^{32}P-POSTLABELLING ANALYSIS OF DNA ADDUCTS WITH 1-(PHENYLAZO)-2-NAPHTHOL (SUDAN I, SOLVENT YELLOW 14) FORMED *in vivo* IN FISHER 344 RATS

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We report the analysis of DNA adducts with 1-(phenylazo)-2-naphthol in the liver and urinary bladder of Fisher 344 rats treated orally with this dye. DNA adducts were detected and quantitated using the nuclease P1-enhanced version of the ^{32}P-postlabelling assay. Two variations of multidirectional chromatographic systems were used to resolve either bulky and/or smaller (polar) ^{32}P-labelled adducts by TLC. In the present study, a double oral administration of the dye (500 mg/kg) for one day yielded negative results in ^{32}P-postlabelling assay of liver DNA (24 h after dosing). However, three DNA adducts in the urinary bladder were detected under the same conditions of treatment. Chromatography experiments indicated that the two principal DNA adducts detected in the urinary bladder of Fisher 344 rats were the same as those detected in DNA modified by 1-(phenylazo)-2-naphthol and its metabolite 1-(phenylazo)naphthalene-2,6-diol after their activation with peroxidase *in vitro*. The results presented here strongly suggest that peroxidase itself or in a combination with cytochrome P450 participates in the initiation phase of 1-(phenylazo)-2-naphthol carcinogenesis in the urinary bladder.

Key words: Carcinogens; Carcinogenesis; Azo dyes; Sudan I; DNA adducts; ^{32}P-postlabelling; Nucleotides; CYP450; Peroxidases.

Sudan I [1-(phenylazo)-2-naphthol, Solvent Yellow 14] was used as a food coloring in several countries[1], but it has been recommended as unsafe, because it causes tumors in the liver or urinary bladder in rats, mice, and rabbits[1–4]. Nevertheless, it is widely used to color other materials, such as hydrocarbon solvents, oils, fats, waxes, and shoe and floor polishes[1].

Sudan I gives positive results in *Salmonella typhimurium* mutagenicity tests with S-9 activation[5,6] and is mutagenic to mouse lymphoma L5178Y TK$^{+/-}$ cells *in vitro*, with S-9 activation[6]. It is clastogenic compound, inducing

micronuclei in the bone marrow of rats[3]. *In vivo* studies on the metabolism of Sudan I in rabbits revealed that this compound is metabolized primarily in the liver by oxidative or reductive reactions[7,8]. C-Hydroxylated metabolites 1-(4-hydroxyphenylazo)-2-naphthol (4′-OH-Sudan I) and 1-(phenylazo)naphthalene-2,6-diol (6-OH-Sudan I) were found to be major products of Sudan I oxidation *in vivo*[1,7] and those of its oxidation by microsomal cytochromes P450 (CYP450) *in vitro*[9]. These derivatives were found major excreting products in urines[7]. Besides the C-hydroxylated metabolites, which are considered detoxication products, the benzenediazonium ion (BDI) formed by CYP450-dependent enzymatic splitting of the azo group of Sudan I was found to react with DNA *in vitro*[9–11]. The major DNA adduct formed in this reaction has been characterized and identified as an 8-(phenylazo)guanine adduct[11]. In addition to CYP450, Sudan I and its C-hydroxylated metabolites are also oxidized by peroxidase[12–15]. In these reactions, DNA, RNA and protein adducts are also formed[12–16]. Microsomal CYP450s are assumed to be responsible for the activation of Sudan I in liver[10,11]. However, a limited role of CYP450s in the *in vivo* metabolic activation of Sudan I in the urinary bladder is suggested. This organ has little or no detectable CYP450, but peroxidases are present at relatively high levels in this tissue[17]. In this regard, peroxidase-mediated Sudan I activation in the urinary bladder has been suggested similarly to that reported for several other urinary bladder carcinogens[17–22]. Moreover, we have suggested the CYP450- or peroxidase-mediated activation of Sudan I or a combination of both mechanisms as an explanation for the organ specificity of this carcinogen for liver and urinary bladder in animals[11]. Problems, however, arise in establishing the real contribution of CYP450s or peroxidases in activation pathways of Sudan I *in vivo*. A suitable approach involves the analysis of DNA adducts specifically derived from either CYP450 or peroxidase activation *in vitro* and a comparison of these adducts with those formed *in vivo*. While DNA adducts derived from Sudan I *in vitro* were detected and partially characterized[10–16], nothing is known about the DNA adduct formation from this carcinogen in organisms *in vivo*, because such *in vivo* studies have not been yet performed. Therefore, our study is focused on investigation of the formation of DNA adducts by Sudan I in tissues of target organs for Sudan I carcinogenesis (liver and urinary bladder) of Fisher 344 rats treated with Sudan I.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: Sudan I [1-(phenylazo)-2-naphthol] from BDH; horseradish peroxidase from Sigma; DNA (calf thymus) from Boehringer. All chemicals were reagent grade or better. 1-(4-Hydroxyphenylazo)-2-naphthol (4′-OH-Sudan I) was synthesized from 4-aminophenol and 2-naphthol, 1-(phenylazo)naphthalene-2,6-diol (6-OH-Sudan I) from aniline and 2,6-dihydroxynaphthalene, as described in refs[23–25]. They were purified by column chromatography on basic alumina and by TLC on silica gel[24,25] (Woelm). Enzymes and chemicals for the ^{32}P-postlabelling assay were obtained commercially from sources described previously[26].

Analytical Method

Incubation mixtures used for the modification of calf thymus DNA with Sudan I, 4′-OH-Sudan I and 6-OH-Sudan I oxidized with peroxidase (horseradish peroxidase used as a model) contained in 1.0 ml of 50 mM Tris-HCl buffer (pH 8.4): 0.2 mg horseradish peroxidase, 0.15 μmol Sudan I or 4′-OH-Sudan I or 6-OH-Sudan I dissolved in methanol (50 μl/1 ml incubation), 0.5 μmol hydrogen peroxide and 1 mg calf thymus DNA. Control incubations were carried out either without peroxidase or without Sudan I or its hydroxy derivatives. Incubations were performed at 37 °C for 60 min. Reactions were stopped by extraction with 2 volumes of ethyl acetate (twice). DNA was isolated by the phenol/chloroform procedure (twice) as described by Kirby[27], modified by Schoepe *et al.*[28] and precipitated with ethanol[11,29]. The DNA content was quantitated spectrophotometrically at 260 nm.

Animal Experiments

Gavage with Sudan I dissolved in sunflower oil (500 mg/kg body weight) was administered to four male Fisher 344 rats (100–150 g) twice a day for one day. Two control animals received an equal volume of oil. Rats were placed in cages in temperature- and humidity-controlled rooms. Standardized diet and water were provided *ad libitum*. Animals were killed 24 h after the last treatment by cervical dislocation. Liver and urinary bladder of animals were excised immediately after sacrifice, quickly frozen in liquid nitrogen, and stored at –80 °C untill the DNA isolation. DNA was isolated from the organs by the phenol/chloroform procedure as it is described for *in vitro* experiments (see above). DNA was dissolved in sterile water and the solution stored at –80 °C.

^{32}P-Postlabelling and Recovery of Individual Nucleotide Adducts

For DNA modified with Sudan I and its metabolites after the reaction with peroxidase and for DNA modified with Sudan I *in vivo*, the nuclease P1 version[29] of the ^{32}P-postlabelling assay[30] was used. DNA was hydrolyzed to deoxyribonucleoside 3′-monophosphates using micrococcal nuclease and spleen phosphodiesterase. Nuclease P1-treated samples were labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP under conditions described previously[13,14,31]. The same ^{32}P-postlabelling procedure was used for DNA of controls. In several cases, the version of ^{32}P-postlabelling assay using the butanol extraction[20] was also used. No differences in pattern and levels of adducts were determined. The labelled digests were

chromatographed on thin layer plates of poly(ethylenimine)-cellulose (PEI-cellulose) by two methods: (i) essentially that described previously[29], except that D3 solvent was 3.5 M lithium formate, 8.5 M urea (pH 3.5); D4 solvent was 0.8 M lithium chloride, 0.5 M Tris-HCl buffer, 8.5 M urea (pH 8.0), followed by a final wash with 1.7 M sodium phosphate (pH 6.0). D2 was omitted (method *A*). (ii) ^{32}P-Labelled adducts were also resolved by the modification described by Reddy *et al.*[33]. This procedure has been shown to be suitable for resolution of benzoquinone-derived adducts[33]. The solvents used in this case were: D1, 2.3 M sodium phosphate (pH 5.77); D2 was omitted; D3, 2.7 M lithium formate, 5.1 M urea (pH 3.5); D4, 0.36 M sodium phosphate, 0.23 M Tris-HCl buffer, 3.8 M urea (pH 8.0). After D4 development and brief water wash, the sheets were developed (along D4) in 1.7 M sodium phosphate (pH 6.0) (D5), to the top of the plate, followed by an additional 30–40 min development with the TLC tank partially opened, to allow the radioactive impurities to concentrate in a band close to the top edge (method *B*). The specific activity of [γ-^{32}P]ATP and efficiency of the kinase reaction were assayed as described[29]. Typical values for specific activity were 2 000 ± 300 Ci/mmol. Autoradiography and evaluation of relative adduct labelling (RAL) values were performed as described previously using Cerenkov counting[29,30].

Chromatography on PEI-Cellulose

Adduct spots of DNA modified with compounds *in vitro* and *in vivo* detected by the ^{32}P-postlabelling assay and showing similar properties on TLC were excised from chromatograms and extracted as described[32] (recovery was around 85–90%). For co-chromatographic analysis the dried extracts were dissolved in water so that equal amounts of radioactivity could be applied from each sample. Developments of these adducts were carried out by the procedure described above (method *A*) in D3 and D4 directions.

RESULTS

In order to resolve whether Sudan I induces the formation of DNA adducts in target organs *in vivo*, samples of hepatic and urinary bladder DNA, obtained from Fisher 344 rats treated with Sudan I, were analyzed. DNA was isolated by procedures, which included phenol/chloroform extraction[27,28]. Using this procedure, stable covalent adducts in DNA should be determined[18]. DNA isolation by phenol/chloroform extraction is, however, not suitable for detection of noncovalent or unstable covalent adducts[34]. The nuclease P1 version of the ^{32}P-postlabelling assay was used for detection and quantitation of DNA adducts. Two variations of multidirectional chromatographic systems were utilized to separate ^{32}P-labelled adducts by TLC: (i) the method *A* using essentially the chromatographic systems originally described by Randerath *et al.*[30] is suitable for resolution of lipophilic bulky adducts and (ii) the method *B* using different chromatographic systems is appropriate for the analysis of more polar adducts[33]. In contrast to the original Randerath method[30], the second variation (method *B*) developed by Reddy *et al.*[33] makes visible adducts containing only one benzene ring[33].

Such system has been succesfully utilized to detect the polar 8-(phenylazo)guanine adduct in DNA formed with BDI derived from Sudan I activated by microsomal CYP450 *in vitro*[11]. Hence, it should be suitable for *in vivo* experiments with this studied carcinogen.

Using the method *A*, no adduct spots were found in DNA isolated from livers of rats treated with Sudan I and from livers of control (untreated) rats (Fig. 1). When the TLC plates were developed using the eluting solvents described by Reddy *et al.*[33] (the method *B*), one major and three minor adduct spots close to the bottom of the plate were observed in liver DNA of treated rats (Fig. 1). These radioactive spots are background spots that are observed also in the DNA samples of control rats (Fig. 1). The spots of the DNA adducts from both control and Sudan I-treated animals are always located in

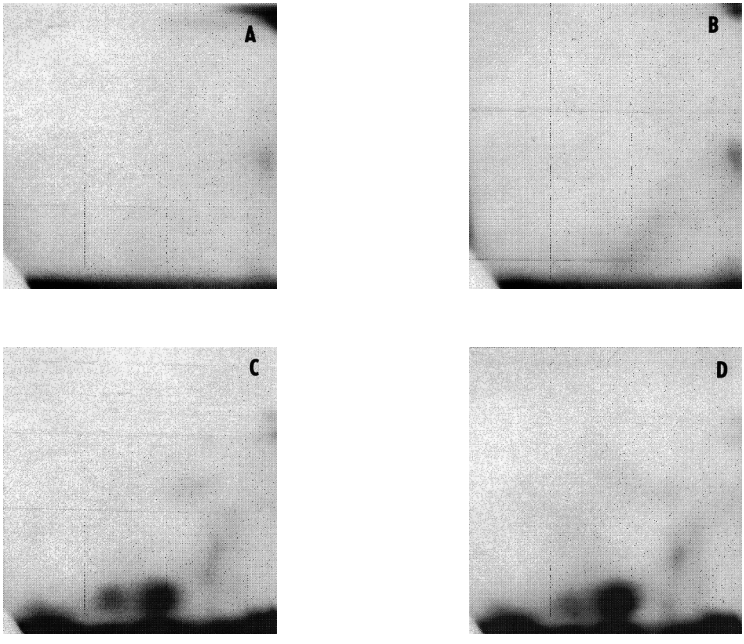

FIG. 1
Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labelled DNA digests from liver of Fisher 344 rats treated with: Sudan I in oil (A, C); oil only (control rats) (B, D). The nuclease P1 enhancement procedure was used for analysis (chromatographic method *A* for A and B and the method *B* for C and D, see Experimental). The origins are in the bottom left hand corners (D3 from bottom to top and D4 from left to right). Screen enhanced autoradiography was 12 h at –80 °C

identical positions. No adducts (induced by Sudan I) corresponding to the 8-(phenylazo)guanine adduct and/or two additional adducts detected *in vitro* by Sudan I activated with CYP450 (ref.[11]) were determined. A possibility, however, remains that adducts are formed but are not detectable, being, for example, beyond the limits of detection.

The pattern of adducts induced by Sudan I in DNA of urinary bladder of rats treated with this carcinogen resolved by the method *A* is shown in Fig. 2. Three adduct spots were detected in these DNA samples (Fig. 2). In contrast, the urinary bladder DNA of control (untreated) rats was free of adduct spots even after prolonged exposure times (Fig. 2). Here, ^{32}P-postlabelled adducts were separated by the four-directional PEI-cellulose TLC technique suitable for resolution of lipophilic adducts. These chromatographic conditions have also been sucessfully used for detection and resolution of adducts formed upon incubation of DNA with Sudan I and the peroxidase system *in vitro* (Fig. 3 and our previous work[14]). Eight major (closed circles in Fig. 3B) and more than eight minor adduct spots were detected in calf thymus DNA reacted with Sudan I activated by the peroxidase system *in vitro*. Autoradiographs of control incubations either without peroxidase or without Sudan I, did not show the presence of radioactivity spots (Figs 3C and 3D). Although the exact nature of these *in vitro* and *in vivo* adducts has not been elucidated yet, the pronounced lipophilicity of the adducts indicates that the whole Sudan I molecule is covalently linked to DNA (refs[13,14]).

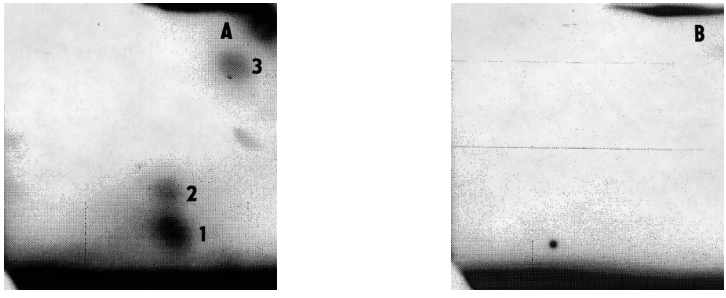

FIG. 2

Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labelled DNA digests from the urinary bladder of Fisher 344 rats treated with: Sudan I in oil (A); oil only (control rats) (B). The nuclease P1 enhancement procedure was used for analysis (chromatographic method *A*, see Experimental). The origins are in the bottom left hand corners (D3 from bottom to top and D4 from left to right). Screen enhanced autoradiography was 10 and 20 h at –80 °C for A and B, respectively

Like the parent Sudan I, both its hydroxy derivatives (formed by CYP450-dependent metabolism[7,9]), which are supposed to be detoxication metabolites, are also bound to DNA upon incubation with the peroxidase enzyme system (Fig. 4). After inspection of autoradiographs, we detected four poorly resolved adducts derived from 4′-OH-Sudan I and five adducts derived from 6-OH-Sudan I (Fig. 4). Autoradiographs of control incubations without hydroxy derivatives of Sudan I were free of radioactive adduct spots (Fig. 4C).

Quantitative analysis of adducts revealed that the extent of modification of DNA by Sudan I activated by peroxidase was 5.1 and 2.8 times greater than for 4′-OH-Sudan I and 6-OH-Sudan I, respectively (Table I). The level

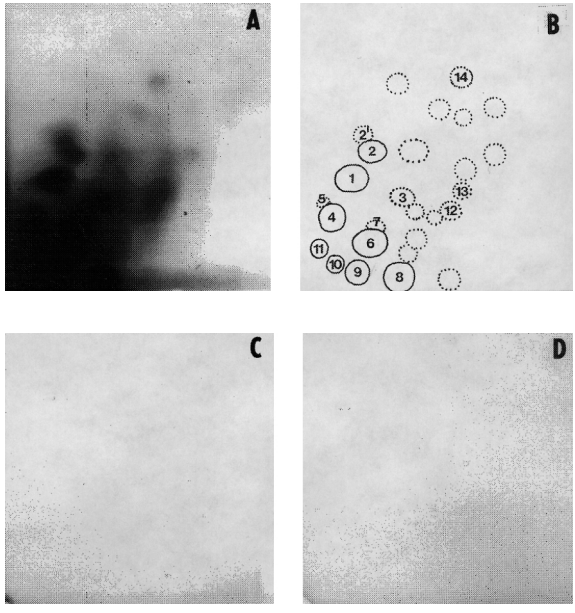

FIG. 3
Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labelled digests of DNA reacted with peroxidase, hydrogen peroxide and Sudan I (A), with the same system but without Sudan I (C) and with the same system but without peroxidase (D) (control samples). Analysis was performed by the nuclease P1 version of the assay (chromatographic method *A*, see Experimental). Autoradiography was at ambient temperature for 1 h. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right). B Schematic autoradiogram of adducts with assigned numbers, major adducts being represented by full-line circles

of *in vivo* DNA adducts was two order of magnitude lower than the levels of *in vitro* adducts (Table I).

We compared the adduct patterns obtained from the urinary bladder DNA samples and DNA modified with Sudan I, 4′-OH-Sudan I and 6-OH-Sudan I *in vitro*. Two major *in vivo* adducts (in urinary bladder DNA) exhibited migrations similar to those of some aducts in DNA modified with Sudan I and 6-OH-Sudan I. Indeed, co-chromatography of these adducts showed that *in vivo* DNA adduct 1 has the same chromatographic properties as the adduct 1 formed in DNA treated with 6-OH-Sudan I activated with peroxidase (Fig. 5), and that *in vivo* adduct 2 corresponds to the adduct 12 found in DNA treated with Sudan I (Fig. 5). It follows from the same chromatographic properties of the adducts that they may be adducts with the same structure. The levels of the two *in vivo* adducts were $7.2/10^9$ and $3.8/10^9$ nucleotides, respectively. These adducts accounted for about 68% of total adducts induced by Sudan I in the urinary bladder DNA under the conditions used (Table I).

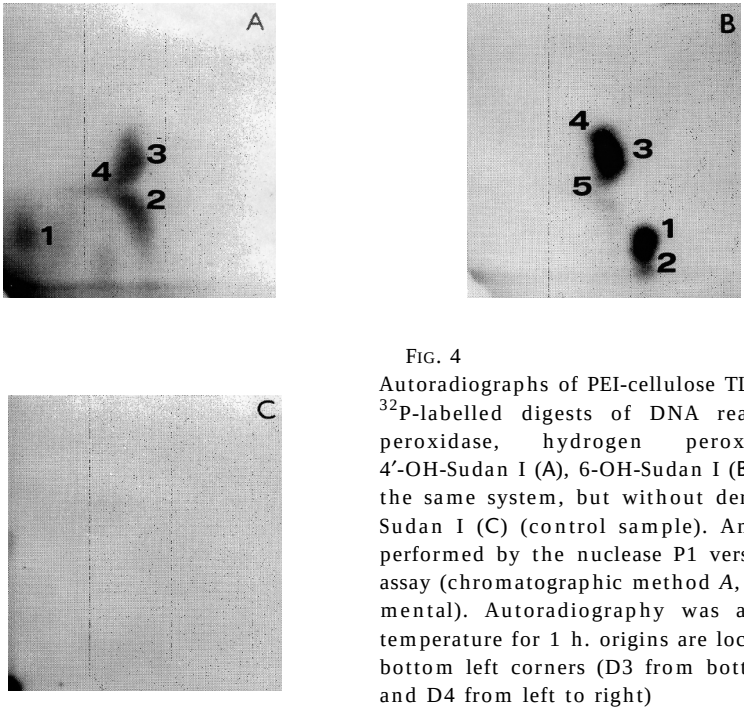

FIG. 4
Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labelled digests of DNA reacted with peroxidase, hydrogen peroxide and 4′-OH-Sudan I (A), 6-OH-Sudan I (B) and with the same system, but without derivatives of Sudan I (C) (control sample). Analysis was performed by the nuclease P1 version of the assay (chromatographic method *A*, see Experimental). Autoradiography was at ambient temperature for 1 h. origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right)

DISCUSSION

This study shows that treatment of Fisher 344 rats with Sudan I results in the formation of stable DNA adducts in the urinary bladder as detected by ^{32}P-postlabelling. Formation of these DNA adducts was observed after short-term treatment of rats with this carcinogen (two doses of Sudan I in one day).

We identified two out of the *in vivo* DNA adducts as adducts formed *in vitro* with peroxidase from Sudan I and its hydroxy derivative, 6-OH-Sudan I. They account for approximately 68% of total *in vivo* adducts. These results

TABLE I
Quantitative analysis of DNA adducts with Sudan I formed *in vivo* and DNA adducts with Sudan I, 4′-OH-Sudan I and 6-OH-Sudan I formed after activation with peroxidase *in vitro*

Activation system	DNA adduct content[a], pmol/mg	
	RAL · 10^7	DNA
In vivo		
– Total DNA adduct content	0.161 ± 0.012	0.048 ± 0.003
Adduct 1	0.072 ± 0.008	0.022 ± 0.002
Adduct 2	0.038 ± 0.004	0.011 ± 0.001
Adduct 3	0.051 ± 0.005	0.015 ± 0.001
Peroxidase with Sudan I		
– Total DNA adduct content	52.800 ± 0.890	15.840 ± 0.260
Adduct 12	0.870 ± 0.170	0.260 ± 0.051
Peroxidase with 4′-OH-Sudan I		
– Total DNA adduct content	10.300 ± 1.210	3.090 ± 0.363
Peroxidase with 6-OH-Sudan I		
– Total DNA adduct content	18.800 ± 1.983	5.640 ± 0.595
Adduct 1	5.300 ± 0.781	1.590 ± 0.234

[a] The numbers are averages and standard deviations of four determinations in separate experiments. Relative adduct labelling (RAL) denotes the number of adducts per normal nucleotides in modified DNA (see refs[20,30]). The total adduct content is sum of RAL of individual adducts.

strongly suggest that Sudan I and its detoxication metabolite formed by CYP450-mediated metabolism (6-OH-Sudan I) may be activated in the urinary bladder by the peroxidase enzyme systems present in this target organ. This finding also confirms our suggestion[11] that peroxidase itself or in a combination with CYP450 enzymes may be responsible for the organ specificity of Sudan I for urinary bladder in animals.

In contrast to the urinary bladder, in liver DNA we have been unable to detect DNA adducts by the ^{32}P-postlabelling assay. Liver CYP450 was assumed to be responsible for both detoxication and activation of Sudan I *in vivo*[1,4,7] and *in vitro*[9–13]. The participation of CYP450 in the activation of Sudan I in liver was suggested because the BDI derived from Sudan I by oxidation with CYP450 is bound to DNA (refs[9,10]), forming 8-(phenylazo)guanine and other adducts *in vitro*[11]. However, using our present *in vivo* experiments

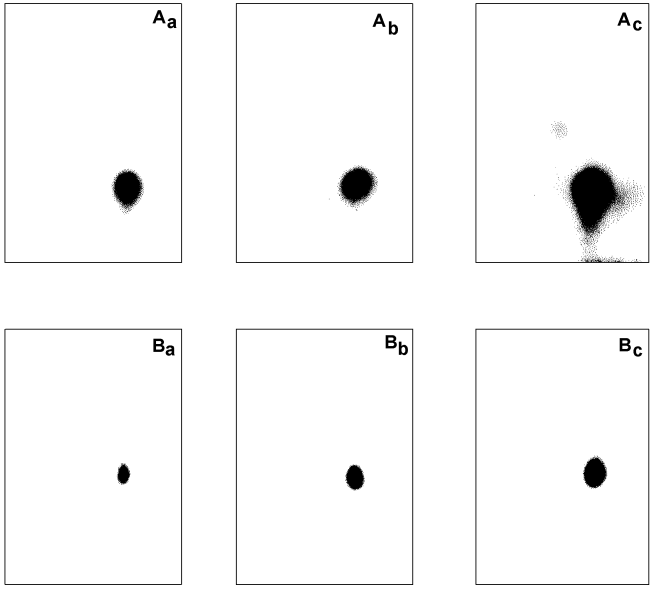

FIG. 5

Autoradiography of PEI-cellulose TLC maps for co-chromatographic analysis of ^{32}P-labelled adducts obtained from digests of urinary bladder DNA of rats treated with Sudan I and incubations of Sudan I and/or 6-OH-Sudan I activated by peroxidase with calf thymus DNA. Adduct spots were excised and eluted from TLC plates and equal amount of radioactivity were spotted and chromatographed in D3 and D4 directions (chromatographic method *A*). A_a Spot 1 from Fig. 2A; A_b spot 1 from Fig. 4B; A_c equal amounts of A_a and A_b; B_a spot 2 from Fig. 2A; B_b spot 12 from Figs 3A/3B; B_c equal amount of B_a and B_b

we have not been able to confirm the participation of CYP450 in Sudan I activation *in vivo*. The failure to detect any amounts of covalent DNA binding to Sudan I in liver *in vivo* is probably due to the arrangement of the experiments (the dose and duration of treatment with Sudan I).

The amount of DNA adducts formed by Sudan I with peroxidase activation *in vitro* is $0.53/10^5$ nucleotides. The level of an 8-(phenylazo)guanine adduct in DNA evolved from Sudan I by activation with CYP450 *in vitro* was determined to be $1/10^7$ nucleotides[11]. Hence, the efficiency of peroxidase-mediated activation of Sudan I to form DNA adducts *in vitro* is one or almost two orders of magnitude higher than for CYP450. The sum of the RALs of the adducts found *in vivo* in the urinary bladder, which should be formed by the peroxidase activation, is approximately 1 adduct per 10^8 nucleotides (Table I). If we assume that the amount of DNA adducts derived from Sudan I with these activation systems *in vivo* is proportional to the amount of adducts formed by the same enzymes *in vitro*, the levels of DNA adducts formed in liver *in vivo* should be almost two orders of magnitude smaller than those formed in DNA of the urinary bladder (the RAL should be equal or less than $1/10^{10}$ nucleotides). Although the ^{32}P-postlabelling technique used for detection of adducts is very sensitive[35–37], its detection limit is just $1/10^{10}$ nucleotides. Therefore, under the conditions used, the *in vivo* adducts derived from activation of Sudan I with CYP450 are probably beyond the limits of detection and can, hence, hardly be detectable by this technique. The results from the present *in vivo* experiments indicate that the treatment of rats with double dose of Sudan I per one day was not sufficient for formation of DNA adducts (derived from its activation by CYP450) in amounts detectable by the ^{32}P-postlabelling method. It is possible that changed conditions *in vivo* experiments could produce a stronger response – *e.g.*, use of different individual dose levels in subchronic or chronic treatment. Therefore, another testing scheme (the different dose and duration) for detecting adducts formed by Sudan I in liver DNA *in vivo* will be performed in future studies.

The present paper shows for the first time the detection of covalent adducts formed by Sudan I *in vivo*.

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