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SELECTIVE INDUCTION OF RENAL MICROSOMAL CYTOCHROME P-450-LINKED MONOOXYGENASES BY 1,1-DICHLOROETHYLENE IN MICE

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Abstract—Intraperitoneal administration of a single dose of 1,1-dichloroethylene (DCE) to C57 B1/6N mice (125 mg/kg) caused a selective 6- to 10-fold increase in renal microsomal 7-ethoxyresorufin *O*-deethylase (EROD) and 7-ethoxycoumarin *O*-deethylase (ECOD), without affecting benzo[*a*]pyrene hydroxylase activity (AHH) or total microsomal cytochrome P-450 content. The observed increases did not result from *in vitro* activation of the enzymes or from any analytical artifact. Moreover, studies with actinomycin D and cycloheximide demonstrated that the increases resulted from *de novo* enzyme synthesis. Maximal enzyme induction was observed after a DCE dose of approximately 125 mg/kg, and the induced enzyme decayed rapidly, returning to control levels in about 3 days. Compared to female mice, male mice had higher basal levels of renal EROD and ECOD and were more responsive to the inductive effects of DCE; this correlated with corresponding differences in microsomal cytochrome P-450 levels. Starvation of mice for 24 or 48 hr increased renal EROD and ECOD activities in both male and female mice, but not the extent observed after DCE. The present results support the view of multiple renal cytochrome P-450 isozymes.

Extrahepatic metabolism of xenobiotics has been relatively neglected since the total capacity for hepatic drug metabolism is many times that of extrahepatic tissues. Therefore, many deficiencies exist in our knowledge of the mixed-function oxidase (MFO) system in extrahepatic organs. Nevertheless, various organs appear to have a microsomal monooxygenase system that is qualitatively similar to the one in liver. However, the MFO system can exhibit marked organ differences in composition and concentration of the pool of cytochrome P-450 isozymes. Many other organ differences have been described, such as responses to inducers and inhibitors, sex and developmental differences, and responses to a number of other physiological and pathological stimuli [1]. In addition, in contrast to the rather homogenous distribution of the MFO system in the liver, extrahepatic organs may have their MFO system concentrated in only a few of the many different cell types present. The major MFO-containing cell types in the kidney seem to be the proximal tubule cells [2–4]; in the lung, MFO has been found predominantly in the non-ciliated bronchiolar (Clara) cell and, to a lesser extent, in the alveolar type II cell [5, 6]. This may imply that in those cells the concentration of MFO enzymes is comparable to or even higher than in hepatocytes. It is to be expected, then, that these cell types would be more vulnerable to toxins that require activation by cytochrome P-450-linked monooxygenases. This selective cell injury indeed seems to occur in the kidney after exposure to 4-ipomeanol [7] and acetaminophen [8], and in the lung with, for example, 4-ipomeanol [9] and naphthalene [10]. Induction of MFO activities may intensify toxicity; thus, environmental pollutants like the halogenated aromatic hydrocarbons enhance the nephrotoxicity of, for example, chloroform and carbon tetrachloride [11].

Although in liver at least six or possibly more isozymes of cytochrome P-450 are present [12], the possible multiplicity of cytochrome P-450 in extrahepatic tissues has been studied less extensively. For kidney, the available data indicate that at least two different constitutive isozymes exist [13–15]; one is primarily active in fatty acid hydroxylation, while the other is more active toward xenobiotic substrates [14].

Although many compounds have been recognised as inducers of liver drug metabolism, relatively little is known of their effects on renal metabolism. Although phenobarbital induces monooxygenases in rabbit [16], hamster [17] and pig kidney [18], it failed to do so in rat, mouse and guinea pig [17, 19]; 3-methylcholanthrene-type compounds induce renal monooxygenases in all the species thus examined.

In a preliminary study on the effects of 1,1-dichloroethylene (DCE) on MFO activities in mouse lung, liver and kidney, a selective increase in 7-ethoxyresorufin *O*-deethylase (EROD) was found in renal microsomes, while DCE caused approximately 50% reduction of the pulmonary monooxygenases and had no effect in the liver [20]. DCE is widely used as a copolymer in the fabrication of plastics. In rats and mice it can cause acute liver and kidney

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damage, expecially in starved animals [21–23]. However, at the dose and route of administration used in the present study (125 mg/kg, i.p.) no renal or hepatic toxicity was observed based on morphological (light microscopy) and monooxygenase enzyme activities [20].

The present study indicates that DCE causes a short-lasting dose-dependent increase in renal microsomal *O*-deethylation of 7-ethoxyresorufin and 7-ethoxycoumarin; however, cytochrome P-450 content and benzo[*a*]pyrene hydroxylase activity were unaffected. The increase in enzyme activities appears to be dependent on *de novo* protein synthesis.

MATERIALS AND METHODS

Animals. C57 B1/6N mice (body weight 20–26 g males, 20–22 g females) had free access to food and water, unless stated otherwise. Animals were treated with DCE by intraperitoneal injection of the drug dissolved in olive oil. Control animals received the vehicle only (5 ml/kg). Treatment with the metabolic inhibitors actinomycin D and cycloheximide was performed in three separate injections: 1 hr before, as well as 5 and 11 hr after, DCE treatment. Both actinomycin D (500 μg/kg per injection) and cycloheximide (10 mg/kg per injection) were injected i.p. in saline; control mice received saline only (10 ml/kg). Mice were killed by cervical dislocation, and the kidneys of five to ten mice were pooled. Microsomes were isolated by differential centrifugation in 150 mM KCl–50 mM Tris–HCl buffer, pH 7.4 [24]. Protein concentration was estimated by the method of Lowry *et al.* [25] with bovine serum albumin as standard.

Enzyme assays. 7-Ethoxyresorufin *O*-deethylase activity was determined by a modification of the method of Burke and Mayer [26]: 0.6 mg of microsomal protein, an NADPH-generating system (see below), 7-ethoxyresorufin in 20 μl methanol (6 μM final concentration) and 0.3 M Tris–HCl buffer, pH 7.4, were incubated at 37° for 5 min in a final volume of 1.3 ml. The vessels were placed on ice, and the reaction was stopped by addition of 0.15 ml of a solution of 10% Triton X-100 in 1 N NaOH. The appearance of the product resorufin was measured fluorometrically (586 nm emission and 570 nm excitation).

For measurement of 7-ethoxycoumarin *O*-deethylase activity, 0.6 mg of microsomal protein, an NADPH-generating system (see below), 1.0 mM substrate and 0.3 M Tris–HCl buffer were incubated in a total volume of 0.6 ml for 10–15 min at 37°. The reaction was stopped on ice, and 3.0 ml diethyl ether (containing 1.5% isoamyl alcohol) was added. The tubes were vortexed vigorously for about 20 sec, and 2.5 ml of the ether layer was transferred to tubes containing 3.0 ml of 1.6 M glycine/NaOH buffer, pH 10.3. The tubes were again vortexed and the fluorescence of 7-hydroxycoumarin in the aqueous phase was estimated at 390 nm (excitation) and 440 nm (emission). The *in vitro* effect of DCE was investigated by addition of DCE (final concentration 0.1 to 100 μM) to the incubation mixtures in an aqueous solution of Tween 80 (1.5%, v/v). These particular incubations were performed in stoppered tubes. Tween 80 itself did not influence *O*-deethylation rates. Benzo[*a*]pyrene (AHH) activity was measured according to the fluorometric assay of Nebert and Gelboin [27]. The NADPH-generating system used in these assays consisted of (final concentrations) 1.9 mM NADP, 20 mM glucose-6-phosphate, 9 mM $MgCl_2$ and 1.1 I.U./ml glucose-6-phosphate dehydrogenase. Microsomal cytochrome P-450 content was determined by its dithionite difference spectrum according to Omura and Sato [28], employing an Aminco DW-2a recording spectrophotometer. All enzyme assays were conducted under zero order kinetics, and standards were included to permit quantification.

Chemicals. DCE (99% purity), 7-ethoxy- and 7-hydroxycoumarin were obtained from the Aldrich Chemical Co., Milwaukee, WI. 7-Ethoxyresorufin and resorufin were purchased from the Pierce Chemical Co., Rockford, IL, and Eastman Organic Chemicals, Rochester, NY, respectively. Benzo[*a*]-pyrene, bovine serum albumin and cofactors used in enzyme assays were obtained from the Sigma Chemical Co., St. Louis, MO. Cycloheximide and actinomycin D were obtained through the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. All other chemicals were of analytical grade.

RESULTS

Selective dose-dependent increase of renal microsomal O-deethylation by DCE. In a preliminary study on the effects of DCE on the microsomal MFO system in lung, liver and kidney of the mouse [20], we observed, 24 hr after DCE treatment, a very consistent increase in renal 7-ethoxyresorufin *O*-deethylase activity. The other renal microsomal parameters measured in that study, i.e. cytochromes P-450 and b_5, NADPH cytochrome *c* reductase, benzphetamine *N*-demethylase and benzo[*a*]pyrene hydroxylase were not affected by DCE treatment. A dose–response study (Fig. 1) revealed that a single dose of DCE caused marked dose-dependent increases in 7-ethoxyresorufin *O*-deethylase (EROD) and slightly lesser increases in 7-ethoxycoumarin *O*-deethylase (ECOD) activities. The data indicated a

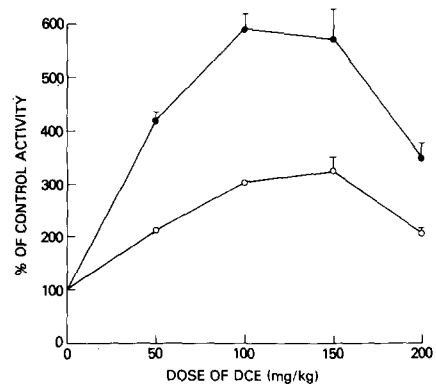

Fig. 1. Dose–response relationship for the increase in renal microsomal EROD (●——●) and ECOD (○——○) after a single intraperitoneal injection of DCE in mice. Enzyme activities are expressed as precentage of control activity (N = 4; bars indicate S.E.M.).

Table 1. Effect of a single dose of DCE (125 mg/kg, i.p.) on renal microsomal monooxygenase activities in male and female mice 24 hr after treatment*

	7-Ethoxyresorufin *O*-deethylase	7-Ethoxycoumarin *O*-deethylase	Benzo[*a*]pyrene hydroxylase
Male			
Control	10.6 ± 0.2 (100)	179 ± 8 (100)	27 ± 1
DCE-treated	79.3 ± 3.9† (748)	685 ± 26† (383)	32 ± 1
Female			
Control	3.5 ± 0.3 (100)	100 ± 5 (100)	N.D.‡
DCE-treated	8.0 ± 1.1† (229)	193 ± 6† (193)	N.D.

* Data are expressed as pmoles/mg protein per min; mean ± S.E.M.; N = 4. Percentage of control activity is given in parentheses.
† Significantly difference from control ($P < 0.01$).
‡ Not determined.

peak effect at DCE doses between 100 and 150 mg/kg. With doses exceeding 150 mg/kg, the DCE-induced increase clearly diminished. Thus, the optimal dose of DCE, 125 mg/kg, was used throughout this study. At 24 hr after treatment with this dose of DCE, EROD and ECOD were increased approximately 8- to 10-fold and 4- to 5-fold, respectively, while benzo[*a*]pyrene hydroxylase (Table 1) and the microsomal cytochrome P-450 content (Fig. 2) were not different from control values; this confirmed the selectivity of the DCE effect, as observed in the preliminary study.

Basal *O*-deethylase activities in female mice of this strain were significantly lower than those in male mice (Table 1). This corresponded roughly to lower levels of cytochrome P-450 in microsomes from female vs male mice (0.08 ± 0.003 and 0.20 ± 0.01 nmole/mg microsomal protein for untreated female and male mice, respectively; mean ± S.E.M.). Although a stimulatory effect of DCE was observed in kidneys of both males and females, the magnitude of the increase in *O*-deethylation was less pronounced in the female mice (Table 1).

Demonstration of induction of renal microsomal O-deethylation via de novo *protein synthesis. In vitro* experiments with microsomes from control and DCE-treated mice indicated no artifactual interference of DCE, or metabolites of DCE, with the assay procedure for EROD and ECOD. Moreover, *in vitro* addition of DCE to the incubation mixtures at concentrations of 0.1 to 100 μM caused no activation of either enzyme activity, nor did preincubation of the mixture with DCE (and no substrate) affect the measured activities. No interference was observed of DCE and possible metabolites with the fluorescence of the generated reaction products. Liver microsomes do further metabolize one of the measured reaction products, namely 7-hydroxycoumarin; DCE might inhibit this secondary metabolism and thereby cause an artifactual apparent increase in ECOD. However, incubation of renal microsomes with 7-hydroxycoumarin caused no change in fluorescence measured before and after incubation, indicating that renal microsomes do not further metabolize 7-hydroxycoumarin (data not presented).

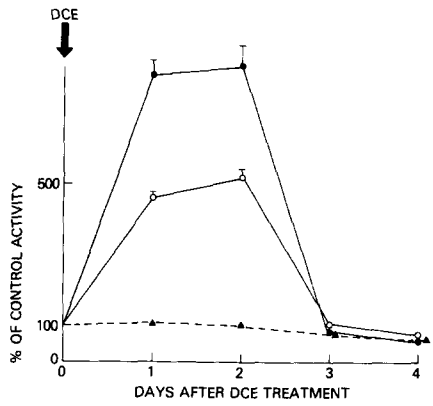

Fig. 2. Time course for the increase in mouse renal microsomal EROD (●——●) and ECOD (○——○) induced by a single injection of DCE (125 mg/kg, i.p.). Simultaneously, total renal microsomal cytochrome P-450 content was estimated (▲- - -▲). Data are expressed as percentage of control (N = 4, bars indicate S.E.M.).

Having thus exhausted what appeared to be the major arguments favoring an analytical artifact or *in vitro* activation as an explanation for the marked stimulatory effects of DCE on renal microsomal EROD and ECOD activities, we sought to directly demonstrate enzyme induction. To this end we used actinomycin D, a classic inhibitor of DNA-directed synthesis of RNA polymerase [29], and cycloheximide, an equally classic pure inhibitor of protein biosynthesis [30]. These concerted reactions are required for induction of *de novo* protein biosynthesis. Use of these metabolic inhibitors, actinomycin D and cycloheximide, in combination with DCE almost completely prevented the DCE-induced increase in EROD and ECOD (Table 2). No change in phospholipid content of renal microsomes was detectable after DCE treatment of the mice.

Duration of the induction of renal microsomal O-deethylation after single and multiple injections of DCE. Treatment of the mice with a single injection of DCE caused a maximal increase in renal *O*-deethylation after 24–48 hr; however, during the third day a rapid return to control levels occurred (Fig. 2). Although no significant changes in microsomal P-450 content were observed during the first 2 days after treatment, at days 3 and 4 cytochrome P-450 levels actually decreased below control values (74 ± 3% and 64 ± 5% of control, at days 3 and 4, respectively; mean ± S.E.M., N = 4; $P < 0.01$).

Table 2. Inhibition of the effect of DCE on renal microsomal *O*-deethylation by actinomycin D and cycloheximidine*

	7-Ethoxyresorufin *O*-deethylase	7-Ethoxycoumarin *O*-deethylase
Actinomycin D		
Control	11.0 ± 0.8 (100)	150 ± 9 (100)
Act. D	8.5 ± 0.5 (77)	128 ± 7 (85)
DCE	109.6 ± 3.9 (996)	766 ± 29 (511)
Act. D + DCE	14.3 ± 0.9 (130)	183 ± 3 (122)
Cycloheximide		
Control	11.0 ± 0.2 (100)	175 ± 3 (100)
Cycloheximide	6.8 ± 0.5 (62)	99 ± 6 (57)
DCE	106.2 ± 2.6 (965)	913 ± 42 (522)
DCE + cycloheximide	18.4 ± 1.2 (167)	247 ± 13 (141)

* Mice were injected with DCE (125 mg/kg, i.p.) 24 hr before being killed. Both actinomycin D (500 μg/kg per injection) and cycloheximide (10 mg/kg per injection) were injected three times: at 1 hr before, as well as 5 and 11 hr after, DCE administration. Both inhibitors were administered i.p. in saline; mice that received no inhibitor received saline (10 ml/kg). Data are expressed as means ± S.E. (N = 4) in pmoles per min per mg protein; percentage of control activity is given in parentheses.

A second injection of DCE, 24 hr after the first treatment, appeared to further increase the extent of induction of EROD and ECOD (Fig. 3). Three daily injections, however, apparently reduced the magnitude of induction and 48 hr after the third DCE administration EROD and ECOD were returned to control levels. Although the second treatment with DCE caused 40–50% mortality, most of the animals that survived two DCE injections survived a third challenge with DCE (80%).

Effect of starvation on renal microsomal O-deethylation. After administration of DCE, the body weight of the mice decreased by approximately 10–15% during the first 24 hr, suggesting that the animals experienced anorexia as a result of DCE treatment. This confirms a reported reduction in food consumption by mice that were exposed to DCE by inhalation [31]. Since, in the rat, starvation has been reported to increase renal microsomal cytochrome P-450 content and related metabolism of fatty acids [32] and xenobiotics [33], the possible contribution of starvation to the induction of *O*-deethylation was investigated further.

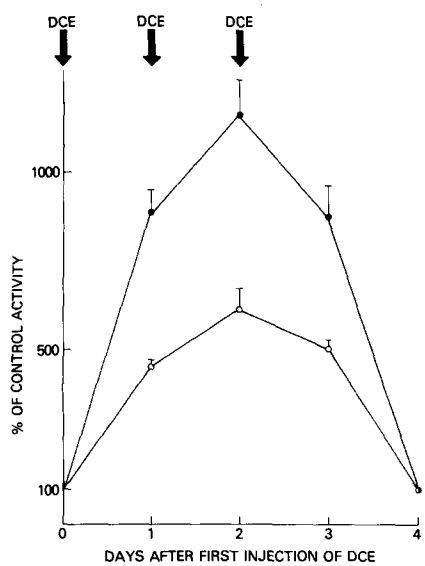

Fig. 3. Effect of multiple DCE injections on EROD (●——●) and ECOD (○——○) in mouse renal microsomes. DCE (125 mg/kg, i.p.) was injected daily for 3 days (see arrows). Enzyme activities are expressed as percentage of control activity (N = 4; bars indicate S.E.M.).

Starvation of mice for 24 hr increased the specific activity of ECOD and EROD, and the effect became more pronounced after 48 hr of food deprivation (Table 3). However, DCE, combined with starvation, caused a much larger increase in enzyme activities than starvation alone. In female mice, starvation caused a trend similar to the one in male mice (Table 3). Although 125 mg/kg DCE caused virtually no deaths in fed mice, starvation for 24 hr prior to DCE injection caused a significant mortality (approximately 40% of the treated animals died).

DISCUSSION

DCE injection caused a pronounced and short-lasting increase in the *O*-deethylation of 7-ethoxyresorufin and 7-ethoxycoumarin in renal microsomes from C57 B1/6N mice. Blockade of the increase in activity by both actinomycin D and cycloheximide indicated that enzyme induction via *de novo* RNA and protein synthesis was involved. The rapid return of induced enzyme to control levels in 3 days after treatment implies a rapid turnover rate for this newly synthesized protein. Prolonged exposure to the inducer by repeated DCE treatment sustained the effect and led to an even further decrease in *O*-deethylation. Three daily injections presumably evoked some nephrotoxicity and thereby diminished the effect. Although during the first 2 days after single DCE treatment no significant change was observed in cytochrome P-450 concentration the

Table 3. Comparison of the effect of starvation and DCE (125 mg/kg, i.p.) on renal microsomal *O*-deethylation in male and female mice*

	7-Ethoxyresorufin *O*-deethylase	7-Ethoxycoumarin *O*-deethylase
Male		
Control	14.9 ± 0.5 (100)	252 ± 7 (100)
Starved, 24 hr	24.9 ± 0.8† (167)	385 ± 9† (153)
48 hr	46.4 ± 2.1† (311)	617 ± 20† (245)
Starved + DCE, 24 hr	121.5 ± 2.3† (815)	1017 ± 31† (404)
48 hr	111.3 ± 3.2† (747)	1001 ± 48† (397)
Female		
Control	< 3‡	47 ± 2 (100)
Starved, 24 hr	< 3	65 ± 3† (138)
Starved + DCE, 24 hr	< 3	108 ± 2† (230)

* Mice were killed 24 hr after injection of DCE. Deprivation of food was started at 24 hr or 48 hr before sacrifice. Data are expressed as mean ± S.E. (N = 4), in pmoles per min per mg microsomal protein. Percentage of control activity is indicated in parentheses.

† Significantly greater than control ($P < 0.01$).

‡ Activities were below detection limit.

slight reduction of cytochrome P-450 during the following days to levels below control values suggests that DCE may simultaneously reduce total cytochrome P-450 content while it increases a specific P-450-isozyme that is particularly involved in *O*-deethylation. This isozyme appears to have a high substrate specificity: benzo[*a*]pyrene hydroxylase activity, which is often highly correlated with both *O*-deethylases, was not increased, nor was benzphetamine *N*-demethylase activity [20]. It is of interest that pretreatment of mice with β-naphthoflavone also stimulates the *O*-deethylation of 7-ethoxyresorufin and 7-ethoxycoumarin by renal microsomes [34].

Little is presently known about the cytochrome P-450 isozymes present in kidney. An early study suggested the possibility of multiple forms in rat kidney based on the observation that pretreatment with benzo[*a*]pyrene induced AHH activity without affecting laurate hydroxylation [35, 36]. For rabbit kidney, two cytochrome P-450 peptides have been reported [10, 37], which were enhanced by 3-methylcholanthrene (3-MC) treatment. Recently Ogita *et al.* [14] purified two forms of cytochrome P-450 from kidneys from 3-MC-pretreated rabbits: a cytochrome P-450 fraction with fatty acid hydroxylase activity and no benzo[*a*]pyrene hydroxylase activity, and a cytochrome P-448 fraction which preferentially hydroxylated benzo[*a*]pyrene. With immunofluorescence techniques, comparison with liver P-450 forms indicated two constitutive isozymes, while tetrachlorodibenzo-*p*-dioxin (TCDD) appeared to induce two other forms of cytochrome P-450 [38]. The selectivity in the inductive effect of DCE suggests that more than one form of cytochrome P-450 is present also in mouse kidneys.

More recently, Ogita *et al.* [39] purified two forms of cytochrome P-450 from the kidneys of phenobarbital-treated rabbits. P-450$_a$ catalyzed the hydroxylation of PGA_1 and myristate but was inactive against exogenous compounds, while P-450$_b$ hydroxylated PGA_1 and myristate poorly or not at all but efficiently metabolized benzphetamine, aminopyrine, 7-ethoxycoumarin, and *p*-nitroanisole. In addition, separate cytochrome P-450s purified from pig kidney have been shown to catalyze the ω- and ω-1 hydroxylation of PGA_1 [15]. Finally, administration of hexachloro-1,3-butadiene to rats [40] caused renal necrosis and a 64% decrease in renal cytochrome P-450. Concomitantly, there was a 60–70% decrease in cytochrome P-450-mediated aldrin oxidation but no change in laurate hydroxylation.

In the present study, a clear sex difference was observed in the renal microsomal cytochrome P-450 content in the mice which correlated with the lower basal *O*-deethylase activities in female mice. Thus, when the enzyme activities are expressed per nmole cytochrome P-450, similar EROD activities are found in male and female renal microsomes; ECOD activity per nmole cytochrome P-450 appears to be even higher in female than in male mice. Nonetheless, female mice seem to be less responsive to DCE induction of *O*-deethylase activity than males.

DCE is known to be hepatotoxic and nephrotoxic [21–23], and very recently acute pulmonary injury has been reported [20, 41]. Oral [^{14}C]-DCE becomes concentrated mainly in liver and kidneys, which retain the ^{14}C-label for the longest time after dosing [42]. The role of metabolism in DCE toxicity is still unclear. It is thought to involve the mixed-function oxidase system, which may lead to reactive metabolites such as dichloroethylene oxide and chloroacetylchloride, which can be inactivated in various ways. Identified metabolites in rats are monochloroacetate, conjugates, thiodiglycollic acid and related products [43]. Recently dichloroacetaldehyde has been reported as a metabolic intermediate [44]. Clearly, glutathione can have a detoxifying effect since glutathione depletion by fasting or diethylmaleate increases the hepatotoxic effect [45]; moreover, hepatic glutatione concentration is decreased by DCE exposure [45, 46]. Nephrotoxic effects of orally administered DCE have been ob-

served particularly in rats that were fasted [23]. In the present study, mice were fed which may have prevented kidney injury. Histological examination of the renal tissue did not reveal any injury (data not shown). When the mice were fasted for 24 hr prior to DCE administration, increased DCE toxicity became obvious as 40% of the treated mice died; DCE caused essentially no lethality in fed mice. Thus, like carbon tetrachloride and other xenobiotics [47], starvation apparently potentiates the acute toxicity of DCE.

In summary, a single i.p. dose of DCE had paradoxical effects on various tissues, i.e. no effect in the liver, necrosis of the Clara cells in the lung and an inductive effect in the kidneys. The presence of multiple forms of renal cytochrome P-450, as suggested by the selectivity in the renal induction by DCE, warrants further investigation.

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