IDENTIFICATION OF 1-(3,4-DIHYDROXYPHENYLAZO)-2-HYDROXYNAPHTHALENE AS THE PRODUCT OF OXIDATION OF 1-PHENYLAZO-2-HYDROXYNAPHTHALENE (SUDAN I, SOLVENT YELLOW 14) BY RAT LIVER MICROSOMES

Marie STIBOROVA[a], Befekadu ASFAW[a], Eva FREI[b], Heinz H. SCHMEISER[b] and Manfred WIESSLER[b]

[a] *Department of Biochemistry, Charles University, 128 40 Prague 2, The Czech Republic*
[b] *Department of Molecular Toxicology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*

Received March 24, 1994
Accepted September 27, 1994

The carcinogenic azo dye 1-phenylazo-2-hydroxynaphthalene (Sudan I, Solvent Yellow 14) is oxidized by rat liver microsomes to a number of azo-containing metabolites. One of the products of hitherto unknown structure has been shown to be identical with 1-(3,4-dihydroxyphenylazo)-2-hydroxynaphthalene. The identification of this product completed the pattern of major products of Sudan I oxidation by microsomal cytochrome P-450.

Sudan I (1-phenylazo-2-hydroxynaphthalene, Solvent Yellow 14, *VII*) has been studied as a model non-aminoazo dye. This dye was used in several countries[1] for food colouring, however, it was found to be carcinogenic leading to tumors in the liver and/or urinary bladder in rats, mice and rabbits[1–3]. In vivo studies on the metabolism of Sudan I in rabbits and mice revealed that this compound is oxidized by cytochrome P-450 to derivatives hydroxylated in the aromatic rings. The resulting major products 1-(4-hydroxyphenylazo)-2-hydroxynaphthalene (4′-OH-Sudan I, *VI*), 1-phenylazo-2,6-dihydroxynaphthalene (6-OH-Sudan I, *V*) and 1-(4-hydroxyphenylazo)-2,6-dihydroxynaphthalene (4′,6-di(OH)-Sudan I, *III*) were detected as such or conjugated either with glucuronic acid or with sulfate in both urine and bile of animals exposed to this dye[4]. In addition to these *C*-hydroxy derivatives arising from oxidation reactions, Sudan I (*VII*) is in vivo also metabolized by reductive reactions to 1-amino-2-naphthol, aniline and *p*-aminophenol[5].

The same *C*-hydroxylation products as in vivo were formed by rat liver microsomal enzymes containing cytochrome P-450 (refs[6,7]). Oxidative splitting of the azo group of Sudan I was also detected and the product of this reaction, benzenediazonium ion (*I*), was found to be responsible for the formation of adducts with DNA in vitro[6–9].

The previous studies did not identify the whole spectrum of products arising in rat liver microsome catalyzed oxidation of Sudan I. The structure of two metabolites was so far not determined[6,7]. The present paper describes the elucidation of the structure of the major of these two unknown products.

EXPERIMENTAL

Chemicals and Radiochemicals

Chemicals were obtained from the following sources: calf thymus DNA, NADPH, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase from Boehringer (Mannheim, Germany); Sudan I from British Drug Houses (Poole, U.K.), β-naphthoflavone (β-NF) from Sigma (St. Louis, MO, U.S.A.); all other chemicals were reagent grade or better. The derivatives 2′-OH-Sudan I (*VIII*), 3′-OH-Sudan I (*IX*), 4′-OH-Sudan I (*VI*) and 4′,6-di(OH)-Sudan I (*III*) were synthesized from the corresponding aminophenols and β-naphthol (or 2,6-dihydroxynaphthalene in the case of *III*), 6-OH-Sudan I (*V*) from phenol and 2,6-dihydroxynaphthalene as described[10], and purified by column chromatography on basic alumina and by thin-layer chromatography (TLC) on silica gel (Woelm). The ^{14}C-labelled Sudan I (*VII*) (20 MBq/mmol) was synthesized and purified as described earlier[6].

Subcellular Preparations

Microsomes from male Sprague–Dawley rats, induced i.p. with β-NF, were prepared as described elsewhere[6,9,11] and stored at −70 °C.

Incubations and Metabolic Analysis

Unless stated otherwise, incubation mixtures for the study of the oxidation of [^{14}C]-Sudan I or unlabelled Sudan I contained in the final volume of 1.5 ml: 50 mM potassium phosphate (pH 7.7), 2 mM NADPH (or the NADPH regeneration system; 2 mM $NADP^+$, 2.95 mM glucose-6-phosphate and 0.045 units glucose-6-phosphate dehydrogenase), 3 mg of microsomal protein and 0.05 – 0.3 mM [^{14}C]-Sudan I (or unlabelled Sudan I) dissolved in methanol (50 μl/incubation). The same incubation mixtures were used to study the oxidation of *C*-hydroxylated Sudan I metabolites, however, unlabelled derivatives *VIII*, *IX*, *VI* and *V* of Sudan I were used instead of [^{14}C]Sudan I. The mixtures were incubated at 37 °C (0 – 120 min) in open tubes, whereupon [^{14}C]Sudan I and its products (or Sudan I *C*-hydroxy derivatives and their products) were extracted with ethyl acetate (2 × 2 ml). The extracts were evaporated, dissolved in a minimum volume of methanol, chromatographed on a thin layer of silica gel and developed in either hexane–diethyl ether–acetone (1 : 0.7 : 0.3, v/v, TLC-I) or diethyl ether–hexane (3 : 1, v/v, TLC-II). The same TLC was performed with the 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. The benzenediazonium ion (*I*) was detected by azo coupling with 1-phenyl-3-methyl-5-pyrazolone as described in our previous paper[6]. Alternatively, the products, dissolved in methanol, were separated by high-performance liquid chromatography (HPLC) on a Separon SGX C18 (Tessek, The 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. The eluted peaks were compared with standards. The retention times of derivatives *III*, *II*, *IX*, *V*, *VI* and *VII* were 3.0, 3.3, 5.0, 5.7, 6.8 and 15.3 min, respectively.

Metabolites, which were used for UV-VIS and/or mass spectroscopy, were isolated by TLC on preparative silica gel plates (1 mm, Merck). The bands were scraped from the TLC plate, eluted with methanol, rechromatographed on a column of silica gel (elution with redistilled methanol), and then analyzed by mass and/or UV-VIS spectroscopy. UV-VIS spectra were measured on a Perkin–Elmer Lambda 5 UV/VIS spectrophotometer, mass spectrometry was performed on a FINNIGAN MAT INCOS 50 mass spectrometer (electron impact, 70 eV, low-resolution mode, direct inlet).

RESULTS AND DISCUSSION

Microsomal metabolites of Sudan I [4′-OH-Sudan I (*VI*), 6-OH-Sudan I (*V*), 4′,6-di(OH)-Sudan I (*III*), benzenediazonium ion (*I*) (or compounds formed from it) and two unknown products *II* and *IV*] (Scheme 1) were separated by TLC on silica gel or by HPLC (Table I). The product *II* was characterized by mass (Fig. 1) and UV-VIS spectroscopy (Fig. 2). The mass spectrum shown in Fig. 1 suggests that the compound is a dihydroxy derivative of Sudan I (m.w. 281). This product was also formed from 4′-OH-Sudan *I* (*VI*), but not from another major Sudan I hydroxy derivative, 6-OH-Sudan I (*V*). Furthermore, the UV-VIS spectrum of the product *II* was more similar to that of 4′-OH-Sudan I (*VI*) than of 6-OH-Sudan I (*V*) (Fig. 2). We therefore suggest that in this dihydroxy metabolite [formed from Sudan I (*VII*) and 4′OH-Sudan I (*VI*)] both the OH groups are attached to the benzene ring. Only the carbon atoms 2 and 3 of the benzene ring can be the targets for hydroxylation. Unfortunately, we could not characterize the product by NMR, as only insufficient amounts of this metabolite could be isolated even from many incubations. Moreover, the preparation of the corresponding standards [2′,4′-di(OH)-Sudan I and 3′,4′-di(OH)-Sudan I (*II*)] by chemical synthesis from 2′,4′- or 3′,4′-dihydroxybenzenediazonium compounds could not be realized as these ions are extremely unstable. The microsomal enzymes were used for preparation of the suggested standards. 2′-OH-Sudan I (*VIII*), 3′-OH-Sudan I (*IX*) and 4′-OH-Sudan I (*VI*) were used as the substrates for microsomal enzymes. Whereas 2′-OH-Sudan (*VIII*) did

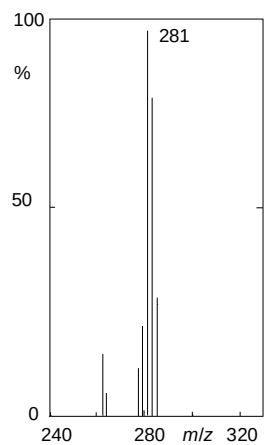
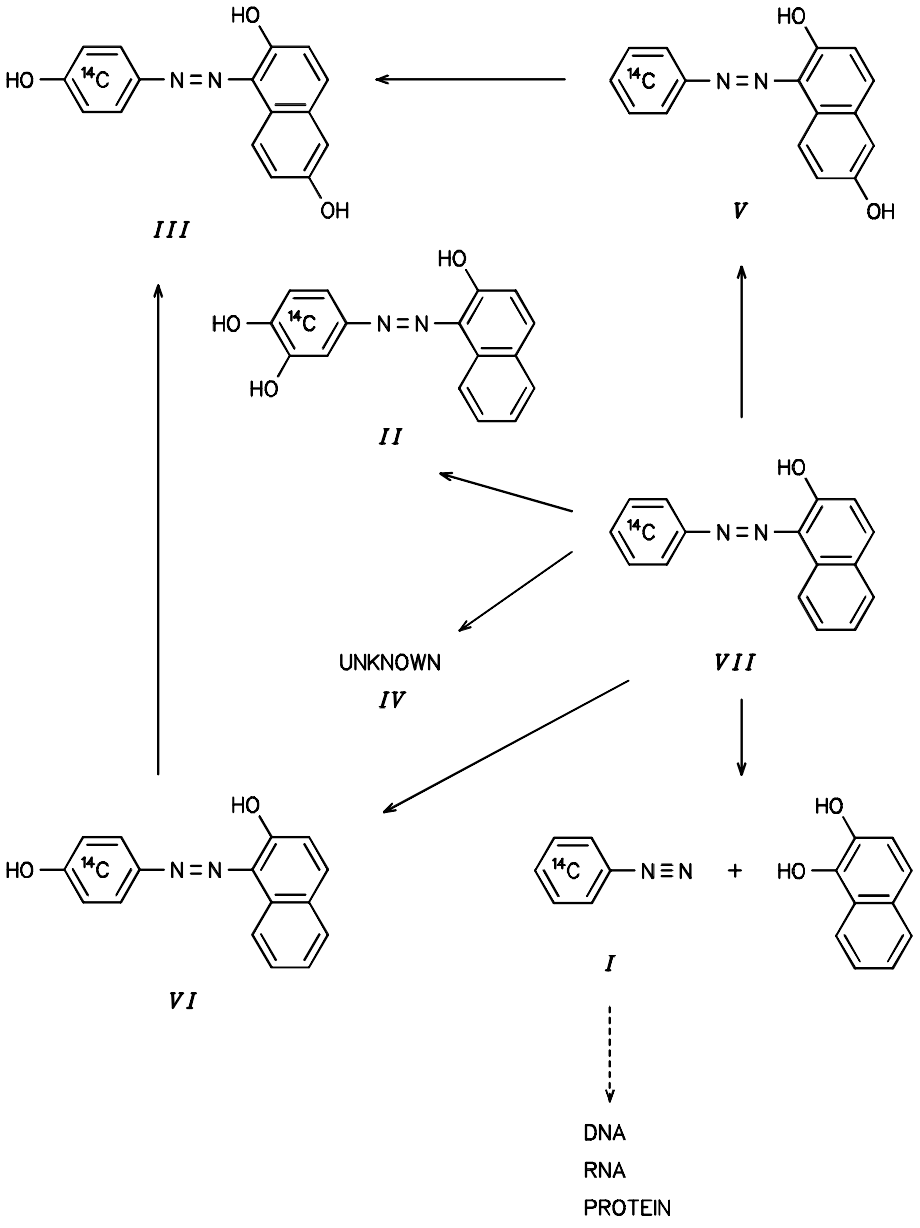


FIG. 1
Mass spectrum of product *II*



SCHEME 1

not react, 3′-OH-Sudan I (*IX*) and 4′-OH-Sudan I (*VI*) were oxidized, both to two coloured products (Table I). Oxidation of 4′-OH-Sudan I (*VI*) afforded 4′,6-di(OH)-Sudan I (*III*) and a compound having identical chromatographic and spectral properties with the unknown dihydroxy metabolite (*II*) [formed from Sudan I (*VII*), Table I, Fig. 2]. The dihydroxy derivative *II* was also formed by oxidation of 3′-OH-Sudan I (*IX*) (Table I, Fig. 2), the second product (*X*) being not identified (Table I).

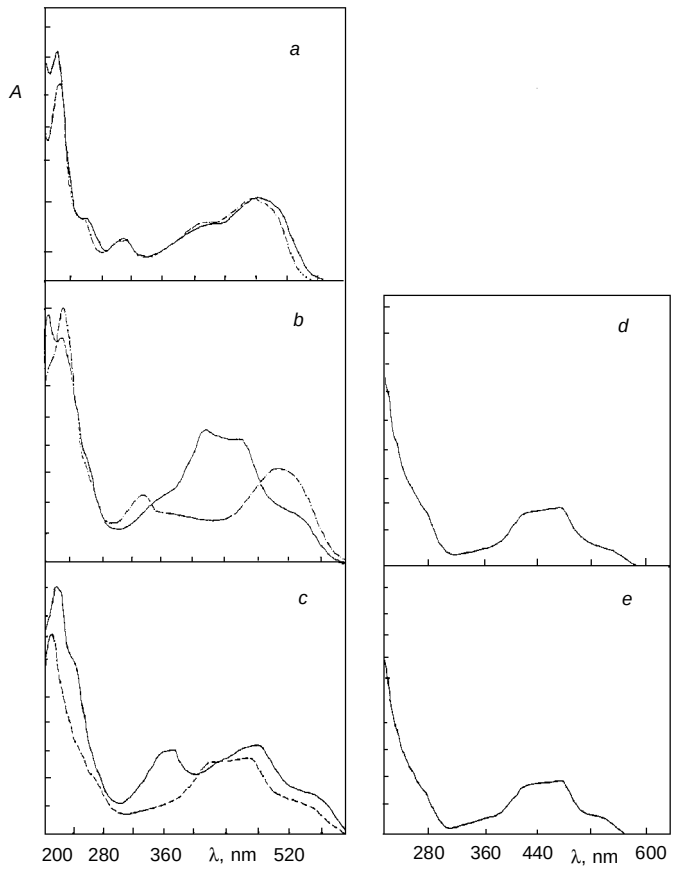


FIG. 2

Absorption spectra of Sudan I and its hydroxy derivatives. *a* Sudan I (*VII*) (– · – · –), 3′-OH-Sudan I (*IX*) (——); *b* 4′-OH-Sudan I (*VI*) (——), 6-OH-Sudan I (*V*) (– · – · –); *c* 4′,6-di-(OH)-Sudan I (*III*) (——), 3′,4′-di(OH)-Sudan I (*II*) (– · – · –); *d* 3′,4′-di(OH)-Sudan I (*II*) formed from 3′-OH-Sudan I (*IX*); *e* 3′,4′-di-(OH)-Sudan I (*II*) formed from 4′-OH-Sudan I (*VI*)

The finding that the unknown product (*II*) was formed from 3′-OH-Sudan I (*IX*) as well as from 4′-OH-Sudan I (*VI*) but not from 2′-OH-Sudan I (*VIII*) suggests that it is 3′,4′-di(OH)-Sudan *I* (*II*) (Table I).

The characterization of this product completed the pattern of the major products arising in microsomal oxidation of Sudan I. The questions concerning the involvement of Sudan I in initiation of liver carcinogenesis, however, still persist. It was found previously that the benzenediazonium ion is responsible for the modification of DNA by Sudan I, activated by microsomes[7,8]. The carcinogenicity of this ion has been established earlier[12–14]. We found that the principal target of benzenediazonium ion formed from Sudan I in DNA was deoxyguanosine and a stable adduct was formed[8]. Koepke et al.[12] recently showed that benzenediazonium ion generated from *N*-nitrosomethylaniline forms an unstable triazene adduct with adenine in DNA. Further studies will be

TABLE I

Characteristics of metabolites formed from Sudan I (*VII*), 3′-OH-Sudan I (*IX*) and 4′-OH-Sudan I (*VI*) in hepatic microsomes from β-NF-treated rats. For details see Experimental

Corresponding standards	R_F^{TLC-I}	R_F^{TLC-II}	R_t^{HPLC}, min	A_{max}, nm
		From Sudan I		
I	0.020	0.030	–	–
III	0.235	0.180	3.0	493.5
II	0.340	0.150	3.3	467.0
VI	0.470	0.520	6.8	414.8 (457.5)[a]
V	0.530	0.390	5.7	506.3
VII	0.870	0.810	15.3	478.3
		From 3′-OH-Sudan I		
X	0.230	0.185	N.D.	508.0
II	0.340	0.150	3.3	467.0
IX	0.505	0.380	5.0	485.3
		From 4′-OH-Sudan I		
III	0.235	0.180	3.0	493.5
II	0.340	0.150	3.3	467.0
VI	0.470	0.520	6.8	414.8 (457.5)[a]

[a] Number in parentheses refers to the second absorption maximum of *VI*.

carried out to determine the structure of the stable and/or unstable adducts in DNA and to resolve the reactions which are crucial for the binding of benzenediazonium ion to DNA bases.

We thank Dr M. Smrcina and Dr B. Maca (Department of Organic Chemistry, Charles University, Prague) for the mass spectral analysis of our samples.

REFERENCES

1. *IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Man*, Vol. 8, p. 25. International Agency for Research on Cancer, WHO Monograph Sci., Lyon 1974.
2. Garner R. C., Martin C. N., Clayson D. B. in: *Chemical Carcinogens* (CH. E. Searle, Ed.), 2nd ed., Vol. 1, p. 175. American Chemical Society, Washington D. C. 1984.
3. Westmoreland C., Gatehouse D. G: Carcinogenesis *12*, 1403 (1991).
4. Childs J. J., Clayson D. S.: Biochem. Pharmacol. *15*, 1247 (1966).
5. Daniel J. W.: Toxicol. Appl. Pharmacol. *4*, 572 (1962).
6. Stiborova M., Asfaw B., Anzenbacher P., Leseticky L., Hodek P.: Cancer Lett. *40*, 319 (1988).
7. Stiborova M., Anzenbacher P.: Collect. Czech. Chem. Commun. *57*, 1537 (1992).
8. Stiborova M., Asfaw B., Anzenbacher P., Hodek P.: Cancer Lett. *40*, 327 (1988).
9. Stiborova M., Frei E., Schmeiser H. H., Wiessler M., Hradec J.: Carcinogenesis *11*, 1843 (1990).
10. Matrka M., Pipalova J.: Collect. Czech. Chem. Commun. *47*, 2711 (1982).
11. Kimura T., Kodama M., Nagata Ch.: Jpn. J. Cancer Res., Gann *73*, 455 (1982).
12. Koepke S. R., Kroeger-Koepke M. B., Michejda Ch. J.: Chem. Res. Toxicol. *3*, 17 (1990).
13. Preussmann R., Ivankovic S., Landschutz C., Gimmy J., Flohr E., Griesbach U.: Krebsforsch. *81*, 285 (1974).
14. Dipple A., Michejda Ch. J., Weisburger E. K. in: *Mechanism of Cell Transformation by Carcinogenic Agents* (D. Grunberger and S. Goff, Eds), p. 1. Pergamon Books Ltd., New York 1987.

Translation revised by M. Tichy.