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

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Abstract—Injection of [^{3}H]-2,4-diaminoanisole (2,4-DAA) between 10 and 200 mg/kg to rats led to its irreversible binding to liver and kidney proteins. The binding was increased by prior treatment of the animals with phenobarbital and β-naphthoflavone and was decreased after cobaltous chloride-pretreatment. Irreversibly bound material was preferentially localized in the microsomal fraction. Pretreatment with diethyl maleate slightly increased liver binding, but was without effect on kidney binding. Non-bound [^{3}H]-2,4-DAA was rapidly cleared from plasma, liver and kidneys, whereas considerable irreversibly bound 2,4-DAA could still be demonstrated 24 hr after a dose of 100 mg/kg i.p. No irreversible binding to hepatic RNA or DNA could be detected.

Many chemicals elicit their toxic effects after being converted in the body to reactive forms. Several hepatotoxic drugs [1], mutagens and carcinogens [2] are chemically stable compounds, but mammalian metabolism activates these substances to products which bind irreversibly to important macromolecules in the cells.

2,4-Diaminoanisole (2,4-DAA), a component of many commercial hair-dyes, is a potent mutagen *in vitro* in the presence of a rat liver activating system [3]. We have previously shown that cytochrome P-450 metabolism is involved in the formation of mutagenic products from 2,4-DAA [4]. Recently, we have also shown that cytochrome P-450 converts 2,4-DAA to metabolites which bind irreversibly to microsomal protein *in vitro* [5]. This paper presents evidence that 2,4-DAA is converted in the body to reactive intermediates which bind irreversibly to protein.

MATERIALS AND METHODS

Chemicals. Synthesis of [^{3}H]-ring-2,4-DAA was performed as described [5]. Unlabelled 2,4-DAA was purchased from ICN Pharmaceuticals, U.S.A.; β-naphthoflavone from Aldrich, Germany; cobaltous chloride and diethyl maleate from Merck, Germany; phenobarbital from the Norwegian Medicinal Depot.

Treatment of animals. Male Wistar rats (150–250 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), β-naphthoflavone (80 mg/kg in corn oil 48 hr before death), cobaltous chloride (60 mg/kg in saline s.c. 48 and 24 hr before death) or diethyl maleate (600 mg/kg i.p. 30 min before death).

Irreversible binding to macromolecules. Rats were injected with [^{3}H]-2,4-DAA (1000–5000 d.p.m./nmol) 10–200 mg/kg in dimethylsulfoxide (0.5 ml per 200 g) and killed at various times after injection. Samples of heparinized blood, plasma and various organs were homogenized in 10–20 volumes of water and 1 ml homogenate was added to 2 ml of 15% trichloroacetic acid (TCA) to precipitate the protein for determination of irreversibly bound radioactivity. Another ml of homogenate was shaken with 2 ml of Soluene (Packard) at 37° for 16 hr for determination of total radioactivity. For determination of distribution of irreversibly bound material in the liver, subcellular fractionation was performed as described by Hogeboom [6]. The precipitated proteins were washed exhaustively with TCA, methanol:water (4:1) and ethanol:ether (1:1) until no more radioactivity could be extracted, as described [7]. The pellets were dissolved in 1.0 ml 1 M NaOH and aliquots were counted in 15 ml Dimilume scintillation fluid (Packard) and corrected for background and quench (external standardization). Protein concentration was determined according to Lowry *et al.* [8] using crystalline bovine serum albumin as standard.

For determination of binding to nucleic acids, pieces of liver were homogenized in 6% Na-*p*-aminosalicylate and extracted with a phenol/*m*-cresol-mixture according to the method of Irving and Veazey [9].

Liver and kidney glutathione levels were determined

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by the method of Ellman [10] as described by Mitchell *et al.* [11].

RESULTS

After intraperitoneal administration of [^{3}H]-2,4-DAA 100 mg/kg to rats, irreversible binding to liver, kidney and muscle protein was determined at various time intervals (Fig. 1A). Both liver and kidney protein contained considerable amounts of irreversibly bound material, kidney nearly as much as liver, whereas binding to muscle, thought to represent non-specific binding, was much lower. Binding increased rapidly with time during the first two hours after administration; thereafter it levelled off. Injection of various doses of [^{3}H]-2,4-DAA showed a linear dose dependency of binding to liver protein between 10 and 200 mg/kg measured 4 hr after injection (Fig. 1B). At the highest dose, binding to kidney protein was even higher than in liver. Attempts were made to demonstrate a possible irreversible binding of [^{3}H]-2,4-DAA to hepatic nucleic acids. However, after phenol extraction and isolation of nucleic acids by the method of Irving and Veazey [9] 4 hr after a dose of 100 mg/kg (5000 d.p.m./nmol), no indication of bound material to either RNA and DNA could be found.

Pretreatments of animals with the cytochrome P-450 inducers phenobarbital and β-naphthoflavone increased binding to protein both in livers and kidneys (Table 1), whereas cobaltous chloride-pretreatment lowered binding in both organs significantly. On the other hand, i.p. injections of 2,4-DAA 100 mg/kg daily for 3 days did not increase microsomal cytochrome P-450 content or ethylmorphine *N*-demethylase above values from DMSO-treated controls.

The livers of rats that had been treated with [^{3}H]-2,4-DAA 100 mg/kg i.p. for 4 hr were fractionated to determine the subcellular distribution of irreversible

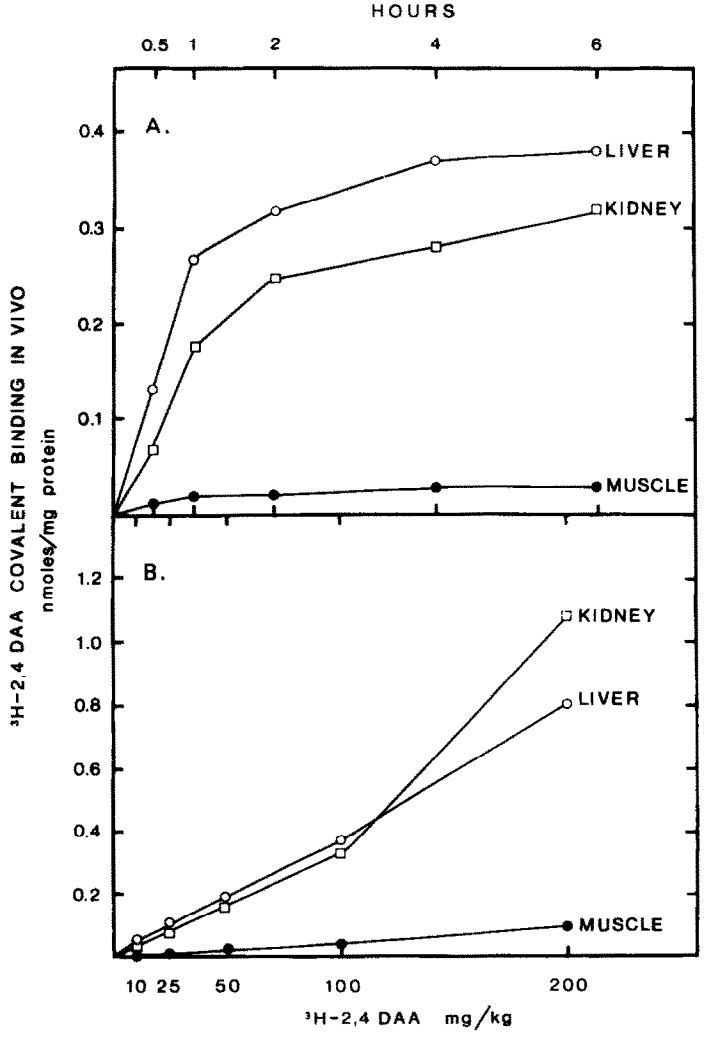

Fig. 1 A-B. Time course (A) and dose dependency (B) of [^{3}H]-2,4-DAA irreversible binding to rat liver, kidney and muscle protein *in vivo*. Irreversible binding was determined as described in Materials and Methods and assayed at various time points after a dose of 100 mg/kg i.p. (A) or 4 hr after various doses i.p. (B). Values are means of 2 animals for each point.

Table 1. Effect of pretreatments on irreversible binding of [^{3}H]-2,4-DAA to rat liver and kidney protein *in vivo*

Pretreatment	Liver binding nmol/mg protein	Kidney binding nmol/mg protein
Control	0.30 ± 0.02	0.29 ± 0.03
Phenobarbital	0.55 ± 0.02*	0.40 ± 0.09
β-Naphthaflavone	0.43 ± 0.04*	0.41 ± 0.03*
Cobaltous chloride	0.24 ± 0.03*	0.21 ± 0.01*

Irreversible binding was determined in tissues from control or pretreated animals 4 hr after a dose of 100 mg/kg i.p. Muscle protein binding has been subtracted from the individual values. Values represent means ± S.D. from 4 animals. *$P < 0.02$ vs controls.

Table 2. Subcellular distribution of irreversibly bound [^{3}H]-2,4-DAA in rat liver *in vivo*

Cell fraction	[^{3}H]-2,4-DAA bound nmol/mg protein
Nuclei + cell debris	0.25 ± 0.00
Mitochondria	0.25 ± 0.02
Microsomal	0.59 ± 0.09
Soluble	0.39 ± 0.04

Irreversible binding was determined in the subcellular fractions 4 hr after a dose of 100 mg/kg i.p. Muscle protein binding has been subtracted from the individual values. Values represent means ± S.D. from 4 animals.

binding (Table 2). Irreversible binding was preferentially localized in the endoplasmic reticulum of the liver cell. Considerable binding was also found in the proteins of the cytosol, whereas lesser amounts were found in proteins of nuclei, cell debris and mitochondria.

The effect of pretreatments of animals with diethyl maleate, a substance which depletes tissue contents of glutathione [12], on the irreversible binding of [^{3}H]-2,4-DAA to liver and kidney proteins was also studied. Diethyl maleate pretreatment led to a 40 per cent increase in liver binding, but was without effect on

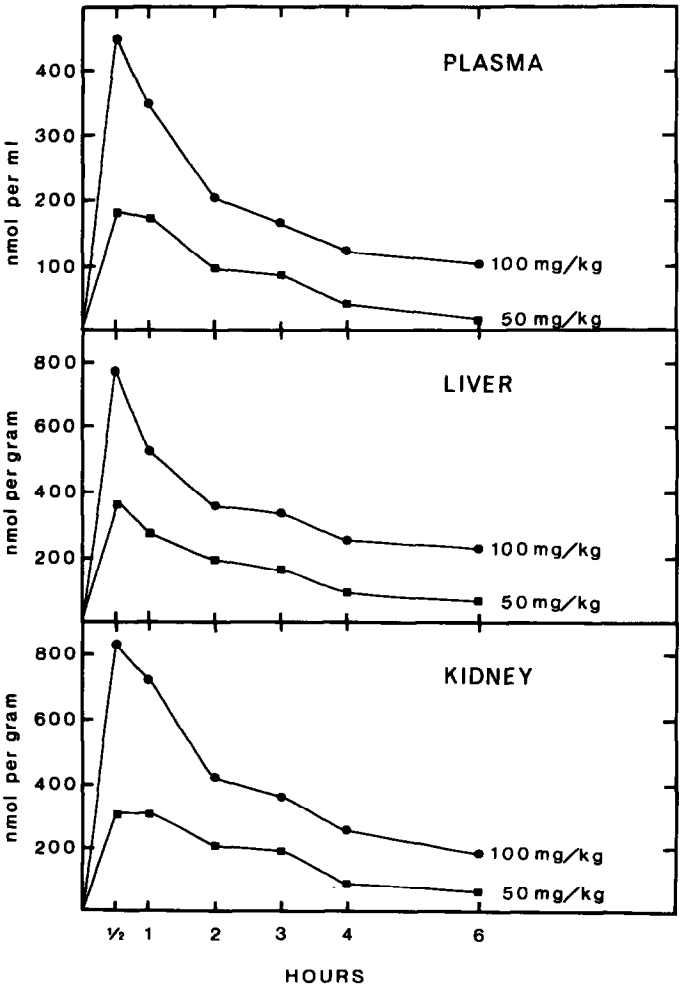

Fig. 2. Disappearance of radioactivity from plasma, liver and kidney after i.p. administration of [^{3}H]-2,4-DAA 50 mg/kg and 100 mg/kg. Values represent means of 2 animals.

kidney binding. Glutathione levels were also determined 0.5, 1, 2 and 4 hr after a dose of 2,4-DAA 200 mg/kg i.p. This treatment did not markedly affect glutathione levels, neither in liver nor in kidneys.

The disappearance of radioactivity from plasma, liver and kidneys was followed with time after i.p. injections of [^{3}H]-2,4-DAA 50 mg/kg and 100 mg/kg (Fig. 2). Peak values in both plasma, liver and kidneys were seen at the earliest time point measured ($\frac{1}{2}$ hr), and elimination was rapid from all tissues. Liver and kidneys contained comparable amounts of radioactivity after each of the two dose levels.

Twenty-four hours after a dose of [^{3}H]-2,4-DAA 100 mg/kg i.p., very little radioactivity could be found in the various tissues (Table 3). The level found in the liver represented approximately 0.3 per cent of the dose. However, considerable amounts of irreversibly bound material could still be demonstrated in the liver and kidneys. Some irreversibly bound radioactivity was also found in the proteins of lungs, adrenals and blood.

Table 3. Total concentration and irreversibly bound [^{3}H]-2,4-DAA in various tissues 24 hr after administration of 100 mg/kg i.p. (0.68 mmol/kg)

Tissue	Total [^{3}H]-2,4-DAA nmol/g wet wt	Bound [^{3}H]-2,4-DAA nmol/mg protein	Bound as per cent of total
Liver	79.4 ± 6.5	0.22 ± 0.02	0.28
Kidney	77.1 ± 1.6	0.32 ± 0.02	0.42
Lung	31.3 ± 3.9	0.13 ± 0.02	0.42
Adrenal	43.8 ± 8.0	0.22 ± 0.03	0.50
Testes	26.4 ± 2.1	0.03 ± 0.01	0.11
Ileum	24.8 ± 5.3	0.08 ± 0.03	0.32
Blood	24.1 ± 6.3	0.17 ± 0.04	0.71
Spleen	33.8 ± 5.3	0.09 ± 0.02	0.27
Muscle	24.7 ± 2.9	0.02 ± 0.01	0.08
Brain	20.9 ± 2.6	0.02 ± 0.00	0.10

Values represent means ± S.D. from 4 animals.

DISCUSSION

In preceding papers, we have demonstrated that liver microsomal cytochrome P-450 oxidation of 2,4-DAA leads to the formation of mutagenic [4] and irreversibly protein-bound [5] metabolites *in vivo*. The finding of irreversible binding *in vitro* does not necessarily mean that the same event will take place *in vivo*, in that the favorable conditions leading to the reactive intermediate *in vitro* very often do not exist in the whole organism. However, with the aromatic diamine, 2,4-DAA, considerable binding was found in the proteins of the liver as well as the kidney. The effect of inducers and inhibitors of the cytochrome P-450 system on binding suggest that this binding is dependent on cytochrome P-450 metabolism as was the case with *in vitro* binding [5]. Phenobarbital-treatment increased *in vivo* binding to the same degree as it did *in vitro* binding. On the other hand, β-naphthoflavone did not increase binding *in vitro* but did give a small increase *in vivo*. At any rate, the effect of the inducers on the extent of binding either *in vitro* or *in vivo* is quantitatively very different from the effect these inducers have on mutagenicity *in vitro* [4], where β-naphthoflavone-treatment increased mutagenic activity up to 8-fold. It is conceivable that BNF induction alters the pathways of metabolism such that the percentage of the metabolized 2,4-DAA that binds is increased, but not the rate of formation of the active metabolites nor the rate of DNA binding. However, BNF induction did not alter the total 2,4-DAA metabolism *in vitro* [6].

2,4-DAA is rapidly cleared from plasma, liver and kidneys. Twenty-four hours after an i.p. dose, very little radioactivity could be demonstrated in any organ; however, some was found to be irreversibly bound to proteins. The finding of irreversibly bound material in blood could be due to a superoxide anion activation pathway, since the *in vitro* binding of 2,4-DAA is in part superoxide dismutase-sensitive [5]. Bound oxygen in oxy-hemoglobin can be envisioned as superoxide [13]; previously, human red cells have been found to activate the catechol α-methyldopa to irreversibly protein-bound products [14].

No indication of an irreversible interaction with hepatic nucleic acids could be found *in vivo*. [^{3}H]-ring-2,4-DAA did not bind to exogenously added DNA *in vitro*, but did bind to microsomal RNA [6]. In analogy with this, we could not demonstrate any binding of the hepatocarcinogen 2,4-diaminotoluene [15] to DNA, either *in vitro* or *in vivo* (unpublished results). On the other hand, 2,4-diaminotoluene did bind to hepatic RNA *in vivo*, in contrast to 2,4-DAA. Whereas 2,4-DAA binding to protein *in vitro* was approximately 6–8 times higher than that of 2,4-diaminotoluene, both diamines caused the same degree of irreversible binding to protein *in vivo*.

The relevance of the finding of irreversible binding of 2,4-DAA to hepatic protein *in vivo* is uncertain. Several hepatotoxins are thought to cause acute liver necrosis because they interact with macromolecules essential to the life of the hepatocyte [1]. Most carcinogens are known to be converted to electrophilic forms which bind irreversibly to protein, as well as DNA and RNA [2]. Preliminary evidence indicates that 2,4-DAA is carcinogenic in rats and mice after incorporating high doses of the diamine in their diet [16]. In preliminary experiments, we have not been able to demonstrate any clear cut acute liver necrosis with 2,4-DAA. 2,4-DAA shows many similarities to the hepatocarcinogenic 2,4-diaminotoluene. Both substances are activated to mutagens *in vitro*, both form irreversibly protein-bound products via cytochrome P-450 metabolism *in vitro* and *in vivo* and neither can be demonstrated to interact with DNA *in vitro* or *in vivo* when the usual phenol-extraction procedures are employed.

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