Biochemical Pharmacology, Vol. 26, pp. 729–734. Pergamon Press, 1977. Printed in Great Britain.

METABOLIC ACTIVATION OF 2,4-DIAMINOANISOLE, A HAIR-DYE COMPONENT—I

ROLE OF CYTOCHROME P-450 METABOLISM IN MUTAGENICITY *IN VITRO*

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(*Received* 30 *August* 1976; *accepted* 25 *November* 1976)

Abstract—The activation of 2,4-diaminoanisole to a mutagen in the *Salmonella* test system by liver fractions of rats and mice is increased by treatments of animals with inducers of the cytochrome P-450 system and is decreased by *in vivo* and *in vitro* inhibitors of cytochrome P-450. Higher revertant rates were seen with 2,4-diaminoanisole in aromatic hydrocarbon responsive mice than in nonresponsive mice after treatment with β-naphthoflavone. Mutagenic activity of 2,4-diaminoanisole is also induced in kidney and lung as well as transplacentally in fetal liver after treatment with β-naphthoflavone. We suggest that metabolic activation of 2,4-diaminoanisole to the hydroxylamine(s) may be the underlying reaction for the formation of mutagenic intermediates.

The importance of environmental contaminants in the etiology of human cancer has recently been emphasized [1, 2]. It has been concluded on the basis of epidemiological studies that a majority of human cancers may be caused by chemicals in the environment [3, 4]. It is therefore a great neeed for detecting and identifying the environmental contaminants which are capable of inducing cancer in man and animals.

Recently, Ames and coworkers have developed a sensitive bacterial test system for the detection of chemical mutagens *in vitro* [5, 6, 7]. This system has been used as a screening test for carcinogens, and 90 per cent of the chemical carcinogens were shown to be mutagens in this assay [8], even if a causal relationship between mutagenesis and carcinogenis remains to be proven. Ames *et al.* have shown that commercial hair dyes, estimated to be used regularly by 20 million people in the USA, are potent mutagens [9]. The mutagenic components contained in the hair dyes were shown to be substituted aryldiamines, which were activated by the liver microsomes into strong frameshift mutagens.

Acetylarylamines, a related group of compounds, are known to be activated via cytochrome P-450 dependent *N*-oxidation into reactive metabolites capable of causing acute liver necrosis [10, 11] and carcinogenesis [12]. Felton and coworkers [13], using the Ames-system for the detection of activated promutagens, showed that *N*-oxidation of 2-acetylaminofluorene is important in the mutagenic activity of that *N*-acetylarylamine. This paper is concerned with the role of cytochrome P-450 metabolism in the *in vitro* mutagenicity of the aryldiamine hair dye component 2,4-diaminoanisole.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following sources: 2,4-diaminoanisole from ICN Pharmaceuticals, USA; 2,4-diaminotoluene, 3,4-diaminobenzoic acid, 2,4-dinitroanisole, α-naphthoflavone, and β-naphthoflavone from Aldrich, W. Germany; 3,4-diaminotoluene, 2,6-diaminotoluene, *o*-phenylenediamine, *m*-phenylenediamine, *p*-phenylenediamine, 3,5-diaminobenzoic acid, and 2,4-diaminophenol from Koch-Light, W. Germany; NADP, glucose 6-phosphate, yeast glucose 6-phosphate dehydrogenase, and l-cysteine from Sigma, USA; diethyl maleate and cobaltous chloride from Merck, W. Germany; metyrapone from Ciba-Geigy, Switzerland; Aroclor 1254 from Monsanto, USA; piperonyl butoxide from Cooper, McDougall & Robertson, England; and phenobarbital from the Norwegian Medicinal Depot. Other chemicals were reagent grade.

Treatment of animals. Male Wistar rats (200 g), male NMRI mice (25 g), and pregnant Wistar rats (250 g) were obtained from Møllegård Breeding Laboratories, Denmark. Male C57BL/6J/BOM/spf mice (B 6, 25 g) and male DBA/2J/BOM/spf mice (D 2, 25 g) were purchased from Bomholtgård Breeding and Research Centre, Denmark. Animals were pretreated with phenobarbital (75 mg/kg in 0.9% NaCl i.p. 72, 48 and 24 hr before death), β-naphthoflavone (80 mg/kg in corn oil i.p. 48 hr before death), Aroclor 1254 (500 mg/kg in corn oil i.p. 5 days before death), cobaltous chloride (40 mg/kg in saline s.c. 48 and 24 hr before death in rats, 60 mg/kg i.p. 72, 48 and 24 hr before death in mice), piperonyl butoxide (1360 mg/kg i.p. 30 min before death), controls received vehicle alone. Control, phenobarbital, and β-naphthoflavone-pretreated mice were injected with diethyl maleate (600 mg/kg i.p. 30 min before death) or cysteine (200 mg/kg i.p. at 2 hr and 30 min before death).

Preparation of enzyme fractions. All steps were carried out at 0–4° with cold sterile solutions and sterile equipment. Animals were killed by decapitation, the organs were removed and minced with an Ultra-Tur-

rax homogenizer (max 2 sec) and subsequently homogenized with a motor driven glass-Teflon homogenizer in 2 vol. of 1.15% KCl containing 20 mM Tris-buffer, pH 7.4, using 5 strokes. The homogenate was centrifuged for 20 min at 9000 *g*, the supernatant (the S9-fraction) was diluted to the appropriate protein concentration (usually 40 mg/ml) after protein determination according to Lowry *et al.* [14], and used as enzyme source. Microsomes were prepared after centrifugation of the 9000 *g* supernatant at 105,000 *g* for 60 min, the microsomal pellet was washed in the Tris-KCl buffer and centrifuged once more at 105,000 *g* for 60 min. The washed microsomal pellet was resuspended in the Tris-KCl buffer.

Mutagenesis assay. Mutagenesis was carried out essentially as described by Ames *et al.* [6]. To 2 ml of molten top agar at 45 were added 0.1 ml of a 17 hr culture of the bacterial tester strain TA 1538 (a generous gift from Dr. Bruce N. Ames, Berkeley, USA; 6×10^8 bacteria/ml), 0.1 ml dimethyl sulfoxide containing chemicals to be tested, and 0.5 ml of the S9-mixture, containing 0.1 ml of S9-fraction (2 mg protein per plate unless otherwise stated), 8 μmoles of $MgCl_2$, 33 μmoles of KCl, 5 μmoles of glucose 6-phosphate, 4 μmoles of NADP, and 100 μmoles of sodium phosphate buffer, pH 7.4, per milliliter. In experiments where microsomes were used as enzyme source, the glucose 6-phosphate concentration was increased to 20 μmoles/ml and each milliliter of reaction mixture also contained 2 units of glucose 6-phosphate dehydrogenase. The colonies on each plate (histidine revertants) were counted after a 2-day incubation at 37 . Values from plates without mutagen (representing spontaneous revertants, averaging 25 colonies per plate) were always subtracted. For bacterial toxicity tests, mutagen and bacteria (after a 1:200,000 dilution) were plated on full agar plates containing 8 g/liter of Difco nutrient broth (15).

RESULTS

Characterization of 2,4-diaminoanisole mutagenesis in vitro. The relationship between varying concentrations of 2,4-diaminoanisole, concentration of liver homogenate, and the histidine revertant rate in control and Aroclor-pretreated rats and mice is shown in Fig. 1 A–D. Whereas mice showed the highest rate of revertants compared to rats with control liver, rats had the highest rates compared to mice with induced liver. No inhibition of the absolute bacterial counts was seen in the concentration range used.

Effect of pretreatment with inducers and inhibitors of cytochrome P-450. Pretreatments of animals with chemicals that are known to alter the concentration or activity of microsomal cytochrome P-450 enzymes were tested for their effect on the mutation rate with

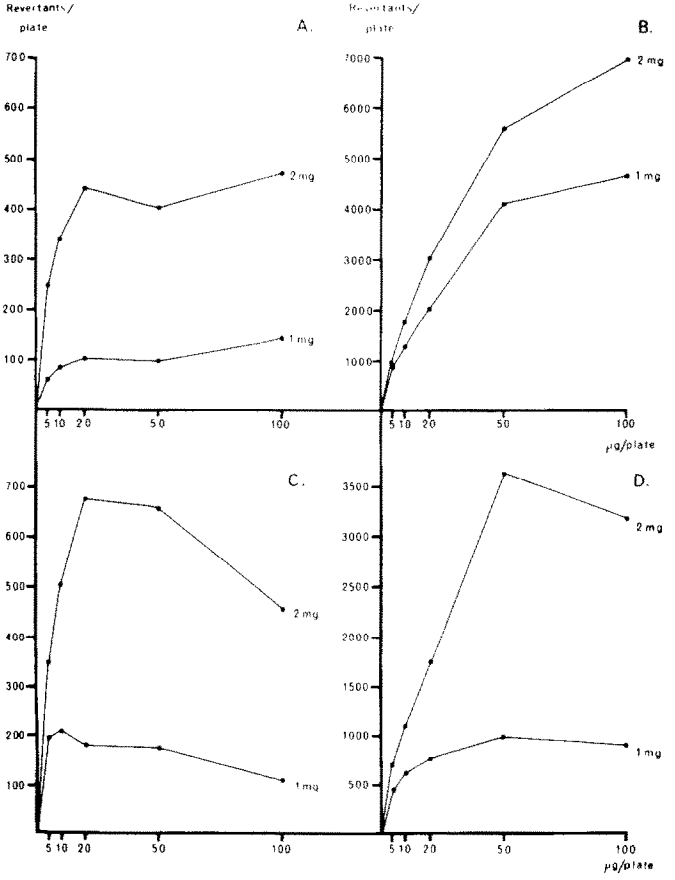

Fig. 1. A–D. Dependence of histidine revertant rate on concentration of 2,4-diaminoanisole *in vitro* in control (A) and Aroclor-pretreated (B) rats and in control (C) and Aroclor-pretreated (D) mice. Two S9 protein concentrations (milligrams per plate) for each treatment group are shown. Values are means of duplicate estimations with pooled livers from 2 rats and 5 mice.

Table 1. Effect of pretreatments on 2,4-diaminoanisole mutagenicity *in vitro* in rats and mice

Pretreatments	Rats Revertants per plate	Mice Revertants per plate
Control	252 ± 41	473 ± 27
Phenobarbital	485 ± 73	630 ± 51
Beta-Naphthoflavone	2077 ± 114	920 ± 96
Cobaltous chloride	97 ± 5	481 ± 85
Piperonyl butoxide	361 ± 51	623 ± 95

Each plate contained 10 μg 2,4-diaminoanisole and 2 mg protein. Values are means ± S.D. of 4 estimations with pooled livers from 2 rats and 5 mice.

2,4-diaminoanisole (Table 1). Pretreatment with phenobarbital or β-naphthoflavone gave increased mutation rates in both rats and mice, β-naphthoflavone was the more effective inducer of 2,4-diaminoanisole mutagenicity in both rats and mice. Cobaltous chloride, a compound thought to inhibit cytochrome P-450 synthesis [16], reduced the mutation rates in rats, whereas the mice were resistant to this treatment. Piperonyl butoxide, an inhibitor of many cytochrome P-450 mediated reactions [17], however, increased 2,4-diaminoanisole mutagenicity in both rats and mice. An explanation for this could be that piperonyl butoxide only inhibits a non-mutagenic metabolic pathway thereby increasing the substrate concentration available for the mutagenic pathway.

Addition of cytochrome P-450 inhibitors in vitro. The cytochrome P-450 inhibitors α-naphthoflavone and metyrapone have been used to differentiate between the various forms of the cytochrome [18]. α-Naphthoflavone was shown in mice to inhibit the 3-methylcholanthrene-inducible aryl hydrocarbon hydroxylase activity more than the phenobarbital-inducible hydroxylase activity, whereas metyrapone inhibited the phenobarbital-inducible hydroxylase activity more than the 3-methylcholanthrene-inducible hydroxylase activity [18]. A similar pattern to the one seen on hydroxylase inhibition was seen when the two inhibitors were tested on 3-methylcholanthrene mutagenicity *in vitro* with S9-liver fractions from DBA/2N mice [19]. Figures 2 A–F show the effects of α-naphthoflavone and metyrapone on 2,4-diaminoanisole mutagenicity in control, phenobarbital-, and β-naphthoflavone-pretreated rats and mice. α-Naphthoflavone was most inhibitory in the polycyclic hydrocarbon-induced animals, least inhibitory in control mice and phenobarbital-pretreated rats. Metyrapone was hardly inhibitory in any of the mouse preparations, whereas it showed the greatest inhibition in phenobarbital-pretreated rats.

Genetic differences in 2,4-diaminoanisole mutagenicity. The mutagenicity of 2-acetylaminofluorene has been shown [13] to be increased by liver postmitochondrial fractions from 3-methylcholanthrene-treated B6 inbred mice, but not from 3-methylcholanthrene-treated D2 inbred mice. Furthermore, the aromatic hydrocarbon induced metabolic activation of 2-acetylaminofluorene to a mutagen *in vitro*

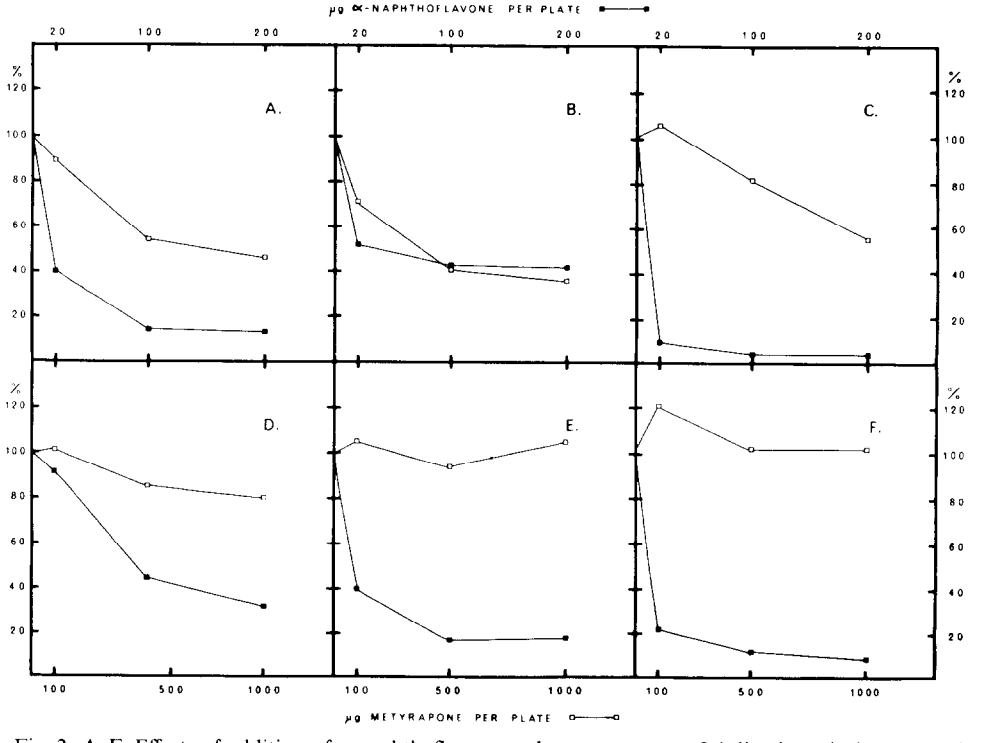

Fig. 2. A–F. Effects of addition of α-naphthoflavone and metyrapone on 2,4-diaminoanisole mutagenicity *in vitro* in control (A), phenobarbital-pretreated (B), and β-naphthoflavone-pretreated (C) rats and in control (D), phenobarbital-pretreated (E), and β-naphthoflavone-pretreated (F) mice. The designated amount of each inhibitor was added directly to the top agar. Each plate contained 10 μg 2,4-diaminoanisole and 2 mg protein. Values are means of duplicate estimations with pooled livers from 2 rats and 5 mice.

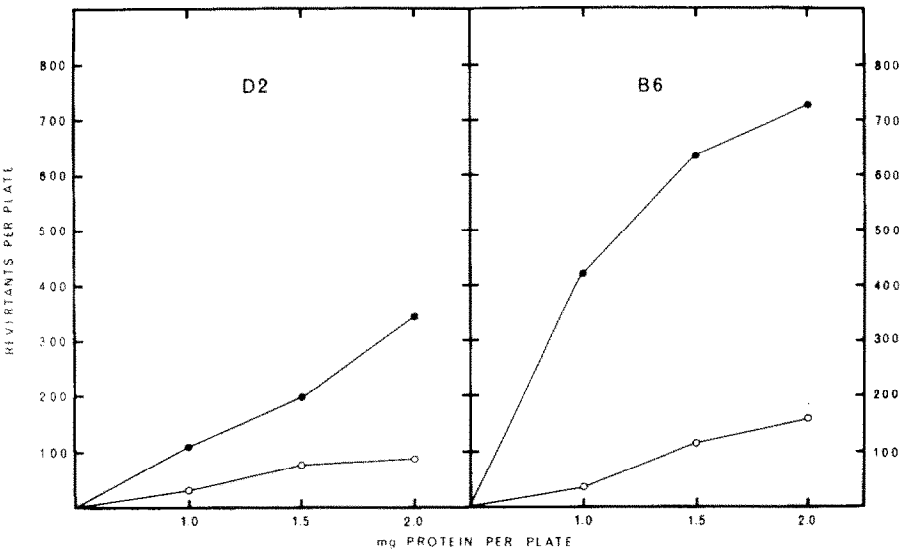

Fig. 3. 2,4-Diaminoanisole mutagenicity *in vitro* in control and β-naphthoflavone-pretreated nonresponsive DBA/2J (D 2) and responsive C57BL/6J (B 6) mice. Each plate contained 10 μg 2,4-diaminoanisole and 1.0, 1.5, or 2.0 mg protein. Open circles, control mice; filled circles, β-naphthoflavone-pretreated mice. Values are means of duplicate estimations with pooled livers from 10 mice.

appeared to be expressed as an additive trait in appropriate backcrosses and intercross involving B6 and D2 mice [13]. Figure 3 shows the histidine revertant rate with 2,4-diaminoanisole using liver fractions from untreated and β-naphthoflavone pretreated nonresponsive D2 and responsive B6 mice. Although β-naphthoflavone pretreatment did increase the revertant rate with liver fractions from the nonresponsive mice, a higher increase was seen with liver fractions from the responsive mice in this experiment.

2,4-Diaminoanisole mutagenicity with extrahepatic tissues and fetal liver. Aroclor 1254, which very effectively induced 2,4-diaminoanisole mutagenic activity with liver preparations, was also tested for its possible effect on extrahepatic tissues (Table 2). In rats, both kidney and lung 9000 *g* supernatant fractions had low basal activity compared to the liver; however, the activity could be induced both in kidneys and lungs, but the effect was especially striking in the kidney preparation.

Induction of aryl hydrocarbon hydroxylase activity has been shown to occur in fetal rat liver [20, 21]. Recently, induction of benzo(a)pyrene mutagenicity with fetal rat liver has also been demonstrated [15]. Table 3 gives the results of experiments in which fetal liver was tested for 2,4-diaminoanisole mutagenic activity. Twenty-day-old fetal liver did not show any activity, however, when the pregnant mothers had been pretreated 24 hr previously with β-naphthoflavone considerable activity was present.

Subcellular localization of activating enzymes. Various subcellular liver fractions from control and β-naphthoflavone-pretreated rats and mice were tested for their ability to form mutagenic products from 2,4-diaminoanisole (Table 4). Washed microsomes were found to be active in this system, whereas very little activity was found in the 105,000 *g* supernatant fraction. Combining microsomes and 105,000 *g* supernatant together increased the mutation rate considerably compared to the rate with microsomes only. For some reason, the absolute mutation frequency rate with 2,4-diaminoanisole in the β-naphthoflavone-induced animals were considerably lower than usual in these experiments.

Comparison of the mutagenic activity of several aryldiamines. Several aryldiamines were tested for mutagenicity with rat and mouse control and β-naphthoflavone-induced liver S9-fractions (Table 5). 2,4-Diaminoanisole showed by far the greatest activity.

Table 2. 2,4-Diaminoanisole mutagenicity *in vitro* with liver and extrahepatic tissues in rats

Enzyme source	Controls Revertants per plate	Aroclor-pretreated Revertants per plate
Liver	258 ± 20	2291 ± 204
Kidney	19 ± 4	685 ± 176
Lung	12 ± 5	55 ± 5

Each plate contained 10 μg 2,4-diaminoanisole and 2 mg protein of each enzyme preparation. Values are means ± S.D. of 4 estimations with pooled organs from 2 rats.

Table 3. 2,4-Diaminoanisole mutagenicity *in vitro* with fetal rat liver

Enzyme preparation	Control Revertants per plate	BNF-Pretreated Revertants per plate
Fetal liver	0*	235 ± 11
Maternal liver	187 ± 18	2389 ± 207

* 20–23 colonies per plate without mutagen, 18–27 colonies per plate with mutagen. Pregnant rats were pretreated with β-naphthoflavone 80 mg/kp i.p. on day 19 of gestation, they were killed 24 hr later. Each plate contained 10 μg 2,4-diaminoanisole and 2 mg protein. Values are means ± S.D. of 4 estimations with pooled livers from 2 litters and dams.

Table 4. 2,4-Diaminoanisole mutagenicity *in vitro* with various subcellular liver fractions in control and β-naphthoflavone-pretreated rats and mice

Subcellular fraction	Rats		Mice	
	Control rev./plate	BNF rev./plate	Control rev./plate	BNF rev./plate
9000 *g* Supernatant	163 (153–172)	839 (758–980)	449 (402–495)	656 (632–701)
Microsomes	45 (28–73)	237 (164–302)	136 (105–151)	226 (211–244)
105,000 *g* Supernatant	5 (0–12)	23 (19–25)	8 (5–12)	3 (0–7)
Microsomes + 105,000 *g* Supernatant	360 (339–399)	873 (826–946)	379 (361–397)	359 (318–391)

Preparation of liver cell fractions are described in Materials and Methods. Each plate contained 10 μg 2,4-diaminoanisole and 2 mg protein of each fraction. Values are means of 3 estimations with pooled livers from 2 rats and 9 mice, range in parenthesis.

Table 5. Histidine revertant rate with 2,4-diaminoanisole analogs in control and β-naphthoflavone-pretreated rats and mice

Aryldiamine	Rats		Mice	
	Control rev./plate	BNF rev./plate	Control rev./plate	BNF rev./plate
2,4-Diaminoanisole	186	2304	410	1299
2,4-Diaminotoluene	10	16	8	23
2,4-Diaminophenol	0	77	0	9
m-Diaminobenzene	6	49	36	58
p-Diaminobenzene	0	73	26	55
o-Diaminobenzene	0	5	4	7
2,6-Diaminotoluene	0	25	18	17
3,4-Diaminotoluene	0	5	0	0
3,4-Diaminobenzoic acid	0	0	0	0
3,5-Diaminobenzoic acid	0	1	3	0

Each plate contained 10 μg aryldiamine and 2 mg protein. Values are means of duplicate estimations with pooled livers from 2 rats and 5 mice.

whereas alteration of the anisole moiety to a methyl- or hydroxyl-group or a hydrogen markedly reduced activity. Even less activity was seen with the ortho-aryldiamines; and the diaminobenzoic acids were not mutagenic at the concentration tested. None of these aryldiamines were cytotoxic at this concentration. 2,4-Dinitroanisole also showed slight mutagenic activity (65 colonies per plate with 10 μg), however, this rate was also found when the S9-Mix was omitted. The explanation for this could be the activation of the dinitro-compound by bacterial reductases.

Effects of pretreatments which alter the availability of hepatic glutathione. Experiments with control, phenobarbital-, and β-naphthoflavone-pretreated animals were performed to see if the availability of hepatic glutathione would influence the rates of 2,4-diaminoanisole mutagenicity with rat and mouse liver S9-fractions. Pretreatments with diethyl maleate, which depletes hepatic glutathione, and cysteine, a glutathione precursor [22], did not markedly alter the revertant rate (data not shown), neither did the direct addition of 0.9 μmole diethyl maleate per plate.

DISCUSSION

The use of a bacterial mutagenesis assay as an indicator for the study of cytochrome P-450 mediated metabolic reactions has successfully been demonstrated using polycyclic hydrocarbons [19] and 2-acetylaminofluorene [13] as substrates. Although such an assay does not allow a kinetic analysis of the reactions studied, much can be learned about the activation pathways by *in vivo* and *in vitro* manipulations of the cytochrome P-450 system.

Our data indicate that activation of the aryldiamine 2,4-diaminoanisole to a mutagenic intermediate(s), occurs via cytochrome P-450 metabolism. Pretreatments that alter the concentration and activity of cytochrome P-450 have similar effects on 2,4-diaminoanisole mutagenicity. The difficulties encountered with cobaltous chloride-pretreatment in mice have also been seen with other cytochrome P-450 parameters in another mouse strain [23]. Addition of cytochrome P-450 inhibitors *in vitro* also inhibits 2,4-diaminoanisole mutagenicity, the pattern of inhibition in rats being very similar to the effects seen with these inhibitors on aryl hydrocarbon hydroxylase [18] and 3-methylcholanthrene mutagenicity [19] in mice. The pattern of relative activity and inducibility of 2,4-diaminoanisole mutagenicity in extrahepatic tissues are similar to the pattern of many other cytochrome P-450 mediated ractions. The differences in 2,4-diaminoanisole mutagenicity with liver fractions from β-naphthoflavone-treated D2 and B6 mice suggest that the induction of cytochrome P-450 mediated 2,4-diaminoanisole activation by polycyclic aromatic

hydrocarbons is controlled in the mouse by the same (or closely linked) gene(s) as that regulating induction of 2-acetylaminofluorene activation to a frameshift mutagen and aromatic hydrocarbon responsiveness [13].

No definite answers to the question of the nature of the mutagenic intermediate(s) of 2,4-diaminoanisole can be given. However, it is tempting to suggest the involvement of a N-hydroxylated metabolite(s). *N*-Acetylarylamines have been shown to be *N*-oxidized by a cytochrome P-450-dependent mixed-function oxidase [24]. This reaction is linked to aromatic hydrocarbon responsiveness in mice [11], and the metabolic activation of 2-acetylaminofluorene to a mutagen *in vitro* is controlled by the same gene as that regulating aromatic hydrocarbon responsiveness [13]. Further activation of the *N*-hydroxy-2-acetylaminofluorene is thought to occur via deacetylation and formation of a sulfate ester [13]. As with 2-acetylaminofluorene, 2,4-diaminoanisole is found to be mutagenic when microsomes are used as the enzyme source in the assay. A considerable increase over the activity in control microsomes alone are seen when the cytosol fraction is combined with the microsomes. This might be due to further activation of *N*-hydroxylated products by soluble enzymes, but could also simply be due to the stabilization of the microsomes.

Activation via demethylation of the anisole moiety is another possibility. However, judging from the low activity seen with 2,4-diaminophenol, this seems rather unlikely. The anisole-group must be important for activity perhaps by making the molecule more lipophilic, or possibly for steric reasons when the mutagen intercalate in the DNA-base-pair stack, since the other 2,4-diamino-derivatives were much less active. 2,5-Diaminoanisole [9] is also much less active.

The demonstration of inducibility of benzo(a)pyrene mutagenesis with fetal rat liver [15] correlates well with the observed transplacental induction of mono-oxygenase activity [20, 21]. The results reported here show that also the enzymes responsible for the formation of a reactive intermediate from an aryldiamine can be synthesized in fetal liver.

What is the possible *in vivo* significance of the finding of 2,4-diaminoanisole mutagenic activity *in vitro*? Very little is known about the toxicology of 2,4-diaminoanisole. A two year study with rats that had been painted on the skin twice weekly with 0.25 g of a mixture containing 0.75% 2,4-diaminoanisole together with 3% 2,5-diaminotoluene and 0.75% resorcinol apparently did not cause any adverse effects [25]. The test substances were applied after mixing with an equal vol. of 6% hydrogen peroxide immediately before use. A similar topical application study in mice, where 2,4-diaminoanisole was tested as a 0.38% mixture together with 2,5-diaminotoluene, *p*-phenylenediamine, vehicles and hydrogen peroxide, did not show any evidence of systemic toxicity or carcinogenicity [26]. Whereas 2,4-diaminotoluene is reported to give hepatocellular carcinomas in feeding experiments in rats [27], and rhabdomyosarcomas after repeated subcutaneous injections in rats [28], this compound did not give signs of carcinogenicity in a chronic skin-painting study in mice [29]. To be able to fully assess the safety of 2,4-diaminoanisole much needs to be learned regarding its toxicokinetics.

Acknowledgements—This study was supported by a grant from "The Norwegian Society For Fighting Cancer". The expert technical assistance of Mrs. Lise Timm Haug is very much appreciated.

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