Microsomal Oxidation of *N,N*-Diethylformamide and Its Effect on P450-Dependent Monooxygenases in Rat Liver

Giada Amato, Vincenzo Longo, Arturo Mazzaccaro, and Pier Giovanni Gervasi*

Laboratory of Genetic and Biochemical Toxicology, Istituto di Mutagenesi e Differenziamento, C.N.R., via Svezia 10, 56124 Pisa, Italy

Received November 29, 1995[⊗]

N,N-Diethylformamide (DEF) is a hepatotoxic polar solvent in which metabolism has not been investigated. In this study we examined the following: (a) the oxidative metabolism of DEF using both liver microsomes from rats pretreated with selected P450 inducers and purified P450 enzyme (2B1, 2E1, 2C11); and (b) the effect of administration of DEF and its metabolite, the monoethylformamide (MEF), on induction and/or inhibition of the P450 isoforms in rats. DEF was deethylated by microsomal P450-dependent oxidation forming acetaldehyde and MEF according to Michaelis−Menten kinetic parameters. Microsomes from rats pretreated with acetone and pyrazole (selective P4502E1 inducers) or rats pretreated with dexamethasone and 200 mg/kg DEF were able to deethylate DEF in a biphasic manner, showing a low K_m component with a V_{max} of about 0.2 nmol/(min·mg of protein) and a K_m between 70 μM and 250 μM. The low K_m component was not present in control microsomes or in microsomes from rats treated with phenobarbital, β-naphthoflavone, or clofibrate, where linear kinetics were observed. The use of purified P4502E1 and 2C11 in a reconstituted system showed that 2E1, which oxidized DEF with a V_{max} of 4.5 nmol/(min·nmol of P450) and a K_m of 0.7 mM, can partially account for the low K_m DEF deethylase, whereas 2C11, which oxidized DEF with a V_{max} of 4.8 nmol/(min·nmol of P450) and a K_m of 17 mM, might be the high K_m deethylase. The purified 2B1 was barely able to deethylate DEF. A confirmation of the role of 2E1 in DEF metabolism was obtained by using various selective inhibitors of P450 isoforms and immunoprecipitation experiments with anti P4502E1 IgG. The low K_m component of DEF deethylation in acetone- or pyrazole-induced microsomes was strongly inhibited (∼90%) by diethyldithiocarbamate, 4-methylpyrazole, and anti-2E1 IgG, but in 200 mg/kg DEF-induced microsomes the inhibition was partial, suggesting that other P450(s) may be involved. Administration of DEF 200 mg/kg ip for 4 days induced hepatic microsomal P4502E1-dependent aniline hydroxylase, P4502B1/2-linked pentoxyresorufin *O*-depentylase, 16β-testosterone hydroxylase P4503A1/2-associated erythromycin *N*-demethylase, and 6β-testosterone hydroxylase. Alternatively, the same dose regimen of MEF induced only the aniline hydroxylase and depressed the 3A1/2-linked activities. Immunoblot experiments verified these data. These findings indicate that DEF, at low concentrations, is predominantly oxidized by P4502E1 and that this enzyme may be induced in rodents by repeated MEF or DEF treatment, thereby increasing their own metabolism and potentially their cytotoxicity through the formation of ethyl isocyanate.

Introduction

N-Alkylformamides, such as diethylformamide (DEF) and dimethylformamide (DMF), are polar solvents widely used in the manufacturing of synthetic fibers, leathers, and films and in chemical laboratories as carriers of water-insoluble compounds (*1, 2*).

Hepatotoxicity induced by DMF and by its demethyl metabolite, *N*-methylformamide (NMF), has been observed in several species including rats, mice, and humans (*3*−*5*). The mechanism by which DMF and NMF exert their toxicity was shown to be mediated by their metabolism (*6*). DMF is hydroxylated on one methyl moiety by the P450 system to yield *N*-(hydroxymethyl)-*N*-methylformamide (HMMF),[1] which chemically decomposes to NMF and formaldehyde. NMF, in turn, may undergo further P450-mediated oxidation of the *N*-formyl group, leading to methyl isocyanate (MIC). This compound and its reactive glutathione (GSH) derivatives are thought to be responsible for the toxicity of DMF and NMF (*6, 7*).

MIC is a potent electrophilic carbamoylating agent which rapidly (i) conjugates with GSH forming two thiocarbamates: *S*-(*N*-methylcarbamoyl)glutathione (SMG) and ultimately urinary *N*-acetyl-*S*-(*N*-methylcarbamoyl)-cysteine (AMCC), both able to perform transcarbamoylating reactions with the nucleophilic cell constituents; (ii) reacts with water, yielding the urinary methylamine

* To whom correspondence should be addressed.
[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1996.

[1] Abbreviations: AC, acetone; AECC, *S*-(*N*-ethylcarbamoyl)-*N*-acetylcysteine; AMCC, *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine; AnH, aniline hydroxylase; APD, aminopyrine demethylase; β-NF, β-naphthoflavone; BzD, benzphetamine demethylase; CLO, clofibrate; DEDTC, diethyldithiocarbamate; DEX, dexamethasone; EIC, ethyl isocyanate; ErD, erythromycin demethylase; EtD, ethylmorphine demethylase; EROD, ethoxyresorufin *O*-deethylase; HEEF, (hydroxyethyl)ethylformamide; HMF, (hydroxymethyl)formamide; HMMF, (hydroxymethyl)-methylformamide; MIC, methyl isocyanate; P450, cytochrome P450; PB, phenobarbital; PROD, pentoxyresorufin *O*-depentylase; PYR, pyrazole; PYR-, PB-, DEX-, AC-, βNF-, CLO-, and DEF-microsomes correspond to hepatic microsomes from PYR-, PB-, DEX-, AC-, βNF-, CLO-, and DEF-treated rats, respectively; T, testosterone; 17-OT, 4-androstene-3,17 dione; 16α-OH, 16β-OH, 7α-OH, 6α-OH, 6β-OH, 2α-OH, and 2β-OH correspond to 16α-, 16β-, 7α-, 6α-, 6β-, 2α-, and 2β-hydroxytestosterone, respectively; TAO, triacetyloleandromycin.

S0893-228x(95)00201-3 CCC: $12.00

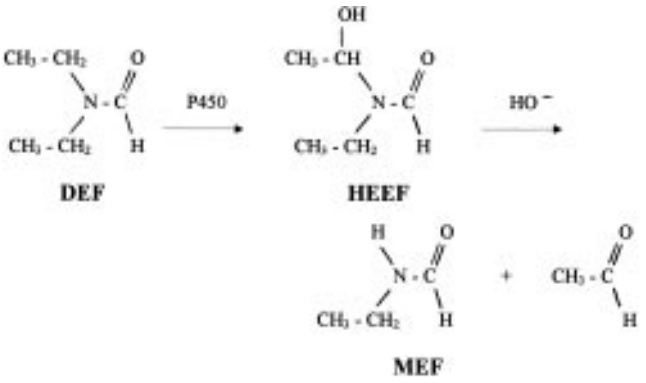

Figure 1. Metabolism of DEF.

and CO_2; and (iii) binds nucleophilic sites of cellular macromolecules (*7*, *8*).

Gescher's group (*9*, *10*) has presented evidence that rat P4502E1, an effective oxidant catalyst of low molecular weight compounds including many solvents (*11*), plays a crucial role in both the hydroxylation of DMF to HMMF and in the oxidation of HMMF and NMF formyl moiety to MIC.

There are some indications that the metabolism and hepatotoxicity of NMF and monoethylformamide (MEF) are similar (*4*, *12*). In mice, MEF and NMF have been reported to undergo metabolism to a carbamoylating intermediate (EIC and MIC) by microsomal oxidation. In addition, EIC was formed from MEF at a rate faster than MIC from NMF (*13*). However, to our knowledge, no data are provided on the biotransformation of DEF. Regarding its hepatotoxicity, a generic indication of an acidophile necrosis caused by DEF treatment in mouse and rat liver has been reported (*14*). Although the occupational exposure to DEF is not as widespread due to a much lower production of this compound compared to DMF, studies on DEF can help to better understand the mechanism of DMF toxicity. Thus, we have studied the *in vitro* oxidative metabolism of DEF, postulating that it is biotransformed by P450 via the same pathway as DMF (Figure 1).

The first step is the α-hydroxylation of one ethyl moiety of DEF, which gives rise to *N*-(hydroxyethyl)ethylformamide (HEEF). This compound is unstable under basic conditions and decomposes rapidly to acetaldehyde and MEF. By assaying the formation of acetaldehyde, we have investigated the role of P450 isoforms in the oxidation of DEF, using both hepatic microsomes from rats treated with inducers of selected P450s and three purified P450s (2B1, 2C11, and 2E1). Since it has been observed that the substrate for a particular P450 isoform is also an inducing agent of the same isoenzyme, the influence of DEF and MEF on P4502E1 and other P450-dependent monooxygenase activities in rats *in vivo* was investigated.

Materials and Methods

Chemicals. Benzphetamine was from the Upjohn Co. (Kalamazoo, MI, USA.). Corticosterone, 16β-OH, 17-OT, T, nitrocellulose filters (0.45 μm), 4-chloro-1-naphthol, and erythromycin were purchased from Sigma Chemicals (St Louis, MO, USA); 2α-OH, 2β-OH, 6α-OH, 6β-OH, 7α-OH, and 16α-OH were obtained from the Steroids Reference Collection (D. N. Kirk, Department of Chemistry, Queen Mary College, London, U.K.). Rabbit anti-rat P4502B1, P4502E1, and P4503A1 polyclonal antibodies were obtained from Oxygene (Dallas, TX, USA). Goat anti-rabbit IgG were purchased from Dako (Copenhagen, Denmark). DEF, MEF, β-NF, DEX, PB, CLO, PYR, DEDTC, and resorufin were supplied by Fluka (Buchs, Switzerland). Enzymes and coenzymes were obtained from Boehringer (Mannheim, FRG). Ethoxyresorufin and pentoxyresorufin were synthesized from resorufin by ethylation with ethyl iodide and by pentylation with pentyl iodide, respectively (*15*). All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Animal Treatment and Preparation of Microsomes. Male Sprague-Dawley rats (6−8 weeks old, Charles River, FRG) were injected ip with DEF or MEF at doses of 50, 200, and 400 mg/kg for 4 days. Other inducers administered ip were: phenobarbital (PB) at 80 mg/kg for 3 days; β-naphthoflavone (β-NF) 40 mg/kg for 3 days; dexamethasone (DEX) 50 mg/kg for 4 days; clofibrate (CLO) 250 mg/kg for 3 days; pyrazole (PYR) 200 mg/kg for 4 days. Acetone (AC) 5% (v/v) was administered in the drinking water for 10 days.

Animals were killed by CO_2 asphyxia; the livers were collected and microsomes were prepared as described previously (*16*). The washed microsomal pellets were resuspended in 100 mM phosphate buffer, 1 mM EDTA (pH 7.4), and stored at −80 °C. Protein content was determined according to Lowry et al. (*17*) using bovine serum albumin standards.

Histopathological Studies. Control male Sprague-Dawley rats and male CD-1 mice (7−9 weeks old, Charles River, FRG) were injected ip with DEF (0.5, 1, or 2 g/kg in 0.9% saline). Animals were killed 48 h after solvent administration; livers were collected, rapidly fixed in Carnoy fluid, and thereafter processed for histology.

Enzyme Assays. Hepatic cytochrome P450 was measured with the Omura and Sato method (*18*). Microsomal aniline hydroxylase (AnH) was determined by measuring the formation of *p*-aminophenol as descibed by Ko et al. (*19*). The aminopyrine (APD), erythromycin (ErD), benzphetamine (BzD), and ethylmorphine (EtD) demethylase activities were assayed by measuring the formation of formaldehyde (*20*). Ethoxyresorufin *O*-deethylase (EROD) and pentoxyresorufin *O*-dependentylation (PROD) activities were determined by measuring the formation of resorufin in a Perkin-Elmer spectrofluorimeter (*21*). Testosterone hydroxylase was assayed as reported previously (*22*) according to an HPLC method described by Platt et al. (*23*).

DEF deethylation was determined using a method for diethylacetamide (*24*) by measuring the acetaldehyde formation after quenching the reaction with NaOH (0.1 M final concentration). Under alkaline conditions or during GLC analysis, the HEEF formed as a stable intermediate decomposes to yield acetaldehyde and MEF. Acetaldehyde was trapped with the addition of semicarbazide, as described by Yoo et al. (*25*). The resulting semicarbazone was *reacted* with (2,4-dinitrophenyl)-hydrazine, and its (2,4-dinitrophenyl)hydrazone derivative was assayed by HPLC as reported previously (*16*).

Reconstituted System. Cytochrome P4502B1 and NADPH−cytochrome P450 reductase were purified from microsomes of male Sprague-Dawley rats treated with PB as described previously (*26*). P4502E1 was purified from pyrazole-treated diabetic rats following a procedure similar to that described for hamsters (*27*). Rats were made diabetic by a single injection of streptozotocin (90 mg/kg dissolved in 0.1 M citrate buffer, pH 4.5) in the caudal vein (*28*). Two weeks after streptozotocin treatment, diabetes was confirmed by analysis of blood glucose and rats were treated ip with 150 mg/kg of PYR for 2 days to fortify the P4502E1 induction (*29*). P4502C11 was purified from control male SD rats with the Fujita method (*30*). The reconstituted system contained 0.1 nmol of purified P450, 0.3 nmol of P450 reductase, 30 μg of dilauroylphosphatidylcholine (DLPC), and substrate at the concentration described in 1 mL of 0.1 mM phosphate buffer, pH 7.4. DLPC was prepared in water and sonicated immediately before use. After 30 min preincubation at room temperature, the reaction was started with NADPH (1 mM) and carried out at 37 °C for 30 min. Acetaldehyde was quantitated by HPLC as previously described (*16*). The activity of the purified 2B1 and 2E1 was checked in a reconstituted system with saturating concentrations of benzphetamine (2B1)

and aniline (2E1) as selective substrates; their turnover numbers were 75 and 12 nmol/(min·nmol of P450), respectively. The purified 2C11 selectively catalyzed the 2α- and 16α-T hydroxylation with turnover numbers of 3.2 and 3.5, respectively.

Gel Electrophoresis and Immunoblotting. SDS gel electrophoresis was performed using the discontinuous system of Leammli (*31*), with a 1.5 mm thick gel and 3% and 7.5% acrylamide in the stacking and separation gel, respectively. Proteins were transferred from the slab gel to nitrocellulose filters following the method of Towbin et al. (*32*). Immunodetections were performed using rabbit anti-rat P4502B1, P4502E1, and P4503A1 polyclonal antibodies. The bands on the nitrocellulose membranes were quantified by a laser densitometer (Ultrascan 2202 LKB).

Preparation of Antibodies. Female New Zealand white rabbits were immunized, as described previously (*33*), with P4502E1 purified antigen. Preimmune serum was collected prior to any injections. For the first immunization, about 0.2 mg of antigen was mixed with complete Freund's adjuvant. After 6 and 10 weeks, injections of 0.1 mg of antigen in incomplete Freund's adjuvant were performed. The immune serum was collected 10 days after the last injection. Immunoglobulin G fractions from preimmune serum and immune serum were purified by precipitation of non-IgG proteins with caprylic acid at pH 4.5, followed by precipitation of the IgG fraction with ammonium sulfate at pH 7.4 (*34*). In immunoblot experiments the purified anti P4502E1 IgG recognized the same antigen as the commercial P4502E1 polyclonal antibodies purchased from Oxygene.

Chemical Inhibition Assay. Specific P450 inhibitors were added at 0.1 mM. All the inhibitors were dissolved in methanol, except sulfaphenazole and metyrapone which were dissolved in water. As methanol could have an inhibitory effect, it was evaporated under N_2. To facilitate the inhibitor redissolution, the residue was resuspended in assay buffer by sonication; then microsomes were added and vortexed vigorously before adding the other components.

Immunoprecipitation Assay. Microsomes (0.75 mg/mL) were mixed with different amounts of a given antibody (IgG fraction) or preimmune rabbit IgG in 100 mM potassium phosphate buffer (pH 7.4) and allowed to preincubate for 20 min at 4 °C. Cofactors and substrate were then added and incubated for 30 min at 37 °C under the conditions previously described.

Enzyme Kinetics. Kinetic parameters (K_m and V_{max}) for DEF deethylation were estimated using a simple computer program (deviced by Dr. Ambrosetti) designed for a nonlinear least-squares regression analysis of a Michaelis−Menten equation. In the case where two enzymes could better explain the measured rates, the kinetic parameters were estimated by fitting the data in the following equation (*35*): $V = V_{max1}S/(K_{m1} + S) + V_{max2}S/(K_{m2} + S)$, where V is the rate of product formation, S is the substrate concentration, K_{m1} and K_{m2} are the constants for low and high K_m components, and V_{max1} and V_{max2} are the maximum rates for the apparent low and high K_m component, respectively.

Results

Histological Effects of DEF Administration. In preliminary experiments we have observed that the administration in rats of a single dose ip of DEF (up to 2 g/kg) did not provoke any clear hepatotoxic effect, whereas in mice a single dose ip of 1 or 2 g of DEF/kg was able to result in a modest centrilobular liver damage (Figure 2). In our experimental conditions, the liver necrosis was clearly observed in both species only after MEF (not shown) but not DEF administration. This pattern of hepatotoxicity of DEF is not surprising as in both rats and mice similar results for DMF have been reported (*12*, *36*).

Effects of DEF and MEF Administration on P450 Activities. Results of studies on the effect of DEF and MEF administration on P450-dependent monooxygenase activities are shown in Tables 1 and 2.

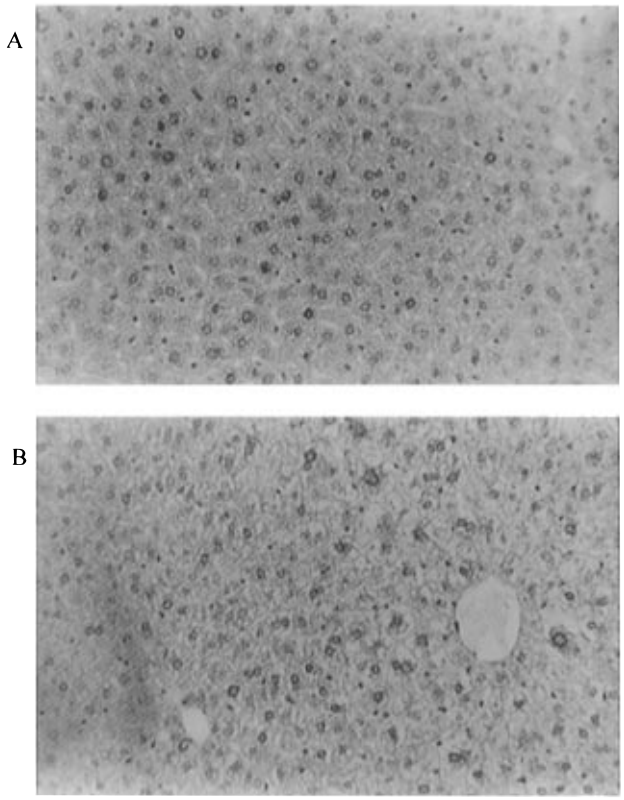

Figure 2. Effects of DEF administration in liver mice. (A) Liver of untreated mouse [H & E; original magnification was 85× (figure reduced 50% for publication)]. (B) Liver of DEF-treated mouse (2 g/kg ip) 48 h after administration: an evident vacuolization involves the centri-mediolobule, while the perilobule is quite normal [H & E; original magnification was 85× (figure reduced 50% for publication)].

DEF was administered to rats at doses of 50, 200, and 400 mg/kg/day ip for 4 days (Table 1). At lower dose (50 mg/kg) DEF did not significantly affect either the microsomal monooxygenase activities AnH, PROD, BzD, APD, EROD, EtD, and ErD, or the P450 specific content.

At a dose of 200 mg/kg/day, DEF resulted in a significant increase, compared to control values, of all the monooxygenase activities tested, except EROD. The activities of AnH, ErD, and especially PROD linked to P4502E1, 3A1/2, and 2B1, respectively (*37*−*39*) were enhanced. These changes were also accompanied by a significant increase in the P450 specific content.

At the highest dose (400 mg/kg), DEF did not additionally enhance the monooxygenase activities assayed, rather some of them (AnH, EtD) and the P450 content fell back toward the control values, suggesting that this solvent concentration could be hepatotoxic (Table 1).

MEF administration to rats at doses of 50, 200, and 400 mg/kg/day ip for 4 days did not produce a significant alteration in the P450 specific content (Table 2). At the lowest dose examined, no increase of the microsomal monooxygenase activities was observed. At 200 mg/kg MEF, only the 2E1-dependent AnH activity increased significantly, whereas the EtD and ErD activities both associated to P4503A1/2 were significantly decreased. At the highest dose, MEF resulted in a generalized reduction of all microsomal monooxygenase activities, possibly as a consequence of (i) hepatotoxicity, (ii) microsomal P450 destruction, and (iii) inhibition of hemoprotein synthesis.

Table 1. P450 Content and Monooxygenase Activities in Liver Microsomes from Rats Treated with Various DEF Doses[a]

parameters	P450 enzyme	DEF doses (mg/kg) 0	50	200	400
P450		0.53 ± 0.12	0.69 ± 0.10	0.97 ± 0.11**	0.74 ± 0.15
An H	2E	0.55 ± 0.14	0.65 ± 0.07	1.1 ± 0.15**	0.51 ± 0.1
PROD	2B	0.011 ± 0.005	0.018 ± 0.007	0.090 ± 0.016**	0.114 ± 0.02**
BzD	2B/2C	5.5 ± 1.2	8.6 ± 1.8	14.1 ± 2.5**	15.3 ± 1.3**
APD	2B/2C	6.3 ± 1.1	8.2 ± 2.1	11.5 ± 1.4**	9.1 ± 0.7*
EROD	1A	0.051 ± 0.015	0.052 ± 0.008	0.074 ± 0.02	0.079 ± 0.02
EtD	3A	4.9 ± 0.5	5.5 ± 2.2	9.1 ± 1.8*	7.5 ± 0.9*
ErD	3A	0.75 ± 0.2	0.86 ± 0.16	1.4 ± 0.22*	1.2 ± 0.23*

[a] Data are presented as means ± of three experiments. Each experiment used microsomes pooled from 3 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. *$p < 0.05$.

Table 2. P-450 Content and Monooxygenase Activities in Liver Microsomes from Rats Treated with Various MEF Doses[a]

parameters	P450 enzyme	MEF doses (mg/kg) 0	50	200	400
P450		0.53 ± 0.12	0.71 ± 0.13	0.54 ± 0.11	0.49 ± 0.08
AnH	2E	0.55 ± 0.14	0.69 ± 0.18	1.05 ± 0.21*	0.84 ± 0.3
PROD	2B	0.011 ± 0.005	0.017 ± 0.003	0.016 ± 0.004	0.007 ± 0.002
BzD	2B/2C	5.5 ± 1.2	6.5 ± 1.4	4.4 ± 1.1	3.6 ± 0.7
APD	2B/2C	6.3 ± 1.1	5.9 ± 0.9	4.2 ± 0.8	3.7 ± 0.5*
EROD	1A	0.051 ± 0.015	0.049 ± 0.005	0.033 ± 0.007	0.031 ± 0.008
EtD	3A	4.9 ± 0.5	5.1 ± 0.6	2.9 ± 0.6*	2.3 ± 0.5**
ErD	3A	0.75 ± 0.2	0.82 ± 1.5	0.17 ± 0.06**	0.18 ± 0.07**

[a] Data are presented as means ± SD of three experiments. Each experiment used microsomes pooled from 3 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. *$p < 0.05$.

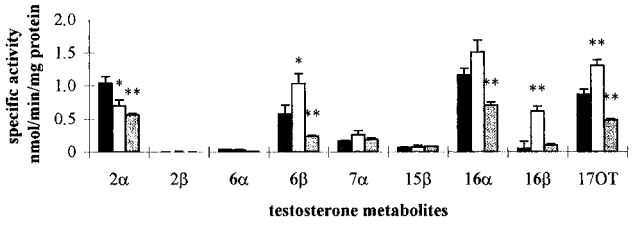

Figure 3. Testosterone hydroxylase activities by microsomes from control (■) and 200 mg/kg DEF- (□) and 200 mg/kg MEF- (shaded bars) treated rats. Data are means ± SD (bar) of three experiments. Each experiment used microsomes pooled from 3 animals. Incubations were carried out at 37 °C for 15 min with 1 mg/mL microsomal protein. **Significantly different from control at $p < 0.01$ by Student's t-test; *$p < 0.05$.

To further investigate the effects of DEF and MEF administration to rats, the microsomal metabolism of testosterone, an endogenous substrate selectively oxidized by many P450 isoforms (*23*), was studied. Neither DEF nor MEF at the lowest dose (50 mg/kg) did alter any T hydroxylase activity (data not shown). At 200 mg/kg, both solvents affected the T hydroxylations (Figure 3). DEF significantly enhanced the 16β-OH and 17-OT and 6β-OH T hydroxylations, corresponding to P4502B1/2 (16β and 17−OT) and 3A1/2 (6β), respectively (*23*). On the contrary, MEF depressed T hydroxylations of the 3A1/2-linked 6β-OH, and the 2α-OH, 17-OT and 16α-OH all associated to the P450 2C11, the major P450 form constitutively present in rat liver (*30*).

Immunoblot Analysis. The hepatic microsomal proteins from control and DEF- and MEF-treated rats were analyzed by western blot using polyclonal antibodies anti-P450 2B1/2, 2E1, and 3A1/2, in order to check if DEF and/or MEF treatment could alter the microsomal content of these P450s.

As illustrated in Figure 4, panel A, in microsomes from rats treated with DEF at doses of 200 and 400 mg/kg, anti-P4502B1 recognized a strong and wide band, which migrates in the same position as purified P4502B1 and possibly 2B2 (not shown). P4502B2 is known to have close molecular weight and a high homology (97%) with 2B1 and a cross-reactivity with antibodies raised against 2B1 (*40*). In control microsomes and in microsomes from MEF-treated rats, the anti-P4502B1 recognized only a very faint band in keeping with the very low constitutive level of 2B1 and 2B2 in rat liver (*41*). Thus, MEF does not appear to induce hepatic P4502B1/2.

As shown in Figure 4, panel B, anti-P4502E1 stained a protein band in control microsomes in agreement with the constitutive presence of 2E1 in rat liver (*42*). In MEF- and DEF-microsomes, this antibody recognized a band, corresponding to 2E1, whose intensity increased with the MEF and DEF doses. Compared to control microsomes, the 2E1 content, as determined by densitometry, increased to 150%, 240%, and 250% after 50, 200, and 400 mg of MEF/kg treatment and to 140% and 170% after 50 and 200 mg of DEF/kg treatment. However, in 400 mg/kg DEF-microsomes the 2E1 apoprotein content was depressed to about 80% of the value of control microsomes in agreement with the lack of induction of the 2E1-dependent AnH activity observed after the DEF treatment (see Table 1). Thus, both DEF and MEF seem to induce the P4502E1 up to a dose of 200 mg/kg.

Figure 4, panel C, shows the western blot analysis with anti-P4503A1. This antibody recognized a wide band ascribable to 3A1 and 3A2, which are constitutively present in rat liver and are immunochemically indistinguishable as they have a high homology (90%) and the same molecular mass (*43*). In microsomes from 200 mg/kg DEF treated rats, the P4503A1/2 content was enhanced by 360%. MEF did not induce the 3A1/2 content at any dose. Thus, only DEF appears to be an inducer of P4503A1/2 isoforms at a dose of 200 mg/kg.

DEF Deethylation by Liver Microsomes and by P4502B1, 2E1, and 2C11. The results of the metabolic studies by control or induced microsomal enzymes in the presence of an NADPH-generating system showed that

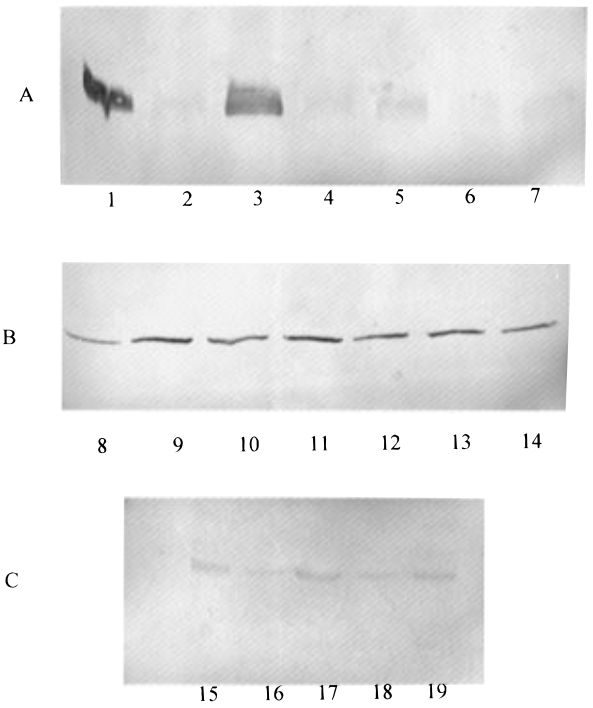

Figure 4. Western blot analysis of liver microsomes from control and DEF- and MEF-treated rats. Microsomes (12 μg of protein in each lane) were subjected to electrophoresis followed by immunoblot analysis, using polyclonal anti-2B1 IgG (see panel A), anti-2E1 IgG (see panel B), or anti-3A1 IgG (see panel C). Lanes 7, 14, and 19 contained microsomes from control rats; lanes 1, 8, and 15 contained microsomes from 400 mg/kg DEF-treated rats; lanes 2, 9, and 19 contained microsomes from 400 mg/kg MEF-treated rats; lanes 3, 10, and 17 contained microsomes from 200 mg/kg DEF-treated rats; lanes 4, 11, and 18 contained microsomes from 200 mg/kg MEF-treated rats; lanes 5, 12 and 6, 13 contained microsomes from 50 mg/kg DEF- and 50 mg/kg MEF-treated rats, respectively.

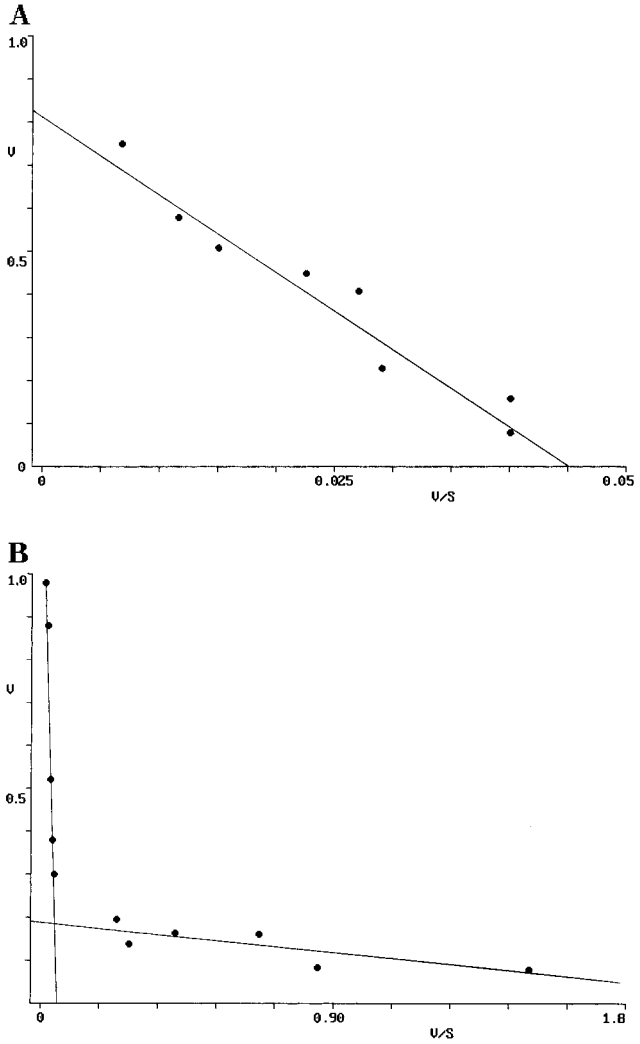

Figure 5. Eadie–Hofstee plot of DEF deethylation catalyzed by liver microsomes from (A) control and (B) AC-treated rats. The substrate concentrations ranged from 0.05 to 100 mM; the individual values were 2, 4, 8, 15, 20, 35, 50, and 100 mM and 0.05, 0.1, 0.2, 0.4, 0.6, 2, 5, 12, 20, 50, and 100 mM for control and AC-microsomes, respectively. V (nmol of CH_3CHO formed/(min·mg of protein)) and S (mM) represent the reaction rate and substrate concentration, respectively. Experimental details are described in Materials and Methods.

DEF was oxidized to an intermediate, most likely being HEEF, which, under basic conditions, decomposed giving rise to the DEF deethylation products: acetaldehyde and MEF (Figure 1). This reaction was catalyzed by P450 as it had an absolute requirement for molecular oxygen and NADPH; NADH was ineffective as a source of electrons and in presence of NADPH did not show any synergy. Carbon monoxide was able, when bubbled into incubation mixture, to inhibit the acetaldehyde formation by 86%.

Kinetic Parameters. In order to establish which form(s) of P450 were most active in the catalysis on the deethylation of DEF to acetaldehyde, incubations were performed using liver microsomes from control, DEF-treated (200 mg/kg) rats and rats treated with various classic inducers of the P450 superfamily (PB, AC, PYR, DEX, βNF, CLO). With control microsomes the dealkylation of DEF followed simple Michaelis–Menten kinetics and was linear up to 30 min and 1.5 mg of microsomal protein/mL. Also with PB-, βNF-, and CLO-microsomes (not shown) the DEF deethylation had linear kinetics. On the contrary, the production of acetaldehyde with AC-, PYR-, DEX-, and DEF-microsomes, when examined at substrate concentrations ranging from 0.05 mM (limit of detection of product) to 100 mM, followed biphasic kinetics. Typical Eadie–Hofstee plots of DEF deethylation catalyzed by control and AC-microsomes are illustrated in Figure 5. From the analysis of the DEF deethylation data performed as described in the Materials and Methods section, we obtained the apparent kinetic constants illustrated in Table 3. The simple apparent K_m for the metabolism of DEF of control microsomes was high (18 ± 6 mM) and not statistically different from that of PB-microsomes (15 ± 5 mM).

With microsomes from β-NF- and CLO-treated rats, in which the 1A and 4A P450 subfamilies are strongly induced (*40*), kinetic patterns were similar to those of microsomes from control rats (data not shown), implying that the above-mentioned P450 enzymes are not involved in the oxidation of DEF.

The kinetic analysis performed with PYR-, AC-, DEX-, and DEF-microsomes revealed a new low K_m component not present in control microsomes with a K_{m1} ranging from 70 to 250 μM, i.e., about 2 orders of magnitude lower than the K_{m2} or the single K_m observed in uninduced microsomes. The V_{max1} value was ~0.2 nmol/(mg·min), which was about one fourth of the V_{max} value seen in control microsomes. The V_{max2} of DEX-, AC-, PYR-, and DEF-pretreated microsomes was similar to the V_{max} value of control microsomes. It should be also noted that when the rates were expressed per nanomole of P450, the

Table 3. Values of Apparent Kinetic Constants for DEF Deethylation by P4502B1, P4502E1, P4502C11, and Microsomes from Control and Pretreated Rats[a]

microsomes or purified P450	major isoform P450	K_{m1} (mM)	K_{m2} (mM)	V_{max1} (nmol/(min·mg of protein))	V_{max2} (nmol/(min.mg of protein))
control			18 ± 6		0.82 ± 0.23
PB	2A/2B		15 ± 5		0.74 ± 0.21
DEX	3A1	0.25 ± 0.10	33 ± 15	0.21 ± 0.09	0.97 ± 0.58
AC	2E1	0.07 ± 0.02	28 ± 12	0.18 ± 0.05	1.32 ± 0.36
PYR	2E1	0.08 ± 0.03	25 ± 8	0.24 ± 0.08	0.96 ± 0.25
DEF		0.21 ± 0.11	27 ± 10	0.19 ± 0.06	1.22 ± 0.14
P450 2B1		ND		0.16	
P450 2E1		0.7		4.5	
P450 2C11		17		4.8	

[a] Values are reported as means ± SD for 3 or more experiments performed with different microsomal preparations. Each preparation contained pooled livers from 2−4 rats. The incubations were performed at 37 °C with a protein concentration of 1 mg/mL. The incubation of the reconstituted system containing 2B1, 2E1, and 2C11 was carried out as described in Materials and Methods. The activities expressed as nmol/(min·nmol of P-450) are the means of two experiments. ND = not determined. *Significantly different from control microsomes, $p < 0.5$ Student's *t*-test.

production of acetaldehyde was significantly lower with PB-microsomes than with control microsomes (0.67 ± 0.19 versus 1.54 ± 0.45 nmol/(min·nmol of P450)). This finding may indicate that 2B1 and 2B2, the major PB-inducible P450 forms along with 2A1, 2C6, and 3A, also induced by PB (*44*), are not specifically involved in the oxidation of DEF.

Furthermore, these results suggest that P4502E1, induced in DEF-, AC-, and PYR-microsomes, may account at least partially for the low K_m component of DEF oxidation.

The apparent involvement of 2E1 in the DEF deethylation is also supported by the finding that this enzyme, in a reconstituted system, was able to catalyze the deethylation at a rate about 20-fold higher than that (V_{max1}) observed in AC- or PYR-microsomes (Table 3). The higher apparent K_m in the reconstituted system with 2E1 compared to K_{m1} found in PYR- or AC- microsomes possibly reflects the lack of an important microsomal component as cytochrome b_5.

Purified 2C11, the most expressed constitutive P450 in rat liver (*30*), catalyzed the DEF oxidation with a substantial turnover number but with a high K_m similar to that of control microsomes. On the contrary, the purified 2B1 was able to catalyze the reaction at a low rate (0.16 nmol/(min·nmol of P450)), suggesting that this enzyme plays a minor role in DEF oxidation.

Inhibition Studies. Hepatic microsomal oxidation of DEF was performed at a substrate concentration of 0.5 mM to probe the low K_m component of DEF deethylase in both AC- and DEF-microsomes.

α-NF-, metyrapone-, and TAO-selective inhibitors for the P450 1A, 2B, and 3A subfamily, respectively (*45*−*48*), did not or only slightly diminished the DEF deethylase activity in either AC- or DEF-microsomes (Table 4). DEDTC, 4-methylpyrazole, and sulfaphenazole were able to reduce the DEF deethylase activities, although to a different extent. It should be noted that both DEDTC and 4-methylpyrazole are specific rat 2E1 inhibitors (*49*, *50*), whereas sulfaphenazole is a potent inhibitor of 2C enzymes primarily in humans (*46*); its inhibition efficacy toward P4502C of rat is not clear. DEDTC and 4-methylpyrazole were more effective than sulfaphenazole in AC-microsomes, whereas in DEF-microsomes these agents inhibited the reaction to 40−50% of control value. These findings may be indicative of the primary involvement of 2E1 in the oxidation of DEF in AC-microsomes, whereas in DEF-microsomes an important contribution could be due to the sulfaphenazole-inhibitable P450(s) presumibly belonging to 2C subfamily.

Table 4. Effects of Various Inhibitors on Microsomal Oxidation of DEF by AC- and DEF-Microsomes[a]

inhibitors (0.1 mM)	major isoform	rate of acetaldehyde production (% of control)	
		AC-microsomes	DEF-microsomes
control		100	100
α-NF	1A	104	93
metyrapone	2B	83	88
DEDTC	2E1	19	51
sulfaphenazole	2C (?)	79	47
4-methylpyrazole	2E1	18	42
TAO	3A	85	92

[a] The oxidation of DEF was carried out for 30 min at a substrate concentration of 0.5 mM to study the low K_m component of DEF deethylase in AC- and DEF-microsomes; the specific control activities were 0.21 and 0.25 nmol/(min·mg of protein), respectively. The inhibitors were solubilized as described in Materials and Methods. The data are expressed as means of two experiments.

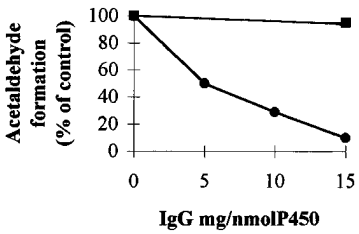

Figure 6. Effect of anti-rat P4502E1 IgG (●) or preimmune IgG (■) on DEF deethylation by microsomes from AC-treated rats. The microsomes were preincubated with the IgG fractions for 20 min. The incubations were performed with 0.5 mM DEF. Values, which are expressed as a percentage of control activity (0.23 nmol/(min·mg of protein)), are the mean of two experiments.

The contribution of 2E1 to the metabolism of DEF was further evaluated by immunoinhibition experiments. The 0.5 and 1 mM substrate concentrations were used in order to saturate only the low K_m component in AC- and DEF-microsomes. Increasing amounts of anti-P4502E1 IgG caused a progressive inhibition of acetaldehyde production from DEF by AC- and DEF-treated rat microsomes (Figures 6 and 7). However, in the case of AC-microsomes (Figure 6) the maximum inhibition was about 90%, whereas in DEF-microsomes (Figure 7) the maximum inhibition was only 70%, supporting the hypothesis that P450s other than 2E1 may account for the remaining DEF deethylase activity in DEF-microsomes.

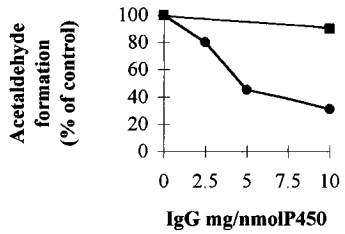

Figure 7. Effect of anti-rat P4502E1 IgG (●) or preimmune IgG (■) on DEF deethylation by microsomes from DEF-treated rats. The microsomes were preincubated with the IgG fractions for 20 min. The incubations were performed with 1 mM DEF. Values, which are expressed as a percentage of control activity (0.22 nmol/(min·mg of protein)), are the mean of two experiments.

Discussion

Hepatic P4502E1 plays a key role in the bioactivation of a variety of environmental low molecular weight protoxicants and procarcinogens (*11*), including benzene (*51*), *N*-nitrosodimethylamine (*25*), *N*-nitrosodiethylamine (*52*), pyridine (*53*), DMF, and NMF (*10*).

The objectives of this study were to elucidate the oxidative biotransformation of DEF (an alkyl homologue of DMF) and to verify whether DEF was metabolized predominantly by P4502E1 and if DEF and/or its proximal metabolite, MEF, were able to induce P4502E1. These solvents, when administered acutely to rats, markedly affected some hepatic microsomal monooxygenase activities. DEF and MEF treatment depressed the P450 activities at doses of 400 mg/kg, probably due to hepatotoxicity.

At concentrations of 200 mg/kg, DEF increased the amount of P450 content and induced the P4502B1/2, 3A1/2 and 2E1. The induction of 2B1/2 was demonstrated by a significant increase in the 2B1/2-dependent PROD and 16β-OH T hydroxylation activities. The induction of 2E1 and 3A1/2 was demonstrated by a significant enhancement of the 2E1-linked AnH and the 3A1/2-mediated EtD, ErD, 2β-OH, and 6β-OH T hydroxylation activities, respectively. At a concentration of 50 mg/kg, DEF did not induce the activity of any monooxygenase including the 3A-linked ErD and the most inducible 2B1-dependent PROD and 16β-T hydroxylase. Thus DEF, as compared to PB, is a modest inducer of 2B and 3A isoforms. Indeed, the ip administration in rats for 4 days of an equimolar dose of PB produces a various-fold induction of the above-mentioned activities (data not shown).

MEF, unlike DEF, at doses of 200 mg/kg induced only the AnH and selectively depressed the EtD and ErD activities. The reason for the selective depression of these 3A-dependent activities is not known, but it could be linked to the P4503A subfamily functioning as suicide enzyme in the biotransformation of MEF.

Immunodetectable P4502B1/2, 3A1/2, and 2E1 were elevated in microsomes from 200 mg/kg DEF-treated rats, consistent with the alteration of the above monooxygenase activities. In the MEF-microsomes only the amount of 2E1 apoprotein was elevated. Thus, DEF, like AC and other solvents (*41*), appears to be an inducer of both 2E1 and 2B1, whereas MEF appears to be a specific 2E1 inducer. Unlike DEF, it has been demonstrated that acute administrations of DMF to rats did not increase or rather decreased, depending on the dose (*36*, *54*), the microsomal concentration of P450. The 2C-mediated aminopyrine demethylase along with other activities were found decreased by DMF, whereas AnH was increased, suggesting that DMF, like DEF, might be a 2E1 inducer. To our knowledge, no data regarding the effect of NMF on the P450 system have been reported.

When the *in vitro* metabolism of DEF was studied, it was clear that this solvent was oxidized by the microsomal P450 system to a stable intermediate, likely HEEF, which, under basic conditions, decomposed in acetaldehyde and MEF, in a time- and protein-dependent manner. A role for P450 in the overall pathway of DEF oxidation by microsomes was demonstrated by the dependence on oxygen and NADPH and by inhibition by CO.

Microsomes isolated from AC-, DEF-, DEX-, β-NF-, PB-, CLO-, and PYR- treated rats were all capable of deethylating DEF, but only AC-, DEF-, PYR- and DEX-microsomes did it at rates higher than that of control microsomes.

In control, PB-, β-NF-, and CLO-microsomes, the deethylation kinetics of DEF were linear and had a high K_m (about 15 ± 22 mM). In AC-, DEF-, DEX-, and PYR-microsomes, this deethylation exhibited two separate kinetic curves, indicating the existence of at least two P450 isoenzymes that dealkylate DEF with different affinities. The P450 responsible for the appearance in AC- and PYR-microsomes of a new low K_m component for DEF deethylase (K_m 70−80 μM) seems to be the P4502E1, which was strongly induced in these microsomes. In DEX-microsomes the P450(s) responsible remain(s) veiled, as the 2B1/2 and 3A induction (*47*) does not appear to have an important role. In DEF-microsomes, in which 2E1 was moderately induced, the contribution of this enzyme can account at least partially for the low K_m component (K_m = 0.21 mM) of DEF deethylase.

The constitutive modest presence of 2E1 (5−8%) in the microsomal P450 population of control adult rat liver (*42*) and the HPLC limits of acetaldehyde detection do not allow detection of a very low DEF deethylase activity in control microsomes (<50 pmol/(min·mg of protein)). A similar low K_m component (K_m = 0.2 mM), at least partially dependent on 2E1, was also reported for the microsomal biotransformation of DMF to HMMF (*9*). Furthermore, it was observed, that at saturating substrate concentrations (DMF = 10 mM) for this low K_m component, the DMF demethylation rate in hepatic microsomes from AC-treated rats was about 3 times that of control microsomes (*9*). Thus, the activity of 2E1 toward DMF oxidation could be higher than that found for the oxidation of DEF. Of course, aside from 2E1, the contribution of other unidentified isozymes can also occur in the oxidative metabolism of DMF.

That 2E1 is a principal catalyst of DEF deethylation at low substrate concentration is further supported by (a) the selective inhibition by DEDTC and 4-methylpyrazole, quite specific inhibitors for 2E1, (b) the high catalytic turnover exhibited, in a reconstitutive system, by purified rat liver 2E1, and (c) the complete inhibition (90%) of DEF deethylase activity by anti-P4502E1 in AC-microsomes and partial inhibition (70%) in DEF-microsomes. On the other hand, 2B1, also inducible by DEF, appeared to exert a minor role on DEF deethylase, as purified 2B1 showed a low turnover and metyrapone elicited a scanty inhibition. Regarding the high K_m component of DEF deethylation, the 2C11 appeared to play an important role. The purified 2C11 was found to catalyze the DEF oxidation with a high turnover but with

a high K_m (17 mM) similar to that of control microsomes. Thus, this enzyme may account at least partially for the DEF deethylase activity in untreated microsomes and for the high K_m component in microsomes from induced rats. Of course, other P450 constitutive isoforms and especially the major ones belonging to 2C subfamily (2C6, 2C7, 2C13) may contribute to this reaction.

It is well documented that many compounds augment the rate of their own metabolism by elevating the activity of certain P450 enzymes. The data presented here demonstrate that 2E1 has a major contribution to the high affinity component of DEF metabolism and that 2E1 is inducible by DEF and its proximal metabolite, MEF. Thus, reiterative or continuous exposure to DEF could potentiate its own oxidative metabolism and potentially its bioactivation to toxic intermediates mainly through the induction of P4502E1. It remains to be elucidated if HEEF and MEF are both oxidized to EIC by 2E1 as in the case of HMMF and NMF (*9*).

In this regard, it is worth noting that MEF, in mice, has been found to undergo more rapid biotransformation than NMF, yielding higher amounts of the corresponding alkylamine, mercapturic acid, and unchanged parent compound (*12*). Possibly, this reflects a faster formation of EIC from MEF than MIC from NMF.

Thus, the link between the P450 isoforms involved in the oxidation of the above solvents, and the production of corresponding alkyl isocyanates, warrants further study, especially in humans. It could be the case that humans are particularly prone to DEF and MEF hepatotoxicity.

Acknowledgment. We acknowledge Dr. M. Minks for critical review of the manuscript, Dr. E. Chieli for the histological data, and Dr. R. Ambrosetti for having devised the computer program for the analysis of enzyme kinetics. This work was partially supported by "Progetto Finalizzato FATMA" of C.N.R.

References

(1) IARC (1989) Dimethylformamide. *IARC Monogr.* **47**, 171−197.
(2) Katritzky, A. R., Chang, H. X., and Yang, B. (1995) N-Formylbenzotriazole: a stable and convenient N- and O-formylating agent. *Synthesis* **5**, 503−505.
(3) Mraz, J., Cross, H., Gescher, A., Threadgill, M. D., and Flek, J. (1989) Differences between rodents and human in the metabolic toxification of *N,N*-dimethylformamide. *Toxicol. Appl. Pharmacol.* **98**, 507−516.
(4) Shaw A. J., Gescher, A., and Mraz, J. (1988) Cytotoxicity and metabolism of the hepatotoxin N-methylformamide and related formamides in mouse hepatocytes. *Toxicol. Appl. Pharmacol.* **95**, 162−170.
(5) Kennedy, G. L. (1985) Biological effect of acetamide, formamide, and the monomethyl and dimethyl derivatives. *CRC Crit. Rev. Toxicol.* **17**, 129−182.
(6) Gescher, A. (1993) Metabolism of *N,N*-dimethylformamide: key to understanding of its toxicity. *Chem. Res. Toxicol.* **6**, 245−251.
(7) Slatter, J. G., Rashed, M. S., Pearson, P. G., Han, D.-H., and Baillie, T. A. (1991) Biotransformation of methyl isocyanate in the rat. Evidence for glutathione conjugation as a major pathway of metabolism and implications for isocyanate-mediated toxicities. *Chem. Res. Toxicol.* **4**, 157−161.
(8) Threadgill, M. D., Axworthy, D. B., Baillie, T. A., Farmer, P. B., Farrow, K. C., Gescher, A., Kestell, P., Pearson, P., and Shaw, A. J. (1987) Metabolism of N-methylformamide in mice: primary kinetic deuterium isotope effect and identification of S-(N-methylcarbamoyl)glutathione as a metabolite. *J. Pharmacol. Exp. Ther.* **242**, 312−319.
(9) Mraz, J., Jheeta, P., Gescher, A., Hyland, R., Thummel, K., and Threadgill, M. D. (1993) Investigation of the mechanistic basis of *N,N*-dimethylformamide toxicity. Metabolism of *N,N*-dimethylformamide and its deuterate isotopomers by cytochrome P4502E1. *Chem. Res. Toxicol.* **6**, 197−207.
(10) Hyland, R., Gescher, A., Thummel, K., Schiller, C., Jheeta, P., Mynett, K., Smith, A. W., and Mraz, J. (1992) Metabolic oxidation and toxification of N-methylformamide catalyzed by the cytochrome P450 isoenzyme CYP2E1. *Mol. Pharmacol.* **41**, 259−266.
(11) Guengerich, F. P., Kim, D. H., and Iwasaki, M. (1991) Role of human cytochrome P4502E1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.* **4**, 168−179.
(12) Kestell, P., Threadgill, M. D., Gescher, A., Gledhill, A. P., Shaw, A. J. and Farmer, P. B. (1987) An investigation of the relationship between hepatotoxicity and the metabolism of N-alkylformamides. *J. Pharmacol. Exp. Ther.* **240**, 265−270.
(13) Cross, H., Dayal, R., Hyland, R., and Gescher, A. (1990) N-Alkylformamides are metabolized to *N*-alkylcarbamoylating species by hepatic microsomes from rodents and humans. *Chem. Res. Toxicol.* **3**, 357−362.
(14) Chann, P. H., Xuong, N. D., and Arnn-Gelade, M. C. (1971) Etude toxicologique de la formamide et de ser dérivés N. Méthylés et N. Ethylés. *Thérapie* **26**, 409−429.
(15) Klotz, A. V., Stogeman, J. J., and Walsh, C. (1984) An alternative 7-ethoxyresorufin assay: a continuous visible spectrophotometric method for measurement of cytochrome P450 monooxygenase activity. *Anal. Biochem.* **140**, 138−145.
(16) Longo, V., Citti, L., and Gervasi, P. G. (1986) Metabolism of diethylnitrosamine by nasal mucosa and hepatic microsomes from hamster and rat: species specificity of nasal mucosa. *Carcinogenesis* **7**, 1323−1328.
(17) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265−275.
(18) Omura, T., and Sato, R. (1964) The carbon-monoxide binding protein of liver microsomes. I. Evidences for its hemoprotein nature. *J. Biol. Chem.* **293**, 2370−2378.
(19) Ko, I. Y., Park, S. S., Song, B. J., Patten, C., Tan, Y., Han, Y. C., Yang, C. S., and Gelboin, H. V. (1987) Monoclonal antibodies to ethanol-induced rat liver cytochrome P450 that metabolize aniline and nitrosamines. *Cancer. Res.* **47**, 3101−3109.
(20) Tu, Y. Y., and Yang, C. S. (1983) High-affinity nitrosamine dealkylase system in rat liver microsomes and its induction by fasting. *Cancer. Res.* **43**, 623−629.
(21) Krijgsheld, K. R., and Gram, T. E. (1984) Selective induction of renal microsomal cytochrome P450-linked monooxygenases by 1,1-dichloroethylene in mice. *Biochem. Pharmacol.* **33**, 1951−1956.
(22) Longo, V., Mazzaccaro, A., Naldi, F., and Gervasi, P. G. (1991) Drug metabolizing enzymes in liver olfactory and respiratory epithelium of cattle. *J. Biochem. Toxicol.* **6**, 123−128.
(23) Platt, K. L., Molitor, E., Dohemer, J., Dogra, S., and Oesch, F. (1989) Genetically engineered V79 Chinese hamster cell expression of purified cytochrome P450IIB1 monooxygenase activity. *J. Biochem. Toxicol.* **4**, 1−6.
(24) Menicagli, S., Longo, V., Mazzaccaro, A., and Gervasi, P. G. (1994) Microsomal metabolism of N,N-diethylacetamide and N,N-dimethylacetamide and their effects on drug-metabolizing enzymes of rat liver. *Biochem. Pharmacol.* **48**, 717−726.
(25) Yoo, J. N., Ishizaki, H. and Yang, C. S. (1990) Roles of cytochrome P450IIE1 in the dealkylation and denitrosation of N-nitrosodimethylamine and N-nitrosodiethylamine in rat liver. *Carcinogenesis* **11**, 2239−2243.
(26) Guengerich, F. P. (1982) Microsomal enzymes involved in toxicology-analysis and separation. In *Principles and Methods of Toxicology* (Hayes, W., Ed.), pp 609−634, Raven Press, New York.
(27) Puccini, P., Menicagli, S., Longo, V., Santucci, A., and Gervasi, P. G. (1992) Purification and characterization of an acetone-inducible cytochrome P450 from hamster liver microsomes. *Biochem. J.* **287**, 863−870.
(28) Donahue, B. S., and Morgan, E. T. (1990). Effects of vanadate on hepatic cytochrome P450 expression in streptozotocin-diabetic rats. *Drug Metab. Dispos.* **18**, 519−526.
(29) Wu, D., and Cederbaum, A. I. (1993) Combined effects of streptozotocin-induced diabetes plus 4-methylpyrazole treatment on rat liver cytochrome P4502E1. *Arch. Biochem. Biophys.* **302**, 175−182.
(30) Fujita, S., Morimoto, R., Chiba, M., Kitani, K., and Suzuki, T. (1989) Evaluation of the involvement of a male specific cytochrome P450 isozyme in a senescence-associated decline of hepatic drug metabolism in male rats. *Biochem. Pharmacol.* **38**, 3225−3231.
(31) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680−685.
(32) Towbin, H., Stachelin, P., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* U.S.A. **76**, 4350−4354.

(33) Kaminsky, L. S., Fasco, M. J., and Guengerich, F. P. (1981) Production and application of antibodies to rat liver cytochrome P-450. *Methods Enzymol.* **74**, 262−272.
(34) McKinney, M. M., and Parkinson, A. (1987) A simple, non chromatographic procedure to purify immunoglobins from serum and ascites fluid. *J. Immunol. Methods* **96**, 271−278.
(35) Dixon, M., and Webb, E. C. (1972) In *Enzimi*, pp 85, Istituto Editoriale Universitario, Milano.
(36) Scailteur, V., and Lauwerys, R. R. (1987) Dimethylformamide (DMF) hepatotoxicity. *Toxicology* **43**, 231−238.
(37) Reinke, L. A., Sexter, S. H., and Rikans, L. E. (1985) Comparison of ethanol and imidazole pretreatments on hepatic monooxygenase activities in the rat. *Res. Commun. Chem. Pathol. Pharmacol.* **47**, 97−106.
(38) Lubet, R. A., Mayer, R. T., Cameron, J. W., Nims, R. W., Burke, M. D., Wolf, T., and Guengerich, F. P. (1985) Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P450 by phenobarbital and other xenobiotic in rat. *Arch. Biochem. Biophys.* **238**, 43−48.
(39) Arlotto, M. P., Sonderfan, A. J., Klassen, C. D., and Parkinson, A. (1987) Studies on the pregnenolone-16α-carbonitrile-inducible of rat liver microsomal cytochrome P450 and UDP-glucuronyltransferase. *Biochem. Pharmacol.* **36**, 3859−3866.
(40) Ryan, D. E., and Levin, W. (1990) Purification and characterization of hepatic microsomal cytochrome P450 *Pharmacol. Ther.* **45**, 153−239.
(41) Imaoka, S., Terano, Y., and Funae, Y. (1990) Changes in the amount of cytochrome P450s in rat hepatic microsomes with starvation. *Arch. Biochem. Biophys.* **278**, 168−178.
(42) Johansson, I., Ekstrom, G., Sholte, B., Puzycki, D., Jornvall, H., and Ingelman-Sundberg, M. (1988) Ethanol-fasting and acetone-inducible cytochrome P450 in rat liver: replication and characterization of enzymes belonging to the IIB and IIE gene subfamilies. *Biochemistry* **27**, 1925−1934.
(43) Gemzik, B., Greenway, D., Nevins, C., and Parkinson, A. (1992) Regulation of two electrophoretically distinct proteins recognized by antibody against rat liver cytochrome P4503A1. *J. Biochem. Toxicol.* **7**, 43−52.
(44) Waxman, D. J., and Azaroff, L. (1992)Phenobarbital induction of P450 gene expression. *Biochem. J.* **281**, 577−592.
(45) Murray, M., and Reidy, G. F. (1990) Selectivity in the inhibition of mammalian cytochromes P450 by chemical agents. *Pharmacol. Rev.* **42**, 85−101.
(46) Halpert, J. R., Guengerich, F. P., Bend, J. R., and Correia, M. A. (1994) Contemporary issue in toxicology. Selective inhibitors of cytochrome P450. *Toxicol. Appl. Pharmacol.* **125**, 163−175.
(47) Namkung, M. J., Yang, H. L., Hulla, J. E., and Juchau, M. R. (1988) On the substrate specificity of cytochrome P4503A1. *Mol. Pharmacol.* **34**, 628−637.
(48) Roos, P. H., Golub-Ciosk, B., Kallweit, P., Kauczinski, D., and Hanstein, W. B. (1993) Formation of ligand and metabolite complexes as a means for selective quantitation of cytochrome P450 isozymes. *Biochem. Pharmacol.* **45**, 2239−2250.
(49) Brady, J. F., Xiao, F., Wang, M. H., Li, Y., Ning, S. H., Gapac, J. M., and Yang, C. S. (1991) Effects of disulfiram on hepatic P4502E1, other microsomal enzymes and hepatotoxicity in rats. *Toxicol. Appl. Pharmacol.* **108**, 366−373.
(50) Feierman, D. E., and Cederbaum, A. I. (1987) Increased sensitivity of the microsomal oxidation of ethanol to inhibition by pyrazole and 4-methylpyrazole after chronic ethanol treatment. *Biochem. Pharmacol.* **36**, 3277−3283.
(51) Nakajima, T., Wang, R. S., Elovaara, E., Park, S. S., Gelboin, H. V., and Vainio, H. (1992) A comparative study on the contribution of cytochrome P450 isozymes to metabolism of benzene, toluene, and trichloroethylene in rat liver. *Biochem. Pharmacol.* **43**, 251−257.
(52) Puccini, P., Fiorio, R., Longo, V., and Gervasi, P. G. (1989) High affinity diethylnitrosamine-deethylase in liver microsomes from acetone-induced rats. *Carcinogenesis.* **10**, 1629−1634.
(53) Kim, S. G., Philpot, R. M., and Novak, R. F. (1991) Pyridine effects on P4502E1, 2B and IVB expression in rabbit liver: characterization of high- and low-affinity pyridine N-oxygenases. *J. Pharmacol. Exp. Ther.* **259**, 470−477.
(54) Imasu, K., Fujishiro, K., and Inoue, N. (1992) Effects of dimethylformamide on hepatic microsomal monooxygenase system and glutathione metabolism in rats. *Toxicology* **72**, 41−50.

TX950201U