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METABOLIC ACTIVATION OF 2,4-DIAMINOANISOLE, A HAIR-DYE COMPONENT—II. ROLE OF CYTOCHROME P-450 METABOLISM IN IRREVERSIBLE BINDING *IN VITRO**

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Abstract—Incubation of rat liver microsomes with radiolabeled 2,4-diaminoanisole (2,4-DAA) in the presence of NADPH and oxygen led to the formation of irreversibly bound products to microsomal protein. The binding was inhibited by a $CO:O_2$ atmosphere and by an antibody against NADPH cytochrome *c* reductase. *In vivo* and *in vitro* inhibitors of cytochrome P-450 decreased the binding and phenobarbital-pretreatment increased binding, whereas β-napthoflavone-pretreatment was without effect. Binding of ring-labeled 2,4-DAA was much higher than with methyl-labeled-2,4-DAA. Experiments with [^{3}H]-ring- and [^{14}C]-ring-labeled-2,4-DAA indicated some loss of tritium; this was confirmed by isolation of labile tritium. Substitution of the hydrogens in the methyl group with deuterium led to increases in both binding and mutagenicity of 2,4-DAA. Formation of formaldehyde and a small amount of methanol could be demonstrated during the oxidative metabolism of methyl-labeled-2,4-DAA. Addition of superoxide dismutase and ascorbic acid inhibited binding, and a small amount of irreversible binding could be demonstrated when NADPH was replaced by a xanthine–xanthine oxidase system. Microsomes from rat kidneys also activated 2,4-DAA in the presence of NADPH. Thin-layer chromatography revealed that 30–40 per cent of 2,4-DAA was oxidized during 10 min of incubation with liver microsomes. And a tentative scheme involving aromatic hydroxylation, oxidative demethylation and *N*-hydroxylation for the microsomal metabolism of 2,4-DAA is presented. Irreversible binding could also be shown with liver microsomal RNA *in vitro*, whereas no binding to exogenously added DNA could be found.

2,4-Diaminoanisole† is a component of many commercial hair-dyes. Since Ames *et al.* [1] demonstrated that this compound and several other aromatic diamines can be activated to potent bacterial mutagens *in vitro*, much concern has arisen regarding the potential for carcinogenic effects in humans of these aromatic diamines.

We have recently implied microsomal cytochrome P-450 metabolism in the activation of 2,4-DAA to a mutagen *in vitro* [2]. Inducers and inhibitors of the cytochrome P-450 system increased and decreased the mutation rates with 2,4-DAA in the *Salmonella* test system, respectively. Enzymes catalyzing the activation of 2,4-DAA to mutagens could also be induced in kidney and lung as well as transplacentally in fetal liver.

Many mutagens and carcinogens are converted in the body to electrophilic reactants which bind irreversibly to DNA, RNA and protein [3]. Whether binding of activated carcinogens to any of these macromolecules is responsible for the subsequent development of cancer remains to be established. Since 2,4-DAA is activated by mammalian metabolism to a bacterial mutagen(s) *in vitro*, we have examined the possibility that cytochrome P-450 metabolism leads to the formation of reactive forms of 2,4-DAA which bind irreversibly to protein and other macromolecules *in vitro*.

MATERIALS AND METHODS

Chemicals. Synthesis of labeled 2,4-diaminoanisoles. Ring-labelled-[^{3}H]-2,4-DAA was prepared in the following manner. A mixture of 2,4-dinitrophenol (Aldrich) and platinum black in tritiated water (25 mCi, New England Nuclear) containing 10 per cent acetic acid, was heated under reflux for 36 hr. The 2,4-dinitrophenol was extracted into ethyl acetate and labile tritium was removed by exhaustive washes with water. The product was then purified by recrystallization from hot hexane to a constant specific activity of 3.1 mCi/mmol, m.p. 105–106°. This material was methylated with methyl iodide and silver oxide [4]. In a typical procedure [^{3}H]-2,4-dinitrophenol (20 mg) was dissolved in a mixture of dimethylformamide (0.1 ml) and chloroform (0.7 ml) and refluxed with silver oxide (50 mg) and methyl iodide (0.05 ml). The reaction was monitored by t.l.c. on silica gel GF (Analtech) using ether as developing solvent, R_F 2,4-dinitrophenol, 0.2, R_F 2,4-dinitroanisole, 0.8. After 2hr the reaction mixture was filtered

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†The following abbreviations are used: 2,4-DAA, 2,4-diaminoanisole; BNF, β-naphthoflavone.

through Celite, which was then thoroughly washed with chloroform. The chloroform extract was successively washed with 10 per cent sodium carbonate and water and dried over Drierite. Solvent was removed and the product purified by recrystallization to a constant specific activity from ethanol, m.p. 88–89° [5]. Finally, [^{3}H]-2,4-DAA was prepared by reducing [^{3}H]-2,4-dinitroanisole with sodium borohydride/palladium on charcoal as previously described for several other nitroaromatic compounds [6]. The compound was recrystallized to constant specific activity from ether:ethanol, m.p. 68° [7]. Mass spectrometric analysis of 2,4-DAA was carried out on a MicroMass VG 16F instrument in the chemical ionization (CI) or electron ionization (EI) mode. Isobutane CI mass spectra showed MH^+ = m/e 139 with no ion at m/e 155 or 171 for mono- or dihydroxylated compounds. In contrast to EI mass spectra of hydroxylamines where hydroxylamines often do not show molecular ions because of a facile loss of oxygen, the milder ionization conditions employed in CI do show molecular ions for hydroxylamines. Therefore, if any had been present, we should have expected additional ions at these higher masses. The compound was stored dessicated at −15° under nitrogen to prevent decomposition. As judged by radiochromatographic scanning (silica gel GF, ether, R_F 0.35) the compound was >98 per cent pure. All thin-layer chromatography and recrystallizations were carried out in an atmosphere of nitrogen using solvent deaerated with nitrogen. Ring-labeled-[^{14}C]-2,4-DAA was prepared by the same procedure starting with [^{14}C]-2,4-dinitrophenol (New England Nuclear). Methyl-labeled-[^{14}C]-2,4-DAA and methyl-labeled-[^{3}H]-2,4-DAA were prepared using 5 mCi of [^{14}C]-methyl iodide and 25 mCi of [^{3}H]-methyl iodide (ICN Radioisotope Division), respectively. The final products had specific activities of 3.5 and 23.3 mCi/mmol, respectively. [^{14}C]-ring-(CD_3)-methyl-2,4-DAA was prepared as described for [^{14}C]-ring-2,4-DAA starting with 50 μCi [^{14}C]-2,4-dinitrophenol (New England Nuclear) and (D_3)-methyl iodide (Merck Isotopes). The final product had a specific activity of 0.21 mCi/mmol. Mass spectrometric analysis of the deuterated 2,4-DAA was carried out in the EI mode. Calculations based on the M^+ and M-1 peaks showed that 99.2 per cent of 2,4-DAA was present as its trideutero-methyl analog.

Other chemicals were obtained from the following sources: unlabeled 2,4-DAA from ICN Pharmaceuticals, USA; α-naphthoflavone and BNF from Aldrich, Germany; SKF 525-A from Smith, French and Kline Laboratories, USA; metyrapone from Ciba-Geigy, Switzerland; calf thymus DNA, glutathione (reduced), ascorbic acid, NADP, glucose 6-phosphate, yeast glucose 6-phosphate dehydrogenase, xanthine and xanthine oxidase from buttermilk from Sigma, USA; superoxide dismutase from bovine erythrocytes from Miles Laboratories, USA; piperonyl butoxide from Cooper, McDougall and Robertson, England; and phenobarbital from The Norwegian Medicinal Depot. Other chemicals were of the best available commercial grades.

Treatment of animals. Male Wistar rats (150–200 g) were obtained from Møllegård Breeding Laboratories, Denmark. They were pretreated with phenobarbital (75 mg/kg in 0.9 % NaCl i.p. 72, 48 and 24 hr before death), BNF (80 mg/kg in corn oil i.p. 48 hr before death), cobaltous chloride (60 mg/kg in 0.9 % NaCl s.c. 48 and 24 hr before death) or piperonyl butoxide (1360 mg/kg i.p. 30 min before death). Controls received vehicle alone.

Preparation of microsomes. Animals were killed by decapitation. Livers and kidneys were removed, minced and homogenized with a motor driven glass-Teflon homogenizer in 2 volumes of ice-cold 1.15 % KCl containing 20 mM Tris-buffer, pH 7.4. Washed microsomes were prepared as described [8], and resuspended in the KCl Tris-buffer before incubation.

Microsomal incubations. Ice-cold reaction vessels contained (final concentrations): 2 mg/ml liver or kidney microsomal protein, 0.5 mM labeled 2,4-DAA (1000–1500 dpm/nmol) in 25 μl dimethylsulfoxide and a NADPH-generating system (0.64 mM NADP, 15.5 mM glucose 6-phosphate, 2 units/ml glucose 6-phosphate dehydrogenase and 10 mM $MgCl_2$) in a total volume of 3.0 ml. In experiments with antibody prepared against NADPH-cytochrome *c* reductase [9], 1.5 ml of incubation mixture contained 10 mg/ml goat preimmune or immune γ-globulin, 1.0 mg/ml microsomal protein and the NADPH-generating system described above. In other experiments the NADPH-generating system was replaced by 0.22 mM xanthine and 10 μg/ml xanthine oxidase. Incubations were carried out at 37° in a shaking water bath incubator (Infors AG, Switzerland, 150 rot./min) under air unless otherwise stated. They were usually stopped after 10 min by adding 1 ml of 30 % trichloroacetic acid (TCA). In some experiments 2 mg of DNA was added together with 0.5 mM ^{3}H- or ^{14}C-labels (containing up to 270,000 dpm/nmol), microsomes and the NADPH-generating system. These reactions were stopped after 15 min with 3 ml of a 12 % 4-aminosalicylate-2 % Na-dodecylsulfate mixture, and DNA was extracted with 3 ml of a phenol/8-hydroxyquinoline *m*-cresol mixture as described by Alexandrov *et al.* [10]. In other experiments microsomal RNA was extracted after 15 min incubation with 0.5 mM ^{3}H- or ^{14}C-labels (containing 6000–9000 dpm/nmol), microsomes and the NADPH-generating system with 6 ml water-saturated phenol containing 0.1 % Na-dodecylsulfate and 0.1 % 8-hydroxyquinoline as described by Pietropaolo and Weinstein [11]. Release of tritium from [^{3}H]-methyl-2,4-DAA was determined after passing aliquots of microsomal incubation mixtures through columns of XAD-2 resin as described by Thompson and Holtzman [12]. For the estimation of formation of glutathione conjugates, microsomal incubations containing 0.5 mM [^{3}H]-ring-2,4-DAA (1000 dpm/nmol), the NADPH-generating system and 0.5 mM glutathione, were stopped with 4.0 ml cold methanol. The supernatants were evaporated with a Rotavapor, the residues taken up in a small volume of methanol, and aliquots were developed on silica gel GF in ether and 1 cm bands were eluted with 0.4 ml methanol and counted in 15 ml of Dimilume (Packard) scintillation fluid. To assess the total metabolism of 2,4-DAA in microsomal incubations, residues of supernatants from 10 min incubations of 0.5 mM [^{3}H]-ring-2,4-DAA were taken up in 2.0 ml of H_2O

Table 1. Conditions for NADPH-dependent irreversible binding of [^{3}H]-ring-2,4-DAA to rat liver microsomal protein *in vitro*

Reaction mixtures	[^{3}H]-2,4-DAA bound pmol/mg protein/min
A. Complete	198 ± 9
− NADPH*	50 ± 0
Boiled microsomes	6 ± 2
B.-O_2 (100% N_2 atmosphere)	44 ± 1
+ CO:O_2 (4:1 atmosphere)	119 ± 5
+ Preimmune γ-globulin†	85 ± 1
+ Immune γ-globulin† (NADPH cytochrome *c* reductase antibody)	48 ± 1
C.+ SKF 525-A 1.0 mM	96 ± 6
+ Metyrapone 5.0 mM	121 ± 10
+ α-Naphthoflavone 0.1 mM	229 ± 14
+ Glutathione 1.0 mM	48 ± 1
D. Phenobarbital-treated	347 ± 20
β-Naphthoflavone-treated	223 ± 12
Cobaltous chloride-treated	100 ± 4
Piperonyl butoxide-treated	206 ± 9

For each incubation, microsomes were prepared as described in Materials and Methods from control and treated animals and incubated with [^{3}H]-ring-2,4-DAA (0.5 mM) and an NADPH-generating system, except as noted. Values represent means ± S.D. from 4 incubations from pooled livers of 2 rats.

*This value of 50 pmol/mg protein/min for NADPH-independent binding has been subtracted to obtain the other values listed above.

†Each incubation contained 10 mg of partially purified preimmune or immune γ-globulin per milligram of microsomal protein.

and extracted with 5 ml of ethyl acetate 4 times and aliquots of ethyl acetate- and water-soluble fractions were counted before and after development in silica gel GF in ether.

Extraction of protein. The TCA-precipitated protein pellets from the microsomal incubations were washed repeatedly with TCA, methanol and ethanol–ether until no more radioactivity could be extracted [8]. The extracted protein was then dissolved in 1.0 ml of 1 M NaOH, and 0.5 ml was transferred to 15 ml scintillation fluid and counted in a Packard liquid scintillation spectrometer. Radioactivity was corrected for quenching (external standardization). Binding values from incubations without NADPH or xanthine oxidase, representing nonspecific binding, were always subtracted. The protein concentration was determined according to Lowry *et al.* [13] using crystalline bovine serum albumin as standard.

Mutagenesis assay. The *Salmonella* mutagenesis test of Ames *et al.* [14] was carried out as described [2]. Each plate contained 0.1 ml of an overnight culture of the bacterial tester strain TA 1538, 10 μg of test compound in 100 μl of dimethylsulfoxide, cofactors and 2 mg of 9000 *g* rat liver supernatant protein.

RESULTS

Involvement of cytochrome P-450 in NADPH-dependent irreversible binding to microsomal protein in vitro. Incubation of [^{3}H]-ring-2,4-DAA with rat liver microsomes in the presence of NADPH led to its irreversible binding to microsomal protein (Table 1A, NADPH was in excess). The binding reaction had a maximal velocity of approximately 0.2 nmol/mg protein/min (Fig. 1), was linear with respect to protein concentration up to 2 mg protein/ml, and proceeded at a constant rate for at least 10 min. Omitting NADPH led to binding rates which were approximately 20 per cent of the total binding in the presence of NADPH; using boiled microsomes abolished the NADPH-dependent binding. The binding reaction was oxygen-dependent and was inhibited by a CO:O_2 atmosphere (4:1) and by an antibody against NADPH-cytochrome *c* reductase (Table 1B). Addition of typical cytochrome P-450 inhibitors such as SKF 525-A and metyrapone also inhibited the rate of binding, whereas α-naphthoflavone slightly stimulated binding rates (Table 1C). Addition of the nucleophile glutathione strongly inhibited the binding reaction. Pretreatment of animals with the inducer phenobarbital increased binding rates to 175 per cent of controls (Table 1D), whereas the polycyclic aromatic hydrocarbon-inducer BNF apparently did not increase the rates of binding (Table 1D, Table 5). Prior treatment with cobaltous chloride, which lowers cytochrome P-450 levels [15], decreased the binding rates to 51 per cent of control values (Table 1D). Piperonyl butoxide treatment, which inhibits many cytochrome P-450 mediated reactions by forming complexes with the cytochrome [16], was without effect, however.

Irreversible binding of different [^{3}H]- *and* [^{14}C]-2,4-*DAA isotopes.* In three separate experiments using both separate incubations of [^{3}H]-ring-labeled- and [^{14}C]-ring-labeled-2,4-DAA and mixtures of the two there was a significantly greater binding of ^{14}C-label than of ^{3}H-label (Table 2). Double-labeling with equal amounts of [^{3}H]-ring-labeled- and [^{14}C]-ring-labeled-2,4-DAA gave a ratio of 0.76 of ^{3}H- vs

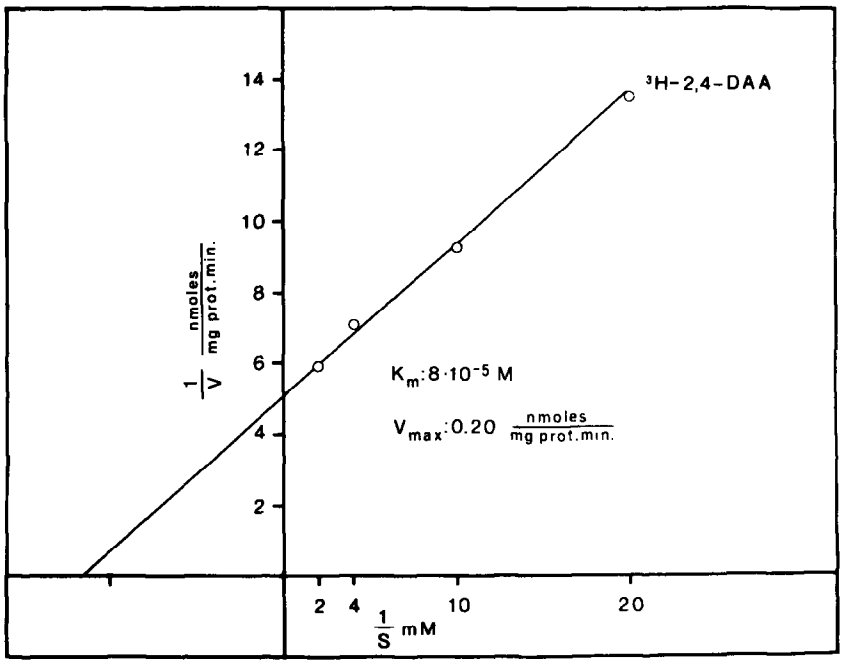

Fig. 1. Lineweaver–Burk plot of irreversible binding of [^{3}H]-ring-2,4-DAA to rat liver microsomal protein *in vitro*.

Table 2. Irreversible binding of ^{3}H, ^{14}C or deuterium labels of 2,4-diaminoanisole to rat liver microsomal protein *in vitro*

Radiolabel added	Radioactivity bound pmol/mg protein/min
[^{3}H]-ring-2,4-DAA	188 ± 11
[^{3}H]-methyl-2,4-DAA	64 ± 0
[^{14}C]-ring-2,4-DAA	247 ± 14
[^{14}C]-methyl-2,4-DAA	86 ± 3
[^{14}C]-ring-deuteromethyl-2,4-DAA	321 ± 30

Microsomes were incubated with labels (0.5 mM) and an NADPH-generating system. Values are means ± S.D. of 4 incubations.

^{14}C-label bound, suggesting loss of tritium. This was confirmed by isolation of labile tritium from incubation supernatants using XAD-2 resin column chromatography [12] and distillation to constant specific activity with water. By this method 1.30 ± 0.21 nmol/mg protein/10 min of tritiated water was formed in incubations containing NADPH-generating system and 0.45 ± 0.07 nmol/mg protein/10 min in the absence of NADPH-generating system.

When 2,4-DAA was labeled in the methyl group with ^{14}C or ^{3}H, less than 40 per cent of the binding observed with ring-labeled-2,4-DAA was seen (Table 2).

Binding studies with [^{14}C]-ring-deuteromethyl-2,4-DAA gave approximately 30 per cent higher values than those observed with the non-deuterated ring-labeled compounds (Table 2).

Experiments were also carried out to test the effect of deuteromethyl-labeling on the *in vitro* mutagenicity of 2,4-DAA (Table 3). Introduction of deuterium in the methyl group caused a 6-fold increase in the activation of 2,4-DAA to a mutagen with liver 9,000 *g* supernatant from untreated rats, and approximately a 50 per cent increase in the activation with liver 9000 *g* supernatant isolated from rats pretreated with BNF.

Table 3. Mutagenicity of 2,4-DAA and trideuteromethyl-2,4-DAA with control and BNF-treated rat liver 9000 *g* supernatant

Test compound	Control Rev per plate	BNF Rev per plate
2,4-Diamonoanisole	167 ± 24	1801 ± 99
Trideuteromethyl-2,4-diaminoanisole	977 ± 61	2663 ± 166

Mutagenicity was performed as described in Materials and Methods. Each plate contained 10 μg test compound and 2 mg 9000 *g* supernatant protein from control or BNF-treated animals. Values are means ± S.D. of 3 estimations with pooled livers from 2 rats.

Table 4. Effect of superoxide dismutase and ascorbic acid on NADPH-dependent or xanthine oxidase-dependent irreversible binding of [^{3}H]-ring-2,4-DAA and [^{14}C]-methyl-2,4-DAA to rat liver and kidney microsomal protein *in vitro*

Reaction mixture		[^{3}H]-ring-2,4-DAA bound pmol/mg protein/min		[^{14}C]-mehtyl-2,4-DAA bound pmol/mg protein/min
		Liver microsomes	Kidney microsomes	Liver microsomes
NADPH	Control	189 ± 14	96 ± 8	60 ± 3
	+ Superoxide dismutase 25 μg/ml	86 ± 10	0 ± 0	25 ± 3
	+ Ascorbic acid 1 mM	99 ± 3	3 ± 2	32 ± 3
Xanthine oxidase	Control	52 ± 6	*	4 ± 2
	+ Superoxide dismutase 25 μg/ml	0 ± 0	*	0 ± 0

Microsomes were incubated with labels (0.5 mM) and an NADPH-generating system or a xanthine oxidase system (xanthine oxidase 10 μg/ml + xanthine 0.22 mM). Values are means ± S.D. of 4 incubations.
*Not determined.

Table 5. Effect of superoxide dismutase and 105,000 *g* supernatant on NADPH-dependent irreversible binding of [^{3}H]-ring-2,4-DAA and [^{3}H]-methyl-2,4-DAA to rat liver microsomal protein *in vitro*

Reaction mixture		Control pmol/mg protein/min	BNF pmol/mg protein/min
[^{3}H]-ring-2,4-DAA	Control	186 ± 40	147 ± 4
	+ Superoxide dismutase 25 μg/ml	107 ± 11	88 ± 1
[^{3}H]-methyl-2,4-DAA	Control	106 ± 24	72 ± 1
	+ Superoxide dismutase 25 μg/ml	37 ± 0	35 ± 1
[^{3}H]-methyl-2,4-DAA	+ Boiled supernatant	164 ± 18	110 ± 7
	+ supernatant	79 ± 6	51 ± 6
[^{3}H]-ring-2,4-DAA	+ Boiled supernatant	55 ± 2	50 ± 5
	+ supernatant	42 ± 2	34 ± 6

Microsomes from control and BNF-treated rats were incubated with labels (0.5 mM) and an NADPH-generating system. Incubation with 105,000 *g* supernatant contained microsomal protein 1.5 mg/ml and 105,000 *g* supernatant protein 0.5 mg/ml. Values are means ± S.D. of 3 incubations.

Involvement of superoxide anion in irreversible binding. Cytochrome P-450 is known to produce superoxide anions in the presence of NADPH [8]. Addition of superoxide dismutase, an enzyme which very effectively scavenges superoxide [17], reduced the [^{3}H]-ring-2,4-DAA binding rates to 46 per cent of controls (Table 4). Ascorbic acid, which can reduce superoxide anions [18], also inhibited [^{3}H]-ring-2,4-DAA binding. The lower binding observed when using [^{14}C]-methyl-2,4-DAA is partially inhibited by superoxide dismutase and ascorbic acid. A small amount of irreversible binding of 2,4-DAA to microsomal proteins also occurred when the NADPH-generating system was replaced by xanthine and xanthine oxidase (Table 4), an enzyme that is known to generate superoxide anions [19]. This binding was totally blocked in the presence of superoxide dismutase. Microsomes prepared from rat kidneys also activated [^{3}H]-ring-2,4-DAA to irreversibly bound products in the presence of NADPH (Table 4). In this situation, superoxide dismutase and ascorbate almost completely inhibited binding.

The effect of superoxide dismutase was also tested with microsomes from BNF-treated animals to see whether there were qualitative differences in the formation of irreversible metabolities in this situation (Table 5). Superoxide inhibited 2,4-DAA binding to approximately the same degree both with control and BNF-treated preparations, and both with [^{3}H]-ring and [^{3}H]-methyl-labeled-2,4-DAA. Addition of rat liver 105,000 *g* supernatant to the microsomal incubations markedly inhibited the irreversible binding of both ^{3}H-labels, in the control as well as the BNF-treated situation. Addition of superoxide dismutase and ascorbic acid, on the other hand, did not affect the mutagenicity of 2,4-DAA with control or BNF-treated 9000 *g* fractions (data not shown).

Formation of formaldehyde from 2,4-DAA metabolism in vitro. The lower binding rates seen with 2,4-DAA labeled in the methyl group compared to the ring-labeled compounds suggested loss of the methyl group and possible formation of formaldehyde. Using the Nash-procedure [20] to test for formaldehyde production, no evidence of such a reaction was found. However, when using the much more sensitive radiometric assay of Thompson and Holtzman [12], it was found that 2.22 ± 0.27 nmol/mg protein/10 min labile tritium was formed from [^{3}H]-methyl-2,4-DAA in the presence of NADPH and 0.63 ± 0.04 nmol/mg protein/10 min without cofactors. Out of this 71 per cent

was formaldehyde based on the dimedone derivatization–extraction procedure outlined by Thompson and Holtzman [12], and 5.2 per cent was methanol as determined by preparation of the methyl ester of 3,5-dinitrobenzoic acid using distillates containing carrier methanol [12]. There were no apparent increases in formaldehyde formation from [^{3}H]-methyl-2,4-DAA with BNF- treated microsomes compared to controls.

Total in vitro *metabolism and formation of glutathione conjugates.* Preliminary experiments were performed to determine the total *in vitro* metabolism of 2,4-DAA by thin-layer chromatography of aliquots of ethyl acetate- and water-soluble fractions from microsomal incubation on silica gel and cellulose (Avicel). With control microsomes 26.9 per cent of 2,4-DAA was metabolized in 10 min in the presence of NADPH, whereas 4.8 per cent was oxidized in the absence of cofactor. NADPH-dependent metabolism was thus 5.54 ± 0.34 nmol/mg protein/min; this was not reduced by the addition of superoxide dismutase. With BNF microsomes total NADPH-dependent metabolism was found to be 5.52 ± 0.58 nmol/mg protein/min. At present, no attempt has been made to characterize the metabolites. As shown in Table 1C, addition of glutathione markedly reduced the binding of 2,4-DAA to microsomal protein. At the same time at least 3 stable glutathione conjugates were formed, one of which only contains the methyl group. These glutathione conjugates account for about 2–3 per cent of the radioactivity present in the incubations, and are undergoing further structure elucidation at the present time.

Irreversible binding to nucleic acids in vitro. When incubating [^{3}H]-ring-2,4-DAA or [^{3}H]-methyl-2,4-DAA in the presence of NADPH, it could be shown that these labels bound irreversibly to microsomal RNA (Table 6). The extracted RNA was free of contaminating protein. As with protein binding, considerably more of the ring-label than the methyl-label was bound to RNA. Attempts were also made to test whether 2,4-DAA would bind irreversibly to DNA added to microsomal incubations. However, no binding could be demonstrated with [^{3}H]-ring-2,4-DAA. Under the assumption that possibly an interaction with DNA involved only the methyl group of the compound, very hot [^{3}H]-methyl-2,4-DAA was used (23 mCi/mmol), but no indication of binding to DNA was found in this situation either. As a positive control, [^{3}H]-benzo(a)pyrene bound to DNA in levels comparable to those reported in the literature [21, 22].

DISCUSSION

We have previously shown that rat and mouse liver microsomal cytochrome P-450 metabolism converts 2,4-DAA to an intermediate(s) which is mutagenic in the *Salmonella* mutagenicity test system [2], and we have suggested that the reactive species could be the hydroxylamine(s) formed via *N*-hydroxylation. NADPH-dependent oxidation of 2,4-DAA by rat liver microsomes also leads to the formation of reactive metabolites which bind irreversibly to microsomal protein. Rather high NADPH-independent values were also seen. Because of the highly activated nature of 2,4-DAA towards oxidation, even though the compound was purified immediately prior to use, small amounts of oxidation products such as aromatic hydroxylamines and nitroso-compounds were probably present. Only small amounts of such compounds are necessary to cause autocatalytic reactions. The binding reaction is increased with microsomes from phenobarbital-treated animals, and is decreased by various *in vivo* and *in vitro* inhibitors of cytochrome P-450, implying cytochrome P-450 metabolism in the activation reaction. However, the lack of effect of the inducer BNF on 2,4-DAA irreversible binding is in sharp contrast to its strong inducing effect on 2,4-DAA mutagenicity [2]. This indicates that there must be differences in the metabolic pathways leading to irreversible protein binding and mutagenicity. It could have been possible that BNF induction altered the route of metabolism such that the amount of 2,4-DAA bound when expressed as percentage of the total metabolized was increased. However, BNF induction did not alter total 2,4-DAA metabolism.

Cytochrome P-450 generation of superoxide anions has been reported to convert catechols to reactive electrophiles [8]. Superoxide anion formation is also involved in the activation of 2,4-DAA to irreversibly bound products. However, whereas superoxide dismutase and ascorbic acid totally blocked methyldopa activation [8], they only inhibited 2,4-DAA binding by approximately 50 per cent. An explanation for this is that 2,4-DAA binding involves more than one activation pathway some of which are not mediated by superoxide anion. The pathway(s) mediated by superoxide anion are apparently not responsible for mutagenicity caused by 2,4-DAA, since the mutagenicity is not altered by dismutase or ascorbate addition. Results of experiments with xanthine/xanthine oxidase, another source of superoxide anion

Table 6. NADPH-dependent irreversible binding of [^{3}H]-ring-2,4-DAA and [^{3}H]-methyl-2,4-DAA to rat liver microsomal RNA *in vitro*

Test compound	NADPH	Label bound pmol/mg RNA/min
[^{3}H]-ring-2,4-DAA	+	36.1
	–	1.7
[^{3}H]-methyl-2,4-DAA	+	11.0
	–	2.1

Microsomes were incubated with labels (0.5 mM) and an NADPH-generating system for 15 min. Microsomal RNA was extracted as described in Materials and Methods. Values are means of duplicate incubations.

[19], and the lack of effect of superoxide dismutase on total 2,4-DAA metabolism, indicated that a major portion of the irreversible binding of 2,4-DAA mediated by superoxide anion generated from microsomes occurred only after a primary oxidation (ring-hydroxylation or *O*-demethylation?) by the microsomes.

Comparing the binding rates from experiments using ring-labeled compounds with those using methyl-labels, it is apparent that part of the methyl group is lost during NADPH-dependent activation of 2,4-DAA to reactive metabolites. Based on the amounts of formaldehyde formed from the methyl group, less than 2 per cent of the metabolism of 2,4-DAA by rat liver microsomes occurs by oxidative demethylation. However, the methyl group must be important to both the mutagenicity and some of the reactions leading to irreversible binding of 2,4-DAA, since deuterium substitution for hydrogen on the methyl group increases the extent of both mutagenicity and irreversible binding. We might speculate that the rate of *O*-demethylation for 2,4-DAA is decreased as has been observed for the oxidative *O*-dealkylation of other aromatic-alkyl ethers [23, 24]. Thereby, other routes of metabolism, including toxic pathways, may account for a greater proportion of the metabolism, and thus lead to increased mutagenesis and binding. However, we cannot measure the effect of deuterium substitution on the *O*-dealkylation reaction until we prepare 2,4-DAA with a radiolabel and deuterium in the methyl group.

The present experiments have not fully elucidated the metabolic pathways involved in the microsomal activation of 2,4-DAA to irreversibly protein-bound products. However, some tentative steps can be suggested (Fig. 2). The formation of formaldehyde shows that 2,4-DAA is subject to oxidative *O*-demethylation which would produce 2,4-diaminophenol. 2,4-Diaminophenol is a highly activated aromatic compound which has amino groups ortho and para to a phenolic group. Just as catechols are susceptible to oxidation by superoxide anion [8], 2,4-diaminophenol would certainly be expected to be as susceptible or probably more susceptible to oxidation by superoxide anion. Aromatic hydroxylation products would be as reactive as catechols and could be further oxidized by superoxide anion to intermediates that would irreversibly bind to microsomal protein. Finally, *N*-hydroxylation reactions can be postulated. *N*-Hydroxy-metabolites can rearrange to phenolic products or yield electrophilic metabolites than can bind to macromolecules.

Since 2,4-DAA can be metabolized to products which can be detected as frame-shift mutagens in the *Salmonella* mutagenicity test [1, 2], an irreversible interaction of 2,4-DAA with DNA would not have been surprising. However, we were unable with the present methods [10, 21, 22] to detect any binding to DNA, whether using ring-labeled or methyl-labeled material. A possible explanation could be that the DNA-adducts are unstable and lost during the phenol-extraction procedure used, such as would be the case if the interaction would involve the phosphate-groups of the nucleotides in DNA [25]. On the other hand, appreciable amounts of binding to microsomal RNA could be demonstrated, again with considerably more binding when using the ring-labeled compound compared to the methyl-label.

In conclusion, we can say that the results of our experiments indicate that several routes of oxidative metabolism by microsomal cytochrome P-450 enzymes are involved in the formation of reactive metabolites from the mutagenic [1, 2] and carcinogenic [26] hair-dye constituent 2,4-DAA. Although the reactive metabolites that are formed *in vitro* can irreversibly bind to tissue proteins, many of these metabolites apparently have little to do with the initiation of mutagenic events. However, a minor fraction of these metabolites are probably involved in causing mutagenesis and the presence of the methyl group appears to be important as determined by the

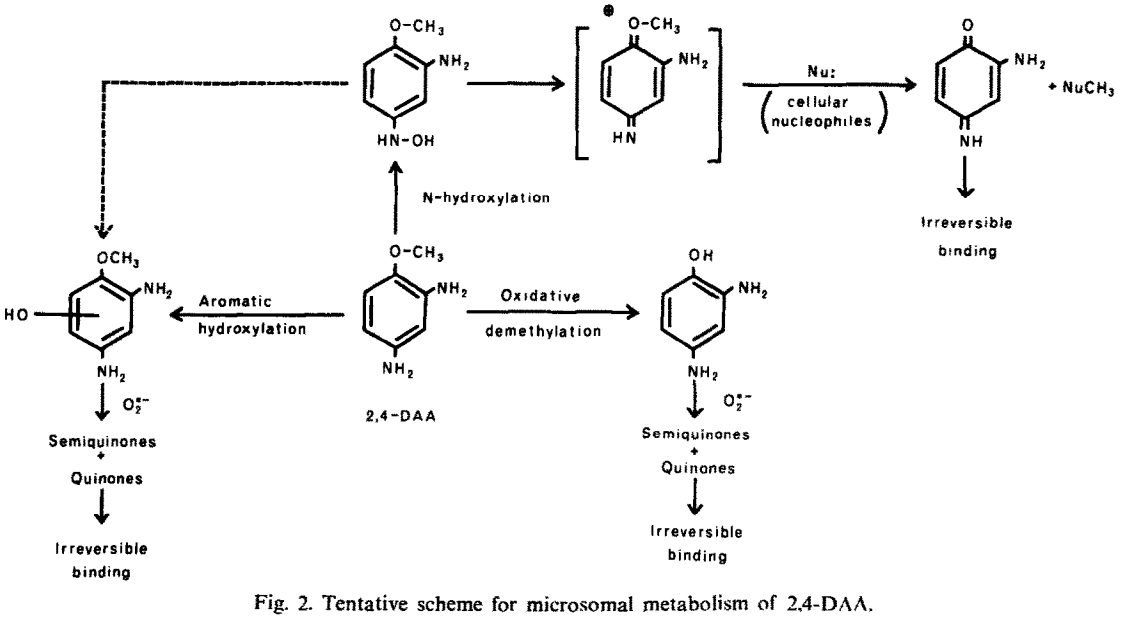

Fig. 2. Tentative scheme for microsomal metabolism of 2,4-DAA.

experiments with 2,4-DAA labeled in the methyl group with deuterium. Work on the biotransformation of 2,4-DAA is in progress to help elucidate the metabolic route(s) leading to the mutagenicity of 2,4-DAA.

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