OXIDATION OF AZO DYES BY PEROXIDASE: ADDITIONAL EVIDENCE OF A ONE-ELECTRON MECHANISM OF OXIDATION OF DIMETHYLAMINOAZOBENZENE AND SUDAN I (SOLVENT YELLOW 14)

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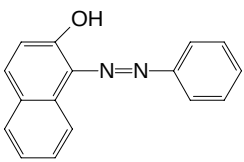
In the presence of hydrogen peroxide, peroxidase oxidized aminoazo dyes, the non-aminoazo dye 1-phenylazo-2-hydroxynaphthalene (Sudan I, Solvent Yellow 14), and its *C*-hydroxy derivatives. The oxidation of azo dyes is a pH-dependent reaction; while slightly acidic conditions are optimal for the aminoazo dyes, a basic pH suits better for Sudan I and its hydroxy derivatives. The oxidation of the carcinogenic Sudan I and dimethylaminoazobenzene catalyzed by peroxidase was investigated in detail. Oxygen consumption was not observed in incubations of peroxidase, azo dyes and hydrogen peroxide. However, oxygen uptake was observed after the addition of glutathione, which indicates that free radical metabolites of these compounds are formed by peroxidase. The results suggest that peroxidase metabolizes Sudan I and dimethylaminoazobenzene through a one-electron oxidation mechanism, giving rise to free radicals. Three of the products of Sudan I oxidation by peroxidase with a hitherto unknown structure were characterized partly by UV/VIS and mass spectroscopy.
Key words: Azo dyes; Oxidation; Peroxidase; Radicals.

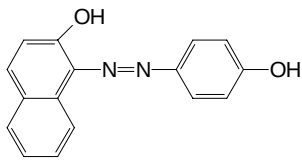
Azo dyes are compounds which are widely used in the textile, cosmetic, printing, drug and food processing industries, as well as in chemical laboratories[1,2]. Azo dyes constitute an important class of xenobiotics, exhibiting toxic and carcinogenic effects[3,4]. They must undergo oxidative conversion to become carcinogenic[3,4]. The mechanism of initiation of chemical carcinogenesis induced by carcinogenic aminoazo dyes (the liver carcinogen *N*,*N*-dimethyl-4-aminoazobenzene (DAB) and its derivatives) has been elucidated. *N*-Hydroxylation catalyzed by cytochrome P-450 or flavin-containing monooxygenases[5,6] is a key activation step for aminoazo dyes in a number of tissues. *N*-Hydroxy metabolites are compounds which are converted to the ultimate carcinogens, i.e. nitrenium and carbenium ions[4,7]. These ions bind to DNA in vivo and in vitro[7], and in this manner are responsible for the carcinogenicity of aminoazo dyes[2,4,7]. DAB (and/or *N*-methyl-4-aminoazobenzene (MAB)) is also converted by the peroxidase system to the detoxification as well as activation intermediate(s) or product(s)[8–10].

The metabolism of non-aminoazo dyes (1-phenylazo-2-hydroxynaphthalene (Sudan I) as the model compound) has also been studied extensively and the carcinogenic activation pathways as well as the detoxication steps have been investigated[11–16]. Sudan I forms the benzenediazonium ion (BDI) during cytochrome P-450 catalyzed metabolism which reacts with DNA in vitro[11,12,16]. The major Sudan I-DNA adduct has been characterized and identified as a 8-(phenylazo)guanine adduct[17]. In addition to cytochrome P-450, Sudan I is also activated by peroxidase[13,14]. We suggest that the cytochrome P-450- or peroxidase-mediated activation of Sudan I and/or a combination of both mechanisms is responsible for the organ specificity of this carcinogen for liver and the urinary bladder in animals[13,18].

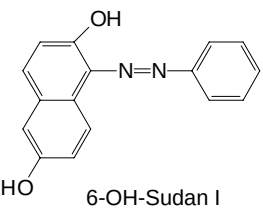
The present paper, which is a continuation of our previous studies, is aimed at contributing to the knowledge of the substrate specificity of peroxidase with respect to azo dyes. The mechanisms of DAB and Sudan I oxidation reactions catalyzed by peroxidase are studied in detail.



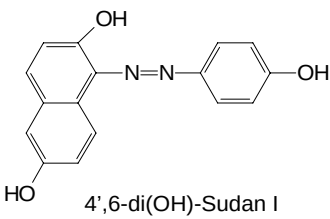
Sudan I



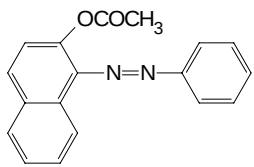
4'-OH-Sudan I



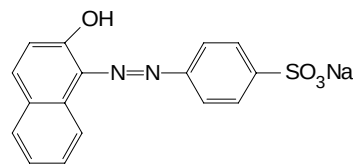
6-OH-Sudan I



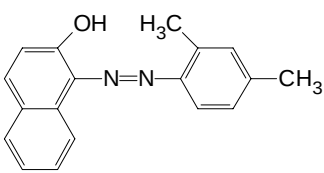
4',6-di(OH)-Sudan I



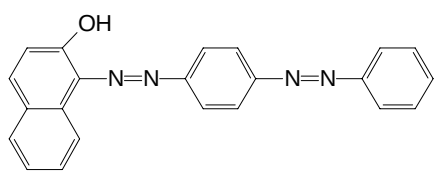
O-acetyl-Sudan I



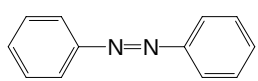
Orange II



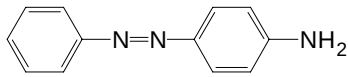
Sudan II



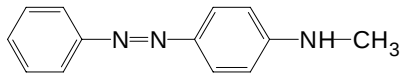
Sudan III



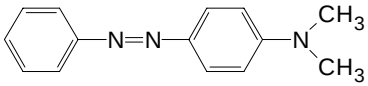
azobenzene



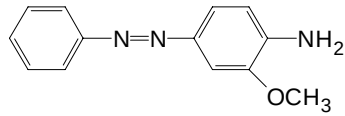
4-aminoazobenzene



4-methylaminoazobenzene



4-dimethylaminoazobenzene



3-methoxy-4-aminoazobenzene

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: Sudan I (1-phenylazo-2-hydroxynaphthalene) from British Drug Houses, DAB, MAB and 4-aminoazobenzene (AB) from Merck, horseradish peroxidase and glutathione (reduced) from Boehringer; all the other chemicals were reagent grade or better. The derivatives 4′-OH-Sudan I and 4′,6-di(OH)-Sudan I were synthesized from the corresponding aminophenols and β-naphthol (or 2,6-dihydroxynaphthalene in the case of 4′,6-di(OH)-Sudan I), 6-OH-Sudan I from phenol and 2,6-dihydroxynaphthalene, as described in refs[16,19], and purified by column chromatography on basic alumina and by thin layer chromatography on silica gel[16] (Woelm). *O*-Acetyl-Sudan I was synthesized from Sudan I (100 mg) dissolved in 20 ml of acetonitrile with 0.5 ml acetanhydride and 5 ml of pyridine. The synthesized *O*-acetyl-Sudan I (50% yield) and residual Sudan I were precipitated from the organic phase with excess distilled water, and washed with water (fivefold). The azo dyes were dissolved in ethyl acetate and purified by thin layer chromatography (TLC) on silica gel (Woelm).

Oxidation of Sudan I by the Peroxidase System

The reaction mixtures contained in a final volume of 1.0 ml: 50 mM Tris-HCl buffer pH 8.4, 0.2 mg of horseradish peroxidase, 0.15 mM Sudan I dissolved in methanol (50 μl/1 ml incubation) and 0.5 mM H_2O_2. The mixtures after incubation (37 °C, 20 min) were extracted twice with ethyl acetate (2 × 2 ml). The ethyl acetate extracts were evaporated under a stream of nitrogen, dissolved in a minimal volume of methanol, and subjected to TLC on silica gel (Kavalier, Czech Republic and/or Schleicher and Schuell, Germany) and estimation of the amount of unconverted Sudan I as described previously[13,14,20]. Spots due to Sudan I and the products were cut out of the thin layer plates and extracted with methanol (2 × 2 ml). Analogous conditions and procedures were used for the preparation of the Sudan I oxidation products for mass spectroscopy, only the concentrations of Sudan I (peroxidase and H_2O_2) were twenty times higher. The products of Sudan I oxidation used for analysis by UV/VIS and/or mass spectroscopy were purified after TLC on a silica gel column (1 × 5 cm) using methanol as the eluent. Spectra were measured on a SPECORD M-40 UV/VIS spectrophotometer and a FINNIGAN MAT INCOS 50 mass spectrometer (electron impact, 70 eV, low resolution mode, direct inlet). The products of Sudan I oxidation with R_F 0.385, 0.575, and 0.690 were analyzed.

Oxidation of Azo Dyes by the Peroxidase System

The structure of the compounds studied are shown below. The reaction mixture contained in a final volume of 2.0 ml: 50 mM Tris-HCl buffer pH 4.71, 7.46 and 8.4, 0.2 mM H_2O_2, and 0.15 mM azo dye dissolved in methanol (100 μl/2 ml incubation). The mixtures after incubation (37 °C, 30 min) were extracted twice with ethyl acetate (2 × 3 ml). The ethyl acetate extracts were evaporated and chromatographed on a thin-layer of silica gel to estimate the amounts of the unconverted azo dyes as described above and in our previous papers[13,20–22].

Measurement of Oxygen Consumption

Oxygen consumption was measured with a Clark-type electrode at 20 °C. The standard reaction mixtures contained in a volume of 1 ml: 50 mM Tris-HCl pH 5.2 and 7.7 for DAB and Sudan I, respectively, 0.2 mM DAB or Sudan I 10 μg of horseradish peroxidase, 100 μM H_2O_2 and 0–1 mM glutathione. The reaction were started by addition of the respective azo dye.

RESULTS AND DISCUSSION

Peroxidase (horseradish peroxidase as a model) catalyzes the oxidation of several azo dyes in vitro. The aminoazo dyes (DAB, MAB and aminoazobenzene (AB)) as well as the non-aminoazo dyes Sudan I and its *C*-hydroxy derivatives are efficiently oxidized (Table I). Compounds structurally similar to Sudan I, namely Sudan II, Sudan III and

TABLE I
Oxidation of azo dyes by the peroxidase system. The values are averages and standard deviations of three parallel experiments. For experimental conditions see the text

Compound	Degree of conversion, %		
	pH 4.71	pH 7.46	pH 8.4
Sudan I	22.0 ± 0.3	56.0 ± 0.6	85.3 ± 0.9
4′-OH-Sudan I	17.2 ± 0.2	34.3 ± 0.3	60.1 ± 0.6
6-OH-Sudan I	33.2 ± 0.3	62.5 ± 0.6	93.3 ± 0.9
4′,6-Di(OH)-Sudan I	34.8 ± 0.4	64.1 ± 0.6	94.2 ± 1.2
O-Acetyl-Sudan I	0	0	0
Orange II	0	0	0
Sudan II	0	0	0
Sudan III	0	0	0
Azobenzene	3.2 ± 0.3	0	0
4-Aminoazobenzene	90.2 ± 0.9	31.1 ± 0.3	12.2 ± 0.1
4-Methylaminoazobenzene	87.3 ± 0.9	30.1 ± 0.3	10.5 ± 0.1
4-Dimethylaminoazobenzene	85.2 ± 0.9	28.3 ± 0.3	8.2 ± 0.1
3-Methoxy-4-aminoazobenzene	52.8 ± 0.3	10.1 ± 0.1	2.1 ± 0.1

Orange II, do not act as peroxidase substrates in the conditions used. If the benzene ring in the molecule of Sudan I is substituted by a methyl, phenylazo, or sulfo group, the resulting compound is not oxidized by peroxidase. The presence of a free hydroxy group in the Sudan I molecule is of key importance for its oxidation by peroxidase: the derivative of Sudan I in which the hydroxy group was acetylated (*O*-acetyl-Sudan I) was not oxidized by peroxidase (Table I).

The oxidation of azo dyes is a pH-dependent reaction. Unlike the hydroxyazo dyes Sudan I and its *C*-hydroxy derivatives, whose optimum pH lies in the basic region (pH 8.0–8.5), the oxidation of DAB or other aminoazo dyes showed an optimum in slightly acidic conditions (Table I).

The oxidation of Sudan I and DAB has been studied previously[10,13,14,20–22]. No explanation of the mechanism of oxidation of the azo dyes exists yet. Herein, we extend the study to the mechanism of their oxidation by peroxidase.

Most of Sudan I or DAB is oxidized by peroxidase within the first five minutes of reaction (Fig. 1). This fast reaction rate as well as the inhibition of oxidation by radical scavengers[13,14,21,22] indicates that the primary reaction products are free radicals.

Peroxidases catalyze the one-electron oxidation of aromatic amines, giving rise to nitrogen- and/or carbon-centered radicals[23]. Although their detection by electron spin resonance is difficult, aromatic amine free radicals can be detected indirectly based on their reaction with glutathione giving the parent compound and a glutathionyl free radical (GS$^{\bullet}$). The radicals can react with oxygen[24–26], and the oxygen consumption in the solution can be monitored by using an oxygen electrode. No oxygen consumption took place during the incubation of peroxidase, DAB and H_2O_2 but it was observed after addition of glutathione to the reaction mixture (Table II). Such observations indicate

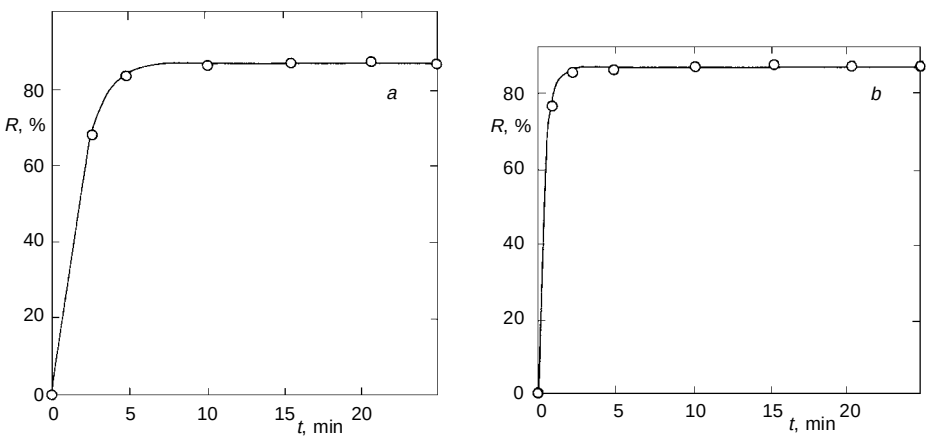


FIG. 1

Time dependence of the oxidation of Sudan I (*a*) and DAB (*b*) by the peroxidase/H_2O_2 system given as a percentage of converted azo dyes (*R*)

that peroxidase oxidizes DAB to a free radical metabolite(s). DAB is oxidized by peroxidase to MAB, AB, and four unknown products[10]. The formation of MAB and AB by *N*-demethylation reactions is supposed to be the one-electron radical reaction. Griffin and Ting[27] and Guengerich[28] postulated that peroxidase-catalyzed *N*-demethylation results in the formation of a free radical cation and an iminium cation by sequential one-electron oxidations, the latter of which is hydrolyzed to formaldehyde and the demethylated amine.

The addition of hydrogen peroxide to an incubation of Sudan I and peroxidase brings about changes in the absorption spectrum of the reaction mixture. During the reaction,

TABLE II

Oxygen consumption by DAB or Sudan I with peroxidase in the presence of various concentrations of glutathione. Oxygen uptake without xenobiotics or H_2O_2 was 0.0016 nmol/s. The values given are averages and standard deviations of three parallel experiments. For experimental conditions see the text

Glutathione, mmol/l	Oxygen uptake (nmol/s) in the presence of	
	Sudan I	DAB
0	0	0
0.25	1.43 ± 0.1	13.21 ± 0.9
0.50	2.95 ± 0.3	26.90 ± 1.8
0.75	4.45 ± 0.4	39.85 ± 2.1
1.00	5.80 ± 0.6	50.80 ± 4.2

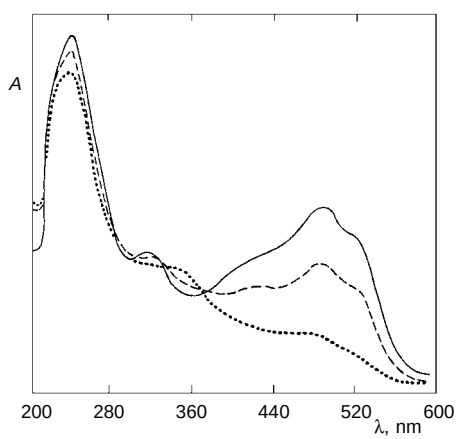


FIG. 2

Oxidation of Sudan I by the peroxidase/H_2O_2 system. The samples (1 ml) contained 50 mM tris-HCl buffer pH 8.4, 0.2 mg horseradish peroxidase, 0.15 mM Sudan I, and 0.5 mM H_2O_2. The spectra were recorded sequentially at 0 min (———), 2.5 min (– – –) and 10 min (· · · ·)

the absorption maximum at 480 nm (due to Sudan I) decreases while the absorbance at about 340 nm increases slightly (Fig. 2).

Sudan I contains a free hydroxy group in its molecule. Many phenolic compounds can serve as substrates for peroxidases, being oxidized to phenoxyl radicals which will undergo secondary reactions in dependence on their individual free radical chemistries[23]. Oxygen-centered free radicals can also oxidize glutathione to a glutathionyl free

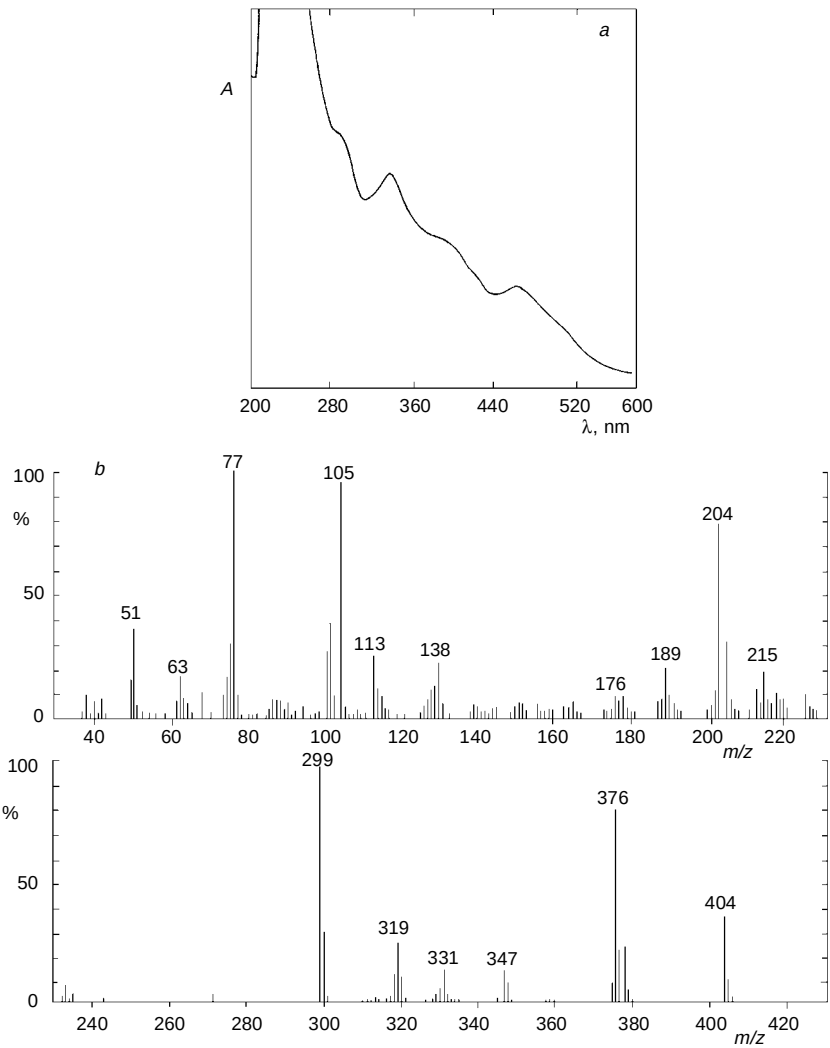


FIG. 3

UV/VIS spectrum (*a*) and mass spectrum (*b*) of the Sudan I oxidation product with R_F = 0.385 (TLC on silica gel)

radical. We found that glutathione is oxidized by Sudan I during the peroxidase-mediated reaction. Again, the oxygen uptake is increased appreciably by the formation of thiyl radicals (Table II). This can again be caused by a mechanism involving interaction of the reduced glutathione with the xenobiotic radical (a Sudan I radical) and subsequent formation of a thiyl radical. The thiyl radical reacts with oxygen (Table II) and eventually produces glutathione disulfide. These data indicate that peroxidase oxidizes Sudan I,

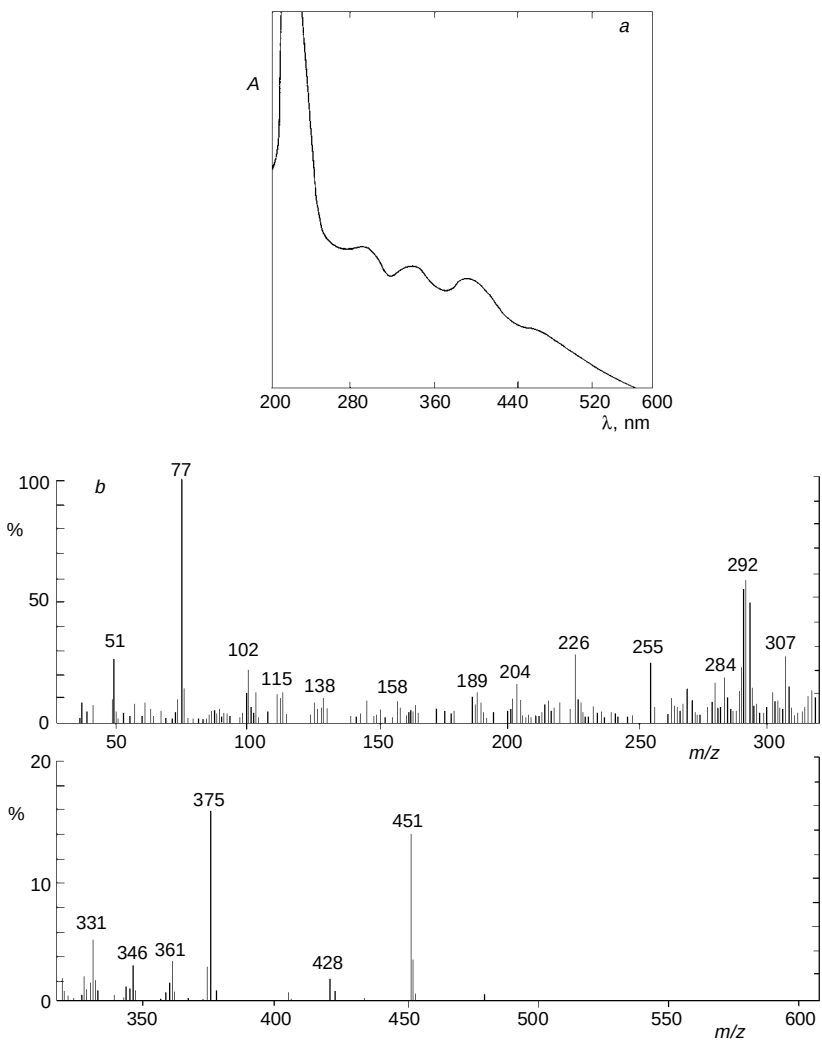


FIG. 4

UV/VIS spectrum (*a*) and mass spectrum (*b*) of the Sudan I oxidation product with R_F = 0.575 (TLC on silica gel)

giving rise to an oxygen-centered radical (naphthoxyl radical) intermediate. This hypothesis is borne out by the fact that the derivative of Sudan I with the OH group acetylated (*O*-acetyl-Sudan I) is not oxidized by peroxidase (Table I).

Ten products emerged from the reaction of Sudan I with peroxidase in our previous study, among which *C*-hydroxy derivatives of Sudan I (6-OH-Sudan I and 4′,6-di(OH)-Sudan I) were minor products and the benzenediazonium ion was one of the major

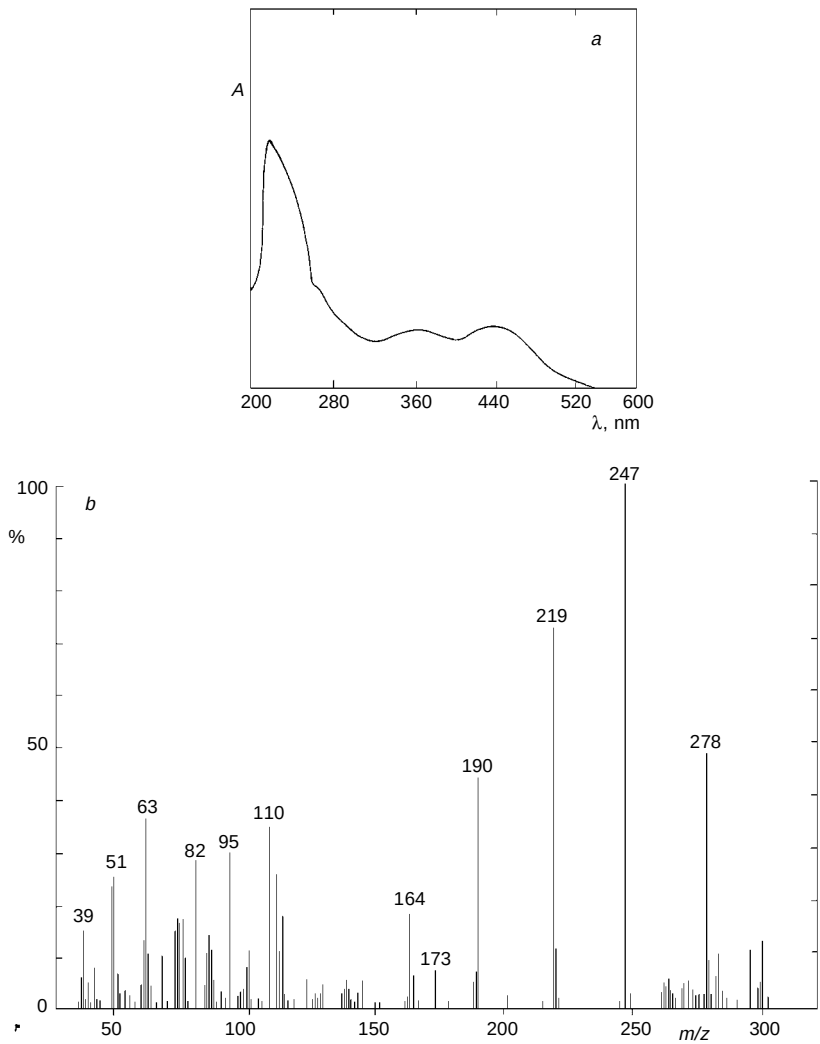


FIG. 5

UV/VIS spectrum (*a*) and mass spectrum (*b*) of the Sudan I oxidation product with R_F = 0.690 (TLC on silica gel)

products[22]. Most of the other unidentified products were unstable (sensitive to light and elevated temperature), due to which their structures have not been elucidated. In the present paper we characterize three of the unstable products by their UV/VIS and mass spectra (Figs 3–5). In all cases the products decomposed during mass spectroscopy. The fragmentation peaks at *m/z* 77 and/or 105 in the mass spectra of two of the products (Figs 3, 4) indicate the presence of the phenyl and phenyldiazonium ion fragments, respectively, whereas the most prominent peak at *m/z* 247 in the mass spectrum of the third product apparently belongs to the Sudan I ion (Fig. 5). We compared the fragmentation patterns of the three products in question 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 three products studied 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.

The results suggest strongly that the one-electron oxidation products (radicals) are the primary intermediates in the peroxidase-mediated oxidation of DAB and Sudan I. Eling et al.[23] postulated that the fate of the primary free radical formed from xenobiotics by peroxidase depends on the environment in which it exists. Indeed, we found both previously and now that the reactive intermediates (free radical(s) formed from DAB or Sudan I): (i) form dimers and/or oligomers; (ii) undergo further oxidation to two-electron oxidation products (*C*-hydroxy derivatives of Sudan I; *N*-demethylation reactions providing MAB and AB from DAB)[10,20,22]; (iii) react with other compounds of potential physiological interest (reactions with NADH, ascorbate)[10,13,21,22]; (iv) react with the SH group of glutathione (reducing the DAB or Sudan I radicals with the formation of a thiyl radical (present paper) or conjugates[22]) and (v) react with macromolecules to form potentially toxic adducts (the DNA, RNA and protein binding)[10,13,14,20,22].

We have already detected the formation of DNA and RNA adducts from Sudan I and DAB activated by peroxidase, and the target nucleotides of DNA (RNA) binding have been identified[10,14,29]. Structure elucidation of the DNA (or RNA) base-adducts will be the objective of a future study.

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