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Effect of phenobarbital pretreatment on the toxicity and metabolism of 2,4-diaminoanisole

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2,4-Diaminoanisole (2,4-DAA), which has both carcinogenic and mutagenic activities, has been a component of commercial hair dyes [1, 2]. The reactive intermediate(s) of this compound, generated via the cytochrome P-450-dependent pathways, may bind irreversibly to protein and RNA and may be responsible for the mutagenic action [3–5]. Pretreatment of rats with inducers or inhibitors of the cytochrome P-450 system caused an alteration in the level of protein binding and mutagenicity [3–6]. Phenobarbital (PB), a broad spectrum microsomal enzyme inducer, has been shown to increase binding of 2,4-DAA to liver and kidney proteins *in vitro* and *in vivo* [4, 5].

It was the purpose of this study to investigate the effects of phenobarbital on the acute toxicity, macromolecular binding, disposition and metabolism of 2,4-DAA in rats.

2,4-Diaminoanisole · H_2SO_4 [ring ^{14}C] with a specific activity of 42.39 mCi/mmoles and chemical purity > 97 per cent was purchased from ICN Pharmaceuticals, Irvine, CA. It gave a single band in two t.l.c. solvent systems (methylene chloride–ethanol, 85:15; and ethyl acetate–methanol–water, 80:35:9), on precoated silica gel (60F-254), 0.25 mm plates. On high performance liquid chromatography (HPLC) (Spectra-Physics model 3500) a single band was obtained with the solvent systems: (A) water–methanol 95/5 and (B) water–methanol 30/70 run as a linear gradient on a reverse phase C-18 μBondapak column (Waters Associates). Young male Fischer rats aged 65 to 75-days old were pretreated with PB (75 mg/kg in 0.9% NaCl), i.p., 72, 48 and 24 hr prior to the i.p. injection of 2,4-DAA dissolved in 0.9% NaCl solution. Control animals received an equal volume of 0.9% NaCl. The dose of 2,4-DAA used in all experiments, except the toxicity study, was 32.86 mg/kg (approximately 25 per cent of the LD_{50} dose), equivalent to 56.2 mg/kg of 2,4-DAA sulfate.

2,4-DAA · dihydrochloride was crystallized from hot water, using Norit, and gave only one band by t.l.c. The mass spectrum showed $m/e = 138$, and no peaks from impurities were noted.

In the acute toxicity study, groups of five rats were injected i.p. with various dose levels of 2,4-DAA · dihydrochloride in 0.9% NaCl and were kept under observation for 7 days. The LD_{50} doses (based on 2,4-DAA, free amine form) were determined according to the method of Litchfield and Wilcoxon [7]. Acute toxicity, as thus assessed, was increased by PB treatment (95 per cent confidence limits) as evidenced by a change in the LD_{50} dose from 116 mg/kg (range 108.6 to 123.9) to 97 mg/kg (90.8 to 103.6) in the pretreated rats.

For metabolism studies, 2,4-DAA[^{14}C]sulfate (56.2 mg/kg, 120 μCi/kg) was injected i.p. The rats were housed individually in stainless steel metabolism cages designed for separate collection of urine and feces (Acme Research Products, Cincinnati, OH). The urine samples were collected at 5 and 24 hr in ice-cold containers and frozen until analyzed. The animals were killed after urine collection, and selected organs were removed, weighed and homogenized. Tissue homogenates were taken for distribution and binding studies.

To analyze urinary metabolites urine was extracted with ethyl acetate (H_2O saturated); the presence of glucuronide and sulfate conjugates was determined by incubation of extracted urine with β-glucuronidase (bacterial, type II, Sigma Chemical Co., St. Louis, MO) and glusulase (β-glucuronidase and sulfatase, Endo Laboratories, Garden City, NJ) as described by Grantham *et al.* [8, 9]. The residue was dissolved in MeOH, and an aliquot was used for radioactivity measurement. Chromatographic analysis and h.p.l.c. fractionation of metabolites were carried out according to the procedure of Grantham *et al.* [8, 9].

To determine the binding to macromolecules, 1 ml of tissue homogenate [1:10 (w/v) in distilled water] was precipitated with 2 ml of ice-cold acetone and 2 ml of MeOH. The precipitate, which was largely protein, was centrifuged and washed with 5 ml of MeOH until no more radioactivity could be extracted [5, 6]. The pellets were dissolved in 0.5 ml of 1 M NaOH by warming at 50° until clear. After 0.5 ml of water was added, the aliquots were neutralized with acetic acid and were counted in Aquasol (New England Nuclear Corp., Boston, MA). Protein concentration was determined according to the method of Lowry *et al.* [10] using crystalline bovine serum albumin as a standard.

The distribution of [^{14}C]-DAA in selected organs at 5 and 24 hr after an i.p. dose of the compound is shown in Table 1. At both time periods the highest concentration in the control and PB-pretreated animals occurred in the kidney. Lower levels were found in the liver, spleen, lung, heart and testis respectively. At the 5-hr period, radioactivity was higher in all control tissues with the exception of the kidney, in which a significantly higher level was found in the samples from PB-pretreated rats. The radioactivity decreased considerably in all tissues from 5 to 24 hr. Retention of the radioactivity at the 24-hr period was higher in all tissues obtained from the PB-pretreated animals. This result suggests two possibilities—a slower process of elimination of 2,4-DAA from tissue depots or higher levels of binding to cellular constituents in PB-pretreated rats.

Results on irreversible binding of [^{14}C]-2,4-DAA to tissue macromolecules, mostly protein, also determined 5 and 24 hr after i.p. administration, are given in Table 1. The highest concentration of bound material was in the kidney, followed by the liver, spleen, lung, heart and testis respectively. Concentrations at 24 hr were approximately half of those observed at 5 hr. Pretreatment with PB significantly increased binding, but only in the liver and kidney and not in other organs.

The urinary excretion of radioactivity and results on extraction and enzymic hydrolyses of urinary metabolites are shown in Table 2. In the control rats, 31 per cent of the ^{14}C-dose was found in the urine in 5 hr, and 69 per cent in 24 hr. By 24 hr the levels of the free, glucuronide, sulfate and water-soluble fractions increased approximately 2-fold over the 5-hr values. In PB-pretreated rats, the rate of excretion of radioactivity was somewhat faster than that of the control animals, for 42 per cent of total radioactivity was in the urine at 5 hr, mostly as free and water-soluble fractions since the levels of glucuronide and sulfate fractions remained unchanged. No difference in the excretion of total radioactivity or of the radioactivity in each fraction was observed between the PB-pretreated and control groups at the 24 hr period.

The h.p.l.c. separation of the five known metabolites of 2,4-DAA was accomplished by the procedure of Grantham *et al.* [9]. The levels of each metabolite formed in the control and PB-treated rats at 5 and 24 hr are given in

Table 1. Tissue concentration of radioactivity and binding to protein *in vivo* after intraperitoneal injection of 2,4-diamino[^{14}C]anisole (56 mg/kg)*

Period	Tissue	Concentration of ^{14}C (nmoles/g wet wt)		Binding (nmoles/mg protein)	
		Control	PB-pretreated	Control	PB-pretreated
5 hr	Liver	179 ± 37.80	143.35 ± 19.10†	0.34 ± 0.06	0.49 ± 0.09‡
	Heart	79.24 ± 18.13	47.51 ± 5.83†	0.11 ± 0.05	0.07 ± 0.02
	Lung	90.43 ± 14.30	60.31 ± 7.23§	0.14 ± 0.03	0.12 ± 0.03
	Kidney	241.46 ± 41.20	303.22 ± 62.46†	0.63 ± 0.20	1.13 ± 0.41‖
	Spleen	90.35 ± 19.27	61.38 ± 10.07‡	0.18 ± 0.08	0.12 ± 0.03
	Testis	75.04 ± 19.60	42.61 ±5.97§	0.05 ± 0.01	0.05 ± 0.01
24 hr	Liver	34.39 ± 6.19	51.90 ± 10.11§	0.15 ± 0.02	0.23 ± 0.04‡
	Heart	9.61 ± 4.99	11.15 ± 3.65	0.04 ± 0.01	0.04 ± 0.01
	Lung	9.13 ± 2.55	9.99 ± 3.53	0.05 ± 0.01	0.06 ± 0.02
	Kidney	64.19 ± 11.85	99.26 ± 26.53‡	0.34 ± 0.07	0.47 ± 0.15‖
	Spleen	14.91 ± 5.16	20.09 ± 9.14	0.06 ± 0.02	0.08 ± 0.03
	Testis	2.94 ± 0.81	3.88 ± 1.73	0.03 ± 0.01	0.03 ± 0.01

* Values are means ± S.D. of eight rats for each time period.
† Significantly different from the control value, $P < 0.05$.
‡ Significantly different from the control value, $P < 0.005$.
§ Significantly different from the control value, $P < 0.001$.
‖ Significantly different from the control value, $P < 0.01$.

Table 3. At 5 hr 4-acetylamino-2-aminoanisole (Metabolite IV) was the major product in the free and glucuronide fractions of both groups. In the free fraction this metabolite was increased over control values in PB-pretreated rats. The second most abundant in the free and glucuronide fractions of the control groups was 2,4-diacetylaminoanisole (Metabolite V). In PB-pretreated rats, however, this metabolite was second most abundant only in the free fraction, whereas 2,4-diacetylaminophenol (Metabolite I) was present to a greater extent in the glucuronide fraction. Pretreatment with PB apparently stimulated the formation of IV with a subsequent increase in the rate of glucuronide conjugation but caused a decrease in the levels of V. In addition, PB pretreatment increased the level of the glucuronide conjugate of I. It appears that PB may stimulate demethylation of V to form I, which may readily form a glucuronide conjugate. The result may be an apparent decrease in the levels of V in the free fraction with a concomitant increase in the level of I in the glucuronide fraction.

In addition to the three major metabolites mentioned, 5-hydroxy-2,4-diacetylaminoanisole (Metabolite II) and 2-methoxy-5-(glycolamido)acetanilide (Metabolite III) occurred to a small extent in both the free and glucuronide fractions of both groups.

At 24 hr the metabolism of 2,4-DAA was nearly completed since at this time approximately 70 per cent of the dose given had already been eliminated in the urine (Table 2). In the free fraction, the levels of all metabolites were relatively similar in both groups. Metabolite IV was the most abundant, followed by V, I, II and III respectively.

In the glucuronide fraction there was little difference in the levels of the various metabolites excreted by both groups when expressed as per cent of fraction. The con-

Table 2. Urinary excretion of radioactivity and fractionation of urinary metabolites 5 and 24 hr after an intraperitoneal injection of 2,4-diamino[^{14}C] anisole*

Period	Fraction	% of dose	
		Control	PB pretreated
5 hr	Total	31.17 ± 6.81	42.49 ± 11.13†
	Free	7.21 ± 1.34	11.48 ± 5.70
	Glucuronides	6.17 ± 2.16	7.61 ± 1.96
	Sulfates	3.21 ± 1.50	3.13 ± 1.43
	'Water-soluble'	10.82 ± 3.86	17.25 ± 3.11‡
24 hr	Total	68.82 ± 13.86	75.69 ± 8.10
	Free	18.10 ± 6.58	22.39 ± 10.69
	Glucuronides	12.27 ± 2.68	11.48 ± 3.02
	Sulfates	5.13 ± 0.94	5.74 ± 1.94
	'Water-soluble'	24.75 ± 3.27	29.45 ± 9.69

* Values are means ± S.D. for eight rats.
† Significantly different from the control value, $P < 0.05$.
‡ Significantly different from the control value, $P < 0.005$.

Table 3. Metabolites identified in free and glucuronide fractions of urine after 2,4-[^{14}C]-DAA

Fraction	Time (hr)	% of Fraction*									
		Metabolite I		Metabolite II		Metabolite III		Metabolite IV		Metabolite V	
		2,4-Diacetyl-aminophenol		5-Hydroxy-2,4-diacetylamino-anisole		2-Methoxy-5-(glycolamido)-acetanilide		4-Acetylamino-2-aminoanisole		2,4-Diacetyl-aminoanisole	
		Control	PB	Control	PB	Control	PB	Control	PB	Control	PB
Free	5	2.0 ± 0.8 (0.14)	2.4 ± 0.8 (0.28)	0.8 ± 0.2 (0.06)	1.0 ± 0.5 (0.11)	1.5 ± 0.7 (0.11)	1.8 ± 0.5 (0.21)	43.5 ± 5.2 (3.1)	61.7 ± 5.5 (7.1)	28.8 ± 4.5 (2.1)	10.3 ± 3.9 (1.2)
Glucuronide		4.2 ± 2.8 (0.26)	10.0 ± 3.2 (1.76)	2.8 ± 0.8 (0.17)	2.0 ± 0.6 (0.15)	2.0 ± 0.8 (0.12)	1.0 ± 0.9 (0.08)	19.5 ± 7.4 (1.2)	25.9 ± 17.0 (1.97)	11.0 ± 5.2 (0.68)	9.0 ± 3.5 (0.68)
Free	24	6.0 ± 1.4 (1.1)	6.6 ± 1.8 (1.5)	5.2 ± 1.9 (0.85)	4.9 ± 1.0 (1.1)	1.86 ± 0.8 (0.33)	1.1 ± 0.6 (0.25)	30.0 ± 8.2 (5.4)	29.0 ± 6.9 (6.5)	28.7 ± 7.0 (5.2)	21.0 ± 7.0 (4.7)
Glucuronide		16.0 ± 4.0 (1.96)	17.3 ± 4.4 (1.99)	9.0 ± 2.1 (1.1)	7.0 ± 1.8 (0.80)	1.8 ± 0.4 (0.22)	2.2 ± 0.1 (0.25)	23.8 ± 9.2 (2.9)	28.4 ± 12.3 (3.3)	3.8 ± 1.3 (0.47)	3.0 ± 0.9 (0.34)

* Data are from the animals of Table 2. Each value is the mean ± S.D. for eight rats. Figures in parentheses represent % of dose.

jugate of Metabolite IV was the major product, followed by the glucuronide conjugates of I, II, V and III. It appeared that the formation of Metabolite II was enhanced considerably between the 5- and 24-hr period. In addition to these five major metabolites, h.p.l.c. separation of urinary extracts showed the presence of other minor metabolites; further investigation and identification of these metabolites are required to obtain a complete picture.

PE pretreatment has been shown to increase the mutagenicity and binding of 2,4-DAA to protein [3–5]. In our studies, PB pretreatment caused significant changes in the rate of elimination, distribution, metabolism, tissue binding and acute toxicity of 2,4-DAA, especially at the 5-hr period.

The levels of radioactivity in all tissues except the kidney were higher in the control rats at 5 hr. Elimination of radioactivity from liver and kidney from control rats, however, appeared to be more rapid than in tissues from PB-pretreated rats at 24 hr. This phenomenon could possibly be due to higher levels of tissue binding in these organs. Irreversible binding of 2,4-DAA to tissue constituents *in vivo* was found to be highest in the kidney at the dose used in this study (32.86 mg/kg). This contrasts with the results of Dybing *et al.*[5], who found that the binding to kidney protein was higher than to liver protein only at a high dose (200 mg/kg) of 2,4-DAA, while at lower doses (10–100 mg/kg) the binding in kidney was significantly less than in the liver.

It is known that PB pretreatment enhances hydroxylation, demethylation and glucuronidation reactions [11]. The present study demonstrated that PB pretreatment stimulated acetylation of 2,4-DAA, resulting in an increase in the formation of the monoacetylated derivative, 4-acetyl-2,4-DAA (Metabolite IV). The glucuronide conjugation of this derivative, believed to result in an *N*-glucuronide, was also enhanced somewhat. A decrease in the level of 2,4-diacetylaminoanisole in PB-pretreated rats suggests that acetylation of the monoacetylated derivatives was decreased or deacetylation of the diacetylated derivative was increased. PB may also stimulate further transformation of 2,4-diacetylaminoanisole to other derivative(s). The last possibility is supported by the observation that urinary excretion of 2,4-diacetylaminophenol was higher in the glucuronide fraction of PB-pretreated rats. PB pretreatment may enhance *O*-demethylation of 2,4-diacetylaminoanisole to form 2,4-diacetylaminophenol, which can be readily conjugated. Ring-hydroxylation and ω-oxidation of the *N*-acetyl group of 2,4-DAA, on the other hand, were unaffected by PB pretreatment. In this respect 2,4-DAA differed from the prototype carcinogenic aromatic amide, *N*-2-fluorenylacetamide (2-FAA). With the latter compound, PB treatment increased the amount of the major hydroxylated derivative of FAA, namely 7-hydroxy-2-FAA [12].

PB pretreatment may cause other changes in metabolic pathway(s) of 2,4-DAA, but further characterization and identification of the minor metabolites will be required. Most carcinogenic aromatic amines are activated by means of *N*-hydroxylation [13], but in general these derivatives are unstable and the urinary levels are very low [14]. Thus, it was considered that *N*-hydroxylated metabolites of 2,4-DAA might be responsible for the toxic and mutagenic effects, but in the present and past studies it was not possible to identify *N*-hydroxylated metabolite(s). The evidence, however, suggests that PB pretreatment increases the formation of toxic metabolite(s) since it enhances acute toxicity, nonspecific covalent binding to liver and kidney macromolecules, and mutagenicity of 2,4-DAA. The latter two variables are believed to be involved in the mechanism of toxicity and carcinogenicity of many compounds.

To summarize, PB treatment of rats increased the toxicity of 2,4-DAA and the initial rate of excretion in the urine. Quantitatively, PB treatment altered somewhat the initial excretion of specific metabolites, especially those derived by demethylation and acetylation of the parent compound. Although the differences in excretion pattern had vanished by 24 hr, binding of 2,4-DAA, or a metabolite, to liver and kidney proteins or cellular constituents was still higher in treated than in control rats at this time period.

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