NMA as a substrate were applied to a C18 SepPak cartridge to quantify the products formed from NMA in the used plant enzyme systems. The cartridge was washed with 0.1 mM NaOH and the products eluted with 50% metha-

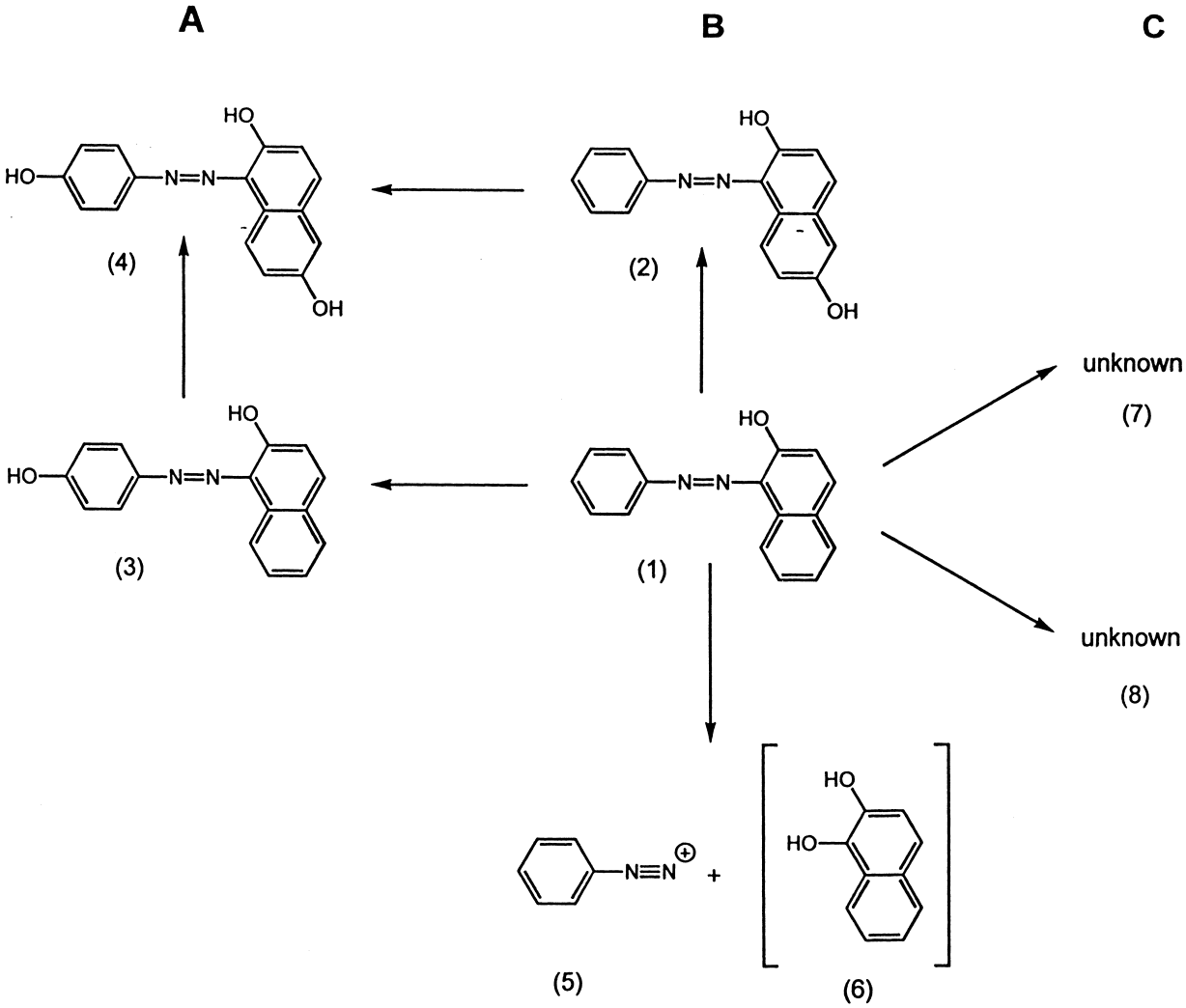

Fig. 2. Proposed metabolic scheme of Sudan I in the tulip CYP reconstituted system (A,B) and tulip peroxidase (B,C). (1) Sudan I; (2) 6-OH-Sudan I; (3) 4′-OH-Sudan I; (4) 4′,6-di(OH)-Sudan I; (5) benzenediazonium ion; (6) 1,2-dihydroxynaphthalene; (7) unknown; (8) unknown. The metabolite in parentheses was not detected.

nol in water. The products were separated by high-performance liquid chromatography (HPLC) on a Separon SGX C18 column as described (Hansíková et al., 1995). Reaction products were identified by comparison of their retention times with authentic standards (*p*-aminophenol, *p*-methylaminophenol, phenol, aniline and *N*-methylaniline, having the retention times 2.2, 3.5, 9.15, 10.6 and 17.1 min, respectively). The amounts were calculated by peak areas by comparison with external standards. Recoveries of products were around 80% after 10 min incubation in the presence of enzymes without cofactors (NADPH or hydrogen peroxide), except the recovery of phenol (it was around 60%). The recovery of phenol when benzenediazonium hexafluorophosphate was added to the incubation mixture containing peroxidase but without hydrogen peroxide was about 20% after 10 min incubation.

The assay mixture for the oxidation reactions of Sudan I contained, in 1 ml, 60 pmol CYP from tulip bulbs, 90 pmol NADPH:CYP reductase from the same source, 50 mM potassium phosphate pH 7.4, 7.5 mM $MgCl_2$, 4 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase, 100 μg of D,L-dilauroylphosphatidylcholine and 0.01–0.5 mM [^{14}C]Sudan I (or unlabelled Sudan I) dissolved in 20 μl dimethyl sulfoxide. The reaction was started by addition of NADPH (final concentration was 0.5 mM). The same reaction mixtures were used for the oxidation of Sudan I by peroxidases except that 200 μg tulip peroxidase was used instead of the CYP-dependent enzyme system. These mixtures contained 1 mM H_2O_2 as a second substrate (cofactor) instead of the NADPH regenerating system. The mixtures were incubated for 10–60 min at 37°C and then extracted with 1 ml of ethyl acetate. The extracts were evaporated, dissolved in a minimum volume of methanol, chromatographed on a thin layer of silica gel and developed in hexan-diethyl ether-acetone (1:0.7:0.3, v/v). The same TLC was performed with standards. The R_f values of 4′,6-di(OH)-Sudan I, 4′-OH-Sudan I, 6-OH-Sudan I and Sudan I were 0.23, 0.47, 0.53 and 0.87, respectively. Mass spectra of two products of Sudan I oxidation by peroxidase, which have not been characterized as yet, (with R_f 0.35 and 0.7) were measured on a FINNIGAN MAT INCOS 50 mass spectrometer (electron impact, 70 eV, low resolution mode, direct inlet). The products of [^{14}C]Sudan I oxidation and the residual parent compound were scrapped from the layers and placed into scintillation vials. Packard Ultra Gold X liquid scintillator cocktail was added and the radioactivity was counted in a Packard Tri-Carb 2000 CA scintillation counter (Stiborová et al., 1988a, 1988b, 1990). The benzenediazonium ion was detected by azo coupling with 1-phenyl-3-methyl-5-pyrazolone as described (Stiborová et al., 1988a, 1988b). Alternatively, the products, dissolved in methanol, were separated by HPLC on a Separon SGX C18 column with a linear gradient of methanol in water (v/v): 90% methanol 0–6 min, 90–91% methanol 6–9 min and 91–100% methanol 9–18 min; flow rate 0.5 ml/min, UV detection at 260 nm. The retention times of 4′,6-di(OH)-Sudan I, 6-OH-Sudan I, 4′-OH-Sudan I and Sudan I were 3.0, 5.7, 6.9 and 15.1 min, respectively. Recoveries of products were around 90% in the presence of enzymes without cofactors (NADPH or hydrogen peroxide).

The 1 ml assay mixture for oxidation reactions of Sudan I catalyzed by microsomes contained 50 mM potassium phosphate, pH 7.4, 0.2 nmol of CYP, 0.4 units of glucose-6-phosphate dehydrogenase, 4 mM glucose-6-phosphate, 7.5 mM $MgCl_2$, 0.01–0.5 mM [^{14}C]Sudan I. The procedure was the same as that described for the experiments with the pure enzymes (see above).

Carbon monoxide inhibition of oxidation of substrates was performed as shown previously (Hansíková et al., 1995).

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