Evidence for Activation of Carcinogenic *o*-Anisidine by Prostaglandin H Synthase: ^{32}P-Postlabelling Analysis of DNA Adduct Formation

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Abstract. 2-Methoxyaniline (*o*-anisidine) is a urinary bladder carcinogen in both mice and rats Since the urinary bladder contains substantial peroxidase activity, we examined the ability of prostaglandin H synthase (PHS), a prominent enzyme in the urinary bladder, to activate this carcinogen to metabolites binding to macromolecules Using [^{14}C]-labeled *o*-anisidine, we observed substantial PHS-dependent binding of *o*-anisidine to protein, DNA and polydeoxyribonucleotides [poly(dX)] This binding is inhibited by radical scavengers glutathione, ascorbate and NADH The nuclease P1 and 1-butanol extraction enrichment procedure of the ^{32}P-postlabeling analysis of DNA modified by activated *o*-anisidine provide evidence that covalent binding to DNA is the principal type of DNA modification Deoxyguanosine is determined to be the major target for binding of *o*-anisidine in DNA The possibility that *o*-anisidine is carcinogenic to the rodent urinary bladder *via* its activation by bladder PHS is suggested The results presented here are the first report demonstrating a PHS-mediated activation of *o*-anisidine to reactive species forming covalent DNA adducts

Key words: Carcinogen — *o*-Anisidine — Prostaglandin H synthase — DNA adducts — ^{32}P-postlabeling

Abbreviations: CT-DNA, calf-thymus DNA, HPLC, high pressure liquid chromatography, HRP, horseradish peroxidase, PEI, polyethylenimine, PHS, prostaglandin H synthase, poly(dX), polydeoxyribonucleotides, RAL, relative adduct labeling, RSV, ram seminal vesicle, TLC, thin-layer chromatography

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Introduction

2-Methoxyaniline (*o*-anisidine) is widely used as an intermediate in the manufacture of many azo dyes, pigments and synthetic guaiacol (National Cancer Institute 1978, Garner et al 1984) Such a wide use of this aromatic amine could result in occupational exposure *o*-Anisidine is a potent carcinogen, causing the tumors of the urinary bladder in both genders of F344 rats and B6C3F1 mice (National Cancer Institute 1978, IARC 1982) Its strong carcinogenicity makes this substance very dangerous also for humans Beside its carcinogenicity it exhibits other toxic effects, including hematological changes, anemia and nephrotoxicity (IARC 1982)

o-Anisidine is mutagenic to *Salmonella in vitro* (Dunkel et al 1985), an effect that has been associated with both peroxidative oxidation and the involvement of *N* acetyltransferase enzymes (Ashby and Tennant 1988, Thompson and Eling 1991, Thompson et al 1992, Ashby et al 1994) The chemical induces chromosomal aberrations in Chinese hamster ovary cells *in vivo* (Galloway et al 1987) gene mutations in mouse lymphoma cells (Wangenheim and Bolcsfold 1988) and intrachromosomal recombination in *Saccharomyces cerevisiae* (Brennan and Schiestl 1999) A statistically significant DNA damage in urinary bladder of CD-1 mice exposed to *o*-anisidine determined by the alkaline single-cell gel electrophoresis (Comet) assay was detected (Sasaki et al 1998) Moreover, Ashby and coworkers (1994) demonstrated that a weak, but significant, increase of frequency of mutations was induced in urinary bladder in transgenic *lac*I (Big Blue^{TM}) mice treated with this carcinogen The mutant phage DNA recovered from *o*-anisidine treated bladders was sequenced and revealed the induction of novel (absent in controls) mutations (Glickman et al 1993, Ashby et al 1994) In spite of the potent rodent carcinogenicity and mutagenicity in *Salmonella* and in these *in vivo* tests, the chemical is however negative in other *in vivo* genotoxicity assays, including the mouse micronucleus test and the DNA single-strand break assay in rat liver, spleen, kidney, and bladder (Ashby and Tennant 1988, Ashby et al 1991, Morita et al 1997) One possible mechanism to explain the different mutagenicity observations involves potential different efficiencies of enzymes metabolizing (activating or detoxicating) *o*-anisidine in different mutagenicity tests

The metabolism of *o*-anisidine has not been studied in detail yet This carcinogen was found to undergo *O*-demethylation (Smith and Williams 1949) In addition, the *in vitro* experiments utilizing rat liver microsomes containing cytochromes P450 showed that *o*-anisidine is *O*-demethylated (Schmidt et al 1973), but products formed due to this microsomal oxidation have not yet been characterized

Additional studies *in vitro* have suggested a possible role for one or more of peroxidation enzymes in the metabolic activation of *o*-anisidine (Thompson and Eling 1991, Thompson et al 1992) Indeed, Thompson and Eling (1991) clearly demonstrated that *o*-anisidine is oxidized by horseradish peroxidase (HRP) *via* radical mechanism We have found that in addition to this enzyme, *o*-anisidine is oxidized by other two peroxidases, mammalian prostaglandin H synthase (PHS) that is a prominent enzyme in the urinary bladder, and lactoperoxidase (Stiborova

et al 2000a,b, Stiborova et al manuscript in preparation) During oxidation, o-anisidine is both activated to form reactive intermediates (radicals, diimine and quinone imine) and detoxicated to a stable metabolite (2,2'-dimethoxyazobenzene) (Thompson and Eling 1991, Stiborova et al 2000a,b) While the mechanism of o-anisidine binding to DNA mediated by lactoperoxidase and HRP was studied in detail (Stiborova et al 2000a,b, Stiborova et al manuscript in preparation), a role of PHS in this process is unknown Therefore, we extend the investigation to this peroxidase

Here, evidence for the formation of covalent DNA adducts by o-anisidine activated with PHS as revealed by the ^{32}P-postlabeling method is shown for the first time

Materials and Methods

Chemicals and enzymes

Chemicals were obtained from the following sources o-anisidine [>99% purity based on high pressure liquid chromatography (HPLC)] from Fluka Chemical Co (Germany), arachidonic acid from Sigma Chemical Co (St Louis, Mo, USA) and calf thymus DNA (CT-DNA) from Roche Diagnostics Mannheim (Germany) Polydeoxyribonucleotides [poly(dX)] were from Pharmacia LKB, Uppsala (Sweden) All other chemicals were of analytical purity or better *Ortho*-[ring-U-^{14}C]anisidine hydrochloride, with a purity of 99% as estimated by HPLC (which was used for repurification of the compound), and a specific activity of 134 mCi (4 96 GBq/mmol), was kindly provided by Dr John Ashby and Dr Paul A Lefevre (Zeneca Central Toxicology Laboratory, Alderley Park, Cheshire, UK) Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained commercially from source described previously (Schmeiser et al 1997, Bieler et al 1997)

Preparation of ram seminal vesicle (RSV) microsomes and assays

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 (Sivarajah et al 1981) and used as a source for PHS Protein concentrations in RSV microsomes were assessed according to Bradford (1976) PHS-cyclooxygenase activity was determined by measuring the arachidonic-dependent oxygen uptake in a 2 0 ml chamber equipped with a Clark-type oxygen electrode as described earlier (Stiborova et al 1999)

Incubations with o-anisidine

The incubation mixtures used for modification of DNA by o-anisidine activated by a PHS system contained in a final volume of 1 0 ml 50 mmol/l potassium phosphate buffer (pH 7 0), 0 1 mmol/l arachidonic acid, 1 0 mg of RSV microsomal protein, 5 mmol/l $MgCl_2$, 1 0 mmol/l [^{14}C] o-anisidine dissolved in DMSO (20 µl/1 ml incubation) and 1 mg of CT-DNA The reaction was initiated by adding

arachidonic acid Incubations were carried out at 37°C for 30 min Control incubations were carried out either without activating system (RSV microsomes) or with activating system but without arachidonic acid After incubation (37°C, 30 min), the mixtures were extracted twice with ethyl acetate (2 × 2 ml) DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described earlier (Stiborová et al 1990, 1994, 1995, Schmeiser et al 1997) Ethanol-precipitated DNA was treated with activated charcoal to remove noncovalently bound radioactivity (Yamazoe et al 1988), but no change in bound radioactivity compared to untreated DNA was observed The same experimental conditions were used to modify poly(dX) except that poly(dG)(dC), poly(dA), poly(dT) and poly(dC) were used instead of DNA Proteins present in the residual water phases after the phenol/chloroform extraction were precipitated by addi tion of 4 ml of acetone, precipitates were washed with acetone, ethanol and diethyl ether (always twice with 2 ml) and dissolved in one ml of 0 1 mol/l NaOH The ^{14}C radioactivity of RSV microsomal proteins, DNA and poly(dX) modified by [^{14}C] *o*-anisidine activated by PHS was counted in 0 1 ml aliquots in a Packard Ultra Gold X liquid scintillation cocktail on a Packard Tri-Carb 2000 CA scintillation counter The DNA and poly(dX) content was quantified spectrophotometrically at 260 nm

Inhibition by glutathione, ascorbic acid and NADH was performed by the addition of 0 5 mmol/l or 1 mmol/l glutathione or ascorbic acid or NADH dissolved in 50 mmol/l phosphate buffer (pH 7 0)

^{32}P-postlabelling analysis

The nuclease P1 enrichment version (Reddy and Randerath 1986) and the 1-butanol extraction-mediated enrichment procedure (Gupta and Early 1988) were performed by digesting DNA samples (12 5 μg) with micrococcal nuclease (750 mU) and spleen phosphodiesterase (12 5 mU) in digestion buffer (20 mmol/l sodium succinate, 8 mmol/l $CaCl_2$, pH 6 0) for 3 h at 37°C in a total volume of 12 5 μl Here, 2 5 μl of the digests were removed and diluted 1 1500 to determine the amount of normal nucleotides A parallel sample of DNA modified by [^{14}C] *o*-anisidine was used to estimate the efficiency of enzymes used for hydrolysis of this DNA This sample was extracted with water saturated 1-butanol at pH 3 5 (100 mmol/l ammonium formate) in the presence of 10 mmol/l tetrabutylammonium chloride as in the 1 butanol extraction enrichment procedure (Gupta and Early 1988) Only ~10% of DNA employed was available for the ^{32}P-postlabelling assay In the nuclease P1 version digests (10 μl) were enriched for adducts by incubation with 5 μg (5 U) of nuclease P1 in 3 μl of a buffer containing 0 8 mol/l sodium acetate, pH 5 0, 2 mmol/l $ZnCl_2$ for 30 min at 37°C The reaction was stopped by adding 3 μl of 427 mmol/l tris base The extraction with 1-butanol to enrich adducts was carried out as described earlier (Gupta and Early 1988) Four microlitres of labelling mix consisting of 400 mmol/l bicine pH 9 5, 300 mmol/l dithiothreitol, 200 mmol/l $MgCl_2$, 10 mmol/l spermidine, 100 μCi [γ-^{32}P]-ATP (15 pmol), 11 25 μmol/l ATP and 10 U T4 polynucleotide kinase were added After incubation for 30 min at

room temperature, 20 µl were applied to a polyethylenimine (PEI)-coated cellulose thin-layer chromatography (TLC) plate (Macherey-Nagel, Duren, Germany) and chromatographed as described (Schmeiser et al 1988) except that D3 and D4 were adjusted to pH 4 0 and 8 0 for better resolution Chromatographic conditions were as follows D1, 1 mol/l sodium phosphate, pH 6 8, D3, 3 5 mol/l lithium formate, 8 5 mol/l urea, pH 4 0, D4, 0 8 mol/l LiCl, 0 5 mol/l Tris-HCl, 8 5 mol/l urea, pH 8 0, D5, 1 7 mol/l NaH_2PO_4, pH 6 0 For determination of the amount of normal nucleotides 5 µl of the 1 1500 dilution of digests were mixed with 2 5 µl of tris buffer (10 mmol/l, pH 9 0) and 2 5 µl of labeling mix (see above) and incubated for 30 min at room temperature The labeling mixture was diluted by mixing 4 µl with 750 µl of 10 mmol/l tris buffer, pH 9 0 5 µl of this solution were applied to a PEI-cellulose TLC plate and run in 0 28 mol/l $(NH_4)_2SO_4$, 50 mmol/l NaH_2PO_4, pH 6 5

Adducts and normal nucleotides were detected and quantified by storage phosphor imaging on a Packard Instant Imager Count rates of adducted fractions were determined from triplicate maps after subtraction of count rates from adjacent blank areas Excess [γ-^{32}P]ATP after the postlabeling reaction was confirmed Adduct levels were calculated in units of relative adduct labeling (RAL) which is the ratio of cpm of adducted nucleotides to cpm of total nucleotides in the assay

Results and Discussion

RSV microsomes in the presence of arachidonic acid or hydrogen peroxide are effective in supporting the activation of [^{14}C] *o*-anisidine to metabolites binding to protein and DNA (Table 1) About 3 6% and 0 3% of [^{14}C] *o*-anisidine, which was present in the incubation mixture, was determined as protein- and DNA-bound radioactivity, respectively The ability of several structurally diverse compounds to catalyze PHS-mediated *o*-anisidine binding to macromolecules was examined (Table 1) The highest levels of binding were obtained using arachidonic acid, followed by hydrogen peroxide The NADPH, which serves as a cofactor of cytochrome P450 enzymes present in microsomes (Eling et al 1990) was not effective Similarly, cumene hydroperoxide, which replaces NADPH in cytochrome P450-dependent reactions, was not effective, either (Table 1) These results indicate that cytochromes P450 in RSV microsomes do not activate *o*-anisidine, but that PHS-catalyzed reactions are primarily responsible for [^{14}C] *o*-anisidine activation Indeed, presence of the well-known PHS-cyclooxygenase inhibitor indomethacin (Wells and Marnett 1993) clearly inhibited the [^{14}C] *o*-anisidine binding to macromolecules when arachidonic acid was used as a substrate for cyclooxygenase, but not with hydrogen peroxide as a substrate (Table 1) This indicates that indomethacin interferes with the synthesis of prostaglandin G_2, i e in the cyclooxygenase component of PHS

The PHS mediated binding of [^{14}C] *o*-anisidine to DNA and protein was inhibited by glutathione, ascorbate and NADH (Table 2) Two mechanisms for the explanation of glutathione and ascorbate inhibition are established Glutathione and ascorbate serve as cosubstrates for PHS peroxidase They are oxidized to rad-

Table 1. PHS-mediated binding of [^{14}C] *o*-anisidine to proteins and DNA

Incubations*	Protein binding (nmol/mg)	DNA binding (nmol/mg)
Complete	35 81 ± 3 31	0 33 ± 0 03
+ indomethacin (0 1 mmol/l)	3 39 ± 0 53	0 04 ± 0 01
without arachidonic acid	1 16 ± 0 30	0 02 ± 0 01
heat denatured microsomes	0 09 ± 0 02	nd
H_2O_2 instead of arachidonic acid	33 23 ± 3 12	0 32 ± 0 04
+ indomethacin (0 1 mmol/l)	33 20 ± 3 14	0 31 ± 0 04
Cumene hydroperoxide instead of arachidonic acid	1 18 ± 0 30	nd
NADPH instead of arachidonic acid	1 01 ± 0 25	nd

*Triplicate incubations were carried out in total volumes of 1 0 ml sodium phosphate (50 mmol/l, pH 7 0) containing 1 0 mg of ram seminal vesicle microsomal protein, $MgCl_2$ (5 mmol/l), [^{14}C] *o*-anisidine (1 mmol/l) dissolved in DMSO (20 μl/1 ml incubation), 1 mg of calf thymus DNA, arachidonic acid (100 μmol/l) or H_2O_2 (100 μmol/l) or cumene hydroperoxide (0 1 mmol/l) or NADPH (2 mmol/l) and indomethacin (100 μmol/l) 30 min at 37 °C Mixtures were extracted with ethylacetate, DNA and protein isolated and [^{14}C]radioactivity of modified DNA and protein measured as described in Materials and Methods The numbers are averages and standard deviations of three parallel experiments nd – not detectable

icals (Eling et al. 1986) and hence they can compete with *o*-anisidine, causing lowering *o*-anisidine oxidation The second explanation is the following· the peroxidase first oxidizes *o*-anisidine, which may be a more efficient reducing cosubstrate than glutathione or ascorbate. The *o*-anisidine radical produced in this oxidation is then reduced by glutathione or ascorbate forming both glutathione or ascorbate radicals and the parent *o*-anisidine.

Similarly, very low levels of *o*-anisidine binding were detected when 1 mmol/l NADH was present in the incubation medium. NADH is not converted by PHS as its cosubstrate under the conditions used. We, however, found that this compound acts as an inhibitor of the cyclooxygenase activity of PHS. It decreases the cyclooxygenase-mediated oxygen incorporation into the arachidonic acid (to 7% of the control). Radicals formed from arachidonic acid in the cyclooxygenase activity (the C-11 or the C-15 radicals of arachidonic acid) (Eling et al. 1990; Stiborová et al. 2000b) may be reduced by NADH back to the parent compound. Reduced radicals are subsequently not able to consume oxygen molecule to form a cyclic 9,11-endoperoxide (from a C-11 radical) or a peroxy radical which then abstracts hydrogen to form prostaglandin G_2 (from C-15 radicals). Second explanation of the inhibition of the *o*-anisidine binding to macromolecules by NADH is the same as that described for glutathione and ascorbate. The primarily formed *o*-anisidine radical is reduced by NADH to parent *o*-anisidine and NADH is simultaneously oxidized to NAD^+. Indeed, we determined that NAD^+ is formed during the reaction.

Table 2. The effect of radical scavengers and physiological donors on the PHS-mediated binding of [^{14}C] *o*-anisidine to DNA and protein

Compound	Protein binding (nmol/mg)	DNA binding (nmol/mg)
None	35 81 ± 3 31	0 33 ± 0 03
Glutathione		
0 5 mmol/l	10 02 ± 1 11	0 17 ± 0 02
1 0 mmol/l	1 02 ± 0 10	0 08 ± 0 01
Ascorbate		
0 5 mmol/l	0 45 ± 0 05	0 07 ± 0 01
1 0 mmol/l	nd	nd
NADH		
1 0 mmol/l	8 42 ± 1 11	0 08 ± 0 02

The values given are averages and standard deviations of three experiments The incubation mixture (1 0 ml) contained 50 mmol/l sodium phosphate buffer (pH 7 0), 1 0 mg of ram seminal vesicle microsomal protein, 5 mmol/l $MgCl_2$, 1 0 mmol/l [^{14}C] *o*-anisidine, 1 mg of calf thymus DNA, 100 μmol/l arachidonic acid and compounds tested in concentrations indicated in the table Mixtures were extracted with ethyl acetate, DNA and protein were isolated, and [^{14}C]radioactivity of modified DNA and protein was measured as described in Materials and Methods
nd – not detectable

Table 3. PHS-mediated binding of [^{14}C] *o*-anisidine to polydeoxyribonucleotides

Polydeoxynucleotide	poly(dX) binding nmol bound/mg of poly(dX)
poly(dG)(dC)	0 35 ± 0 04
poly(dA)	0 05 ± 0 01
poly(dC)	nd
poly(dT)	nd

Triplicate incubations were carried out as described in the Materials and Methods section The numbers are averages and standard deviations of three parallel experiments
nd – not detectable

When most of the NADH (in the reaction mixture) was oxidized, the reaction was adjusted to pH 8.8 and an NADH-generating system (alcohol dehydrogenase and ethyl alcohol) was added, NADH was fully reformed, indicating that the product was NAD^+.

To identify the target deoxyribonucleotides for the PHS-mediated *o*-anisidine binding, poly(dX) were used instead of DNA. As shown in Table 3, *o*-anisidine activated by RSV microsomes is bound to poly(dG)(dC) and partially to poly(dA). No binding to poly(dC) and poly(dT) was detectable (Table 3). Taken together,

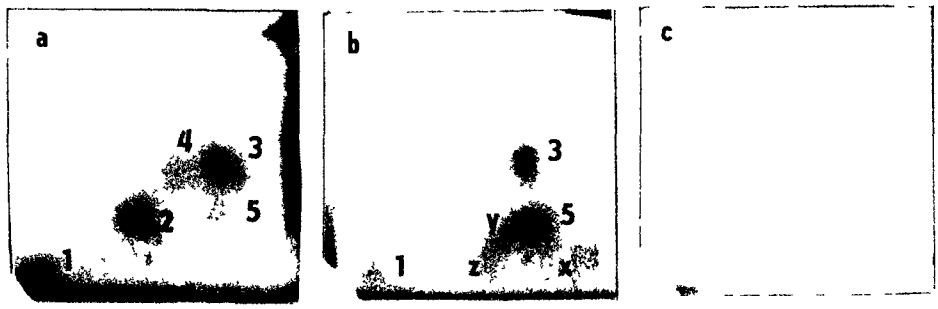

Figure 1. Autoradiographic profiles of *o*-anisidine adducts obtained from CT-DNA after activation by RSV microsomes (**a, b**) (**c**) is a control sample with the same system but without RSV microsomes The nuclease P1-enrichment procedure (**a, c**) and the extraction of adducts with the 1-butanol (**b**) were used for analysis Screen enhanced autoradiography was 12 h at −80 °C Origins, in the bottom left-hand corner, were cut off before exposure, D3 direction from bottom to top and D4 direction on four directional TLC on PEI-cellulose from left to right Quantification of adducts 1–5 and x, y, z is shown in Table 4

these results show that *o*-anisidine gives majority of adducts on guanine residues of DNA.

DNA modified by metabolites of *o*-anisidine was isolated from the reaction mixture by the procedures which included phenol/chloroform extraction (Stiborová et al. 1990, 1994, 1995). This suggests that a covalent binding of ultimate form(s) of the compound is the predominant modification The covalent binding of metabolites was confirmed by the ^{32}P-postlabelling analysis Both enhancing versions of the ^{32}P-postlabelling assay (the nuclease P1 version and enrichment of DNA adducts by 1-butanol extraction) were utilized in the experiments.

After inspection of autoradiographs, three major adduct spots (spots 1, 2, 3) and two minor ones (spots 4, 5) were detected in DNA reacted with *o*-anisidine activated by PHS using the nuclease P1 version of the ^{32}P-postlabelling analysis (Fig 1a) Enrichment by 1-butanol extraction led to a different pattern of adducts as compared to the nuclease P1 version of the ^{32}P-postlabelling assay. While adduct spots 2 and 4 became undetectable, the adduct spot 5 (determined the minor adduct by the nuclease P1 enhancing method) was detected as the prominent adduct spot (Fig. 1b). Moreover, three other minor radioactive spots that were almost undetectable using the nuclease P1 version of the assay (spots x, y, z), became visible (Fig. 1b) Autoradiographs of control incubations without RSV microsomes did not show any adduct (Fig. 1c). Further control incubations carried out without *o*-anisidine, or without DNA were also free of spots (results not shown). The adducts were quantified and the levels of the adducts were determined by measurement of the adduct count rates and expressed as RALs. The total adduct levels of 0.77 per 10^7 normal deoxynucleotides (0.23 pmol adduct/mg DNA) and 0.51 per 10^7 nor-

Table 4. Quantitative analysis of adducts formed in DNA by *o*-anisidine activated by RSV microsomes

Adduct*	RAL (means ± S E M /10^7 nucleotides)	
	Nuclease P1	1-Butanol extraction
1	0 173 ± 0 062	0 095 ± 0 010
2	0 228 ± 0 070	nd
3	0 200 ± 0 053	0 198 ± 0 030
4	0 011 ± 0 003	nd
5	0 006 ± 0 010	0 200 ± 0 050
x	nd	0 006 ± 0 002
y	nd	0 004 ± 0 001
z	nd	0 003 ± 0 001
Total	0 771 (0 23 pmol/mg DNA)	0 506 (0 15 pmol/mg DNA)

Numbers are averages ± S E M ($n = 6$) of triplicate *in vitro* incubations, each DNA sample was determined by two post-labeled analyses *See Fig 1 Experimental conditions are described in Materials and Methods
RAL – Relative adducts labeling, nd – not detectable

mal deoxynucleotides (0.15 pmol adduct/mg DNA) were determined for nuclease P1 and 1-butanol versions of the assay, respectively (Table 4).

The total level of adducts determined by the ^{32}P-postlabelling technique was three orders of magnitude smaller than that determined by measuring the total binding of radioactive [^{14}C] *o*-anisidine to DNA. Similar differences were found between the adduct levels formed by another carcinogen, Sudan I, activated by peroxidase when analyses were carried out by the same experimental procedures (measuring the total binding of radioactive [^{14}C]Sudan I to DNA and ^{32}P-postlabelling quantification) (Stiborová et al. 1992). Likewise, considerable discrepancies were found between the levels of adducts detected in placental DNA from smoking women when analyses were carried out by ELISA with antibodies against DNA modified with 7β, 8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene (2 adducts in 10^6 nucletotides) or by ^{32}P-postlabelling (1 adduct per 10^8 nucleotides) (Everson et al. 1986). The obtained discrepancies could be due to incomplete digestion of DNA highly adducted by carcinogens, different degrees of resistance of adducted nucleotides to dephosphorylation, incomplete extraction into 1-butanol, incomplete ^{32}P-postlabelling, losses of material during the experimental manipulations or retaining compounds at the origin of the PEI-cellulose TLCs. The fact that only 10% of [^{14}C] *o*-anisidine adducts were accessible after butanol extraction for labelling (see the Materials and Methods section) suggests that the highly adducted DNA was digested incompletely. This could be the result of an increase in intramolecular or intermolecular DNA-*o*-anisidine-DNA cross-linking, which is known to occur as a consequence of the peroxidative metabolism of some carcinogens (e.g., benzidine) *in vitro* (Fourney et al. 1986) as well as *in vivo* (Yamazoe et al. 1988).

The chromatographic conditions used for resolution of adducts were suitable for separation of adducts formed from carcinogens with a pronounced hydrophobicity (Stiborova et al 1990, 1994, Schmeiser et al 1997) Although the exact nature of the adducts has not been elucidated as yet, the pronounced lipophilicity of the adducts indicates that o-anisidine metabolites containing more than one benzene ring (e g , diimine or quinone imine) (Stiborova et al 2000a,b) are covalently linked to DNA This is in accordance with our previous results The diimine formed from *o*-anisidine by other two peroxidases (lactoperoxidase and HRP) was found to be the *o*-anisidine metabolite which seems to be responsible for formation of deoxyguanosine adducts in DNA (Stiborova et al 2000a,b) Furthermore we found that adduct 5, determined to be the major adduct using the 1 butanol extraction version of the ^{32}P-postlabelling assay, is not resistant to dephosphorylation by nuclease P1 during the nuclease P1 version of the method (see Fig 1) Low resistance to dephosphorylation of 3' phosphate by nuclease P1 is a typical feature for adducts of aromatic amines binding by their NH_2 groups to a C8 atom of guanine residues in DNA (Gupta and Early 1988) Therefore, we can speculate that the adduct 5 might be generated by reaction of diimine with guanine residues in DNA, having a C8 carbon atom modified by the =NH group of a diimine molecule The characterization of its real structure remains, however, to be explored

The results presented in the paper clearly show that PHS mediates the formation of covalent DNA adducts by *o*-anisidine *in vitro* Hence, the activation of *o*-anisidine by PHS present in the urinary bladder might also be responsible for DNA adduct formation *in vivo* and the initiation of *o*-anisidine-mediated carcinogenesis in this organ However, as reported by Ashby et al (1994), there was no evidence observed of *o*-anisidine binding to liver or bladder DNA isolated 6, 12 or 24 h after treating mice with a single dose of [^{14}C] *o* anisidine The failure to detect any amounts of covalent DNA binding *in vivo* might probably be due to the arrangement of the experiments (the dose and duration of treatment with *o* anisidine) (Ashby et al 1994) Therefore we plan to use another testing scheme (the different dose and duration) for detecting adducts formed by *o*-anisidine *in vivo* in our future studies

Finally, it should be noted that cancer in a specific organ may also be a function of promotional pressures on initiated cells in target organs and not only of the levels of DNA adducts (Barret and Wiseman 1987) In view of the formation of radicals during the peroxidase-mediated oxidation of *o* anisidine and of the role played by radicals in processes of tumor promotion (Marnett 1987, O'Brien 1988) we can speculate about their biological consequences in the promotional phase of carcinogenesis However, the exact function of such and/or other promotional pressures in *o*-anisidine-mediated tumorigenesis remains to be resolved

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References

Ashby J , Tennant K W (1988) Chemical structure Salmonella mutagenicity and extent of carcinogenicity as indicator of genotoxic carcinogenesis among 222 chemicals tested in rodents by the US NCI/NTP Mutat Res **204,** 17—115

Ashby J , Lefevre P A , Tinwell H , Brunborg G , Schmezer P , Pool-Zobel B , Shanu-Wilson R , Holme J A , Soderlund E J , Gulati D , Wojciechowski J P (1991) The non- genotoxicity to rodents of the potent rodent bladder carcinogens *o*-anisidine and *p*- cresidine Mutat Res **250,** 115—133

Ashby J , Short J M , Jones N J , Lefevre P A , Provost G S , Rogers B J , Martin E A , Parry K , Burnette J M , Glickmann B W , Tinwell H (1994) Mutagenicity of *o*-anisidine to the bladder of *lacI*$^-$ transgenic B6C3F1 mice absence of ^{14}C or ^{32}P bladder DNA adduction Carcinogenesis **15,** 2291—2296

Barret, J C , Wiseman, R W (1987) Cellular and molecular mechanisms of multistep carcinogenesis relevance to carcinogen risk assessment Environ Health Perspect **76,** 65—70

Bieler C A , Stiborova M Wiessler M , Cosyns J P , van Ypersele de Strihou C , Schmeiser H H (1997) ^{32}P-post-labelling analysis of DNA adducts formed by aristolochic acid in tissues from patients with Chinese herbs nephropathy Carcinogenesis **18,** 1063—1067

Bradford M M (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Anal Biochem **72,** 248—254

Brennan R J , Schiestl R H (1999) The aromatic amine carcinogens *o*-toluidine and *o*-anisidine induce free radicals and intrachromosomal recombination in *Saccharomyces cerevisiae* Mutat Res **430,** 37—45

Dunkel V C , Zeiger E , Brusick D , McCoy E , McGregor D , Mortelmans K , Rosenkranz H S , Simmon V F (1985) Reproducibility of microbial mutagenicity assays II Testing of carcinogens and noncarcinogens in Salmonella typhimurium and Escherichai coli Environ Mutagen , **7** (Suppl 5), 1—248

Eling T E , Curtis J F , Harman L S , Mason R P (1986) Oxidation of glutathione to its thiol free radical metabolite by prostaglandin H synthase a potential endogenous substrate for the hydroperoxidase J Biol Chem **261,** 5023—5028

Eling T E , Thompson D C , Foureman G L , Curtis J F , Hughes M F (1990) Prostaglandin H ynthase and xenobiotic oxidation Annu Rev Pharmacol Toxicol **30,** 1—45

Everson R B , Randerath E , Latella R M , Cefalo R C , Arvitts T A , Randerath K (1986) Detection of smoking related covalent DNA adducts in human placenta Science **231,** 54—57

Fourney R M , O'Brien P J , Davidson W S (1986) Peroxidase catalyzed aggregation of plasmid pBR322 DNA by benzidine metabolites *in vitro* Carcinogenesis **7,** 1535—1542

Galloway S M , Armstrong M J , Reuben C , Colman S , Brown B , Cannon C , Bloom A D , Nakamura F , Ahmed M , Duk S , Rimpo J , Margolin B H (1987) Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells, evaluations of 108 chemicals Environ Mol Mutagen **10** (Suppl 10), 1—175

Glickman B W , Ashmed A , Rogers B J , Hammer R , Short J M , Tinwell H , Lefevre P A , Ashby J (1993) Mutational specificity of *o*-anisidine in the bladder of Big Blue^{TM} mice Environ Mol Mutagen **21** (Suppl 22), 1

Gupta R C , Early K (1988) ^{32}P-adduct assay comparative recoveries of structurally diverse DNA adducts in the various enhancement procedures Carcinogenesis **8,** 1687—1693

IARC (1982) *Ortho-* and *para*-anisidine and their hydrochlorides In IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans **22,** 63—80

Marnett L (1987) Peroxyl free radicals potential mediators of tumor initiation and promotion Carcinogenesis **8,** 1365—1373

Morita T , Asano N , Awogi T , Sasaki Y F , Sato S I , Shimada H , Sutou S , Suzuki T , Wakata A , Sofuni T , Hayashi M (1997) Evaluation of the rodent micronucleus assays in the screening of IARC carcinogens (groups 1, 2A and 2B) The summary report of the 6th collaborative study by CSGMT/JEMS-MMS Collaborative Study of the Micronucleus Group Test Mammalian Mutagenicity Study Group Mutat Res **389,** 3—122

National Cancer Institute (1978) Bioassay of *o*-anisidine hydrochloride for possible carcinogenicity NTP Technical Report No 89, National Cancer Institute, Bethesda, MD

O'Brien, P (1988) Radical formation during the peroxidase catalyzed metabolism of carcinogens and xenobiotics the reactivity of these radicals with GSH, DNA, and unsaturated lipid Free Radical Biol Med **4,** 169—183

Reddy M V , Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabelling test for structurally diverse DNA adducts Carcinogenesis **7,** 1543—1551

Sasaki Y F , Nishidate E , Su Y Q , Matsusaka N , Tsuda S , Susa N , Furukawa Y , Ueno S (1998) Organ-specific genotoxicity of the potent rodent bladder carcinogens *o*-anisidine and *p*-cresidine Mutat Res **412,** 155—160

Sivarajah K , Lasker J M , Eling T E (1981) Prostaglandin synthetase dependent co-oxidation of (+)benzo[a]pyrene-7,8-dihydrodiol by human lung and mammalian tissues Cancer Res **41,** 1834—1839

Schmeiser H H , Frei E , Wiessler M , Stiborová M (1997) Comparison of DNA adduct formation by aristolochic acids in various *in vitro* activation systems by ^{32}P-post-labelling evidence for reductive activation by peroxidases Carcinogenesis **18,** 1955—1062

Schmeiser H H , Schoepe K B , Wiessler M (1988) DNA adduct formation of aristolochic acid I and II *in vitro* and *in vivo* Carcinogenesis **9,** 297—303

Schmidt H L , Moeller M R , Weber N (1973) Influence of substrates on the microsomal dealkylation of aromatic $N-, O-$ and $S-$alkyl compounds Biochem Pharmacol **22,** 2989—2996

Smith J N , Williams R T (1949) The metabolism of *p*-phenetidine (*p*-ethoxyaniline) with some observations on the anisidines (methoxyanilines) Biochem J **44,** 250—255

Stiborová M , Frei E , Schmeiser H H , Wiessler M , Hradec J (1990) Mechanism of formation and ^{32}P-postlabelling of DNA adducts derived from peroxidative activation of carcinogenic non-aminoazo dye 1-phenylazo-2-hydroxynaphthalene (Sudan I) Carcinogenesis **11,** 1843—1948

Stiborová M , Frei E , Schmeiser H H , Wiessler M (1992) ^{32}P-Postlabelling analysis of adducts formed from 1-phenylazo-2-hydroxynaphthalene (Sudan I, Solvent Yellow 14) with DNA and homopolydeoxyribonucleotides Carcinogenesis **13,** 1221—1225

Stiborová M , Fernando R C , Schmeiser H H , Frei E , Pfau W , Wiessler M (1994) Characterization of DNA adducts formed by aristolochic acids in the target organ (forestomach) of rats by ^{32}P-postlabelling analysis using different chromatographic procedures Carcinogenesis **15,** 1187—1192

Stiborová M , Asfaw B , Frei E , Schmeiser H H , Wiessler M (1995) Benzenediazonium ion derived from Sudan I forms an 8-(phenylazo)guanine adduct in DNA Chem Res Toxicol **8,** 489—498

Stiborová M , Schmeiser H H , Frei E (1999) Prostaglandin H synthase-mediated oxidation and binding to DNA of a detoxication metabolite of carcinogenic Sudan I 1-(phenylazo)-2,6- dihydroxynaphthalene Cancer Lett **146,** 53—60

Stiborová M , Mikšanová M , Frei E (2000a) Molecular study of the carcinogenicity of *o*-nitroanisole and *o*-anisidine, carcinogens with unknown mechanism Chem Listy **94,** 1063

Stiborová M , Mikšanová M , Martínek V , Frei E (2000b) Heme peroxidases structure, function, mechanism and involvement in activation of carcinogens A review Collect Czech Chem Commun **65,** 297—325

Thompson D C , Eling T E (1991) Reactive intermediates formed during the peroxidative oxidation of anisidine isomers Chem Res Toxicol **4,** 474—481

Thompson D C , Josephy P D , Chu J W , Eling T E (1992) Enhanced mutagenicity of anisidine isomers in bacterial strains containing elevated *N*-acetyltransferase activity Mutat Res **279,** 83—89

Wangenheim J , Bolcsfold G (1988) Mouse lymphoma L5178Y thymidine kinase locus assay of 50 compounds Mutagenesis **3,** 193—205

Wells I , Marnett L J (1993) Inactivation of prostaglandin endoperoxide synthase by acylating derivatives of indomethacin, Biochemistry **32,** 2710—2716

Yamazoe Y , Zenser T V , Miller D W , Kadlubar F F (1988) Mechanism of formation and structural characterization of DNA adducts derived from peroxidative activation of benzidine, Carcinogenesis **9,** 1635—1641

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