Ce(IV)-MEDIATED FORMATION OF BENZENEDIAZONIUM ION FROM A NON-AMINOAZO DYE, 1-PHENYLAZO-2-HYDROXY-NAPHTHALENE (SUDAN I) AND ITS BINDING TO DNA

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Ce(IV) ions in acidic medium convert a carcinogenic non-aminoazo dye, 1-phenylazo-2-hydroxy-naphthalene (Sudan I) into an ultimate carcinogen, which binds to calf thymus DNA. The principal product of Sudan I oxidation by the Ce(IV) system is the benzenediazonium ion. A minor product is the dihydroxyderivative of Sudan I, 1-(4-hydroxyphenylazo)-2,6-dihydroxy-naphthalene. Other minor coloured products (yellow and brown) were not identified. The principal product (the benzenediazonium ion) is responsible for the carcinogenicity of Sudan I, as it covalently binds to DNA. Ce(IV) ions in acidic medium represent a suitable model system, which imitates the activation route of carcinogenic azo dyes.

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]. The metabolism of carcinogenic aminoazo compounds has been studied extensively. It is known that nitrenium and carbenium ions evolved from aminoazo compounds are supposed to act as the ultimate carcinogens. In aminoazo compounds the activation pathways involve oxidative N-dealkylation, N-hydroxylation and esterification[3]. N-hydroxy metabolites are compounds which are converted into the ultimate carcinogens, i.e. nitrenium and carbenium ions[3,4]. These ions bind to DNA in vivo and in vitro[3], and thus, they are responsible for the carcinogenicity of aminoazo dyes[2-4]. Recently, Matrka and Pípalová[5,6] described that derivatives of one of the aminoazo dyes, N,N-dimethyl-4-aminoazobenzene, are oxidatively split by a model system containing cerium(IV) ions to form arenediazonium ions and the corresponding quinoneimine[5,6]. The formation of arenediazonium ion may be the further step for the activation of azo compounds, because the arenediazonium ion is supposed to be the ultimate carcinogen[2]. It is suggested that the initiation of the chemical carcinogenesis is evolved from the binding of the active forms of carcinogens to nucleic acids in cells[7]. However, information about the binding of azo dyes oxidation products by Ce(IV) ions to nucleic acids is missing.

The subject of this paper is the oxidation of Sudan I (1-phenylazo-2-hydroxy-naphthalene) *I*, which is a typical proximate carcinogen causing tumours of rat

liver and of the urinary bladder[8]. Sudan I is an azo dye which does not contain an amino group in its molecule. Thus, the mechanism of the activation of this non-aminoazo dye to ultimate carcinogens is different from that of the typical aminoazo compounds[9-12]. The present paper is a contribution to the understanding of the activation routes of carcinoegnic azo compounds.

EXPERIMENTAL

Chemicals: Sudan I and 1-phenyl-3-methyl-5-pyrazolone (British Drug Houses) and other chemicals (Lachema, Czechoslovakia) were of analytical grade. The model compound used for the detection of the presumed product of the oxidative splitting of Sudan I was obtained by azo coupling of benzenediazonium chloride with 1-phenyl-3-methyl-5-pyrazolone. Its purity was checked by thin-layer chromatography (TLC) on silica gel Silufol R, (Kavalier, Czechoslovakia) in hexane–diethyl ether (2 : 1), furthermore, by electronic absorption spectrum and melting point. Calf thymus DNA was prepared as described by Kay et al.[13].

^{14}C-labelled 1-([U-^{14}C]phenylazo)-2-hydroxynaphthalene (^{14}C-Sudan I) (20 MBq mmol^{-1}) was synthesized as described in an earlier paper[5] from [U-^{14}C]aniline (The Radiochemical Centre, Amersham, England) and β-naphthol and purified by column chromatography on basic alumina and preparative TLC on silica gel. The labelled compound was stored in a methanol solution at −5°C.

Oxidation of ^{14}C-Sudan I

Titrations of Sudan I by $Ce(SO_4)_2$ were carried out by the slightly modified procedure described by Matrka and Pípalová[5,6]. To a 1·5 ml sample of $3 \cdot 10^{-3}$M ^{14}C-Sudan I in dimethylsulfoxide 50% CH_3COOH was added to reach a total volume of 10 ml; the solution was cooled in an ice bath and titrated by 0·1M-$Ce(SO_4)_2$ (0·2 ml portions were added at 1 min intervals). This titration was followed potentiometrically on a Radelkis (Hungary) pH-meter OP-211/1 with platinum and calomel electrodes.

After the end of the titration, a 3 ml aliquot was withdrawn from the reaction mixture and added to 25 ml of 0·05M-Na_2CO_3. After 4 h incubation, the reaction products were extracted from the mixture with 25 ml of ethyl acetate. The extract was evaporated to dryness, the residue was dissolved in a minimum volume of methanol and separated by TLC on silica gel (Silufol R, Kavalier, Czechoslovakia). The chromatogram was developed in diethyl ether–light petroleum (1 : 1). The products were separated mechanically, by cutting thin layers of silica gel, which were placed in scintillation vials and the radioactivity was counted in a toluene-based scintillation cocktail using and Isocape/300 liquid scintillation counter (Searle, Netherlands) with an efficiency of about 80%. The same chromatography was carried out with standards.

Detection of the Benzenediazonium Ion

Formation of the benzenediazonium ion was identified by its azo coupling with 1-phenyl-3-methyl-5-pyrazolone, which results in the formation of 1-phenyl-3-methyl-4-phenylazo-5-pyrazolone (400 nm). Alternatively, after the end of the titration, 25 ml of 0·5M-Na_2CO_3 containing 0·01M 1-phenyl-3-methyl-5-pyrazolone were added to 3 ml samples of the reaction mixture. After 4 h, the reaction mixture was extracted with 25 ml of ethyl acetate. The extract was evaporated, dissolved in a minimum volume of methanol and chromatographed on the silica gel thin layer and eluted with hexane–diethyl eter (2 : 1). The azo zone, which co-chromatographed

with 1-phenyl-3-methyl-4-phenylazo-5-pyrazolone, and further zones were separated mechanically and the radioactivity was measured as described above. Alternatively, the zones were dissolved in benzene, centrifuged and the clear solutions were used for spectrophotometry (SPECORD M-40, Zeiss Jena, G.D.R.).

Incubation mixtures used for the modification of DNA by products formed from ^{14}C-Sudan I by oxidation with Ce(IV) ions were the same as described above, moreover, they contained 0·092 g l^{-1} DNA, which was added to the 3 ml aliquot of the reaction mixture dissolved in 25 ml of 0·5M-Na_2CO_3. After the incubation (4 h) described above, the azo dyes were extracted twice with ethyl acetate, DNA was extracted from the water layer with phenol–chloroform[14] and precipitated with ethanol. Precipitates of DNA were washed (ethanol, benzene, diethyl ether) and the ^{14}C-radioactivity was measured in the dried DNA dissolved in the Instagel (Amersham, England) cocktail on the Isocape/300 liquid scintillation counter with an efficiency of about 80%.

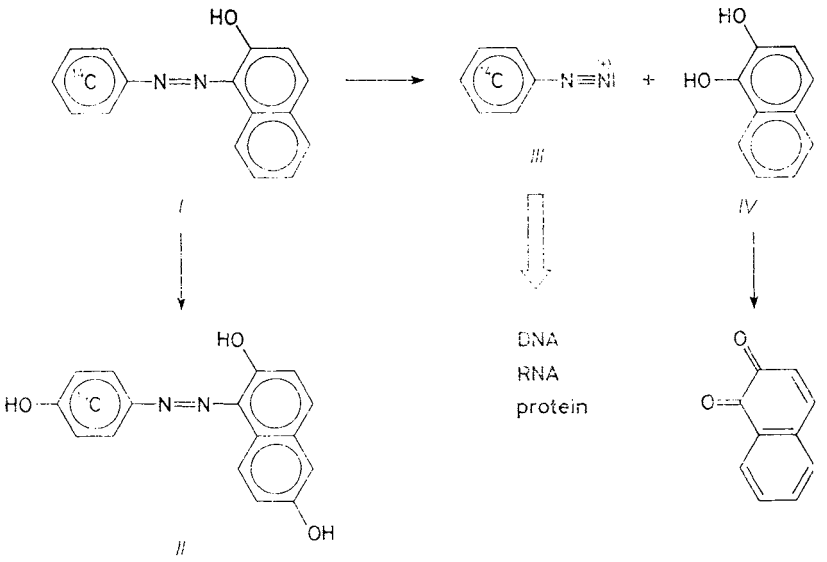
Effect of Azo Coupling of Benzenediazonium Ion with 1-Phenyl-3-methyl-5-pyrazolone on ^{14}C-Labelling of DNA

A solution of 0·5M-Na_2CO_3 (25 ml) or the same solution of Na_2CO_3 containing 0·01 mol l^{-1} 1-phenyl-3-methyl-5-pyrazolone were added to 3 ml aliquots of the reaction mixture, in which the ^{14}C-Sudan I was oxidized by Ce(IV) (see above), without DNA. After 5 h, the DNA was added and mixtures were incubated for further 12 h. Azo dyes were extracted from the mixtures with 25 ml of ethyl acetate. The DNA was extracted from the remaining water phase by phenol–chloroform, neutralized (1M-HCl) and precipitated with ethanol. The precipitates were dissolved in a minimum volume of 0·1M-EDTA, dialyzed against distilled water (500 ml, 8 h) and precipitated with ethanol. After washing (ethanol, diethyl ether) the radioactivity of DNA was measured as described above.

RESULTS AND DISCUSSION

^{14}C-Sudan I is oxidized by Ce(IV) ions in an acidic medium with 97·3% conversion. Thin layer chromatography analyses of ^{14}C-Sudan I oxidation products indicate that Ce(IV) ions converted this azo dye into four products. The major product (colourless) is hydrophilic (on the TLC in hexane–diethyl ether it has R_F 0·03) (Table I). The next product co-chromatographed with 1-(4-hydroxyphenylazo)-2,6-dihydroxynaphtalene (*II*) and other minor coloured products (yellow and brown) were not identified. The identity of the polar product with R_F 0·03 was determined indirectly and it was shown to be the product of the oxidative splitting of Sudan I. This reaction may lead to the formation of the benzenediazonium ion (*III*) and quinone (*IV*). The formation of benzenediazonium ion by Ce(IV) ions was identified by its azo coupling with 1-phenyl-3-methyl-5-pyrazolone, which results in the formation of a yellow coupling product 1-phenyl-3-methyl-4-phenylazo-5-pyrazolone with the absorption maximum at 400 nm. In our experiments, the mixture of products formed from ^{14}C-Sudan I by Ce(IV) reacted with 1-phenyl-3-methyl-5-pyrazolone and the compounds formed were separated by TLC. The radioactive 1-phenyl-3-methyl-4-phenylazo-5-pyrazolone separated by TLC from other products was identified by means of two methods: UV-VIS spectroscopy and TLC, by comparison

with the synthetic standard. After the reaction of the products formed from ^{14}C--Sudan I by Ce(IV) ions with 1-phenyl-3-methyl-5-pyrazolone, the amount of the hydrophilic product decreased and this observed decrease correlated with the increase of the formation of the yellow radioactive coupling product (Table II). Thus, it can be suggested that the polar product evolved from the reaction of ^{14}C-Sudan I with Ce(IV) may be the compound derived from the benzenediazonium ion.



SCHEME 1

TABLE I

Products formed from ^{14}C-Sudan I by Ce(IV) oxidation in acidic medium; TLC in ether–light petroleum (1 : 1)

Products	% of total radioactivity		R_F
	with Ce(IV)	without Ce(IV)	
III (colourless)	61·1	0·5	0·03
II (yellow)	20·5	0·1	0·18
Unidentified (yellow)	12·6	1·9	0·43
Unidentified (brown)	3·1	0·3	0·56
I (yellow)	2·7	97·2	0·81

It is known that the benzenediazonium ions act as the ultimate carcinogens, which bind to DNA (ref.[2]). Thus, the binding of the benzenediazonium ion and/or other products formed from Sudan I by Ce(IV) ions to DNA was further studied in the in vitro experiments.

While practically no radioactivity could be detected in DNA incubated with ^{14}C-Sudan I alone (7·8 Bq mg^{-1} DNA), the DNA became labelled after the incubation with this radioactive carcinogen in the presence of Ce(IV) ions (9 356 Bq mg^{-1} DNA).

As the benzenediazonium ion is formed as the major product from Sudan I by Ce(IV) ions, in further experiments we studied whether this reactive compound (or another compound from the other products) is that one which binds to the DNA. The radioactivity of DNA, which was added into the incubation medium containing ^{14}C-Sudan I and Ce(IV), after the addition of 25 ml of 0·5M-Na_2CO_3 without 1-phenyl-3-methyl-5-pyrazolone was 9 991·8 Bq mg^{-1} (the control samples). Furthermore, the radioadtivity of the DNA added into the same incubation medium, which in this case contained 1-phenyl-3-methyl-5-pyrazolone was 2 748 Bq mg^{-1}. Thus, the binding of the active product formed from ^{14}C-Sudan I to DNA is supressed by this agent to 27·5% of the control, because 1-phenyl-3-methyl-5-pyrazolone reacts effectively and readily with the benzenediazonium ion by azo coupling. Thus, the benzenediazonium ion may be the main product of Sudan I which binds to DNA and, furthermore, is responsible for the carcinogenicity of the studied non-amino azo dye.

In our earlier papers[9-12] we have studied the metabolism of the non-aminoazo dye (Sudan I) by microsomal cytochrome P-450 and by peroxidase. We found that benzenediazonium ion is formed from Sudan I by both above mentioned biological systems and that this ion is bound to DNA in vitro[10-12]. However, in biological

TABLE II

Products of ^{14}C-Sudan oxidation with Ce(IV) followed by coupling with 1-phenyl-3-methyl-5-pyrazolone; TLC in hexane–ether (3 : 1)

Products	% of total radioactivity		R_F
	with PMP[a]	without PMP[a]	
Benzenediazonium ion	16·0	61·1	0·01
Sudan I and its derivatives	31·7	38·2	0·1–0·6
1-Phenyl-3-methyl-4-phenylazo-5-pyrazolone	52·3	0·7	0·63

[a] 1-Phenyl-3-methyl-5-pyrazolone.

systems (mainly microsomes) the C-hydroxy derivatives of Sudan I were found as major products.

The present paper shows that Ce(IV) in acidic medium is a very effective system which converts Sudan I to the benzenediazonium ion. Moreover, this ion is formed as the major product. Thus, this reaction may effectively imitate some reactions found in biological systems. Although the oxidative mechanisms of both systems (biological and Ce(IV)) are probably different (some products formed by microsomes[11] and Ce(IV) (present paper) are different), the Ce(IV)-mediated oxidation is useful as a model because benzenediazonium ion generated by both systems binds to DNA and is responsible for the initiation of carcinogenesis. Moreover, the DNA-labelling by the ultimate carcinogenic form of Sudan I formed by Ce(IV) ions is more effective than that by biological systems[10-12]. Thus, the DNA modified by Ce(IV) system may be used for the further study of the exact modes of benzenediazonium ion binding to DNA. The exact reaction of the arenediazonium ion with DNA which is responsible for the initiation of the carcinogenesis is unknown at the present time[2]. The possible reactions of arenediazonium ions are: (1) azo coupling, (2) displacement of nitrogen by nucleophiles, (3) induction of free radical processes, (4) the formation of triazenes by reactions of nitrogen atoms of purine and pyrimidine bases of DNA. Which of these reactions is responsible for the initiation of carcinogenesis (in other words, for the binding to DNA) should be resoved by further studies.

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