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Activation of carcinogens by peroxidase

Horseradish peroxidase-mediated formation of benzenediazonium ion from a non-aminoazo dye, l-phenylazo-2-hydroxynaphthalene (Sudan I) and its binding to DNA

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Horseradish peroxidase in the presence of hydrogen peroxide (HRP/H_2O_2) oxidizes a carcinogenic non-aminoazo dye, l-phenylazo-2-hydroxynaphthalene (Sudan I) to the ultimate carcinogen, which binds to calf thymus DNA. The principal product of Sudan I oxidation by the HRP/H_2O_2 system is the benzenediazonium ion. Minor products are hydroxy derivatives of Sudan I, in which the aromatic rings are hydroxylated. The principal oxidative product (the benzenediazonium ion) is responsible for the carcinogenicity of Sudan I, because this ion, formed from this azo dye, binds to DNA.

Horseradish peroxidase; Azo dye; DNA binding

1. INTRODUCTION

Mammalian peroxidases are vital to the body's defense systems and hormone synthesis [1]. However, several peroxidases present in mammalian tissues have been implicated in the metabolic biotransformation of xenobiotics [2–4]. Horseradish peroxidase (EC 1.11.1.7) in the presence of hydrogen peroxide (HRP/H_2O_2) is a suitable enzymic system, which is able to mimic the so-called 'oxidation step' (electron abstraction from the substrate) in biotransformation of the exogenous molecules in organisms [5]. Moreover, oxygenation of xenobiotics catalyzed by HRP/H_2O_2 was also detected [6]. Various organic compounds such as phenols, amines, diamines, polycyclic aromatic hydrocarbons have been shown to be oxidized by HRP/H_2O_2 [5–7]. However, the mechanism of these oxidations is not fully explained yet. The formation of free radicals [5,8,9], ions [8,10], polymers [5,7,11] and further unidentified reactive products [5] is due to HRP/H_2O_2-mediated oxidation of organic compounds. Furthermore, some of the peroxidase oxidation products are so reactive that they may interact with nucleophiles under certain conditions [5,8,9,12]. Although the oxidation of several of the above mentioned compounds by HRP/H_2O_2 was detected, the oxidations of azo dyes by this system have not yet been determined. We are especially interested in the problem of whether HRP/H_2O_2 is able to oxidize a non-aminoazo dye, 1-phenylazo-2-hydroxynaphthalene (Sudan I), which is known to act as the proximate carcinogen of rat livers or of the urinary bladder [13]. The biotransformation

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Abbreviations: HRP/H_2O_2, horseradish peroxidase in the presence of hydrogen peroxide; PMP, 1-phenyl-3-methyl-5-pyrazolone; R_f, relative mobility; TLC, thin layer chromatography

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of Sudan I by rat liver microsomes was studied earlier [14–16]. Here, we firstly report that Sudan I is oxidized by HRP/H_2O_2 to the form which binds to calf thymus DNA in vitro.

2. MATERIALS AND METHODS

^{14}C-labelled Sudan I (1′-([*phenyl*-U-^{14}C]phenylazo)-2-hydroxynaphthalene; 20 MBq·mmol^{-1}) was synthesized from [U-^{14}C]aniline (Amersham) and non-labelled β-naphthol, and purified by column chromatography on basic alumina and by thin-layer chromatography (TLC) on silica-gel G. Incubation mixtures for the study of the [^{14}C]Sudan I conversion by HRP/H_2O_2 contained in a final volume of 2 ml: 0.1 M acetate buffer, pH 4.7, 0.03 mM HRP (Fluka), 0.11 mM H_2O_2, 0.3 mM [^{14}C]Sudan I and 0.69 g·l^{-1} calf thymus DNA. Unless stated otherwise, the mixtures were incubated at 37°C (30 min–3 h) in open tubes after which the [^{14}C]Sudan I products were twice extracted with ethyl acetate (2 × 2 ml) and the DNA was further isolated by the phenol-chloroform method [17], and precipitated with ethanol. The ethyl acetate extracts of products were evaporated, dissolved in a minimal volume of methanol and chromatographed on thin layer of silica-gel and eluted with diethyl ether/petroleum ether (1:1, v/v). The products were mechanically separated from layers, placed in a scintillation vial and the radioactivity was counted in an Instagel scintillation cocktail (Amersham) on the Isocape/300 (Searle) scintillation counter with an efficiency of about 80%. The idential TLC was carried out with standards. Precipitates of DNA were washed (ethanol, benzene, diethyl ether) and the ^{14}C radioactivity was measured in dried DNA dissolved in the Instagel cocktail.

The formation of the benzenediazonium ion was identified by its azo coupling with 1-phenyl-3-methyl-5-pyrazolone (PMP), which results in the formation of 1-phenyl-3-methyl-4-phenylazo-5-pyrazolone (λ_{max} = 400 nm). After the end of the above described incubations with HRP/H_2O_2, 25 ml of 0.5 M Na_2CO_3 or the same solution of Na_2CO_3 containing 0.01 M PMP were added to the reaction mixtures. After 12 h incubations, the azo dyes were extracted from the reaction solution with 25 ml of ethyl acetate. The extracts were evaporated and chromatographed on the silica-gel thin layer (*n*-hexane/diethyl ether mixture, 3:1, v/v). The azo zone, which cochromatographed with 1-phenyl-3-methyl-4-phenylazo-5- pyrazolone (the coupling product) and further zones were separated mechanically and the radioactivity was measured. Alternatively, the zones were dissolved in benzene, centrifuged and the clear solutions were used for the spectrophotometry.

The effect of azo coupling of the benzenediazonium ion with PMP on ^{14}C-labelling of DNA was examined: the DNA was isolated from the water phases which remained after the ethyl acetate extraction of the reaction mixtures incubated with or without PMP (see above), by phenol-chloroform extraction [17], neutralized (1 M HCl) and precipitated with ethanol. The obtained precipitates were dissolved in a minimal volume of 0.1 M EDTA, dialyzed against water (1000 ml, 24 h) and precipitated with ethanol. After washing (ethanol, diethyl ether) the radioactivity of DNA was measured as described above.

3. RESULTS

[^{14}C]Sudan I is oxidized by HRP/H_2O_2 to four products. Two minor coloured products cochromatographed with 1-phenylazo-2,6-dihydroxynaphthalene and 1-(4′-hydroxyphenylazo)-2,6-dihydroxynaphthalene. The identity of the third minor product (colourless) with R_f = 0.69 has not yet been established. The major [^{14}C]Sudan I product formed by oxidation with HRP/H_2O_2 (R_f = 0.03, table 1) was shown to be the product of the oxidative splitting of this azo dye; its identity being determined indirectly. The oxidative splitting of Sudan I can be considered to lead to the formation of the benzenediazonium ion and quinone (fig.1). The formation of the benzenediazonium ion by the HRP/H_2O_2 system was identified by its azo coupling with PMP, which results in the formation of the 1-phenyl-3-methyl-4-phenylazo-5-pyrazolone. In our experiments, the mixture of products formed from [^{14}C]Sudan I by HRP/H_2O_2 reacted with PMP and the formed compounds were separated by TLC. The radioactive coupling product (1-phenyl-3-methyl-4-phenylazo-5-pyrazolone) separated by TLC from other products was identified by two methods: UV-VIS spectroscopy and TLC, by comparison with the synthetic standard (table 2). After the reaction of products formed from [^{14}C]Sudan I by HRP/H_2O_2 with PMP, the amount of the major product decreased and this observed decrease correlated with the increase of the formation of the yellow radioactive coupling product (table 2). Thus, it can be suggested that the major product of the oxidation of [^{14}C]Sudan I by HRP/H_2O_2 may be the compound derived from the benzenediazonium ion.

The prolonged incubation of [^{14}C]Sudan I with HRP/H_2O_2 (more than 15 h) led to the formation of further coloured unknown products (not shown).

While practically no radioactivity could be detected in DNA incubated (3 h) with [^{14}C]Sudan I alone (20.0 Bq·mg^{-1}), or with [^{14}C]Sudan I and H_2O_2 (24.6 Bq·mg^{-1} DNA), the DNA became labelled after incubation with this radioactive carcinogen in the presence of the HRP/H_2O_2 (2608.4 Bq·mg^{-1} DNA). The oxidation of Sudan I by HRP/H_2O_2 resulted in the covalent binding of the active metabolite to DNA. The evidence for such binding was provided by the incorporation of

Table 1

Products formed from [^{14}C]Sudan I by HRP/H_2O_2 system

TLC in diethyl ether/ petroleum ether (1:1) R_f	Products[a] obtained by incubations					Corresponding standard
	Without HRP, without H_2O_2, 30 min	Without HRP, with H_2O_2, 30 min	With HRP/H_2O_2 30 min	60 min	360 min	
0.03	0.54	0.88	9.90	12.81	15.69	–
0.18	0.14	0.18	1.24	1.82	1.37	1-(4'-hydroxyphenylazo)-2,6-dihydroxynaphthalene
0.41	0.27	0.49	2.70	2.20	2.45	1-phenylazo-2,6-dihydroxy-naphthalene
0.69	0.69	0.88	1.96	3.59	3.79	–
0.81	98.36	97.58	84.20	79.58	76.70	Sudan I

[a] The ^{14}C-labelled products and [^{14}C]Sudan I were extracted from incubation mixtures by ethyl acetate and separated by TLC as described in section 2. The values given are the mean of 3 experiments and are shown as percentage of ^{14}C total radioactivity

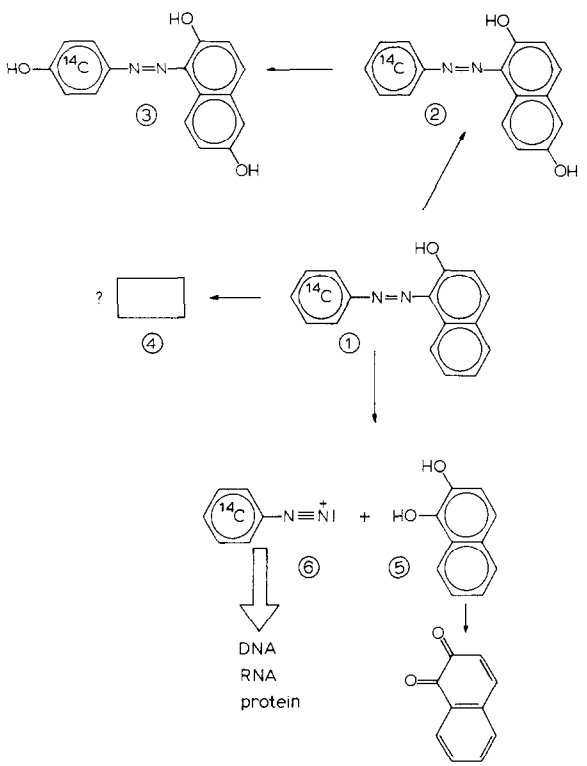

Fig.1. Products of oxidation of [^{14}C]Sudan I by HRP/H_2O_2. (1) 1-Phenylazo-2-hydroxynaphthalene (Sudan I) (^{14}C-labelled in the benzene ring); (2) 1-phenylazo-2,6-dihydroxynaphthalene; (3) 1-(4'-hydroxyphenylazo)-2,6-dihydroxynaphthalene; (4) unknown colourless product; (5) 1,2-dihydroxynaphthalene and 1,2-naphthoquinone; (6) benzenediazonium ion.

radioactivity into the DNA left after ethanol precipitation and dialysis of the DNA radiolabelled by [^{14}C]Sudan I activated by HRP/H_2O_2 (see section 2). The radioactivity of DNA incubated with [^{14}C]Sudan I and HRP/H_2O_2 (2 h) and after addition of 25 ml of 0.5 M Na_2CO_3 without PMP (12 h) was 2030.2 Bq·mg^{-1} (the control sample). On the other hand, the radioactivity of DNA incubated under the same conditions, but in the presence of the PMP was 1076 Bq·mg^{-1}. Thus, the binding of the active product formed from [^{14}C]Sudan I by HRP/H_2O_2

Table 2

Azo coupling of products formed from [^{14}C]Sudan I by HRP/H_2O_2 with 1-phenyl-3-methyl-5-pyrazolone (PMP)

TLC in *n*-hexane/ diethyl ether (3:1) R_f	Products[a] obtained		Corresponding standard
	Without PMP	With PMP	
0.01	12.87	4.71	–
0.1–0.6	85.71	84.59	Sudan I and its hydroxy derivatives
0.63	1.42	10.70	1-phenyl-3-methyl-4-phenylazo-5-pyrazolone (λ_{max} = 400 nm)

[a] The ^{14}C-labelled products formed after 2 h incubations with HRP/H_2O_2 and thereafter in 0.1 M Na_2CO_3 with or without PMP were extracted with ethyl acetate and separated by TLC (see section 2). The values given are the mean of 3 experiments and are shown as percentage of ^{14}C total radioactivity

to DNA is suppressed by PMP to 53% of the control, because PMP reacts effectively and readily with the benzenediazonium ion formed from [^{14}C]Sudan I (by azo coupling). Hence, the PMP decreases the concentration of the benzenediazonium ion in the incubation mixture (see table 2), which leads to a significant decrease of [^{14}C]DNA labelling. Hence, the benzenediazonium ion may be that compound which binds to DNA.

4. DISCUSSION

The importance of oxidative bioactivation of various azo compounds which are carcinogenic is gradually being recognized. Within the biooxidative biotransformation, most of the studies have been devoted to the hepatic metabolism performed by cytochrome P-450 monooxygenases [18] and, furthermore, mainly with the aminoazo dyes [19]. However, the aminoazo dyes are not supposed to be adequate models for the study of the carcinogenicity of azo dyes, because the amino group in their molecules can participate in the mechanism of the carcinogenicity [18]. Nevertheless, the studies on the activation steps as well as the detoxication pathways of non-aminoazo dyes, namely of Sudan I, are scarce [14–16,20,21]. The HRP/H_2O_2 system, which is a suitable promising model for studying of the oxidative bioactivation of various organic compounds in vitro, is used in the present paper to find out whether the non-aminoazo dye, Sudan I, is the substrate of this system. Our experiments indicate that the HRP/H_2O_2 system mainly converts Sudan I by oxidative splitting to form the benzenediazonium ion, which is known as the ultimate carcinogen [19]. The oxidative splitting of Sudan I was also found to be caused by hepatic microsomes of rats [14–16]. This reaction may probably be the main route of activation of the non-aminoazo dyes to the ultimate carcinogen, because the benzenediazonium ion formed from Sudan I by microsomal enzymes binds to deoxyguanosine residues in DNA [15,16]. This paper is the first study showing the activation of carcinogenic azo dye by HRP/H_2O_2 resulting in the binding of metabolite to DNA. Further studies will be carried out to study the parallelism of the metabolism of carcinogens by the microsomal (P-450) and HRP/H_2O_2 systems.

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