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MICROSOMAL METABOLISM OF *N,N*-DIETHYLACETAMIDE AND *N,N*-DIMETHYLACETAMIDE AND THEIR EFFECTS ON DRUG-METABOLIZING ENZYMES OF RAT LIVER

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Abstract—This study was undertaken to investigate: (1) the effect of *N,N*-diethylacetamide (DEAC) and *N,N*-dimethylacetamide (DMAC) administration to rats on drug-metabolizing enzymes in the liver; (2) the *in vitro* dealkylation of DEAC and DMAC by hepatic microsomes from rats treated with various P450 inducers and purified P450 (2B1 and 2E1). DEAC administration at doses of 100–300 mg/kg i.p. for 3 days mostly induced P450 2B1/2-associated hepatic microsomal monooxygenase activities (pentoxyresorufin *O*-depenthylase and the 16 β-testosterone hydroxylase) and its own dealkylation (DEAC deethylase activity). P4502E1-linked monooxygenase activities, such as aniline and *p*-nitrophenol hydroxylases, were not affected. DEAC treatment increased the amount of P4502B1/2 in microsomes in a dose-dependent manner, but depressed the amount of P-4502C11 as assayed by western blotting. DMAC treatment did not alter any microsomal monooxygenases or phase II enzymatic activity. The oxidative metabolism of DEAC and DMAC with control and induced microsomes resulted in the dealkylation of these solvents, giving rise to acetaldehyde and formaldehyde, respectively. The kinetic parameters for these N-dealkylations were investigated. It was found that phenobarbital-, dexamethasone- and DEAC-induced microsomes deethylated DEAC with a V_{max} ~3-fold of control-, ethanol- or β-naphtoflavone-induced microsomes, although with a similar affinity; ethanol- or acetone-induced microsomes demethylated DMAC with a V_{max} higher than that of control microsomes. In a reconstituted system, the purified P4502B1 dealkylated DEAC, but not DMAC, at the rate of 6.2 nmol/min/nmol P450, whereas purified P4502E1 dealkylated DMAC, but not DEAC, at the rate of 7.9 nmol/min/nmol P450. Oxidation of DEAC and DMAC were markedly inhibited in microsomes from DEAC-treated rats by anti-P4502B1 IgG and in microsomes from acetone-treated rats by anti-P4502E1 IgG, respectively. These results indicate that DMAC and DEAC are predominantly oxidated by different P450 isozymes and that only DEAC, when administered to rat, is capable of altering the expression of the hepatic P450 system. This latter feature could be related to the higher toxicity reported for DEAC.

Key words: dealkylation by P450 isoforms; P450 induction, *N,N*-diethylacetamide- and *N,N*-dimethylacetamide-metabolism

N-Alkylacetamides, such as DMAC† and DEAC are excellent polar solvents widely used in industrial processes, such as the manufacture of films and fibres, and in the laboratory as carriers of water-insoluble chemicals.

The general and tissue specific toxicity and the *in vivo* metabolism of DMAC in various experimental animals has been studied but much less information is available on DEAC [1–3]. The toxicity of these *N*-alkylacetamides is less than that of the corresponding *N*-alkylformamides [1], whose metabolic role in their bioactivation has been widely clarified [4]. DEAC has been reported to be more toxic than DMAC [2], but no hypothesis as to the mechanism has been put forward. As regard the *in vitro* metabolism of these compounds only one paper has been published on DMAC [5] and, to the authors' knowledge, there is no literature on DEAC.

Many solvents of low molecular weight, such as ethanol, acetone, benzene, chloroform, styrene, diethyl ether, aniline, pyridine [6] and other substances, including the carcinogenic *N,N*-dimethylnitrosoamine, *N,N*-diethylnitrosoamine, tioacetamide [7–9], have been found to be substrates for the ethanol-inducible P4502E1. Some of these compounds are hydrophylic, many others lipophylic and a low molecular weight rather than polarity seems a necessary prerequisite for these P4502E1

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† Abbreviations: DEAC, *N,N*-diethylacetamide; DMAC, *N,N*-dimethylacetamide; PB, phenobarbital; DEX, dexamethasone; TAO, triacetyloleandomycin; β-NF, β-naphtoflavone; T, testosterone; 17-OT, 4-androsten-3,17-dione; 2α-OH, 2β-OH, 6α-OH, 7α-OH, 16α-OH, 16β-OH correspond to 2α-, 2β-, 6α-, 6β-, 7α-, 16α-, 16β-hydroxytestosterone, respectively; APD, aminopyrine demethylase; BzD, benzphetamine demethylase; PROD, pentoxyresorufin *O*-depentylase; EROD, ethoxyresorufin *O*-deethylase; ECOD, 7-ethoxycoumarin *O*-deethylase; pNPH, *p*-nitrophenol hydroxylase; AnH, aniline hydroxylase; ErD, erythromycin *N*-demethylase; DEACd, *N,N*-diethylacetamide deethylase; DMACd, *N,N*-dimethylacetamide demethylase; P450, cytochrome P450; PB-, DEX-, ETOH-, βNF-, acetone-, DEAC-microsomes correspond to hepatic microsomes from PB-, DEX-, ETOH-, βNF-, acetone- and DEAC-treated rats, respectively.

substrates [6]. It has recently been reported that DEAC and DMAC are capable of inhibiting the P4502E1-mediated metabolism of *N*-methylformamide and it was further suggested that DEAC and DMAC could be metabolized by this P450 form [4] and potentially alter its expression. In this work the effect of DEAC and DMAC administration on the drug-metabolizing enzymes of rat liver have been examined. In addition, the *in vitro* hepatic metabolism of DEAC to acetaldehyde and DMAC to formaldehyde (by microsomes from rat treated with inducers of selected P450 isozymes and by two purified P450 [2B1 and 2E1] have been studied.

MATERIALS AND METHODS

Chemicals. DEAC, DMAC and resorufin were supplied by Fluka (Buchs, Switzerland); 7-ethoxycoumarin was from EGA-Chemie (Steinheim, F.R.G.) and benzphetamine from Upjohn Co. (Kalamazoo, MI, U.S.A.). Enzymes and coenzymes were obtained from Boehringer (Mannheim, F.R.G.). Ethoxyresorufin and pentoxyresorufin were synthesized from resorufin by ethylation with ethyl iodide and by pentylation by pentyl iodide, respectively [10].

T,17-OT, 16 β-OH, TAO and corticosterone were purchased from Sigma Chemicals (St Louis, MO, U.S.A.), 2α-, 2β-, 6α-, 6β-, 7α- and 16α-OH were obtained from the Steroids Reference Collection (D. N. Kirk, Dept of Chemistry, Queen Mary College, London, U.K.). Rabbit anti-rat P4502B1, P4502E1 and P4503A1 polyclonal antibodies were obtained from Oxygene (Dallas, TX, U.S.A.). Goat anti-rat P4502C11 polyclonal antibodies were kindly supplied by Prof. J. B. Schenkman (Farminton, CT, U.S.A.). Goat anti-rabbit IgG and rabbit anti-goat IgG were purchased from Dako (Copenhagen, Denmark). Nitrocellulose filters (0.45 μm), goat serum, 4-cloro-1-naphtol and erytromycin were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals and solvents were of analytical grade and were obtained from common commercial sources.

Animal treatment and microsomal preparation. Male Sprague–Dawley rats (5–6 weeks old; Charles River, F.R.G.) were injected i.p. with DEAC or DMAC in a saline solution at doses of 150 and 300 mg/kg for 3 days. Other inducers administered i.p. were: PB 80 mg/kg for 3 days; β-NF 40 mg/kg for 3 days; DEX 50 mg/kg for 4 days. EtOH 10% v/v and acetone 5% v/v were administered in drinking water. Animals were killed by CO_2 asphyxia; the livers were excised, and 100,000 *g* supernatants and microsomes prepared as described previously [7]. The washed microsomal pellets were resuspended in 100 mM phosphate buffer, 1 mM EDTA, pH 7.4, and stored at −80°. Protein content was determined according to Lowry *et al.* [11] using BSA as standard.

Enzymic assays. Hepatic cytochrome P450 and b_5 content were measured by the method of Omura and Sato [12]. Microsomal AnH was determined by measuring the formation of *p*-aminophenol [13]. pNPH was determined according to Reinke and Moyer [14]. APD, ErD and BzD demethylase activities were assayed by measuring the formation of formaldehyde [15]. DMAC demethylation was determined, as reported for DMF demethylation [16], by assaying formaldehyde [15] after having terminated the reaction by addition of NaOH (0.1 M, final concentration). Under this condition *N*-(hydroxymethyl)-*N*-methylacetamide, formed as stable intermediate, decomposed to yield formaldehyde and *N*-methyacetamide. DEAC deethylation was assayed by HPLC, measuring the formation of acetaldehyde as the 2,4-dinitrophenylhydrazone derivative, as described previously [8], with the addition of semicarbazide to trap acetaldehyde, as reported by Yoo *et al.* [17]. Microsomal ECOD activity was assayed by the fluorimetric determination of 7-ethoxycoumarin [18]. EROD and PROD activities were determined by measuring the formation of the corresponding hydroxy product in a Perkin-Elmer spectrofluorimeter [19]. Microsomal epoxide hydrolase, glutathione *S*-transferase and UDP-glucuronyl transferase activities were quantified as described previously [20], using safrole oxide, 1-chloro-2,4-dinitrobenzene and 1-naphtol, respectively, as substrates. Testosterone hydroxylase was assayed as reported previously [21], according to a HPLC method described by Platt *et al.* [22].

Reconstituted system. Cytochrome P4502B1 and NADPH-cytochrome P450-reductase were purified in this laboratory from liver microsomes of male Sprague–Dawley rats treated with PB, as described previously [23]. Hepatic cytochrome b_5 was purified from PB-treated rats by the method of Spatz and Strittmatter [24]. P4502E1 was purified from liver of acetone-treated rats following the procedure illustrated by Patten *et al.* [25]. The reconstituted system generally contained in 0.1 M potassium phosphate buffer, pH 7.4, 0.1 μM P4502B1 or 0.1 μM P4502E1, 0.3 μM P450 reductase, 30 μg/mL of dilauroylphosphatidylcholine and substrates 0.5–10 mM. Dilauroylphosphatidylcholine was prepared in water and sonicated immediately before use. After 30 min pre-incubation at room temperature the reaction was carried out for 20 min at 37° after the addition of 1 mM of NADPH. The activity of the purified P4502B1 and 2E1 was checked in a reconstituted system with saturating concentrations of benzphetamine (2B1) and aniline (2E1) as selective substrates; their turnover numbers were 82 and 9.3 nmol/min/nmol P450, respectively.

Gel electrophoresis and immunoblotting. Gel electrophoresis was conducted using the discontinuous system of Laemmli [26], with a 1.5 mm thick 7.5% acrylamide gel. Proteins were transferred from the gel to nitrocellulose filters following the method of Towbin *et al.* [27].

Immunodetections were performed using goat anti-rat P4502C11 and rabbit anti-rat P4502B1, P4503A1 and P4502E1 polyclonal antibodies.

The bands on the nitrocellulose membranes were quantified by laser densitometry (Ultrascan 2202 LKB).

Preparation of antibodies. Female New Zealand white rabbits were immunized, as described previously [28], with P4502B1 and 2E1 purified antigens. Pre-immune serum was collected prior to

Table 1. P450 content and monooxygenase activities in liver microsomes from control rats and rats treated with two DEAC doses

Parameters	DEAC dose (mg/kg)		
	0	150	300
P450	0.59 ± 0.12	0.68 ± 0.11	0.69 ± 0.15
APD	7.7 ± 2.3	14.3 ± 2.9**	14.9 ± 3.4**
BzD	5.8 ± 1.6	13.2 ± 1.8**	15.9 ± 3.3**
PROD	0.02 ± 0.007	0.21 ± 0.02**	0.28 ± 0.05**
EROD	0.20 ± 0.03	0.26 ± 0.04	0.18 ± 0.03
ECOD	0.68 ± 0.2	1.5 ± 0.3*	1.8 ± 0.3**
pNPH	1.1 ± 0.2	1.4 ± 0.2	1.3 ± 0.3
ErD	0.8 ± 0.2	1.2 ± 0.2	1.5 ± 0.3*
AnH	1.0 ± 0.3	1.2 ± 0.2	1.4 ± 0.3
DEACd	0.9 ± 0.3	1.8 ± 0.4*	2.2 ± 0.3**

Data are presented as means ± SD of three experiments. Each experiment used microsomes pooled from 3–4 animals. P450 content is expressed as nmol/mg of protein and enzymatic activities as nmol/min/mg of protein. **Significantly different from control microsomes, $P < 0.01$ by Student's t-test. *$P < 0.05$.

any injections. For the first immunization, 0.2 mg of antigen was mixed with complete Freund's adjuvant. After 6 weeks, two subsequent injections 0.1 mg of antigen in incomplete Freund's adjuvant were performed at 4 week intervals. The immune serum was collected 10 days after the last injection. Immunoglobulin fractions from pre-immune serum and immune serum were prepared by caprylic acid and subsequent ammonium sulphate precipitation [29]. The purified anti P4502E1 and 2B1 IgG recognized the same antigens as the commercial P4502E1 and 2B1 polyclonal antibodies purchased from Oxygene.

Immunoinhibition assay. Microsomes (0.75 mg/mL) were mixed with different amounts of a given antibody (IgG fraction) or pre-immune rabbit IgG in 50 mM potassium phosphate buffer, pH 7.4, and allowed to pre-incubate for 20 min at 20°. Cofactors and substrate were then added and incubated for 20 min at 37° under the conditions described for each assay. Both the anti P450 2B1 and 2E1 IgG were effective in inhibiting related-specific monooxygenases. Anti2B1 IgG, at a concentration of 10 mg/nmol P450, inhibited the PROD activity in PB-microsomes by 90% and anti2E1 IgG at the same concentration inhibited AnH activity in acetone-microsomes by 80%.

RESULTS

Effect of DEAC and DMAC administration on rat hepatic drug-metabolizing enzymes

DEAC, when administered to rats at doses of 150 and 300 mg/kg/day i.p. for 3 days, significantly affected microsomal monooxygenase activities such as ECOD, APD, BzD, PROD and DEACd (Table 1). At higher doses a slight increase of ErD activity was also observed. These changes were accompanied by an insignificant alteration in the P450 content, and pNPH and EROD activities. Microsomal protein and cytochrome b5 contents, NADPH-cytochrome P450 reductase activity, and the rates of phase II enzymes, namely GST, EH, DT-diaphorase and UDP-GT, were not altered (results not shown).

Intraperitoneal administration of DEAC at 400–500 mg/kg for 3 days resulted in a marked inhibition of all monooxygenase activities tested (data not shown), suggesting that these concentrations of solvent were hepatotoxic.

When the metabolism of an endogenous substrate, such as T, was investigated in hepatic microsomes from rats treated i.p. for 3 days with 300 mg/kg/day of DEAC, the 16β-OH and 17-OT T hydroxylations were significantly enhanced (Table 2) and 2α-OH T hydroxylation was depressed. 6β-OH and 2β-OH T hydroxylase activities appeared to increase, although not significantly. Liver microsomes from rats treated i.p. for 3 days with 150 mg/kg/day of DEAC produced similar results (data not shown).

DMAC, administered i.p. for 3 days at doses of 150 mg/kg or 300 mg/kg, did not significantly affect any of the above mentioned P450-dependent monooxygenase and phase II enzyme activities (results not shown). *N*-mono-methylacetamide did not induce any hepatic monooxygenases in rat [30].

Gel electrophoretic and western-blot analysis

Hepatic microsomes were subjected to gel SDS electrophoresis. No differences were visible in the electrophoretic pattern of liver microsomal proteins from control rats and rats treated either with 150 or 300 mg/kg DMAC.

DEAC treatment altered the intensity of some protein bands in the molecular weight range of 50–53 KDa (not shown). In order to check if these protein bands corresponded to P4502B1/2, 2E1, 3A1/2 and 2C11, the hepatic microsomal proteins from the DEAC-treated rats were analysed by western blot analysis using polyclonal antibodies anti P4502B1/2, 2E1, 3A1/2 and 2C11.

As shown in Fig. 1A, anti P4502B1/2 cross-reacted with a band enhanced by DEAC treatment, corresponding in migration position to purified P4502B1. In addition to this P450, another faint

Table 2. Hydroxylation of T by hepatic microsomes from control rats and rats treated with 300 mg/kg of DEAC

Metabolites	Control	DEAC-microsomes
6α-OH	0.08 ± 0.02	0.10 ± 0.02
7α-OH	0.32 ± 0.04	0.41 ± 0.05
6β-OH (3A1/2)	1.16 ± 0.27	1.62 ± 0.33
16α-OH (2B1/2-2C11)	2.15 ± 0.21	2.49 ± 0.35
16β-OH (2B1/2)	0.05 ± 0.02	2.02 ± 0.18*
2α-OH (2C11)	1.57 ± 0.28	0.34 ± 0.11*
2β-OH (3A1-1A1)	0.22 ± 0.06	0.33 ± 0.09
17-OT (2B1/2-2C11)	2.20 ± 0.31	4.34 ± 0.48*

Data of T hydroxylase activities expressed as nmol/min/mg of protein are the means ± SD of three experiments. Each experiment used microsomes pooled from 3–4 animals. Incubations were carried out at 37° for 15 min with 1 mg/mL of microsomal protein. *Significantly different from control at $P < 0.01$ by Student's *t*-test. The P450 isozyme(s) implied in the formation of the indicated metabolites of T are shown in parentheses [22].

band with a slightly higher molecular mass, possibly corresponding to P4502B2 [31], was also visible in PB- and DEAC-microsomes. In control microsomes the anti-P4502B1/2 did not recognize any bands, in keeping with the very low constitutive level of P4502B1 and 2B2 in rat liver [32]. In Fig. 1B, anti P450 3A1/2 recognized a protein band in control microsomes according to the relevant constitutive presence of P4503A2 in rat liver [33]. In DEAC-induced microsomes, and more markedly in PB- and DEX-microsomes, this antibody recognized a major band whose electrophoretic mobility corresponded to 3A1/3A2, these isoforms having the same molecular mass and being immunochemically indistinguishable [33]. In DEAC-microsomes, the P4503A1/2 content appeared to be, as determined by densitometry, ~120% of control microsomes. The other band visible in the blot, and not inducible with a lower molecular mass, could be the 50 KDa protein reported previously [33].

When the immunoblot was probed with anti P4502C11 (Fig. 1C) a different pattern emerged. A protein band corresponding to P4502C11 was present in control microsomes and, to a lesser extent, in DEAC-microsomes. The P4502C11 contents, as determined by densitometry, were 55 and 23% of control after 150 and 300 mg/kg of DEAC treatment, respectively.

Anti P4502E1 cross-reacted with a protein band in control microsomes in agreement with the constitutive presence of P4502E1 in the rat liver [34], but this cross-reaction was not enhanced in the DEAC-treated microsomes (not shown). Thus, DEAC does not appear to induce hepatic P4502E1.

DEAC and DMAC dealkylation by liver microsomes and P4502B1 and P4502E1

DEAC and DMAC were dealkylated by rat liver microsomes into acetaldehyde and formaldehyde, respectively. These reactions were catalysed by the P450 system, since they had an absolute requirement for molecular oxygen and NADPH; NADH could not replace NADPH as a source of electrons. Carbon monoxide, a classic P450 inhibitor, was able, when bubbled into the incubation mixture, to inhibit both DEAC and the DMAC dealkylation by 80%. The oxidative dealkylation of the two solvents followed a simple Michaelis–Menten kinetic equation, and were linear up to 30 min and 2.5–3 mg/mL of microsomal proteins with control microsomes, but only up to ~20 min and 1.5–2 mg/mL when DEX-, PB-, DEAC-induced microsomes (in the case of DEACd), or acetone-, ETOH-induced microsomes (in the case of DMACd) were used. With higher microsomal protein concentrations, the DEAC and DMAC dealkylations were linear for shorter periods of time.

From the Lineweaver–Burk plots (not shown), the apparent kinetic constants were determined, and illustrated in Tables 3 and 4 for DEAC and DMAC, respectively. The apparent K_m for the DEAC deethylation of control microsomes was quite high (~7 mM) and very similar to those of induced microsomes (Table 3). Microsomes from DEX-, PB- and DEAC-pre-treated rats exhibited high catalytic activities (~3-fold) for the deethylation of DEAC compared to microsomes from control, ETOH- or β-NF-treated rats.

When the rates were normalized for the different P450 levels (i.e. rates expressed per nmol of P450), the production of acetaldehyde was still 2–3-fold higher with DEX-, PB- and DEAC-induced microsomes than for control microsomes. These results suggest that the P450 isoforms 2B1/2 and 3A may play an important role in DEAC deethylation. Direct evidence for involvement of P4502B1 was obtained using purified cytochrome.

Purified P4502B1 was able, in a reconstituted system, to catalyse the DEAC dealkylation at higher rates than control or EtOH- and β-NF-induced microsomes (Table 3).

An indication, although indirect, that P4503A1/2 were implicated in DEAC deethylation was obtained using TAO as selective inhibitor for P4503A-mediated reactions [35]. In liver microsomes from DEX-treated rats, the DEAC deethylation, at saturating conditions of substrate, was only slightly inhibited (20%) by TAO 1 mM. With regard to the

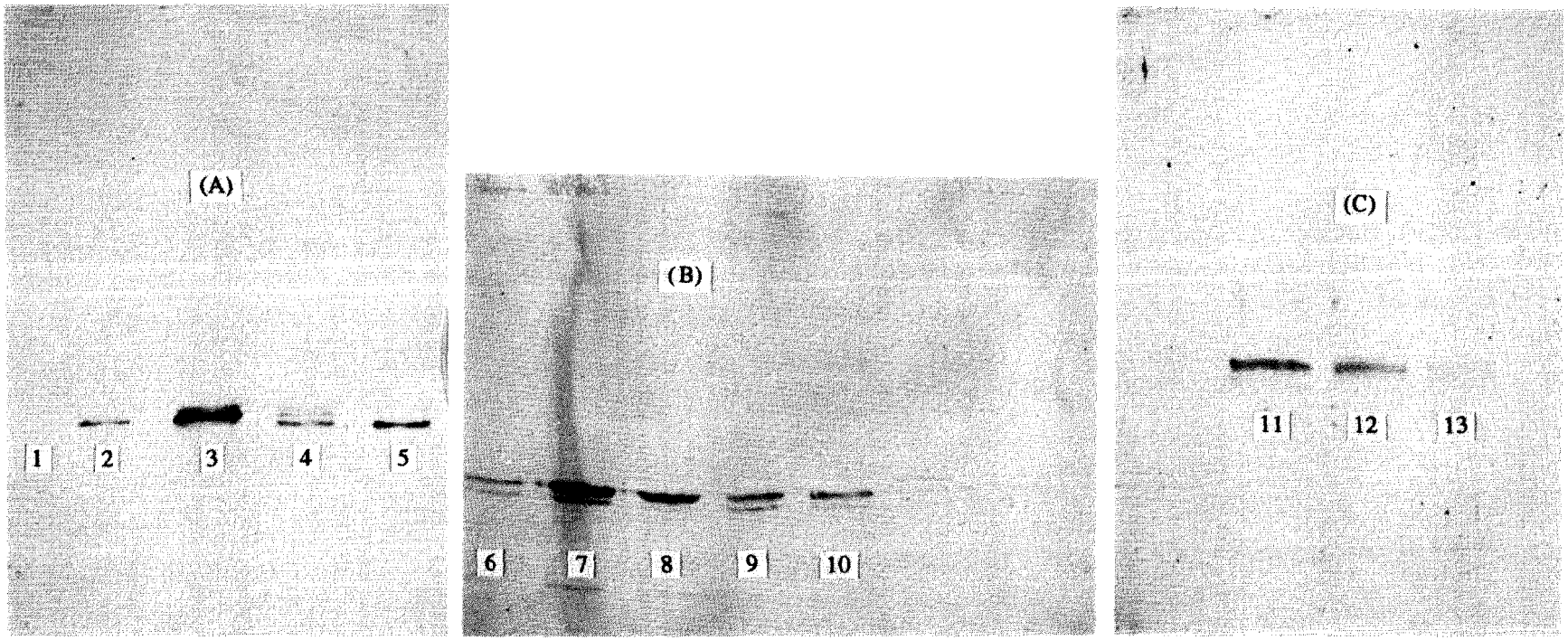

Fig. 1. Western blot analysis of liver microsomes from control and DEAC-treated rats. Microsomes (12 μg of proteins) were subjected to electrophoresis and blotted to nitrocellulose sheets. Each microsomal preparation was probed using polyclonal anti P4502B1/2 IgG (panel A), anti P4503A1/2 IgG (panel B) or anti P4502C11 IgG (panel C). Lanes 1, 6 and 11 contained microsomes from control rats; lane 2 contained purified rat P4502B1; lanes 3 and 8 contained PB-microsomes; lane 7 contained DEX-microsomes; lanes 4, 9 and 12 and 5, 10 and 13 contained microsomes from 150 and 300 mg/kg DEAC-treated rats, respectively.

Table 3. Values of apparent kinetic constants for DEAC-deethylase by P4502B1, P4502E1 and microsomes from control and treated rats

Microsomes or purified P450	K_m (mM)	V_{max} (nmol/min/mg protein)	V_{max} (nmol/min/nmol P450)
Control	7.3 ± 1.6	0.6 ± 0.2	1.1 ± 0.2
ETOH	6.8 ± 1.1	0.8 ± 0.2	1.2 ± 0.2
DEX	5.7 ± 0.8	2.1 ± 0.3**	1.9 ± 0.2*
PB	8.2 ± 2.1	2.0 ± 0.3**	2.0 ± 0.3**
DEAC (300 mg/kg)	5.5 ± 1.3	2.5 ± 0.4**	3.7 ± 0.5**
β-NF	7.7 ± 1.5	0.6 ± 0.1	0.7 ± 0.1
P4502E1	n.d.	–	n.d.
P4502B1	3.5 ± 0.5	–	6.2 ± 0.8**

Values are reported as means ± SD for three experiments performed with different microsomal preparations. Each preparation contained pooled livers from 3–4 rats. The incubations of the reconstituted system containing P4502B1 or P4502E1 were carried out as described in Materials and Methods; these P450 isoforms were very active towards benzphetamine and aniline, respectively (see Materials and Methods). n.d. = not detected. **Significantly different from control microsomes by Student's t-test $P < 0.01$; *$P < 0.05$.

Table 4. Values of apparent kinetic constants for DMAC-demethylase by P4502B1, P4502E1 and microsomes from control and treated rats

Microsomes or purified P450	K_m (mM)	V_{max} (nmol/min/mg protein)	V_{max} (nmol/min/nmol P450)
Control	0.6 ± 0.2	0.6 ± 0.2	1.0 ± 0.3
Acetone	0.3 ± 0.1	4.8 ± 1.5**	5.3 ± 1.6**
ETOH	0.8 ± 0.2	1.4 ± 0.3*	2.0 ± 0.4*
DEX	2.3 ± 0.4**	0.9 ± 0.2	0.8 ± 0.2
PB	1.2 ± 0.3*	0.8 ± 0.3	0.8 ± 0.3
β-NF	3.1 ± 0.4**	0.9 ± 0.2	1.1 ± 0.2
P-4502B1	n.d.	–	n.d.
P-4502E1	2.1 ± 0.4	–	7.9 ± 1.3

Values are reported as means ± SD for three experiments performed with different microsomal preparations. Each preparation contained pooled livers from 3–4 rats. The incubations of the reconstituted system containing P4502B1 or P4502E1 were carried out as described in Materials and Methods. n.d. = not detected. **Significantly different from control microsomes by Student's t-test $P < 0.01$; *$P < 0.05$.

demethylation of DMAC, only microsomes from ETOH- and acetone-treated rats showed enhanced specific activities (expressed either per mg of protein or nmol of P450) relative to control or β-NF-, PB-, DEX-induced microsomes (Table 4). These induced microsomes significantly increased the apparent K_m for the DMACd compared to that of control microsomes. When purified P4502E1 and 2B1 were used in a reconstituted system, only the former isozyme was able to catalyse the demethylation of DMAC with a turnover number higher than that of control microsomes.

Immunoinhibition studies

In order to confirm the role of P4502E1 and 2B1 in DMAC- and DEAC-metabolism, respectively, immunoinhibition experiments were carried out. Increasing amounts of anti P4502B1 IgG produced progressive inhibition of acetaldehyde production from DEAC by microsomes from DEAC-treated rats (Fig. 2); maximum inhibition was 85%. Both rabbit pre-immune serum IgG and anti P4502E1 IgG had no significant effect on DEAC deethylation.

As shown in Fig. 3, varying concentrations of anti P4502E1 IgG, when added to incubations of DMAC in microsomes from acetone-treated rats, inhibited DMAC demethylation with a maximum inhibition of 70%. Neither pre-immune serum IgG or anti P4502B1 IgG affected the oxidation of DMAC.

DISCUSSION

In the present work both the *in vitro* metabolism of DEAC and DMAC and their effects on hepatic drug-metabolizing enzymes have been studied. DEAC, but not DMAC, when administered acutely i.p. to rats, markedly affected some hepatic P450-dependent monooxygenases but not phase II

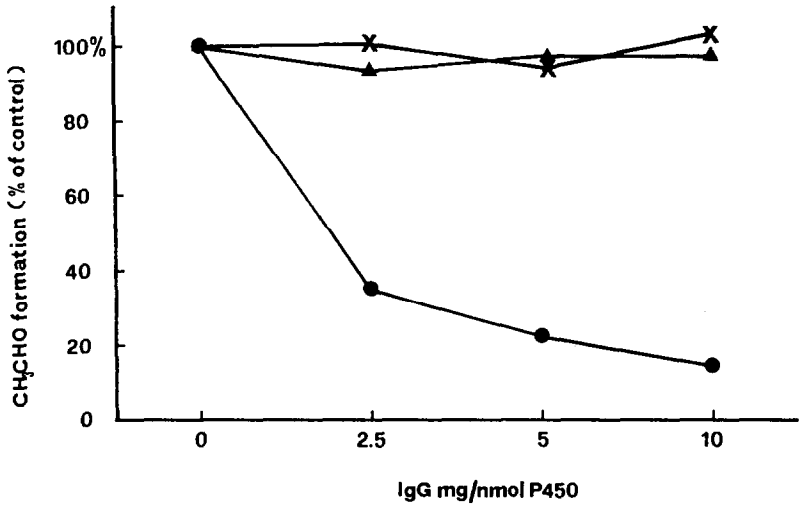

Fig. 2. Effect of anti-rat P4502B1 IgG (●), anti-rat P4502E1 IgG (×) or pre-immune IgG (▲) on DEAC deethylation by microsomes from DEAC-treated rats. Values, which are expressed as a percentage of the control activity (2.3 nmol/min/mg protein, determined in absence of IgG) are the mean of two experiments. Details of incubation conditions and metabolite analysis are described in Materials and Methods.

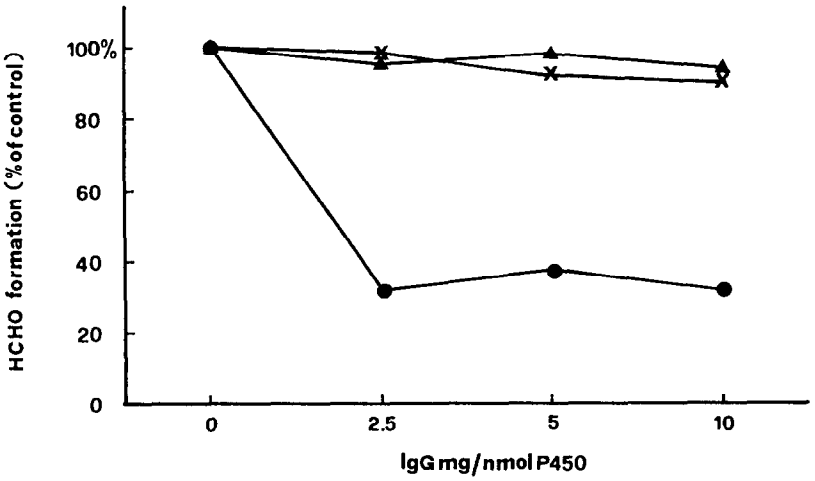

Fig. 3. Effect of anti-rat P4502E1 IgG (●), anti-rat P4502B1 IgG (×) or pre-immune IgG (▲) on DMAC demethylation by microsomes from acetone-treated rats. Values, which are expressed as a percentage of control activity (4.1 nmol/min/mg protein, determined in absence of IgG) are the mean of two experiments. Details of incubation conditions and metabolite analysis are described in Materials and Methods.

enzymes; this property of DEAC may be related to its higher toxicity than that reported for DMAC [2, 3]. At doses higher than 400 mg/kg/day reiterative DEAC treatments caused a generalized depression of microsomal monooxygenase activities, probably as a consequence of hepatotoxicity. A reduction of mixed-function oxidase activities after DEAC administration to mice has previously been observed [36]. Whether this effect is due to the formation of a reactive intermediate during DEAC biotransformation is unknown. DEAC treatment, at doses of 150 and 300 mg/kg/day, was able to increase, compared to control, the BzD and PROD activities, both associated with P4502B1/2. DEACd and ECOD activities also increased, the latter being linked to various P450 isozymes and particularly to P4501A1 [37]. However, it is unlikely that P4501A1 was induced since P4501A1-specific EROD activity [38]

was not enhanced. pNPH and AnH activities, both associated with P4502E1 [39], were not affected, whereas P4503A1/2-linked ErD activity [40] appeared slightly induced only at the highest dose.

Analysis of the metabolites from microsomal oxidation of T, a useful substrate that is regio- and stereoselectively hydroxylated by many P450 isozymes [22], demonstrated that DEAC administrations markedly increased P4502B1/2-dependent 16β-OH T hydroxylation and strongly depressed the 2α-OH T hydroxylation associated with P4502C11, the most expressed P450 form in untreated rat liver [32]. P4503A1/2-mediated 2β-OH T and 6β-OH T hydroxylations were enhanced, but not significantly. It is of interest to note that in liver from 300 mg/kg DEAC-treated rats, both the immunodetectable P-4502C11 level and the P4502C11-linked 2α-OH T hydroxylase activity were decreased in a similar manner (~4-fold) in comparison to that found in control rats. As the total P450 content did not change (Table 1), the lower amount of P4502C11 in the DEAC-treated microsomes probably compensated for the induction of P4502B1/2. A similar compensation between the levels of these P450 isoforms may account for the unchanged 16α-OH T hydroxylase activity linked both to P4502C11 and P4502B1/2 [41], observed in control and DEAC-microsomes. In microsomes from DEAC-treated rats the enhancement of the P4502B1/2-linked monooxygenases was consistent with the increase in a dose-related manner of immunodetectable P4502B1/2. Also, the slight increase in immunodetectable P4503A1/2 observed in DEAC-induced microsomes was in agreement with the scarce induction of P4503A1/2-mediated ErD and 6β-OH/2β-OH T activities. No significant alteration in immunodetectable P4502E1 was found, in keeping with the unaltered P4502E1-linked AnH activity. Thus, DEAC appeared to induce mainly the P4502B1 form.

When *in vitro* metabolism was studied, both the examined polar solvents, DMAC and DEAC, were N-dealkylated by the microsomal P450 system. Their biooxidation was dependent on O_2 and NADPH (not NADH), and was inhibitible by CO, giving rise to formaldehyde and acetaldehyde, respectively, and the corresponding monoalkylderivative. A previous *in vitro* study [4] demonstrated that DMAC was N-demethylated by rat liver homogenates, but at a much lower rate than found in this work. The low N-demethylation rate previously reported appeared to be due to the stability of *N*-(hydroxymethyl)–*N*-methyl acetamide, the direct precursor of formaldehyde during the microsomal oxidation of DMAC. The identification of a carbinolamine as stable metabolite has also been found in the urine from *N*-methylacetamide-administered rats [42] and mice [43]. In the current incubation mixtures the basic conditions (addition of 0.1 M NaOH) after the enzymic oxidation of DMAC allowed the complete decomposition of *N*-(hydroxymethyl)–*N*-methyl-acetamide to formaldehyde, as found for *N*-methylformamide [16]. The oxidative metabolism of DEAC produced an unstable carbinolamine intermediate which promptly decomposed to acetaldehyde, without requiring alkalinization.

Microsomes from PB-, DEX- and DEAC-treated rats, but not from rats treated with other P450 inducers, were capable of deethylating DEAC faster than control microsomes. These results suggest that both P4503A1/2, inducible by DEX [40], and P4502B1/2, inducible either by PB [44] or DEAC, are active in the oxidative metabolism of DEAC. The ability of anti P4502B1 antibodies to inhibit DEAC deethylation in microsomes from DEAC-treated rats point to the role of P4502B1 in this reaction. This data was confirmed by the finding that purified P4502B1 enzyme was able to oxidize DEAC with a high turnover number in a reconstituted system. On the contrary, P4503A1/2 isoforms do not appear to play a role in DEAC deethylation, as TAO did not strongly inhibit the reaction and it is known that the substrates for these P450 are bulky and lipophilic [45]. The higher V_{max} of DEACd observed in DEX-microsomes may be attributed at the higher levels of P4502B1/2 induced in DEX-treated rats [44]. However, P4502B-dependent catalysis cannot fully explain the fact that the DEAC deethylation rate per nmol of P450 was found to be nearly double in DEAC-induced than in PB-induced microsomes. It is possible that DEAC treatment in rat induced other hepatic P450 forms which may catalyse DEAC deethylation.

Neither ETOH-microsomes nor purified P4502E1 showed metabolic activity towards DEAC, indicating that this P450 form is not involved in DEAC deethylation. Thus, multiple DEAC administrations can induce P4502B1/2 and then potentiate its own metabolism. The oxidative dealkylation of DMAC was not catalysed by P4502B1 (as was DEAC), but by 2E1. The rate of DMAC demethylation was higher only in liver microsomes from rats pre-treated with ethanol or acetone, known inducers of P4502E1. Neither the purified hamster [46] nor mouse [47] orthologue P4502E1 catalysed the deethylation of DEAC but demethylated DMAC (unpublished data). The inhibition by P4502E1 antibodies of DMAC demethylation in microsomes from acetone-treated rats further supported the view that this oxidation is primarily catalysed by P4502E1. These findings are striking inasmuch as a simple N-substitution of a methyl with an ethyl group (DMAC vs DEAC) was not expected to provoke an abrupt change in the spectrum of P450 isozyme(s) able to catalyse the N-dealkylations of these simple and structurally similar molecules. As such they [4], as many other solvents [6], were thought to be possible substrates for P4502E1. The reason why DEAC is not a substrate of P4502E1, in spite of its ability to inhibit P4502E1-mediated *N*-methylformamide oxidation [4], is an interesting question which deserves to be explored.

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