TO THE MECHANISM OF 2-NITROANISOLE CARCINOGENICITY: *in vitro* METABOLISM OF 2-NITROANISOLE MEDIATED BY PEROXIDASES AND XANTHINE OXIDASE

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The *in vitro* enzymatic metabolism of carcinogenic 2-nitroanisole was investigated using peroxidases (horseradish peroxidase and prostaglandin H synthase) and xanthine oxidase catalyzing oxidative and reductive reactions, respectively. The oxidation of 2-nitroanisole catalyzed by horseradish peroxidase exhibits the Michaelis–Menten kinetics. The Michaelis constant (K_m) and the maximal velocity (V_{max}) values for this substrate were determined at pH 5.0, 7.0, 7.6 and 8.0. At optimal pH (7.6), the K_m and V_{max} values are 0.219 μmol/l and 34.45 pmol/min per nmol peroxidase, respectively. The oxidation of 2-nitroanisole is inhibited by radical trapping agents (NADH, ascorbate, glutathione and nitrosobenzene). This indicates that the peroxidase-mediated oxidation of 2-nitroanisole proceeds *via* a radical mechanism. Active oxygen species are formed during the horseradish peroxidase-catalyzed reactions in the presence of NADH, hydrogen peroxide and 2-nitroanisole. 2-Nitroanisole is also oxidized by mammalian prostaglandin H synthase. Using the nuclease P1-enhanced variation of the ^{32}P-postlabelling assay, the formation of DNA adducts was detected in DNA treated with 2-nitroanisole and xanthine oxidase. No DNA binding was detected after oxidation of 2-nitroanisole with horseradish peroxidase and prostaglandin H synthase. The results presented (the formation of DNA adducts after 2-nitroanisole activation by xanthine oxidase and that of radicals and/or superoxide radicals during the reactions with peroxidases) strongly suggest the participation of 2-nitroanisole both in the initiation and in the promotion phases of carcinogenesis.

Key words: Carcinogens; Nitro compounds; Oxidation; Reduction; Radicals; DNA adducts; Initiation and promotion phases of carcinogenesis.

Aromatic amines and nitro compounds rank among a group of carcinogenic or potentially carcinogenic compounds, presenting a considerable danger for human population[1,2]. These compounds are often found in a workplace (*e.g.* in chemical industry) and in the environment, where they add to local and regional pollution (car exhausts, technological spills). The compounds of this group are thus intensively studied in many laboratories and are also in the center of interest of many public institutions.

This is also the case for 2-nitroanisole (2-methoxynitrobenzene). 2-Nitroanisole is used primarily as a precursor in the synthesis of 2-anisidine, an intermediate in the

manufacture of many azo dyes[3]. An industrial accident at Hoechst in Germany caused a large-scale leakage of this compound in 1993 and subsequent local and regional contamination. As a result, researchers in Germany and other countries of the European Union started to address in particular the following research problems: (i) the toxicity and carcinogenicity of this compound, (ii) what are the dangerous concentrations of 2-nitroanisole, (iii) the metabolic pathway of 2-nitroanisole and (iv) the persistence of residues of this compound and/or its metabolites in environment and organisms after the accident.

2-Nitroanisole is a toxic compound, causing anaemia, characterized by increased levels of methemoglobin and accelerated destruction of erythrocytes[3]. 2-Nitroanisole exhibits carcinogenic activity, causing neoplastic transformation in urinary bladder and, to a lesser extent, in spleen, liver and kidneys[3]. The compound is weakly mutagenic in the Ames test with the *Salmonella typhimurium* TA100 strains[3]. However, its mutagenic activity could not be proved with other strains[3] (*e.g.* TA97, TA98, TA1535, TA1537). This chemical also exhibits a low activity in cytogenetic tests. It induces a slight increase in chromosomal aberrations and of sister chromatid exchanges, but only at high concentration[3]. However, these weak mutagenic effects sharply constrast with its strong carcinogenicity. The data mentioned above imply a rather enigmatic mechanism of the 2-nitroanisole carcinogenicity. Indeed, up to the present time, it has not been determined exactly, if this compound is a genotoxic or epigenetic carcinogen and in which phase of carcinogenesis it is involved. But these data are necessary to resolve the mechanism of 2-nitroanisole carcinogenicity.

The major route of metabolism of 2-nitroanisole *in vivo* is oxidative demethylation to 2-nitrophenol, which appears in urine predominantly as the sulfate conjugate[4]. A second pathway involves reduction to 2-anisidine (2-methoxyaniline); at blood concentrations at which the metabolism and elimination of 2-nitroanisole are linear, 2-anisidine is a minor metabolite formed in liver[4]. However, at higher doses the 2-nitrophenyl sulfate pathway may reach saturation, leading to the formation of proportionally more 2-anisidine[3].

The efficiences and balance of oxidative and/or reductive enzyme systems catalyzing the 2-nitroanisole biotransformation (detoxication and/or activation) are unknown. These are, however, the first essential data needed to explain the mechanisms, by which 2-nitroanisole is implicated in carcinogenesis. Therefore, to answer this question, the complex study carried out in our laboratories is aimed at finding which enzymes are able to convert 2-nitroanisole. In the present study, we used peroxidases (abundant enzymes catalyzing oxidative reactions of several xenobiotics in urinary bladder)[5–8] to determine their abilities to oxidize 2-nitroanisole *in vitro*. Horseradish peroxidase and mammalian prostaglandin H synthase (PHS) are used as model peroxidases. We report here their kinetic and catalytic characteristics with respect to 2-nitroanisole as the substrate. Moreover, the participation of oxidative and reductive reactions catalyzed by

peroxidases and xanthine oxidase, respectively, in the formation of DNA adducts from 2-nitroanisole, are reported.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: arachidonic acid, indomethacin and horseradish peroxidase from Sigma; L-ascorbic acid from Merck; NADH, yeast alcohol dehydrogenase, nitrosobenzene, DNA (from calf thymus), ferricytochrome c, superoxide dismutase and glutathione (reduced) from Boehringer; 2-nitroanisole from Fluka; xanthine oxidase and hypoxanthine from Serva. All chemicals were reagent grade or better. Enzymes and chemicals for the ^{32}P-postlabelling assay were obtained commercially from sources described previously[9].

Analytical Method

The assay mixture for the demethylation of 2-nitroanisole contained, in 1 ml, 200 μg horseradish peroxidase, 0.01–1.0 μmol 2-nitroanisole dissolved in ethanol, 1 μmol H_2O_2 and 50 μmol sodium acetate (pH 5.0) or 50 μmol potassium phosphate (pH 7.0, 7.6 or 8.0). The mixtures were incubated for 10 min at 37 °C. The reaction was terminated by addition of 500 μl of 20% trichloroacetic acid. The amount of formaldehyde formed was measured as described by Nash[10]. Inhibition of 2-nitroanisole oxidation by radical scavengers was performed in the same reaction mixture, but 0.5 or 1 μmol NADH, or ascorbate, or glutathione dissolved in 50 μmol of potassium phosphate buffer (pH 7.6), or 1 μmol of nitrosobenzene dissolved in ethanol was added.

Kinetic analyses were carried out using the non-linear least-square method described by Cleland[11].

NADH oxidation was measured in a final volume of 1 ml containing: 50 μmol potassium phosphate (pH 7.0, 7.6 or 8.0) or 50 μmol sodium acetate (pH 5.0), 0.1 μmol NADH, 0–0.220 μmol H_2O_2, 0–15 μg horseradish peroxidase and 0–1.5 μmol 2-nitroanisole. The reaction was started by addition of 2-nitroanisole, and the disappearance of NADH with time was followed at 340 nm using a Beckman D-62 spectrophotometer at 20 °C.

The same reaction mixture was used for estimation of ferricytochrome c reduction except that 20 nmol ferricytochrome c was added. Reduction of ferricytochrome c was monitored by the increase in absorbance at 550 nm (ref.[12]) at 20 °C. The molar extinction coefficient of cytochrome c at 550 nm (ref.[12]) was $2.9 \cdot 10^4$ mol/cm.

Oxygen consumption was measured with a Clark-type electrode at 20 °C.

Fresh ram seminal glands were obtained from a local slaughterhouse, trimmed of excess fat and tissue, and stored at –70 °C until use. Microsomes were prepared as described previously[13] and used as a source for PHS. PHS-Cyclooxygenase activity was determined by measuring the arachidonic acid-dependent oxygen uptake in a 2.0-ml chamber equipped with a Clark-type oxygen electrode. The incubation mixture contained (in 1 ml) 1.5 mg microsomal protein, 50 μmol potassium phosphate (pH 7.6) and 10 μl ethanol (2-nitroanisole solvent), with different concentration of 2-nitroanisole, preincubated for 1 min at 37 °C. The reaction was initiated by addition of arachidonic acid (0.1 μmol) dissolved in 2 μl of ethanol. Some reaction mixtures also contained 100 nmol of indomethacin, which is known as an inhibitor of the PHS-cyclooxygenase activity[7]. The initial rate of arachidonic acid oxidation was determined from the slope of the linear portion of the oxygen uptake curve.

Protein concentrations were estimated according to Lowry *et al.*[14] with bovine serum albumin as a standard.

Incubation mixtures used for the modification of DNA by 2-nitroanisole oxidized with peroxidases contained in final volume of 1.0 ml: 50 μmol potassium phosphate (pH 7.6), 200 μg horseradish peroxidase and 1.0 μmol H_2O_2, 1.5 mg microsomal protein and 0.1 μmol arachidonic acid, 1.0 μmol 2-nitroanisole and 1 mg calf thymus DNA. Control incubations were carried out either without peroxidases or without 2-nitroanisole. Incubations were performed at 37 °C for 60 min.

The incubations with xanthine oxidase were carried out according to Howard and Beland[15]. 2-Nitroanisole (1.0 or 10.0 μmol) was added to a deaerated and argon-purged solution of 1.0 mg of calf thymus DNA containing 1 μmol hypoxanthine and xanthine oxidase (1 unit) in 50 μmol potassium phosphate (pH 6.0) (the total volume was 1.0 ml). Mixtures were incubated 4 h at 37 °C under hypoxic conditions in the dark. Both control incubations without xanthine oxidase and without 2-nitroanisole were performed. In both enzymatic systems, 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[16], modified by Schoepe *et al.*[17] and precipitated with ethanol[18,19]. The DNA content was quantified spectrophotometrically at 260 nm.

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

For DNA modified by 2-nitroanisole after reaction with peroxidases or xanthine oxidase, the nuclease P1 version of the ^{32}P-postlabelling assay[17] 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 nad [γ-^{32}P]ATP under conditions described previously[20,21]. The same ^{32}P-postlabelling procedure was used for control incubations (either without enzymes or without 2-nitroanisole). Labelled digests were chromatographed on thin layer plates of poly(ethylenimine)–cellulose (PEI–cellulose), with modifications described by Reddy *et al.*[22]. This procedure is suitable for the resolution of benzoquinone-, phenol-, or hydroquinone derived adducts[22]. 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 thin layer chromatography (TLC) tank partially opened, to allow the radioactive impurities to concentrate in a band close to the top edge. The specific activity of [γ-^{32}P]ATP and efficiency of the kinase reaction were assayed as described[23]. 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[23,24].

RESULTS

Oxidation of 2-Nitroanisole by Peroxidases

Horseradish peroxidase in the presence of hydrogen peroxide oxidizes carcinogenic 2-nitroanisole. The formation of formaldehyde illustrating demethylation of this carcinogen was proved. No activity was observed when any of the component was omitted from the reaction mixture. The rates of demethylation of 2-nitroanisole by peroxidase were measured at pH 5.0, 7.0, 7.6 and 8.0 and were 15.0, 23.8, 28.0 and 26.0 pmol formaldehyde/min per nmol peroxidase, respectively. The highest rate of demethylation was found at pH 7.6.

Formaldehyde formation was measured in the reaction medium which contained peroxidase, hydrogen peroxide and various 2-nitroanisole concentrations. The reactions followed the Michaelis–Menten kinetics (Fig. 1). Under the conditions used, the maximal velocity (V_{max}) and the apparent Michaelis constant (K_m) values were the highest and lowest, respectively, at pH 7.6 (Table I).

The conversion of 2-nitroanisole is strongly inhibited by NADH, glutathione, ascorbate and nitrosobenzene, which are known to be radical scavengers[25] (Table II). These findings indicate that radicals are involved in the reaction mechanism.

We studied the mechanism of inhibition of the 2-nitroanisole oxidation by ascorbate and NADH. Low formaldehyde production was detected when 1 mmol/l ascorbate was present in the incubation mixture. Two possibilities of the mechanism of the ascorbate inhibition of the 2-nitroanisole oxidation could be suggested. Ascorbate may be the substrate of peroxidase, and it may compete with 2-nitroanisole, or it may react with reactive metabolites of 2-nitroanisole. Ascorbate as a subtrate is practically not con-

TABLE I
Kinetic parameters of 2-nitroanisole oxidation with horseradish peroxidase

pH	K_m[a]	V_{max}[a]
	mmol/l	pmol HCHO/min nmmol peroxidase
5.0	0.345 ± 0.020	21.50 ± 1.81
7.0	0.308 ± 0.025	32.25 ± 1.99
7.6	0.219 ± 0.017	34.45 ± 2.01
8.0	0.386 ± 0.021	33.00 ± 1.70

[a] Means and standard deviations of three experiments.

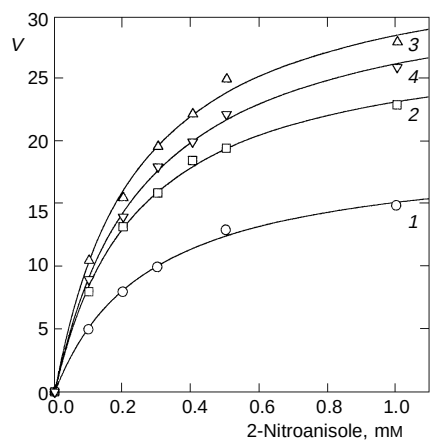


FIG. 1
Dependence of the rate of oxidation of 2-nitroanisole with horseradish peroxidase on the 2-nitroanisole concentration. *V* (pmol/min per nmol peroxidase) at pH: *1* 5.0, *2* 7.0, *3* 7.6 and *4* 8.0

verted by peroxidase (measured at 265 nm) under the conditions used in the experiments with the peroxidase system alone (ΔA/min = 0.03), but a rapid oxidation occurred in the presence of 1 mmol/l 2-nitroanisole (ΔA/min = 0.75). Ascorbate is therefore not a peroxidase substrate but reduces the radicals formed from 2-nitroanisole back to 2-nitroanisole.

Likewise, free radical(s) from 2-nitroanisole act as catalyst in the oxidation of NADH by hydrogen peroxide and peroxidase. In the absence of 2-nitroanisole in the reaction mixture, the oxidation of NADH by the peroxidase system was negligible (0.002 NADH/min per nmol peroxidase); the same was true for the system with this compound but without hydrogen peroxide (0.003 nmol NADH/min per nmol peroxidase). However, in the complete system (peroxidase, H_2O_2, NADH, 2-nitroanisole), NADH was oxidized (Table III). An effective oxygen uptake during the reaction in the above system was also observed (Table III). We found that NAD^+ is formed during the reaction. When most of the NADH (in the reaction mixture) was oxidized, the reaction adjusted to pH 8.8, and an NADH-generating system (alcohol dehydrogenase and ethyl alcohol) was added, NADH was fully recovered, indicating that the product was NAD^+.

As shown in Table IV, 2-nitroanisole enhanced the reduction of ferricytochrome c in the peroxidase–H_2O_2–NADH system. No reduction of ferricytochrome c occurred with 2-nitroanisole alone in the absence of hydrogen peroxide. The enhancement by 2-nitroanisole of the reduction rate was completely prevented by superoxide dismutase (Table IV) suggesting that the superoxide radical was formed, being responsible for the reduction of ferricytochrome c. Ferricytochrome c decreased the oxygen consumption by the peroxidase–H_2O_2–NADH–2-nitroanisole system. Addition of 0.05, 0.1, 0.15 and 0.2

TABLE II
The effect of radical scavengers on the 2-nitroanisole demethylation catalyzed by horseradish peroxidase

Scavenger	Concentration mmol/l	Rate[a] of 2-nitroanisole demethylation pmol HCHO/min nmol peroxidase
None		28.00 ± 1.21
Ascorbate	0.5	14.42 ± 1.12 (51.5)[b]
	1.0	1.51 ± 0.12 (5.4)
Glutathione	0.5	12.21 ± 1.15 (43.6)
	1.0	2.04 ± 0.18 (7.3)
NADH	0.5	20.36 ± 1.76 (72.7)
	1.0	2.88 ± 0.21 (10.3)
Nitrosobenzene	1.0	22.40 ± 2.10 (80.0)

[a] The values are averages and standard deviations of three parallel experiments. [b] The figures in the parentheses are percentage values relative to controls.

mmol/l ferricytochrome c resulted in 51, 75, 89 and 100% inhibition of oxygen consumption, respectively. However, no effect of ferricytochrome c on NADH oxidation by the system was observed. This indicates that superoxide radicals are not responsible for NADH oxidation and that the oxidation of NADH is mediated by the reaction of peroxidase with 2-nitroanisole (see above). These findings confirm that radicals are formed by a one-electron oxidation of 2-nitroanisole catalyzed by peroxidase, which is effective in the oxidation of NADH.

Although horseradish peroxidase has served as a suitable model peroxidase for mammalian peroxidases in the present work and in other studies[25,26], it was of interest to investigate the metabolism of 2-nitroanisole by PHS, another enzyme with peroxidative activity present in several mammalian tissues, including the tissues of lower urinary

TABLE III
NADH oxidation and oxygen uptake rates[a] in the peroxidase–H_2O_2–2-nitroanisole system

System	Conversion rate nmol NADH/min nmol peroxidase	Oxygen uptake rate pmol O_2/min nmol peroxidase
Complete	0.232 ± 0.0091	0.211 ± 0.012
– 2-nitroanisole	0.002 ± 0.0003	0.040 ± 0.003
– H_2O_2	0.003 ± 0.0003	0.045 ± 0.004
– peroxidase	0	0

[a] Values are averages and standard deviations of three parallel experiments.

TABLE IV
Ferricytochrome c reduction rate[a] (v) in the 2-nitroanisole-mediated NADH oxidation with the peroxidase–H_2O_2 system at various 2-nitroanisole concentrations (*c*)

c, mmol/l	*v*, nmol/min
0	0.033 ± 0.010
0.1	0.083 ± 0.050
0.2	0.150 ± 0.013
0.3	0.206 ± 0.018
0.4	0.230 ± 0.021
0.5	0.255 ± 0.022
0.5[b]	0

[a] Values are averages and standard deviations of three parallel experiments; [b] 0.2 nmol of superoxide dismutase added.

tract[5–7,27]. These tissues are the target organs for 2-nitroanisole. Conversion of arachidonic acid requires the incorporation of molecular oxygen catalyzed by PHS cyclooxygenase and the presence of a reducing cofactor for the reduction of endogeneous substrates (prostaglandins) catalyzed by the hydroperoxidase activity of the enzyme[7,13]. Oxygen uptake serves as a means of measuring cyclooxygenase activity of PHS (refs[7,13]). We investigate if 2-nitroanisole serves as a cofactor for PHS by measuring oxygen incorporation into arachidonic acid catalyzed by ram seminal microsomes containing PHS. Table V shows the concentration-dependent stimulation of oxygen uptake produced by 2-nitroanisole, which is completely inhibited by indomethacin, a well-known PHS cyclooxygenase inhibitor. At high concentrations of 2-nitroanisole, the stimulating effect of this compound was decreased (Table V). These findings indicated that 2-nitroanisole serves as a cosubstrate for PHS, radicals being formed during the reaction[7].

The Binding of 2-Nitroanisole to DNA

Using the nuclease P1 version of the ^{32}P-postlabelling assay, we did not detect DNA binding of 2-nitroanisole metabolite(s) after oxidation by horseradish peroxidase of PHS (Fig. 2). Although the reactive intermediates (radicals) are formed in this system, the data mentioned above imply that available amounts of these radicals should be very low or that the radicals might be quenched before the DNA is reached.

Because reduction can be an alternative metabolic pathway for 2-nitroanisole *in vivo*[4], we studied the possible DNA binding of the metabolites, formed in such reduc-

TABLE V
Stimulation of PHS-cyclooxygenase activity (measured as oxygen uptake rate per protein content[a], v) by various concentrations of 2-nitroanisole

2-Nitroanisole concentration μmol/l	v mmol/min kg
0	106.0 ± 9.7
10	165.1 ± 12.1
50	196.8 ± 17.2
100	259.8 ± 21.1
150	278.7 ± 24.3
200	157.2 ± 16.4
50[b]	9.1 ± 0.9

[a] The numbers are averages and standard deviations of triplicate analyses; [b] 100 nmol of indomethacin was added.

tive reactions. Out of the several mammalian reductases (microsomal NADPH : cytochrome P450 oxidoreductase, xanthine oxidase, aldehyde oxidase, lipoyl dehydrogenase, DT-diaphorase) that are known to catalyze reduction of nitro compounds in mammalian tissues[28], xanthine oxidase was chosen for our experiments. Using the same method for the covalent DNA binding determination (the above-mentioned nuclease P1 version of the ^{32}P-postlabelling assay), we investigated formation of DNA adducts by 2-nitroanisole in the system containing this mammalian reductase. After inspection of autoradiographs, five major and several minor adduct spots were detected in DNA treated with 2-nitroanisole and the xanthine oxidase system (Fig. 2). In contrast, control incubations carried out in parallel either without xanthine oxidase, or without 2-nitroanisole were free of adduct spots even after prolonged exposure times. Quantitative analysis of the adducts formed is shown in Table VI.

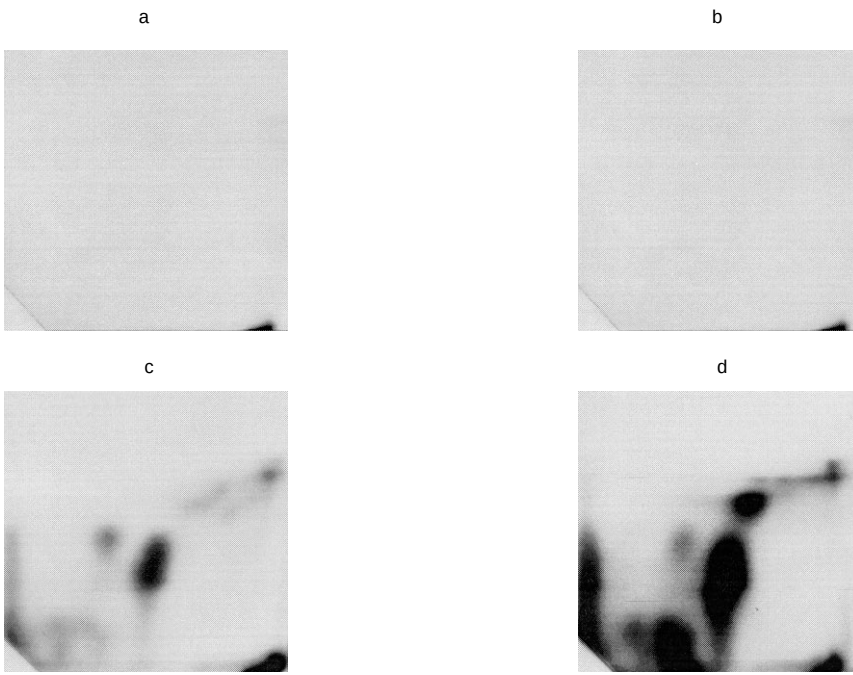


FIG. 2
Autoradiographs of PEI–cellulose TLC maps of ^{32}P-labelled digest of DNA, which was treated with 2-nitroanisole (1 μmol) activated with horseradish peroxidase (a), activated with PHS (b), activated with xanthine oxidase (c) and of that which was treated with 2-nitroanisole (10 μmol) activated with xanthine oxidase (d). Analysis was performed by the nuclease P1 version of the assay. Autoradiography was done at –70 °C for 16 h (a, b) and for 2 h (c, d). The origins are located at the bottom left corner, D3 direction from bottom to top and D4 direction on four directional TLC on PEI–cellulose from left to right (see Experimental or refs[20–24])

DISCUSSION

It is well established that the development of cancer (carcinogenesis) requires multiple steps. In several model systems, the stages of initiation, promotion and progression can be operationally defined through the use of discrete chemical agents[29,30].

Although the mechanism, by which 2-nitroanisole is involved in carcinogenic processes remains to be explained, one could speculate about a possible pathway. A prerequisite for 2-nitroanisole participation in the initiation stage of carcinogenesis is, that this compound forms adducts with DNA. In the present work we observed that activation of 2-nitroanisole leading to formation of DNA adducts *in vitro* is caused by the xanthine oxidase system, which is known to catalyze reductive reactions[15,28]. The ultimate carcinogens formed by the reductive enzyme systems may hence be involved in the initiation of carcinogenesis induced by 2-nitroanisole. These results also strongly suggest that 2-nitroanisole acts as a genotoxic carcinogen.

Here, ^{32}P-postlabelled adducts of 2-nitroanisole–DNA were resolved by the four-directional PEI–cellulose TLC technique modified by Reddy *et al.*[22] for analysis of polar adducts (Fig. 2). In contrast to the original Randerath method[24], this modification makes visible also adducts which contain only one benzene ring[18,22]. Therefore, the ultimate carcinogen formed from 2-nitroanisole probably contains only one benzene ring, too. The nitro group in 2-nitroanisole molecule is in all probability responsible for 2-nitroanisole activation, since it was postulated by Howard and Beland[15] and Kedderis and Miwa[28] that reduction of nitro compounds, such as carcinogenic or mutagenic polycyclic nitroaromatic hydrocarbons[30–32] and nitroheterocyclic compounds used as therapeutic, antimicrobial or antiparasitic agents, is important for their biological activity. At present, we can not determine the exact mechanism of reductive activation of 2-nitroanisole. Nevertheless, we can speculate that similarly to other nitroaromatic com-

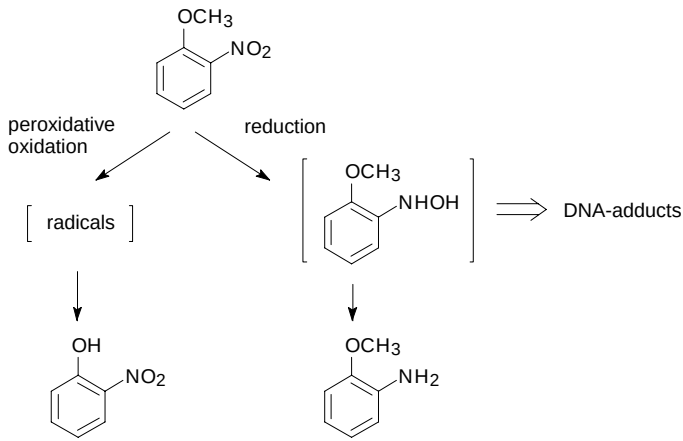
TABLE VI
Quantitative analysis of adducts formed from calf thymus DNA reacting with 2-nitroanisole and xanthine oxidase at various 2-nitroanisole concentrations (*c*)

c, mmol/l	Total DNA adduct content[a]	
	RAL . 10^7	mmol/g
0	0	0
1.0	5.70 ± 0.62	1.71 ± 0.19
10.0	13.80 ± 1.23	4.14 ± 0.37

[a] The numbers are averages and standard deviations of triplicate analyses. Relative adduct labelling (RAL) represents the number of adducts per normal nucleotides in modified DNA (see refs[20–24]). Total adduct content was determined by summing up of RAL of individual adducts.

pounds, the nitro group of 2-nitroanisole should undergo one-electron reduction to form the nitro anion radical, which is than converted by another one-electron reduction forming the nitroso group. This compound can be further reduced by two electrons to *N*-(2-methoxyphenyl)hydroxylamine. Further studies with that compound, aimed at resolving the mechanism of this reductive activation, are already in progress. Proposed metabolic pathway of 2-nitroanisole is shown in Scheme 1.

The reactions catalyzed by peroxidases were studied in detail in our laboratories. At present, 2-nitroanisole has been found to be oxidized by horseradish peroxidase to reactive metabolites with NADH and/or ascorbate oxidation activity. In addition, it mediates an effective oxygen uptake producing active forms of oxygen (superoxide radicals) under the conditions used. These findings could imply that the reactions are one-electron redox processes having free radicals as primary products, similarly to those observed with several other xenobiotics oxidized by peroxidase to radicals[7,8,20,25–27]. The radicals of 2-nitroanisole are also formed in its oxidation reactions catalyzed by PHS.



SCHEME 1

Several lines of evidence suggest a role of free radicals not only in the initiation of carcinogenesis (formation of adducts) but particularly also in processes of tumor promotion[33,34] and/or even progression[35]. We did not prove the participation of radicals derived from 2-nitroanisole by the action of peroxidases in the adduct formation under the conditions used. However, in view of the role played by radicals and superoxide radicals in carcinogenesis, formation of these species during the peroxidase-catalyzed oxidation of 2-nitroanisole can have biological consequences in the second (promotional) phase of carcinogenesis. In addition to peroxidases, other oxidative enzymes could also be effective in 2-nitroanisole oxidation and they might even be responsible for the DNA adduct formation. Therefore, the studies with major oxidative enzymes

metabolizing xenobiotics in organisms (cytochromes P450) in biotransformation of 2-nitroanisole are under way in our laboratories.

The analysis of induction type of bladder cancer caused by 2-nitroanisole, in dependence on different concentrations used, has shown a striking similarity to the induction type previously found for a well-characterized bladder carcinogen, *N*-[4-(5-nitro-2-furyl)thiazol-2-yl]formamide (FAFNT) (ref.[36]). For this compound, a two-stage model of carcinogenesis has been postulated. According to the model, FAFNT is involved both in the initial phase of carcinogenesis, modifying DNA of the target cells, and in the promotion phase, stimulating the target cell proliferation. However, it was not proved experimentally, whether the model is also valid for 2-nitroanisole. The data described in this paper (the formation of DNA adducts after 2-nitroanisole activation by xanthine oxidase and that of radicals or superoxide radicals during the reactions with peroxidases) strongly imply the participation of 2-nitroanisole both in the initiation and promotion phases of carcinogenesis. The model of induction type of bladder cancer caused by FAFNT is therefore probably applicable to 2-nitroanisole. The importance of the data presented here in an *in vivo* system has yet to be determined.

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