Benzenediazonium Ion Derived from Sudan I Forms an 8-(Phenylazo)guanine Adduct in DNA[1]

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1-(Phenylazo)-2-hydroxynaphthalene (Sudan I, Solvent Yellow 14) is a liver and urinary bladder carcinogen in mammals. Sudan I forms benzenediazonium ion during cytochrome P-450 catalyzed metabolism. Calf thymus DNA was reacted with Sudan I activated by microsomal enzymes or with benzenediazonium ion in vitro, and the adducts formed were analyzed by the ^{32}P-postlabeling technique. Both enrichment procedures (1-butanol extraction and nuclease P1 digestion) of this technique were employed for detection and quantitation of the DNA adducts formed. Cochromatographic analyses of adduct spots obtained by reaction with DNA or homopolydeoxyribonucleotides showed that the major Sudan I–DNA adduct was formed with deoxyguanosine. This adduct was also found in DNA directly reacted with benzenediazonium ion. The major Sudan I–DNA adduct was characterized by UV/vis absorbance spectroscopy as well as by the chromatographic properties of the adduct on cellulose or poly(ethylenimine)–cellulose TLC and HPLC. The characteristics are identical to those of the adduct synthesized from benzenediazonium ion and guanine, identified by mass, UV/vis, and ^{1}H-NMR spectroscopy as 8-(phenylazo)guanine. The results suggest strongly that benzenediazonium ion derived from Sudan I reacts with DNA in vitro to form the stable 8-(phenylazo)guanine adduct.

Introduction

Sudan I (1-(phenylazo)-2-hydroxynaphthalene, Solvent Yellow 14; see Figure 1 for structure) 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 a clastogenic compound, inducing micronuclei in the bone marrow of rats (*3*). This azo dye is metabolized primarily in the liver by oxidative or reductive reactions (*7*, *8*). Sudan I is oxidized in vitro by cytochrome P-450 monooxygenases (*9*) and peroxidase (*10*). During these reactions, DNA adducts are formed (*10*, *11*). Besides C-hydroxylated metabolites, which are considered detoxication products, the benzenediazonium ion (BDI)[2] arises by cytochrome P-450-dependent enzymatic splitting of the azo group, as shown by trapping with 1-phenyl-3-methyl-5-pyrazolone (*9*). The principal target of BDI generated from Sudan I is deoxyguanosine in DNA (*11*), and this reaction might represent an alternative activation pathway for azo dyes (*10*, *11*).

The carcinogenicity of BDI was first suggested by the pioneering work of Preussmann and co-workers (*12*). This ion is mutagenic in the Ames assay (*13*). Arenediazonium ions are chemically very different from alkanediazonium ions: they are more stable. Their reactions include azo coupling, displacement of nitrogen by nucleophiles, or induction of free radical processes (*14*, *15*). BDI reacts readily with adenine and guanine (*16*, *17*). Reaction with adenine, adenosine, or adenylic acid resulted in the formation of N^6 triazenes, while that with guanine and its derivatives resulted in the formation of C-8 azo coupled or C-8 arylated products and unstable N^2 triazenes. Koepke et al. (*14*) recently showed that BDI metabolically derived from NMA forms an unstable N^6 triazene adduct with adenine in DNA both in vitro and in vivo. Nevertheless, a detailed study of the reaction of BDI (or compounds producing BDI) with DNA is required.

Materials and Methods

Chemicals and Radiochemicals. Chemicals were obtained from the following sources: calf thymus (ct) DNA and NADPH from Boehringer Mannheim (Germany), Sudan I from British Drug Houses (Poole, U.K.), β-naphthoflavone from Sigma Chemical Co. Ltd. (St. Louis, MO), benzenediazonium hexafluorophosphate from Aldrich Chemical Co. (Milwaukee, WI), adenine and guanine from Serva (Heidelberg, Germany); and all other chemicals were reagent grade or better. Sudan I is a hazardous chemical and was handled accordingly. Materials for DNA digestion and ^{32}P-postlabeling were from sources reported previously (*18*, *19*). Nuclease P1 was from Sigma Chemical Co. ^{14}C-Labeled Sudan I (20 MBq/mmol) was syn-

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[1] Dedicated to Prof. R. Preussmann on the occasion of his 65th birthday.
[2] Abbreviations: BDI, benzenediazonium ion; ct, calf thymus; dGp, deoxyguanosine 3′-monophosphate; poly(dX), homopolydeoxyribonucleotides; NMA, *N*-nitroso-*N*-methylaniline; PEI-cellulose, poly(ethylenimine)–cellulose; RAL, relative adduct labeling; R_F, relative mobility.

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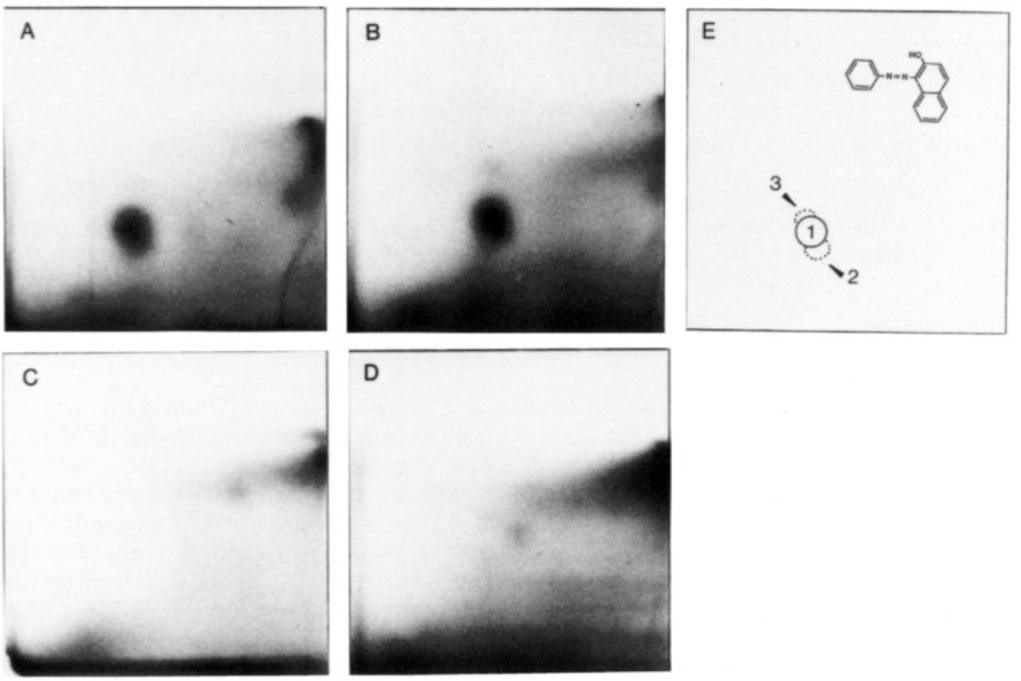


Figure 1. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of the following: calf thymus DNA treated with microsomes, NADPH, and Sudan I (A, B); calf thymus DNA treated with microsomes and NADPH (C); microsomes, NADPH, and Sudan I without DNA (D). Analysis was performed by the nuclease P1 version (A, C, D) and the butanol extraction version of the assay (B). Autoradiography was at 25 °C for 2 h (A, B) and for 4 h (C, D). Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right). (E) Formula of Sudan I and schematic figure of adducts with assigned numbers. The major adduct is represented by the closed circle.

thesized from [U-^{14}C]aniline (The Radiochemical Centre, Amersham, U.K.) as described earlier (*9*). Homopolymers [poly(dX)] were from Pharmacia LKB (Uppsala, Sweden).

Instrumentation. Analytical HPLC was conducted with two Bischoff 2200 pumps, a Bischoff gradient master, and a LDC/Milton spectrophotometric detector set at 254 nm; peaks were integrated with a Waters QA-1 integrator. UV/vis spectra were recorded on a Perkin-Elmer Lambda 5 spectrometer. ^{1}H-NMR spectra were recorded on a Bruker AM 500 spectrometer. Mass spectral analysis was performed on a Finnigan-MAT 90 mass spectrometer. Radioactivity was determined by liquid scintillation counting using Packard Ultra Gold XR liquid scintillator in a Packard Tri-Carb 2000 CA.

Preparation of (*E*)-6-(3-Phenyl-2-triazen-1-yl)purine and 8-(Phenylazo)guanine. N^6 adenine triazene and 8-(phenylazo)guanine were prepared by the methods described by Stock and co-workers (*16*, *17*). Aniline was used, in our experiments, instead of 4-bromobenzamine described earlier (*16*, *17*). Products of the reactions were washed thoroughly with chloroform, diethyl ether, and water and purified separately by chromatography on thin layer plates of cellulose (Merck, Darmstadt, Germany). The adducts were resolved by 1-butanol/1-propanol/NH_4OH (14 N)/H_2O (35:25:35:10 v/v). The separated adduct spots were scraped from the chromatograms and extracted with the same solvent system used for their resolution. After evaporation to dryness under vacuum, they were washed thoroughly with diethyl ether and chloroform. Besides 8-(phenylazo)guanine and N^6 adenine triazene, prepared as described above, further major products formed with guanine were analyzed by mass, ^{1}H-NMR, and UV/vis spectrometry. Degradation of N^6 adenine triazene in acidic solution and formation of Sudan I from degradation products and β-naphthol were carried out as described previously (*16*). The reduction of 8-(phenylazo)guanine by sodium dithionite to 8-aminopurine was performed as described by Hung and Stock (*17*).

Subcellular Preparations. Male Sprague-Dawley rats (100−150 g) were injected with β-naphthoflavone (dissolved in maize oil, 60 mg/kg body weight) once a day for three consecutive days. Animals were starved for 16−18 h before they were killed. Liver microsomes were prepared as described by Kimura et al. (*20*) and stored at −80° C.

Reaction of BDI with DNA. The procedure described by Koepke et al. (*14*) was used. Briefly, 50 mg of DNA was dissolved in 0.1 M sodium phosphate buffer (pH 7.4), and 0.2 mmol (50 mg) of benzenediazonium hexafluorophosphate, dissolved in 5 mL of the same buffer, was added. After incubation (37 °C, 30 min) and cooling to 4 °C, the mixture was extracted with ethyl acetate and the DNA was precipitated with 2 volumes of absolute ethanol and reprecipitated twice by the same procedure.

Incubations. Incubation mixtures used for the modification of DNA by [^{14}C]Sudan I activated by microsomes contained the following, in a final volume of 1.5 mL: 50 mM potassium phosphate buffer (pH 7.7), 2 mM NADPH, 3.5 mg of microsomal protein, 0.2 mM [^{14}C]Sudan I dissolved in methanol (100 μL/1.5 mL incubation), and 1 mg of calf thymus DNA. After incubation (37 °C, 120 min) the mixtures were extracted twice with ethyl acetate (2 × 2 mL) and 1-butanol. DNA was isolated by the phenol/chloroform extraction method (2×) as described by Kirby (*21*) and modified by Schoepe et al. (*19*) and precipitated by ethanol (*10*).

Ethanol-precipitated DNA was treated with activated charcoal to remove noncovalently bound radioactivity (*22*), but no change in bound radioactivity compared to untreated DNA was observed. The control incubations without the activating system (microsomes, NADPH) did not show [^{14}C]Sudan I binding (*10*, *11*). The same experimental conditions were used to modify poly(dX) except that poly(dA), poly(dG), poly(dT), and poly(dC) were used instead of DNA. The DNA and poly(dX) content was quantified spectrophotometrically at 260 nm.

Hydrolysis of DNA Modified by [^{14}C]Sudan I (Activated by Microsomes) and the Resolution of Adducts. DNA samples reacted with microsome-activated [^{14}C]Sudan I were hydrolyzed in 0.1 M HCl at 70 °C for 30 min, as described by Becker et al. (*23*). The ^{14}C-labeled acid hydrolysates were evaporated in a Speed-Vac centrifuge, dissolved in a minimal volume of 1-butanol/1-propanol/NH_4OH (14 N)/H_2O (35:25:25:10 v/v), applied on thin layer plates of cellulose (Merck, Darmstadt, Germany) or PEI-cellulose (Macherey-Nagel, Düren, Germany), and developed in three different solvent systems [1-butanol/1-propanol/NH_4OH (14 N)/H_2O (35:25:25:10 v/v); 1-butanol/methanol/NH_4OH (14 N)/H_2O (60:20:1:10 v/v); and

2-propanol/NH_4OH (4 N) (1:1 v/v)]. The adduct zones, which cochromatographed with standard adducts prepared synthetically [8-(phenylazo)guanine, 8-(biphenylazo)guanine], and further zones detected as UV positive were scraped from the plates, and the radioactivity was determined. Alternatively, the radioactive adduct spots with relative mobility (R_F) corresponding to 8-(phenylazo)guanine were extracted with 1-butanol/1-propanol/NH_4OH/H_2O (35:25:25:10 v/v), evaporated to dryness under vacuum, dissolved in 90% ethanol (pH 11.0), and centrifuged, and the clear solutions were used for UV/vis spectroscopy.

The ^{14}C-labeled acid hydrolysate, dissolved in a minimum volume of methanol, was also separated by HPLC on a reverse phase C_{18} column (Nucleosil, ODS, 250 × 4 mm, 5 μm), using a linear gradient of methanol in 50 mM ammonium formate (pH 4.6), as mobile phase (flow rate 1.5 mL/min), as follows: 5% methanol (v/v) from 0 to 0.5 min, 5−100% methanol from 0.5 to 27 min, and 100% methanol from 27 to 33 min, and monitored for absorbance at 254 nm. One minute fractions were collected, and radioactivity was determined.

^{32}P-Labeling and Recovery of Individual Nucleotide Adducts. DNA was reacted with Sudan I plus microsomes or with BDI and isolated as described above. Nonradioactive Sudan I was, however, used in these experiments. For DNA modified by activated Sudan I, the nuclease P1 version (*24*) or the 1-butanol extraction-mediated enrichment procedure (*25*) of the ^{32}P-postlabeling assay (*18*) was used. For the DNA modified by BDI, only the nuclease P1 version of the ^{32}P-postlabeling assay was used. DNA was hydrolyzed to deoxyribonucleoside 3′-monophosphates, using micrococcal nuclease and spleen phosphodiesterase. Nuclease P1-treated samples and butanol extracts were labeled using polynucleotide kinase and [γ-^{32}P]ATP under conditions described previously (*10*, *26*). The same ^{32}P-labeling procedure was used for control incubations (either without DNA or without microsomes). Ethyl acetate and butanol extracts of incubations containing either Sudan I with microsomal systems or BDI without DNA were evaporated, dissolved in a minimal amount of methanol, and used for ^{32}P-postlabeling analysis (*27*). Labeled digests were chromatographed on thin layer plates of PEI-cellulose, with modifications described by Reddy et al. (*28*).

This procedure is suitable for the resolution of benzoquinone-, phenol-, or hydroquinone-derived adducts (*28*). The solvents used were as follows: 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, 3.8 M urea (pH 8.0). After D4 development and a 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.

Postlabeled adducts were located by autoradiography, adduct spots were excised from the chromatograms (three parallel experiments), and the radioactivity was measured by Cerenkov counting (*18*, *24*, *26*). Blank regions adjacent to the adduct spots were also evaluated, and their count rates were subtracted from adduct count rates. Adduct levels were calculated by relative adduct labeling (RAL) (*24*, *25*). Since adducts were derived from 8.11 μg of DNA (2.36×10^4 pmol of dNp) and the specific activity of [γ-^{32}P]ATP was (3.72 ± 0.23) × 10^6 cpm/pmol (3000 Ci/mmol), adduct levels were calculated according to:

$$\text{RAL} = \frac{\text{cpm in adduct nucleotides}}{\text{sp act. (in cpm/pmol)} \times \text{pmol of dNp}}$$

The same procedure described above was used for analysis of poly(dX) modified by microsomally activated Sudan I. Adduct spots derived from DNA or poly(dX) separations showing similar properties on TLC were excised from the chromatograms and extracted as described (*26*). Cutouts were extracted with two portions (800 μL) of 6 N ammonium hydroxide/2-propanol (1:1), for a total of 40 min. The eluent was evaporated in a Speed-Vac centrifuge. For cochromatographic analyses the extracts were dissolved in water so that equal amounts of radioactivity could be applied for each sample. Chromatography of these adducts was carried out by the procedure described above in the D3 and D4 directions.

Results

^{32}P-Postlabeling of Adducts Formed from Sudan I Activated by Microsomal Enzymes with ct DNA and Polydeoxyribonucleotides. After inspection of autoradiographs, one major (closed circle in Figure 1E) and two minor adduct spots (overlapping one another) were detected in ct DNA reacted with Sudan I activated by rat liver microsomal enzymes, using either of the two enrichment procedures for the ^{32}P-postlabeling analysis.

As reported previously (*10*), we were not successful in detecting adducts in DNA modified by microsomally activated Sudan I by ^{32}P-postlabeling when the chromatographic systems employed were the same as for lipophilic adducts, such as adducts formed by aristolochic acids (*29*) or dimethylbenz[*a*]anthracene (*26*). With these resolution systems, more polar adducts are washed off the plate by the phosphate washes (D1 or D5 directions). Here, ^{32}P-postlabeled adducts of Sudan I−DNA were resolved by the four-directional PEI-cellulose TLC technique modified by Reddy et al. (*28*) for the analysis of more polar adducts (Figure 1). As opposed to the original Randerath method (*18*), this modification (*28*) makes visible adducts which contain only one benzene ring derived from phenol, hydroquinone, or benzoquinone.

The adducts were quantitated by both enrichment procedures (nuclease P1 treatment and butanol extraction). The levels of adducts were determined by measurement of the adduct count rates and expressed as RALs (Table 1). Both versions of the ^{32}P adduct assay used yielded the same DNA adduct pattern (Figure 1), but the recovery of the adducts with the butanol extraction was only 20% of that with nuclease P1 (Table 1). No radioactive spots were detected in autoradiographs of control incubations (Figure 1).

To identify the target deoxyribonucleotides for the microsome-mediated Sudan I binding, polydeoxynucleotides [poly(dX)] were used instead of DNA (Figure 2). Five major and several minor adducts were formed with poly(dA), three major and three minor adducts with poly(dG), and one adduct with poly(dC). All three poly(dX) were modified by Sudan I in the microsomal activating system in vitro to a larger extent than was DNA. No adduct spots were detectable in poly(dT) after its reaction with microsome-activated Sudan I (Figure 2).

As shown in Table 1, the extent of adduct formation was highest with poly(dA) and proceeded in the order: poly(dA) > poly(dC) > poly(dG).

Adducts formed from poly(dG) were analyzed by both enrichment procedures and showed approximately 80% lower recoveries with butanol, as found in DNA (Table 1).

When the adduct patterns obtained from DNA and individual poly(dX), both reacted with microsomally activated Sudan I, were compared, a major adduct in DNA exhibited migration similar to that of one poly(dG) adduct (adduct 1 in Figure 1 A and B and adduct 1 in Figure 2A). Cochromatographic analyses confirmed that this adduct from DNA had the same chromatographic properties as that formed with poly(dG) (Figure 3A).

^{32}P-Postlabeling of Adducts Formed from Benzenediazonium Ion and ct DNA. Sudan I is metabo-

Table 1. Quantitative Analysis of Microsome-Activated Sudan I–DNA and Poly(dX) Adducts by Either Nuclease P1 or Butanol Extraction Version of ^{32}P-Postlabeling Assay[a]

	DNA– or poly(dX)–				
	nuclease P1		butanol		
adduct no.[b]	RAL × 10^7	pmol of adducts/ mg of DNA [poly(dX)]	RAL × 10^7	pmol of adducts/ mg of DNA [poly(dG)]	1-butanol/ nuclease P1 extraction (%)
DNA 1	1.40 ± 0.42	0.42 ± 0.13	0.27 ± 0.03	0.08 ± 0.009	19.3
DNA 2	0.50 ± 0.08	0.15 ± 0.04	0.09 ± 0.01	0.03 ± 0.003	18.0
DNA 3	0.20 ± 0.03	0.06 ± 0.02	0.04 ± 0.01	0.01 ± 0.003	20.0
total	2.10 ± 0.81	0.63 ± 0.19	0.40 ± 0.05	0.12 ± 0.015	19.0
poly(dG) 1G	0.80 ± 0.08	0.24 ± 0.024	0.15 ± 0.02	0.04 ± 0.006	18.8
poly(dG) 2G	0.81 ± 0.09	0.24 ± 0.027	0.15 ± 0.03	0.04 ± 0.009	18.5
poly(dG) 3G	1.30 ± 0.35	0.39 ± 0.105	0.30 ± 0.04	0.09 ± 0.012	23.1
total	2.91 ± 0.92	0.87 ± 0.276	0.60 ± 0.05	0.17 ± 0.015	20.6
poly(dA) 1A	12.40 ± 2.32	3.73 ± 0.697	ND	ND	ND
poly(dA) 2A	2.40 ± 0.91	0.72 ± 0.273	ND	ND	ND
poly(dA) 3A	2.80 ± 1.03	0.84 ± 0.309	ND	ND	ND
poly(dA) 4A	4.10 ± 1.58	1.23 ± 0.474	ND	ND	ND
poly(dA) 5A	2.60 ± 0.95	0.78 ± 0.285	ND	ND	ND
total	24.30 ± 3.51	7.30 ± 1.054			
poly(dC) 1C	5.80 ± 1.80	1.74 ± 0.540	ND	ND	ND

[a] Numbers are averages and SD of triplicate analyses. ND = not determined. [b] See Figures 1 and 2 [e.g., 1G = adduct 1 of poly (dG)].

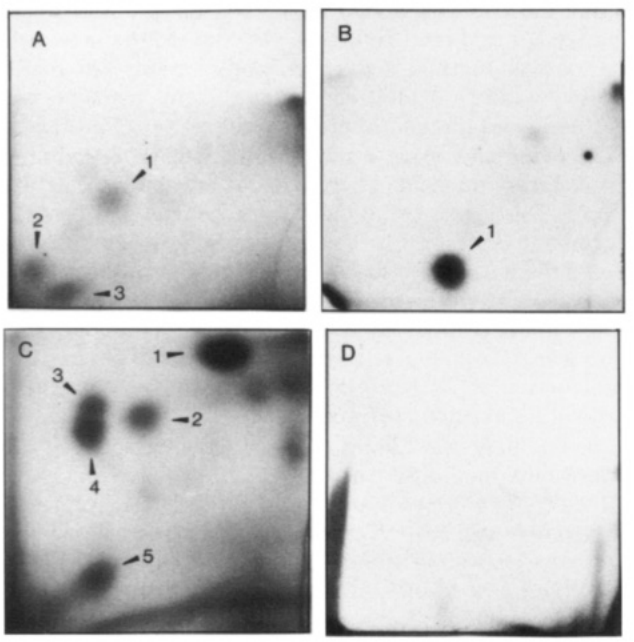


Figure 2. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of the following: poly(dG) (A), poly(dC) (B), poly(dA) (C), and poly(dT) (D) treated with microsomes, NADPH, and Sudan I. Analysis was performed by the nuclease P1 version of the assay. Autoradiography was at 25 °C for 2 h. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

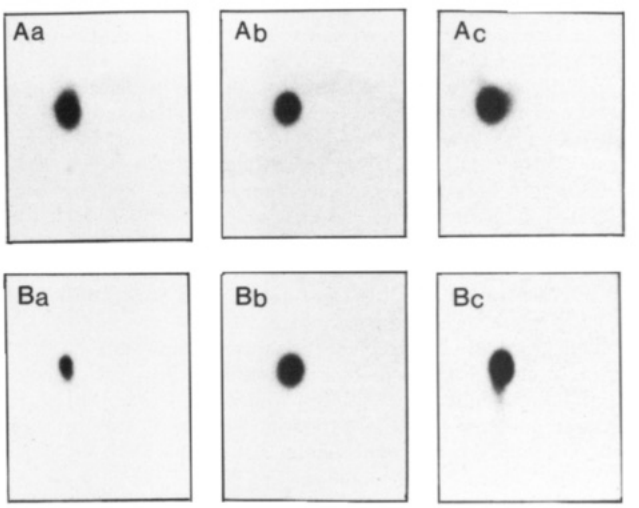


Figure 3. Autoradiographs of PEI-cellulose TLC maps for cochromatographic analysis of the following: adducts obtained from ^{32}P-labeled digest of DNA and poly(dG) treated with Sudan I activated by the microsomal system (A); DNA treated with BDI and Sudan I activated by the microsomal system (B). Adduct 1 in DNA from Figure 1 (Aa), adduct 1 in poly(dG) from Figure 2A (Ab), and cochromatography of both adducts (Ac). Adduct 2 from Figure 4 (Ba), adduct 1 in DNA from Figure 1 (Bb), and cochromatography of both adducts (Bc). Autoradiography was at −70 °C for 24 h. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

lized to the benzenediazonium ion by microsomal cytochrome P-450, and reactive metabolites formed from Sudan I react with DNA (*9*, *11*). In order to test whether BDI does bind to DNA, benzenediazonium hexafluorophosphate was mixed with DNA and the purified DNA was analyzed by the ^{32}P-postlabeling assay. This confirmed that BDI readily modifies DNA in vitro, to form covalent adducts which are stable enough to be detectable by the ^{32}P-postlabeling analysis. The pattern of adducts formed in this reaction is shown in Figure 4. The sum of the RALs of the adducts found with the nuclease P1 version is approximately 1 adduct per 10^5 nucleotides (Table 2). Autoradiographs of control incubations (either without BDI or without DNA) did not show any radioactive spots (Figure 4C,D).

Spot 2 in Figure 4 has chromatographic properties similar to those of the major adduct in DNA modified by Sudan I activated by microsomal enzymes which was already shown to be formed in poly(dG) (adduct 1 in Figure 1). Indeed, cochromatographic analysis confirmed that these two adducts are chromatographically indistinguishable (Figure 3B). This finding strongly suggests that BDI derived from Sudan I binds to deoxyguanosine in DNA.

In order to identify the structure of this adduct, a reference compound for the ^{32}P-postlabeling analysis should be synthesized by reaction of deoxyguanosine 3′-

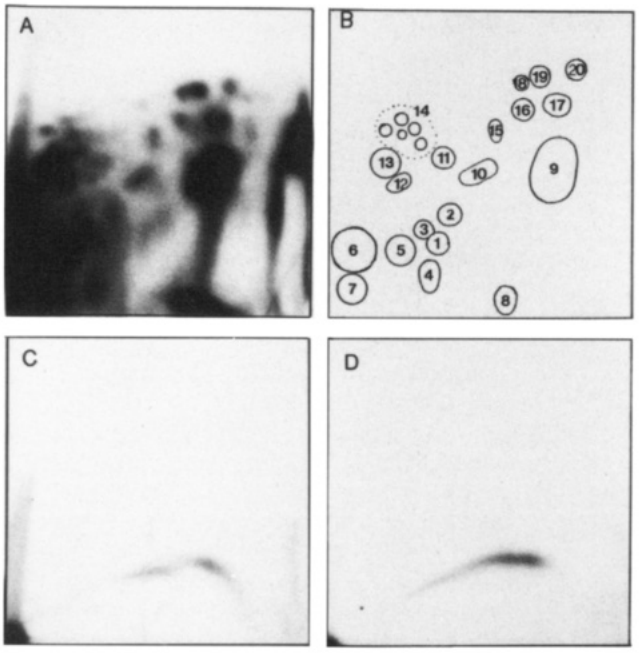


Figure 4. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of calf thymus DNA treated with BDI (A), schematic figure of adducts with assigned numbers of adducts (B), autoradiographs of the control sample without BDI (C), and the control sample without DNA (D). Analysis was perfomed by nuclease P1 version of the assay. Autoradiography was at 25 °C for 30 min (A) and for 2 h (C, D). Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

Table 2. Quantitative Analysis of BDI–DNA Adducts Analyzed by the Nuclease P1 Version of the ^{32}P-Postlabeling Assay

adduct no.[b]	RAL × 10^7	pmol of adducts/ mg of DNA
1	1.43 ± 0.22	0.43 ± 0.07
2	1.47 ± 0.02	0.44 ± 0.01
3	1.01 ± 0.24	0.30 ± 0.07
4	2.91 ± 0.17	0.87 ± 0.05
5	5.30 ± 0.24	1.59 ± 0.07
6	43.60 ± 1.96	13.09 ± 0.59
7	29.58 ± 3.59	8.88 ± 1.08
8	2.81 ± 0.04	0.84 ± 0.01
9	23.33 ± 1.07	7.01 ± 0.32
10	2.10 ± 0.11	0.63 ± 0.03
11	0.65 ± 0.09	0.18 ± 0.03
12	1.59 ± 0.02	0.48 ± 0.01
13	5.27 ± 0.13	1.58 ± 0.04
14	4.78 ± 0.17	1.43 ± 0.05
15	0.93 ± 0.05	0.28 ± 0.01
16	0.96 ± 0.19	0.29 ± 0.06
17	2.35 ± 0.16	0.70 ± 0.05
18	0.46 ± 0.12	0.14 ± 0.04
19	1.16 ± 0.05	0.35 ± 0.01
20	0.82 ± 0.01	0.25 ± 0.003
total	132.51 ± 2.77	39.76 ± 0.83

[a] Numbers are averages and SD of triplicate analyses. [b] See Figure 4.

monophosphate (dGp) with BDI (or Sudan I activated by microsomes) and structurally characterized. In our experiments, dGp was incubated with Sudan I and microsomal enzymes, and adducted dGp was extracted from the reaction mixture with butanol and directly labeled. ^{32}P-postlabeling analysis resulted in the appearance of a single adduct spot with the same chromatographic mobilities on TLC as the adduct determined in DNA or poly(dG) (results not shown). The amount of this dGp adduct analyzed by the sensitive ^{32}P-postlabeling assay was, however, not sufficient for its structural characterization. We therefore used another method suitable for the identification of the structure of the DNA adduct(s).

Preparation and Isolation of Synthetic Adducts Formed from Benzenediazonium Ion with Guanine or Adenine. BDI reacts more readily with guanine (or adenine) bases than with their nucleosides or nucleotides (*16*, *17*). In order to obtain reference compounds (authentic synthetic standards) in amounts sufficient for structural characterization, as well as for comparative chromatographic and spectroscopic analysis, BDI was reacted with purine bases (guanine, adenine), and the products were characterized and used as standards. The chemical structure of DNA adducts(s) formed in DNA from BDI derived from Sudan I was then identified by comparison of the chromatographic mobilities of adducts(s) with those of these synthetic standards.

DNA treated with radioactive [^{14}C]Sudan I and microsomes was hydrolyzed chemically to bases and adducts, and synthetic standards were compared on TLC and HPLC. The experimental procedures and results are described below.

BDI was reacted with adenine or guanine according to the method of Stock and co-workers (*16*, *17*). Adenine reacts with BDI to form one major and three minor products, which were separated by TLC on cellulose plates. The major product (R_F = 0.65) was characterized by UV/vis, mass, and ^{1}H-NMR spectroscopy. The UV/vis spectrum of the compound (Figure 5a) is the one described by Chin et al. (*16*) for (*E*)-6 (3-phenyl-2-triazen-1-yl)purines. The mass spectrum showed the molecular ion (MW 239), and the ^{1}H-NMR spectrum of this compound confirmed the published structure (data not shown). Furthermore, as postulated by Chin et al. (*16*), we found this product to be unstable in acidic solution; it decomposed at pH 3.0 to adenine and BDI, the latter forming Sudan I by azo coupling with 2-naphthol (data not shown).

We have now extended the investigations to the chemistry of the reaction of BDI with guanine. Hung and Stock (*17*) established previously that guanine reacts rapidly with several benzenediazonium ions at pH 10.5 to form 8-(arylazo)guanines in good yields. We determined that the reaction products of BDI with guanine contained two major and three minor products, which were separated by TLC on cellulose. The 8-azo coupling product, 8-(phenylazo)guanine, is formed as the major product (R_F = 0.32, 65% yield) and was confirmed by ^{1}H-NMR, mass, (Figure 6), and UV/vis spectroscopy (Figure 5). The ^{1}H-NMR spectrum corresponds to the published data of this adduct (*17*), including the absence of a singlet signal near 8.4 ppm that would correspond to the C-8 proton of guanine. The reduction of this azo compound with sodium dithionite, giving 8-aminopurine (*17*), completed the characterization of the product. A second product (accounting for approximately 20–30% of the products), R_F = 0.67, had not been isolated by Hung and Stock (*17*). The structural assignment of this azo compound is based upon its spectroscopic properties. The UV/vis spectrum of the compound is similar to that of 8-(phenylazo)guanine (Figure 5C). The mass spectrum shows that the compound has a molecular weight of 331 (Figure 6), which is the sum of the molecular weights of 8-(phenylazo)guanine and one phenyl group. The fragmentation peaks at *m*/*z* 154, 168, and 182 (Figure 6) indicate the presence of biphenyl, N-biphenyl, and N_2-

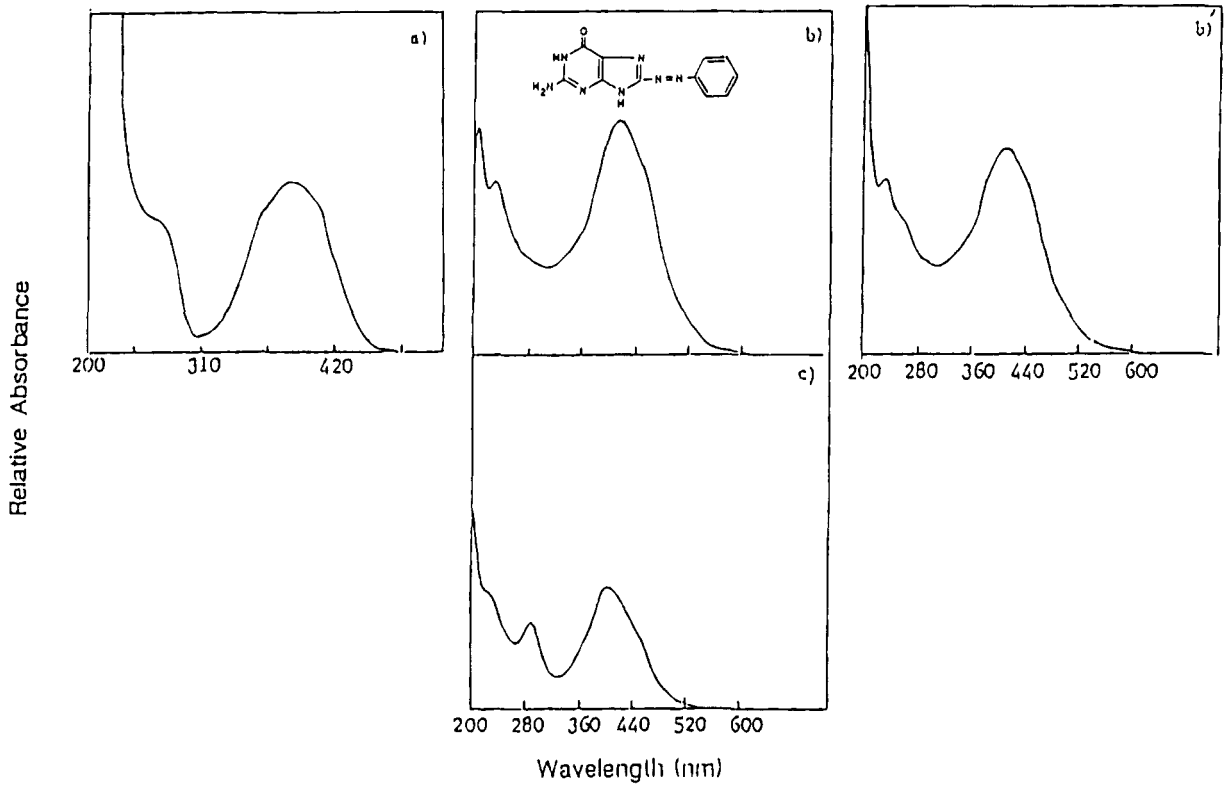


Figure 5. Absorption spectra of (*E*)-6-(3-phenyl-2-triazen-1-yl)adenine (a), 8-(phenylazo)guanine (b), and 8-(biphenylazo)guanine (c) prepared synthethically from BDI with adenine and guanine, respectively, and the adduct separated by TLC on cellulose plates from hydrolysate of DNA reacted with [^{14}C]Sudan I activated by microsomes (b'). The adducts were dissolved in 90% ethanol (pH 11), and the spectra were recorded as described in Materials and Methods.

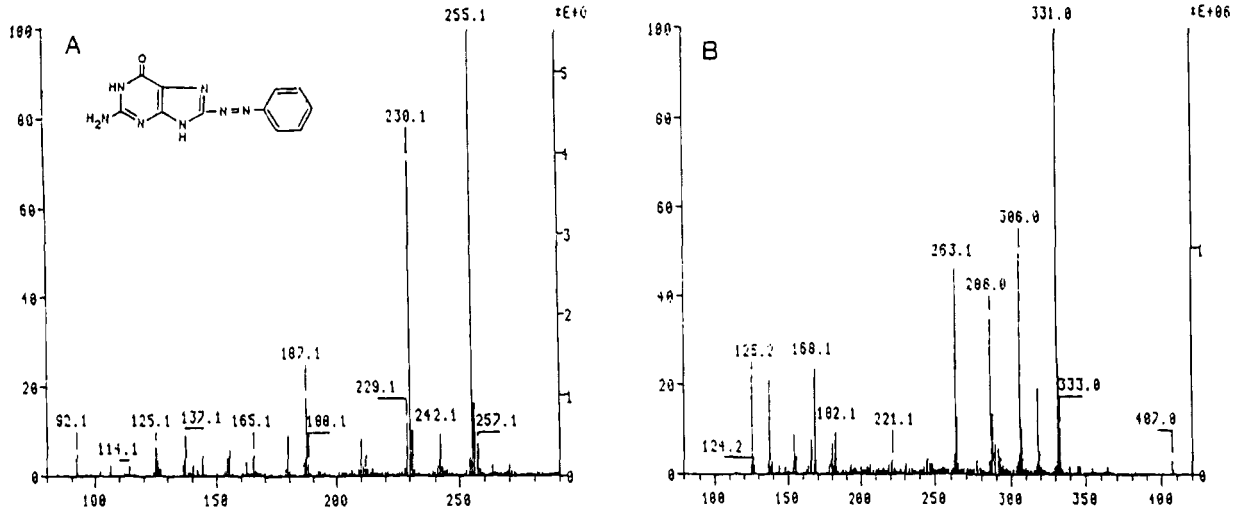


Figure 6. Mass spectra of 8-(phenylazo)gunaine (A) and 8-(biphenylazo)guanine (B) synthesized from BDI and guanine.

biphenyl fragments, respectively. The ^{1}H-NMR spectrum of this compound in dimethyl sulfoxide-d_6 showed the presence of the following protons: δ (ppm) 6.63 (br, s, 2H), 7.42–7.62 (m, 9H), 10.74 (br, s, 1H), and 12.65 (br, s, 1H). Among these protons, the two at 6.63 ppm were assigned to the amino group at the N^2 position of guanine. The nine protons at 7.42–7.62 ppm were assigned to aromatic protons of the biphenyl group, one proton at 10.74 to N1 proton of guanine and one proton at 12.65 ppm to N9 proton of guanine. Furthermore, the NMR data indicated the absence of a C8 proton of guanine. The combined spectroscopic data strongly support the identification of this product as 8-(biphenylazo)guanine.

The minor products formed from BDI and adenine or guanine were not characterized.

Isolation of the DNA Adduct Formed from [^{14}C]-Sudan I Activated by Microsomes and Its Characterization as 8-(Phenylazo)guanine. DNA modified by microsome-activated [^{14}C]Sudan I was hydrolyzed with 0.1 M HCl, and the ^{14}C-labeled adducts were separated by TLC on cellulose or PEI-cellulose in three different solvent systems (Table 3) and by HPLC (Figure 7).

Forty to sixty percent of the total radioactivity cochromatographed with 8-(phenylazo)guanine, in all three solvent systems used (Table 3). The UV/vis spectrum of this radioactive material obtained after elution from cellulose corresponds to that of 8-(phenylazo)guanine prepared synthetically (Figure 5b). We also detected other ^{14}C radioactive zones on TLC-cellulose plates (Table 3). Since mild acid treatment does not completely

Table 3. Values of Relative Mobilities (R_F) of Synthetic Guanine–BDI Adducts and Distribution of Radioactivity in Hydrolysate of DNA Treated with [^{14}C]Sudan I and Microsomes

	adduct				
solvent system[a]	8-(phenylazo)guanine	8-(biphenylazo)guanine	unknown		
A[a] (on cellulose)					
R_F values[b]	0.34	0.82	0.024	0.12	0.88
^{14}C radioactivity[c]	1363		227	396	227
B (on cellulose)					
R_F values	0.32	0.67	0.048	0.16	0.51
^{14}C radioactivity[c]	891		500	283	248
C (on PEI-cellulose)					
R_F values	0.35	0.55	0.03	0.21	0.90
^{14}C radioactivity[c]	1223		225	408	230

[a] Solvent systems: A: 1-butanol/methanol/NH_4OH (14 N)/H_2O (60:20:1:10 v/v); B: 1-butanol/1-propanol/NH_4OH (14 N)/H_2O (35:25:25:10 v/v); C: 2-propanol/NH_4OH (4 N) (1:1 v/v). [b] R_F: relative mobilities of 8-(phenylazo)guanine and 8-(biphenylazo)guanine and unknown adducts observed in DNA hydrolysate. [c] Radioactivity of acid hydrolysate of DNA (dpm) with R_F values shown.

hydrolyze DNA (*23*), this radioactive material may result either from other adducts or from partially hydrolyzed DNA (oligonucleotides). We did not detect a radioactive signal which cochromatographed with 8-(biphenylazo)-guanine (Table 3). The identity of one adduct formed in DNA with 8-(phenylazo)guanine is shown by the above cochromatographic and spectral analysis.

As a second independent chromatographic procedure to confirm identities of the adducts, we employed HPLC analysis. The retention times of 8-(phenylazo)guanine and 8-(biphenylazo)guanine on our HPLC system, at pH 4.6, were 14.6 and 15.9 min, respectively (Figure 7). We also analyzed the synthetic N^6 adenine triazene adduct under these acidic conditions and, as expected, found that the compound decomposes, giving two peaks on HPLC, with retention times of 6.8 and 18.7 min (Figure 7). Acid hydrolysis of [^{14}C]Sudan I–DNA isolated in our experiments gave rise to one major and two or three minor peaks of radioactivity (Figure 7). About 50% of the radioactivity (presumably corresponding to intact or partially hydrolyzed DNA) was not eluted from the column.

The major radioactive peak, with a retention time of 14.5 min, which accounted for approximately 32% of the total radioactivity, cochromatographed with 8-(phenylazo)guanine (Figure 7). No radioactivity, however, was detectable at the retention times of the N^6 adenine triazene decompositon products, nor at that of 8-(biphenylazo)guanine adduct (15.9 min) (Figure 7). These results show that the structure of the adduct formed from BDI derived from Sudan I in vitro is 8-(phenylazo)-guanine.

Discussion

The first direct proof that Sudan I is metabolized to BDI by a cytochrome P-450 monooxygenase-catalyzed cleavage of the azo group was obtained by trapping the intermediate with 1-phenyl-3-methyl-5-pyrazolone, which led to the coupling product 1-phenyl-3-methyl-4-(phenylazo)-5-pyrazolone (*9*). Moreover, pyrazolone inhibited the modification of DNA by microsomally activated [^{14}C]-Sudan I (*11*). These observations suggested that BDI derived from Sudan I was one of the electrophilic species responsible for binding to DNA in vitro (*11*).

The formation of DNA adduct(s) in the reactions of DNA with BDI in vitro, or in those of DNA with the strong esophageal carcinogen NMA, which forms BDI after α-C-hydroxylation, was studied by Koepke et al. (*14*,

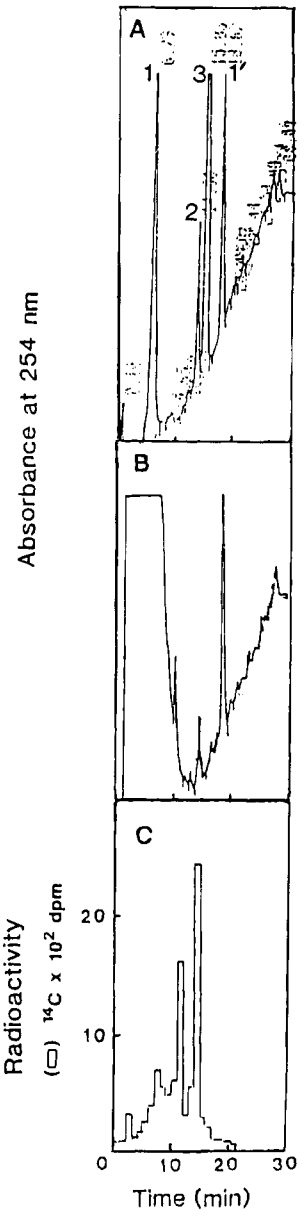
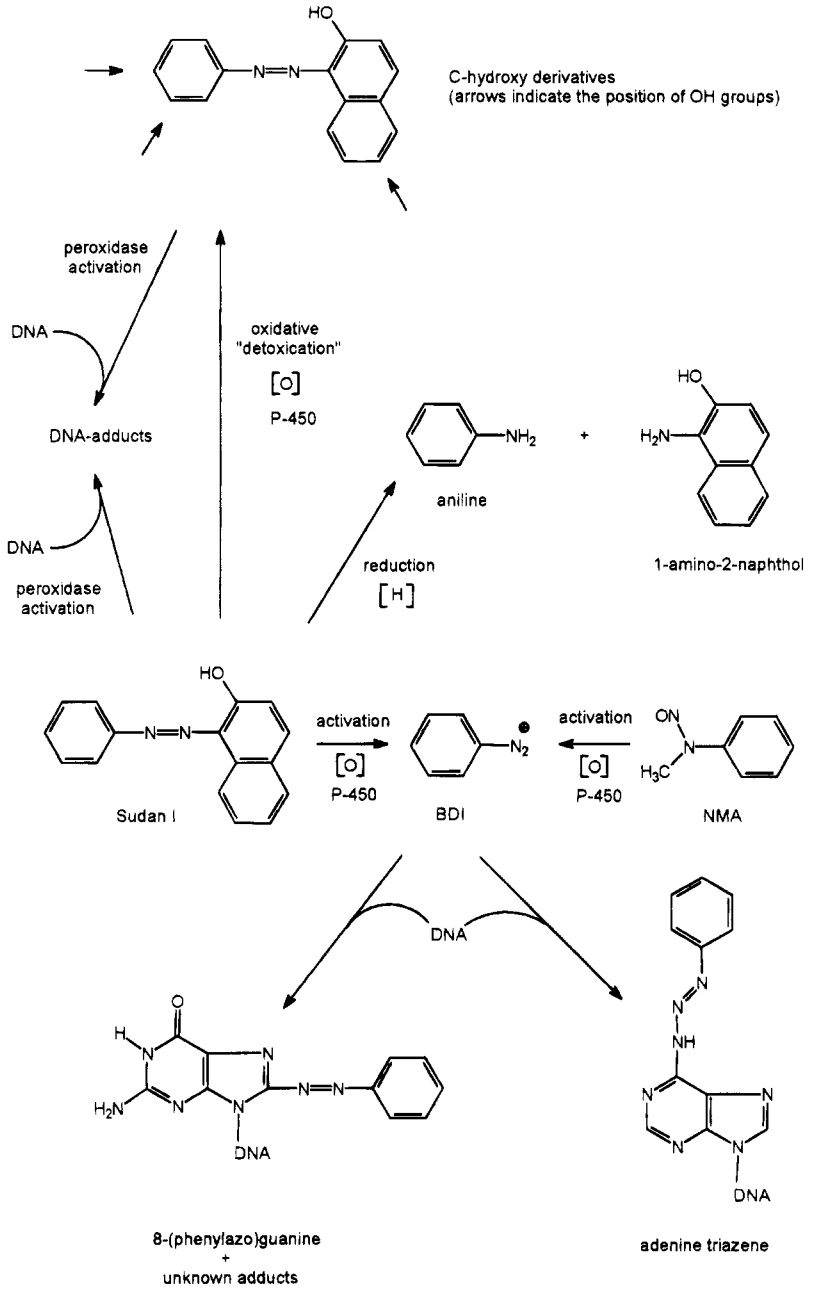


Figure 7. HPLC profile of (A) the mixture of adduct standards prepared syntheticaly (1 and 1': N^6 adenine triazene decomposition products, 2: 8-(phenylazo)guanine, and 3: 8-(biphenylazo)guanine] and (B, C) hydrolysate of DNA obtained from incubations with [^{14}C]Sudan I and the microsomal system containing synthetic adducts as internal standards [UV detection (B) and ^{14}C radioactivity detection (C)]. The experimental conditions are described in Materials and Methods.

Scheme 1. Pathways of Metabolism of Sudan I and NMA Leading to DNA Adducts



30). Their studies (*14*, *30*) were directed to the detection and identification of an unstable N^6 adenine triazene adduct in NMA-treated DNA, which explained the low level of covalent binding of NMA to DNA in vivo, determined previously by Lijinsky (*31*). Koepke et al. (*14*) isolated DNA carefully, without phenol extraction, and detected and identified, indirectly, the unstable N^6 adenine triazene adduct.

The failure to detect high levels of covalent DNA binding by NMA is probably due to the low rate of metabolism of NMA by microsomal cytochrome P-450 (*13*, *30*, *32*–*34*). Therefore, BDI is also formed in a very low amount (*30*, *32*, *33*), and DNA adducts with BDI may, hence, hardly be detectable. Sudan I is effectively oxidized by cytochrome P-450. The amount of [^{14}C]Sudan I converted by microsomal enzymes under the conditions used was 85–90% (*10*, *35*); BDI accounts for more than 20% of the products (*9*, *35*). This effective metabolism of Sudan I can, hence, lead to a stronger DNA binding of activated Sudan I than of NMA metabolites.

Here, we determined the structure of a stable DNA adduct formed by BDI derived from Sudan I. The ^{32}P-postlabeling assay confirmed the formation and polar nature of stable adducts formed between microsomally activated Sudan I and DNA. These adducts behaved similarly to the polar adducts obtained from DNA modified by phenol or benzoquinone (*28*). Hence, metabolite(s) of Sudan I containing one benzene ring (or derivatives) are responsible for the adducts' polar nature. Gupta and Early (*25*) postulated that certain adducts which were recovered in higher yields by nuclease P1 digestion than by butanol extraction are more polar. This is consistent with the results presented here (see Table 1).

The ^{32}P-postlabeling assay is also useful for the identification of individual deoxynucleotides as targets for carcinogen attachment (*36*). Three deoxynucleoside bases [namely, poly(dA), poly(dC), and poly(dG)] are targets for reactive intermediate(s) produced during Sudan I metabolism. The higher reactivities of these three poly(dX) toward Sudan I metabolite(s), as well as the differences

in adduct patterns, compared with DNA, were somewhat surprising. However, the higher level of binding obtained with these single-stranded homopolymers is in accordance with the 1.3-fold and 3.8-fold higher binding levels of activated Sudan I to tRNA and rRNA, respectively, than to DNA, reported earlier (*35*). Single-stranded nucleic acids are hence more accessible for modifications by microsome-activated Sudan I. Using cochromatographic analysis of ^{32}P-postlabeled adducts, we also confirmed our previous report that deoxyguanosine is the target for an electrophile derived from Sudan I (*11*).

By using ^{14}C-labeled Sudan I, we were able to isolate the major adduct formed in DNA by activated Sudan I. This adduct was characterized as 8-(phenylazo)guanine by cochromatography with the synthetic product of the reaction of BDI with guanine on TLC or HPLC and by comparing their UV/vis spectra.

The formation of an 8-azo coupling product with 5'-guanylic acid and 2,5-disulfobenzenediazonium ion was described by Moudrianakis and Beer (*37*). We found that BDI reacts with guanine to form not only the 8-azo coupling product, but another product, which was characterized as 8-(biphenylazo)guanine, by spectroscopic analysis. Our results show that the nucleophilicity of the C-8 carbon atom of guanine in DNA is sufficient to react with the electrophile studied. This is consistent with the formation of other C-8 guanine adducts in DNA by several carcinogens (*24*, *38*). Evidence for the reaction of DNA and RNA with diazonium ions can also be gained from the results of Noyes and Stark (*39*) and Alwine et al. (*40*), who described procedures for linking RNA or DNA covalently to finely divided cellulose through a diazotized arylamine.

The results reported herein have extended the investigations to the adduct formation from BDI derived from the non-aminoazo dye Sudan I. As mentioned above, BDI was formed during the metabolism of this non-aminoazo dye or NMA by cytochrome P-450 (*9*, *14*, *30*). As described earlier (*10*, *36*, *41*), Sudan I is also activated by peroxidases. Scheme 1 summarizes previous (*7*, *9*–*11*, *35*, *36*, *41*) and new findings concerning the detoxication and/or activation reactions for Sudan I mediated by cytochrome P-450 and peroxidase in vitro. For comparison, the N^6 adenine triazene adduct from microsomally activated NMA is also shown (*14*). We suggest the cytochrome P-450- 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 (*10*, *41*). Our present study showing the formation of 8-(phenylazo)-guanine as the major stable adduct by microsomal enzymes does not exclude the formation of other adducts (e.g., C-8 arylations of adenine or guanine) in DNA, since other (minor) stable adducts were detectable by the ^{32}P-postlabeling assay and also in mild acidic hydrolysates of DNA modified by [^{14}C]Sudan I. Moreover, we did not study the formation of unstable triazene adducts with N^6 of adenine formed by BDI derived from NMA (*14*) (see Scheme 1) or N^2 of guanine in DNA, which are unstable under the DNA isolation, digestion, and hydrolysis procedures used in our experiments (*14*). Future studies will show if such unstable triazenes are also formed from Sudan I activated by microsomes and DNA. The chemical synthesis and characterization of 8-(phenylazo)-deoxyguanosine 3'-monophosphate, which can be used as a standard in the ^{32}P-postabeling assay, are in preparation.

The results presented in this paper and future in vivo experiments will provide evidence for the biological significance of either the cytochrome P-450-derived Sudan I–DNA adducts (present paper) and/or peroxidase activation (*10*, *36*, *41*) for Sudan I carcinogenicity.

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