CYTOCHROME P-450 FROM TULIP BULBS (*Tulipa fosteriana* L.) OXIDIZES AN AZO DYE SUDAN I (1-PHENYLAZO-2-HYDROXYNAPHTHALENE, SOLVENT YELLOW 14) IN VITRO

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The microsomal fraction from tulip bulbs (*Tulipa fosteriana* L.) contains cytochrome P-450 enzymes catalyzing the NADPH-dependent oxidation of the xenobiotic substrate, an azo dye Sudan I (1-phenylazo-2-hydroxynaphthalene, Solvent Yellow 14). *C*-Hydroxy derivatives [1-(4-hydroxyphenylazo)-2-hydroxynaphthalene, 1-phenylazo-2,6-dihydroxynaphthalene, 1-(4-hydroxyphenylazo)-2,6-dihydroxynaphthalene] and the benzenediazonium ion are the products of the Sudan I oxidation. The oxidation of Sudan I has also been assessed in a reconstituted electron-transport chain with the isolated cytochrome P-450, isolated plant NADPH-cytochrome P-450 reductase and phospholipid. The results are discussed from the point of view of the role of cytochromes P-450 in the metabolism of xenobiotics in plants.

Key words: *Tulipa fosteriana*; Cytochrome P-450; Oxidation of xenobiotics; Sudan I azo dye.

While the biotransformation reactions of xenobiotics in animals are well known, information on the plant system is scarce. Nevertheless, plants are organisms primarily exposed to many xenobiotics, which are, at the present time, an integral part of the environment. The increasing use of pesticides and other chemicals in modern agriculture causes higher amounts of these compounds to enter into the plants and thus into the trophic chain.

During the phase I of xenobiotics biotransformation in an organism the oxidative reactions lead to the formation of polar compounds, which can be further conjugated during the phase II of biotransformation. Another possibility is formation of toxic, mutagenic or carcinogenic metabolites, which are not conjugated and, due to their reactivity, bind covalently to biological macromolecules[1]. Hence, the oxidative reactions are crucial for the further activity of xenobiotics and for the understanding of their fate in the organism.

The oxidation of xenobiotics in animals is thought to be catalyzed mainly by mixed function oxidases, usually with a cytochrome P-450 as the terminal oxidase. Cytochromes P-450 have also been found in many plants[2–5] and their physiological roles have been investigated[2,3,5]. Hendry postulated[6] that some cytochromes P-450 in plants probably function in the same way as in all other eukaryotic organisms, i.e. in detoxi-

cation mechanisms. Other plant cytochrome P-450 enzymes are known to be involved in a number of biosynthetic pathways leading to formation of monoterpenes, sterols, giberellins etc. as well as in several reactions related to metabolism of xenobiotics[2,5–12]. It is not clear if the number of individual plant cytochrome P-450 enzymes is an large as in animals, or if any plant cytochromes P-450 have a broad, overlapping specificity as do those involved in xenobiotic metabolism especially in animal livers.

The metabolism of xenobiotics by purified plant cytochrome P-450 reconstituted with reductase was studied only in a few cases. A cytochrome P-450 isolated from avocado was found to metabolize xenobiotics, but its endogeneous role may be that of monoterpene hydroxylase[13,14]. Recently we isolated and characterized cytochrome P-450 from tulip bulbs (*Tulipa fosteriana*)[15]. The enzyme preparation was active in oxidation of xenobiotic (*N*-nitrosamines and aminopyrine) by a NADPH-cytochrome P-450 reductase – mediated monooxygenase reaction[15,16].

Here, we report that this cytochrome P-450 preparation catalyzes oxidation of another exogeneous substrate. An azo dye Sudan I (1-phenylazo-2-hydroxynaphthalene) is oxidized by this enzyme when reconstituted with purified NADPH-cytochrome P-450 reductase of the same plant tissue and with dilauroylphosphatidylcholine.

EXPERIMENTAL

Chemicals and Radiochemicals

Chemicals were from the following sources: Sudan I (1-phenylazo-2-hydroxynaphthalene, **5**) (CAS No. 842-07-9) from British Drug Houses, NADH from Boehringer, NADPH and dilauroylphosphatidylcholine from Fluka, dithiothreitol from Koch–Light. All other chemicals were reagent grade or better. The derivatives 1-(4-hydroxyphenylazo)-2-hydroxynaphthalene (4′-OH-Sudan I, **3**), 1-phenylazo-2,6-dihydroxynaphthalene (6-OH-Sudan I, **4**) and 1-(4-hydroxyphenylazo)-2,6-dihydroxynaphthalene (4′,6-di(OH)-Sudan I, **2**) were synthesized as described previously[17,18] and purified by column chromatography on basic alumina and by thin layer chromatography (TLC) on silica gel[17] (Woelm).

^{14}C-Labelled Sudan I (20 MBq/mmol) was synthesized as described earlier[19]. The labelled compound was stored in methanol at –17 °C.

Preparation of Microsomes

Tulip bulbs (*Tulipa fosteriana* L.) were extracted without any inducing pretreatment. Microsomes were prepared as described previously[15] and resuspended in 0.1 M sodium phosphate (pH 7.4), containing 30% (v/v) glycerol and stored at –70 °C.

Purification of Cytochrome P-450

The cytochrome P-450 enzyme used for the reconstitution experiments was isolated from tulip bulb microsomes by the procedure we described previously[15]. The specific content of cytochrome P-450 in the final preparation was 5.2 nmol/mg protein. The molecular mass of this enzyme by SDS-gel electrophoresis is 54 200 (ref.[15]).

Purification of NADPH-Cytochrome P-450 Reductase

A homogeneous preparation of NADPH-cytochrome P-450 reductase was isolated from solubilized tulip bulb microsomes by the procedure as described[15]. The final preparation of NADPH-cytochrome P-450 reductase had a specific activity of 77.5 nmol/min mg. The molecular mass of the enzyme by SDS-gel electrophoresis is 77 600 (ref.[15]).

Analytical Methods

Quantitative determination of cytochrome P-450 was carried out according to Omura and Sato[20]. The concentration of NADPH-cytochrome P-450 reductase was estimated as described earlier[21]. The activity of NADPH-cytochrome P-450 reductase was measured according to Sottocasa et al.[22] using cytochrome c as the substrate (i.e. as NADPH-cytochrome c reductase). Protein concentrations were estimated according to Bradford[23] or Lowry et al.[24] with bovine serum albumin as a standard.

The assay mixture for the oxidation reactions of Sudan I contained, in 1 ml, 50 pmol cytochrome P-450 from tulip bulbs, 80 pmol NADPH-cytochrome P-450 reductase from the same source, 10 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 150 μl of 3 mM [^{14}C]Sudan I (or unlabelled Sudan I) dissolved in methanol. After 1 min preincubation, the reaction was started by addition of NADPH (final concentration was 1 mM). The mixtures were incubated for 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 hexane–diethyl ether–acetone (1 : 0.7 : 0.3, v/v). The same TLC were performed with standards. The products of [^{14}C]Sudan I oxidation and the residual parent compound were scraped 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[19,25,26]. The benzenediazonium ion was detected by azo coupling with 1-phenyl-3-methyl-5-pyrazolone as described in our previous paper[19]. Alternatively, the products, dissolved in methanol, were separated by high-performance liquid chromatography (HPLC) on a Separon SGX C18 (Tessek, Czech Republic) 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 (ref.[17]). The eluted peaks were compared with standards. The retention times of derivatives **2**, **4**, **3** and **5** were 3.0, 5.7, 6.9 and 15.1 min, respectively.

The 1 ml assay mixture for oxidation reactions of Sudan I catalyzed by microsomes contained 10 mM potassium phosphate, pH 7.4, 0.42 nmol of cytochrome P-450 (measured in microsomal preparations containing 3–4 mg/ml protein), 0.4 units of glucose-6-phosphate dehydrogenase, 4 mM glucose-6-phosphate, 7.5 mM $MgCl_2$ 50 μl of 3 mM [^{14}C]Sudan I dissolved in methanol. The procedure was the same as that described for the reconstitution experiments (see above).

Carbon monooxide inhibition of Sudan I oxidation was performed by the addition of a 50 μl aliquot of 50 mM phosphate buffer saturated with CO to the incubation medium either in darkness or under white light from a 15 cm distant 150 W heat filtered lamp.

RESULTS

Sudan I which is oxidized by liver microsomal cytochromes P-450 (refs[17,19,25,26]), can also be oxidized by microsomes of tulip bulbs. The conversion of Sudan I had an absolute requirement for NADPH. The reaction was negligible when the NADPH regenerating system was omitted (Table I). NADH is a less efficient cofactor than NADPH.

The Sudan I conversion was significantly inhibited by CO and this inhibition was partly reversed upon irradiation of the incubation mixture (Table I).

Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), which acts as a heterocyclic ligand, binding to both the oxidized and reduced form of cytochrome P-450 heme, competing with substrate and oxygen, inhibited the oxidation of Sudan I (Table I).

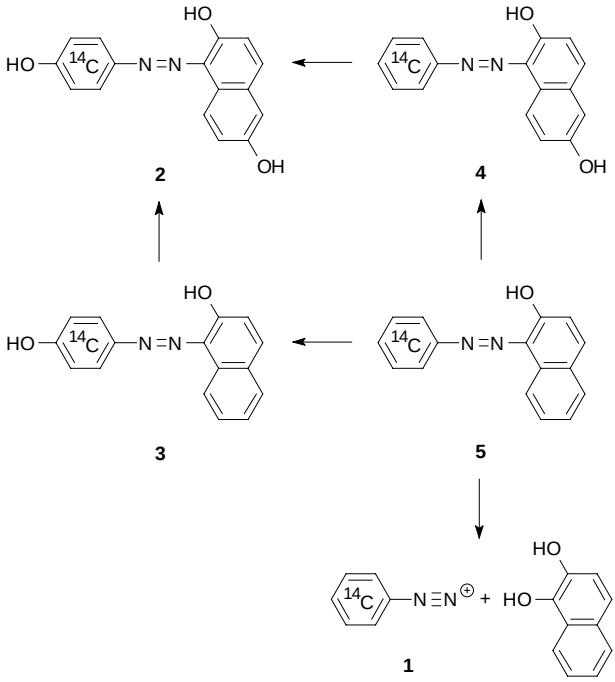
Thin layer chromatography analyses of [^{14}C]Sudan I oxidation products indicate that Sudan I is converted by plant microsomes into five products. Two major products co-chromatographed with 4′-OH-Sudan I (**3**) and 6-OH-Sudan I (**4**). One minor product cochromatographed with 4′,6-di(OH)-Sudan I (**2**). The colourless product which has hydrophylic nature (it practically remained at the start on the TLC and has R_F = 0.02 (Table II) was identified previously[19] as compound derived from the benzenediazonium ion (BDI) (Scheme 1). We have already determined that analogous *C*-hydroxy derivatives of Sudan I and BDI are also formed from Sudan I by microsomal cytochrome P-450 of rat livers[19,25,27]. The results suggest strongly that oxidation of Sudan I is catalyzed by cytochrome P-450 enzymes present in microsomes of tulip bulbs.

In order to examine whether the oxidative reactions are catalyzed solely by the cytochrome P-450 dependent monooxygenase system, a reconstituted system composed of purified cytochrome P-450, NADPH-cytochrome P-450 reductase, and dilauroylphosphatidylcholine was used. Cytochrome P-450 isolated from microsomes of tulip bulbs was mixed with NADPH-cytochrome P-450 reductase purified from the same source and with dilauroylphosphatidylcholine. A molar ratio of 0.65 of cytochrome P-450 to the reductase, which was shown to be the most appropriate for the

TABLE I
Inhibition of Sudan I oxidation catalyzed by a reconstituted and crude cytochrome P-450 system from tulip bulbs. For experimental conditions see the text

Incubation condition	Degree of Sudan I conversion[a], %	
	Microsomes (crude system)	Reconstituted system
Complete	13.8 ± 1.1[b]	19.0 ± 1.5
– NADPH regenerating system	0.7 ± 0.1	0
+ NADH (0.5 mM) instead of NADPH	3.1 ± 0.2	0
+ CO-buffer (50 μl) darkness	5.7 ± 0.5	8.3 ± 0.8
+ CO-buffer (50 μ) light	10.0 ± 0.9	11.2 ± 1.0
+ metyrapone (0.1 mM)	2.9 ± 0.2	7.1 ± 0.6

[a] The amount of [^{14}C]Sudan I was determined by the procedure described in Experimental. [b] Means and standard deviations of three experiments.



SCHEME 1

TABLE II

Products formed from [^{14}C]Sudan I by plant microsomes expressed in the relative radioactivity (%). For experimental conditions see the next

Products[a]	% of total redioactivity[b]		R_F
	microsomes + NADPH regenerating system	microsomes without NADPH regenarating system	
1[c]	3.10 ± 0.30	0.13 ± 0.01	0.02
2	0.80 ± 0.08	[d]	0.23
3	6.67 ± 0.63	0.14 ± 0.01	0.47
4	1.69 ± 0.16	[d]	0.53
5	87.74 ± 8.82	99.73 ± 9.50	0.87

[a] Compounds separated by TLC [hexane–diethyl ether–acetone (1 : 0.7 : 0.3, v/v)] after extraction by ethyl acetate (see Experimental). [b] Means and standard deviations of three experiments. [c] Detected by azo coupling with 1-phenyl-3-methyl-5-pyrazolone[19]. Other products formed from BDI could also be present. [d] Not detected.

catalysis of the metabolism of xenobiotics[16], was also used in the experiments with Sudan I. The requirement of cofactor (NADPH) and inhibition by CO and metyrapone for Sudan I oxidation by the plant cytochrome P-450 reconstituted system are shown in Table I.

The products of Sudan I oxidation generated by the reconstituted plant cytochrome P-450 system were separated by HPLC (Fig. 1). *C*-Hydroxyderivatives **3** (4′-OH-Sudan I), **4** (6-OH-Sudan I) and **2** [4′,6-di(OH)-Sudan I] (which are also formed by the crude microsomal system) were determined to be the metabolites formed by the reconstituted plant P-450 system (Fig. 1). They were identified by comparison of their retention times with those of authentic standards (Fig. 1).

DISCUSSION

The present paper describes the conversion of an exogeneous substrate of the plant microsomal mixed function oxidases, namely, the azo dye Sudan I. The crude microsomes as well as a purified plant cytochrome P-450 reconstituted with NADPH-cytochrome P-450 reductase are able to oxidize this xenobiotic. These results are important for the evaluation of the role of cytochromes P-450 in the metabolism of exogeneous compounds in plants. It was suggested previously that it is questionable whether cytochromes P-450 are the major plant enzymes participating in oxidative reactions converting xenobiotics[1,28]. The activity of a purified cytochrome P-450 protein fraction, reconstituted with its reductase, with respect to xenobiotics was determined only in several cases. An avocado cytochrome P-450 carried out demethylation of *p*-chloro-*N*-methylaniline, by a reductase-mediated monooxygenase reaction[13,14]. Several xenobiotics including the herbicide chlorotoluron are oxygenated by a recombinant plant cinnamate 4-hydroxylase CYP73 (from *Helianthus tuberosus* tubers) produced in yeast[29,30]. Cytochrome P-450 of tulip bulbs isolated in our laboratory is able to oxidize *N*-nitrosamines and aminopyrine[15,16]. As it is shown here, it also converts an azo dye

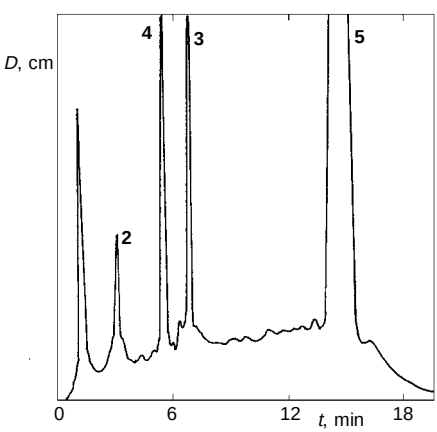


FIG. 1
HPLC profile of the mixture of Sudan I and its products formed by a plant cytochrome P-450 reconstituted system. *D* is recorder deflexion

Sudan I. Therefore, the results presented are the additional confirmation that plant cytochromes P-450 can be responsible for the metabolism of at least several xenobiotics in plants. We previously described[15,16] that isolated tulip cytochrome P-450 interacts with several other compounds. Cinnamic acid, lauric acid and hexobarbital elicited type I binding spectra with a maximum at around 390 nm and a minimum at around 420 nm (refs[15,16]). Therefore, it could be supposed that these compounds may be substrates of tulip cytochrome P-450. We, however, have still not studied their conversion in the plant cytochrome P-450 reconstituted system.

Although an isolated cytochrome P-450 fraction used in our experiments was shown as one band of protein (in one dimensional SDS-electrophoresis[15]), we cannot exclude that this fraction may contain several cytochrome P-450 isoforms with the same apparent molecular weight. This remains to be resolved in further studies.

The products of the Sudan I oxidation in the plant crude microsomes and in the fully reconstituted enzyme systems are *C*-hydroxy derivatives similarly like in the rat liver microsomal enzymes[19,25,26]. However, the liver microsomal enzymes are more effective in conversion of the studied azo dye than plant ones.

Oxidation of Sudan I by another plant enzyme, namely, peroxidase (horseradish peroxidase as a model) was also studied in detail[25,31–33]. Sudan I is oxidized by plant peroxidase much more efficiently than by plant cytochrome P-450. Furthermore, the patterns of products formed by both enzymes are different. *C*-Hydroxy derivatives of Sudan I were minor products, the BDI and other seven products (some of them are dimers or oligomers of Sudan I) were major ones[31–33].

The results presented in this paper and future in vivo experiments will provide evidence for the biological significance of either cytochrome P-450- and/or peroxidase-mediated oxidation for Sudan I metabolism in plants.

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