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METABOLISM OF PARALDEHYDE TO ACETALDEHYDE IN LIVER MICROSOMES

EVIDENCE FOR THE INVOLVEMENT OF CYTOCHROME P-450*

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Abstract—A concentration-dependent acetaldehyde (AcH) generation was observed when paraldehyde was incubated with the mouse liver microsomal fraction. The process, which exhibited a requirement for oxygen and NADPH and was inhibited by carbon monoxide, was found to have a K_m of 17.9 mM with respect to paraldehyde and a V_{max} of 40.1 nmoles/mg protein/min with respect to AcH formation. NADH was much less effective as an electron donor than NADPH, though a more than additive increase in AcH generation was observed when both of these nucleotides were added to the incubation. The rate of microsomal AcH generation from paraldehyde was increased 2.5-fold by pretreatment of the mice with phenobarbital but only 0.6-fold by pretreatment with 3-methylcholanthrene. Pretreatment with 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF-525A) resulted in 54% inhibition of the reaction rate. Addition of metopirone to the incubation inhibited AcH generation in a concentration-related fashion, the inhibition being greatest, proportionately, in microsomes from phenobarbital-pretreated animals. The above results conclusively indicate the involvement of cytochrome P-540 mixed function oxidase in the formation of AcH from paraldehyde by mouse liver microsomes. It is also postulated that this process may be accomplished in the reaction analogous to O-dealkylation.

A century has elapsed since the introduction of paraldehyde into medicine [1]. It continues to be used clinically in the control of seizures and status epilepticus [2–4], particularly in infants [5–7], and in the treatment of alcohol withdrawal and delirium tremens [8, 9]. Nonetheless, knowledge concerning its metabolism and disposition remains very limited.

We have shown recently that mice, administered ^{14}C-labeled paraldehyde i.p., metabolized 72% of the dose to carbon dioxide by what was, pharmacokinetically, a two-step process, and that almost 10% of the dose was excreted in the exhaled air as unchanged paraldehyde [10, 11]. Using D-penicillamine as an *in vivo* AcH§ sequestering agent, we were able to demonstrate the urinary excretion of the labeled condensate of D-penicillamine and AcH: 2,5,5-trimethylthiazolidine-4-carboxylic acid [11, 12]. This observation was the first direct demonstration of *in vivo* formation of AcH from paraldehyde. The above data indicate that AcH is an intermediary product in the metabolism of paraldehyde to carbon dioxide. We have also reported that pretreatment of mice with SKF-525A resulted in an *in vivo* inhibition of the first step in the metabolism of paraldehyde to carbon dioxide, indicating that the degradation of paraldehyde is mediated by an enzymatic process with the probable involvement of the hepatic endoplasmic reticulum [10, 11].

Results described below provide the direct evidence for cytochrome P-450 monooxygenase involvement in the process of generation of AcH from paraldehyde by liver microsomes.

In a preliminary publication, we have reported that in the presence of paraldehyde mouse liver microsomes generate AcH, that the process is NADPH dependent and that phenobarbital pretreatment of mice resulted in a 2-fold increase in the rate of AcH generation [13]. Subsequently, Zera and Nagasawa [14] reported obtaining similar results with rat liver microsomes.

MATERIALS AND METHODS

Chemicals. USP paraldehyde was obtained from Elkins-Sinn Inc., Cherry Hill, NJ; as evidenced by GLC analysis, it contained less than 0.05% AcH. AcH (Eastman Kodak, Rochester, NY) was redistilled, sealed in 5 ml glass ampules, and stored at −25°. SKF-525A was a gift from the Smith, Kline & French Laboratories, Philadelphia, PA. Sodium phenobarbital was purchased from Merck & Co. Inc., Rahway, NJ, and 3-methylcholanthrene from Calbiochem-Behring, La Jolla, CA; sucrose and glucose-6-phosphate dehydrogenase were from the Sigma Chemical Co., St. Louis, MO, and NADH, NADPH and glucose-6-phosphate (Type XII from Torula Yeast, 268 units/mg protein) from Boehringer Mannheim, Indianapolis, IN.

Animals and treatment. Male BALB/c mice (Health Research, West Seneca, NY) weighing 25–30 g were used. Animals were permitted free access to food and water. All drugs were adminis-

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§ Abbreviations: AcH, acetaldehyde; and SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride.

tered i.p. in a 10 ml/kg volume of vehicle. Pretreatments: SKF-525A (40 mg/kg, in 0.9% saline) was administered 90 min before sacrifice; phenobarbital (70 mg/kg in 0.9% saline) was administered every 12 hr for 2 days, and the mice were killed 48 hr after the last injection; 3-methylcholanthrene (80 mg/kg in corn oil) was administered 3 days prior to sacrifice. Control animals received equal volume injections of the vehicle.

Isolation of microsomes. Animals were killed by stunning and decapitation. Livers were quickly removed and homogenized in 4 vol. of ice-cold 0.25 M sucrose containing 10 mM sodium phosphate buffer (pH 7.4). After centrifugation of homogenates at 600 *g* for 10 min at 2°, supernatant fractions were collected; pellets were rehomogenized in original volume of sucrose and recentrifuged. The supernatant fractions from both centrifugations were combined and centrifuged at 7,000 *g* for 10 min. Microsomes were sedimented from 7,000 *g* supernatant fractions by centrifugation at 106,000 *g* for 60 min. The microsomal pellets were washed once and suspended in 0.25 M sucrose, equal in volume to original wet weight of liver (ml/g). The activity of the fresh suspension was determined. These were then stored in small aliquots at −85° and used for incubations as required. Microsome suspensions could be stored in this manner for at least 25 days without change or loss of activity. Protein concentration was determined by the method of Lowry *et al.* [15] using bovine serum albumin as the standard.

Incubations. Incubations were carried out in a shaking water bath at 37° in the presence of atmospheric oxygen using 6 ml sealed (crimp-on) vials. The standard incubation mixture (0.5 ml) contained the following components: 100 mM phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM nicotinamide and 1 mM NADPH. Since preliminary experiments showed that incubation of microsomes either in the presence of 1 mM NADPH or of an NADPH-generating system (1 mM NADP, 10 mM glucose-6-phosphate and 0.5 unit of glucose-6-phosphate dehydrogenase) resulted in identical rates of AcH formation from paraldehyde, NADPH was used in all the reported experiments. Following addition of microsomal protein, the reaction mixture was preequilibrated in the water bath at 37° for 5 min prior to the initiation of the reaction by addition of paraldehyde via a hypodermic needle inserted through the rubber septum. All incubations were carried out in triplicate. To verify the linearity of the reaction for different microsomal preparations, incubations were carried for 5, 10 and 20 min. Incubations were terminated by the transfer of 0.1 ml aliquots of the reaction mixture, using a micro syringe, onto 200 mg of solid NaCl in sealed head-space vials kept at 0°. Zero time samples, containing the complete incubation mixture, microsomal protein, and paraldehyde, were prepared in the cold, concomitantly with each set of experimental samples, and assayed immediately.

Analysis. The quantity of AcH in the samples was determined using a Sigma 1 Chromatography System equipped with an HS-6 head-space sampler and flame ionization detector (Perkin–Elmer, Norwalk, CT). The conditions used were: sample equilibration time at 60°: 20 min; injector temp: 80°; oven temp: 70°; detector temp: 225°; carrier gas (N_2) flow rate: 20 ml/min; column: 6 ft × 2 mm i.d., glass, packed with 5% Carbowax 20 M on 60–80 mesh Carbopack B (Supelco Inc., Supelco Park, Bellefonte, PA). Retention time for AcH was 1.4 min. Peak areas were computed by a Sigma 1 Console Printer/Plotter Perkin–Elmer). The concentration of AcH was calculated by comparing peak areas with AcH standards curves which were run daily in parallel with experimental samples. The identity of the material formed in the microsomal incubation was established as AcH (i) by comparison of its gas-chromatographic retention time with that of reference AcH and (ii) by the disappearance of the gas-chromatographic peaks for both the unknown and reference AcH upon addition of 0.1 M sodium bisulfite to the respective incubation mixtures prior to gas chromatography. Zero time values were subtracted for all calculations. Standard curves for the range 50–500 nmoles AcH were constructed by introducing known amounts of a standard solution of redistilled AcH into sealed vials containing the incubation mixture at 0°. The vials were swirled and thereafter treated in the same way as experimental samples. The recovery of AcH from such samples was 97.5 + 3.0% (N = 20) of that obtained using vials containing only AcH in distilled water.

Statistical analysis was performed using Student's *t*-test for measuring the significance of the difference between the means of independent groups.

RESULTS

Characterization of the formation of AcH from paraldehyde by microsomes. Incubation of the mouse liver microsomal fraction with paraldehyde in the presence of NADPH and air resulted in the formation of AcH. The rate of AcH generation was a linear function of the concentration of protein up to 1 mg/0.5 ml of incubation mixture (Fig. 1a). It was linear with time for the first 10 min of incubation at 50 (Fig. 1b), 25, 10, and 5 mM paraldehyde concentrations. Both of these linearity characteristics continued to be observed when microsomes isolated from mice pretreated with phenobarbital, 3-methylcholanthrene, or SKF-525A were employed. In all subsequent experiments, a 5-min incubation time was used to ensure that the initial rates of AcH formation were being measured.

When the effect of varying concentration of paraldehyde on the rate of formation of AcH by microsomes was investigated, saturation kinetics were observed. The double-reciprocal plot for the formation of AcH was linear (Fig. 2); the apparent K_m calculated for paraldehyde was 17.9 mM while the V_{max} equaled 40.1 nmoles/mg protein/min.

Cofactor requirement for the formation of AcH from paraldehyde by microsomes. The enzymatic nature of the reaction was demonstrated by the lack of any detectable AcH formation in the absence of microsomal protein or when the microsomes had been heated (5 min at 100°) prior to incubation (Table 1).

An absolute requirement for oxygen was render

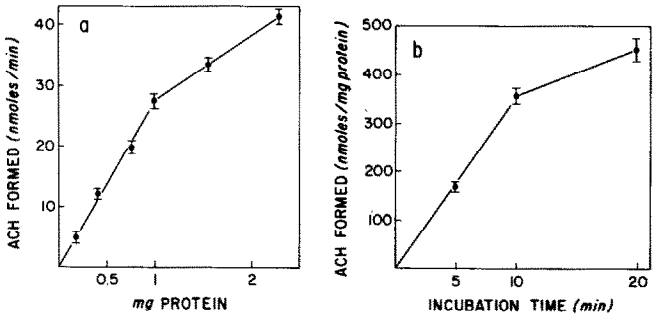

Fig. 1. (a) Formation of AcH from paraldehyde by mouse liver microsomes as a function of protein concentration. Incubations were carried out for 5 min in the presence of 1 mM NADPH, 50 mM paraldehyde and various amounts of microsomal protein from untreated mice. Each point is the mean ± S.E. of three incubations. (b) Formation of AcH from paraldehyde by mouse liver microsomes as a function of time. Incubations were carried out in the presence of 1 mM NADPH, 50 mM paraldehyde and 0.167 mg of microsomal protein from untreated mice. Each point is the mean ± S.E. of four incubations. See Materials and Methods for details of reaction mixture content, incubation, sampling, and analysis.

evident by the complete inactivity of the system when the incubations were carried out in an atmosphere of nitrogen instead of air. Although it was necessary to carry out the incubations in sealed vials, due to the volatility of AcH, the oxygen content of the air in the vials was shown to be sufficient for the maximal rate of the reaction since incubation in a pure oxygen atmosphere did not increase the rate above that observed with air (Table 1).

No generation of AcH was observed in the absence of NADPH. The standard 1 mM NADPH concentration used was sufficient to saturate the system since doubling of the NADPH concentration did not result in a significant increase in the reaction rate. Although NADH also supported the reaction, the rate of AcH production was only 14% of the NADPH-supported rate. When both reduced pyridine nucleotides were present in the incubation medium at a concentration of 1 mM, the rate of AcH formation was significantly higher ($P < 0.05$) than the sum of the rates obtained with each cofactor separately (Table 1).

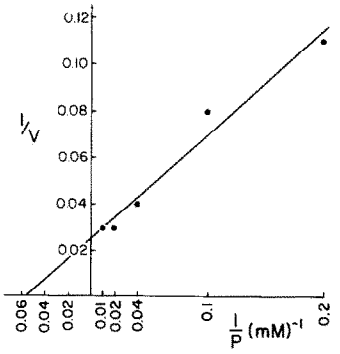

Fig. 2. Double-reciprocal plot of the rate of AcH formation as a function of paraldehyde concentration. The ordinate is the reciprocal of the initial rate expressed in nmoles/mg protein/min. The line was fitted by regression analysis. Incubations were carried out for 5 min as described under Materials and Methods except that substrate concentration was varied as shown in the figure.

Effect of the modifiers of monooxygenase activity. The influence of inducers and inhibitors of the cytochrome P-450 monooxygenase system on the rate of AcH formation from paraldehyde is shown in Fig. 3. Microsomes from phenobarbital-pretreated mice were found to have a 2.5-fold greater rate of AcH generation from paraldehyde than those from saline-pretreated controls. Pretreatment with 3-methylcholanthrene resulted in only a 0.6-fold increase. *In vivo* pretreatment with SKF-525A resulted in the inhibition of the rate of microsomal AcH generation by 54%.

Table 1. Cofactor requirements for the formation of AcH from paraldehyde in mouse liver microsomes*

Experimental condition	AcH formed (nmoles/mg protein/min)	N
Standard incubation†	32.8 ± 3.0	11
Incubation under O_2 atmosphere‡	31.3 ± 3.3	7
Incubation under N_2 atmosphere‡	0	3
Microsomes omitted	0	3
Microsomes heated (5 min at 100°)	0	3
NADPH omitted	0	3
NADPH doubled (2 mM)	39.3 ± 4.0	3
NADPH replaced by 1 mM NADH	4.5 ± 0.8§	3
Standard incubation + 1 mM NADH	47.4 ± 2.6‖	3

* Data are expressed as means ± S.E.; N is the number of experiments.

† Incubations were carried out for 5 min and contained 100 mM phosphate buffer (pH 7.5), 5 mM $MgCl_2$, 5 mM nicotinamide, 1 mM NADPH, 0.2 to 0.5 mg microsomal protein, and 50 mM paraldehyde.

‡ Gas was bubbled through the mixture for 2 min prior to the start of incubation in a sealed vial.

§ Significant difference from standard incubation, $P < 0.001$.

‖ Significant difference from standard incubation, $P < 0.05$.

The incubation of control microsomes under an atmosphere containing 80% carbon monoxide and 20% oxygen resulted in the dramatic decrease in the metabolism of paraldehyde to AcH. Under these circumstances, the rate of AcH formation was only 18% of that observed under standard conditions (Fig. 3).

In the experiments designed to characterize the reaction leading to the formation of AcH from paraldehyde, metopirone [2-methyl-1,2-bis-(3-pyridyl)-1-propanone], an inhibitor of cytochrome P-450-mediated O-dealkylation reactions [16], was used. In low concentration (10 μM) metopirone inhibited significantly ($P < 0.01$) the rate of AcH production only in phenobarbital-induced microsomes (Fig. 4). This inhibition reached 75% when metopirone concentration was increased to 1 mM. Control and 3-methylcholanthrene-induced microsomes were found to be relatively less sensitive to metopirone. The capability of these microsomal preparations to generate AcH from paraldehyde was also diminished significantly, however, with a mean inhibition of 45% being observed in the presence of 1 mM metopirone ($P < 0.005$).

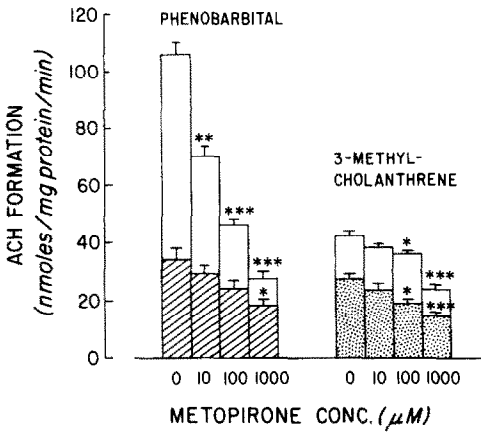

Fig. 4. Effect of metopirone on the rate of AcH generation from paraldehyde by liver microsomes from phenobarbital- and 3-methylcholanthrene-pretreated animals. Pretreatment and incubation details are in the legend of Fig. 3. Open bars: microsomes from inducer-pretreated animals; shaded bars microsomes from vehicle-injected controls. In each instance, the mean ± S.E. of three experiments is given. Significant differences from values obtained in the absence of metopirone (leftmost bar of each group) are represented as follows: (***) $P < 0.001$, (**) $P < 0.01$, and (*) $P < 0.05$.

DISCUSSION

We have reported previously that in the process of paraldehyde metabolism there was *in vivo* formation of AcH, although the levels of AcH were too low to be directly detectable in tissues or in the expired air [10–12]. Our current finding that AcH is produced upon incubation of mouse liver microsomes with paraldehyde is congruent with these earlier findings and the previously presented evidence suggesting the *in vivo* participation of the liver microsomal drug-metabolizing system in the biotransformation of paraldehyde [11, 12]. Hepatic involvement in paraldehyde metabolism has been suggested earlier on the basis of observations that liver injury evoked by deep chloroform anesthesia [17], bile duct ligation, or by carbon tetrachloride [18, 19] resulted in a decrease in the rate of paraldehyde disappearance from blood.

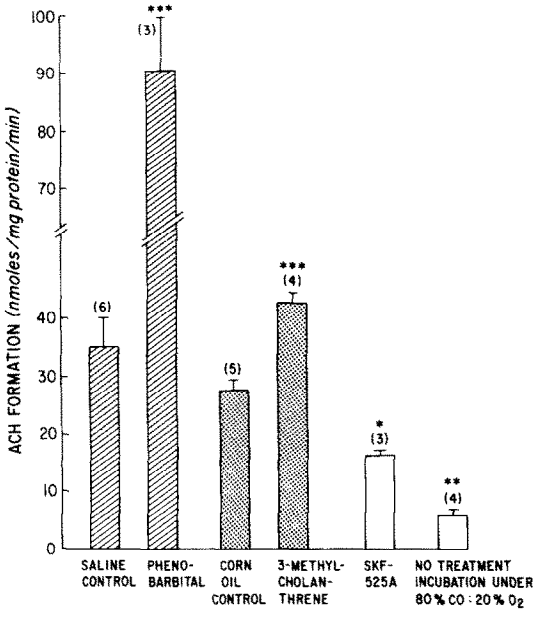

Fig. 3. Effects of modifiers of monooxygenase activity on the rate of microsomal AcH generation from paraldehyde. Incubations were carried out for 5 min in the presence of 1 mM NADPH, 50 mM paraldehyde and 0.2 to 0.6 mg of microsomal protein. See Materials and Methods for phenobarbital, 3-methylcholanthrene, and SKF-525A pretreatment schedules. For incubation under CO/O_2, gas was bubbled through the mixture for 2 min prior to the start of incubation in a sealed vial. Bars represent means ± S.E. Numbers of experiments are shown in parentheses. Significant differences from respective controls are represented as follows: (***) $P < 0.001$, (**) $P < 0.01$, and (*) $P < 0.05$. The values for the two control groups were not significantly different.

Paraldehyde is a trimer of AcH. In the presence of acid, it can depolymerize to AcH [20]. At a neutral pH, the rate of depolymerization has been reported to be infinitely slow [21]. Nonetheless, we were particularly concerned to determine whether the observed generation of AcH from paraldehyde during the incubation was truly enzymatic. We found the rate of formation of AcH to be proportional to the quantity of protein present and saturable with increasing paraldehyde concentration. Omission of microsomes from the incubation mixture abrogated AcH formation, as did denaturation of microsomal protein through heating. It follows that the formation of AcH from paraldehyde is an enzymatic process. The K_m for the process (17.9 mM), although relatively high, is comparable to values observed for other xenobiotic substrates of the cytochrome P-450 monooxygenase system [22], and of the same order of magnitude as the K_m for ethanol metabolism by the microsomal ethanol-oxidizing system [23]. Following the preliminary publication of these results [13], Zera and Nagasawa [14] reported that rat liver microsomes generate AcH when incubated with par-

aldehyde. Since neither the kinetic constants for this process nor the concentration of paraldehyde employed were reported, quantitative comparisons of the data in the two species are precluded.

It was of interest to calculate the degree to which the observed *in vitro* and *in vivo* rates of paraldehyde metabolism corresponded. Given a rate constant of 0.0121 min^{-1} for the first step in the metabolism of paraldehyde [11] and a body burden of, say, 400 mg/kg, one obtains an *in vivo* rate of paraldehyde metabolism of 4.84 mg/kg/min. The volume of distribution for paraldehyde is 840 ml/kg [11] and thus a body burden of paraldehyde of 400 mg/kg would result in a blood concentration of 476 mg/l or 3.60 mM. From the Michaelis–Menten relationships and the observed values for K_m and V_{max} *in vitro* (17.9 mM and 40.1 nmoles/mg microsomal protein/min) it can be calculated that the rate of acetaldehyde formation from paraldehyde at this concentration would be 6.71 nmoles/mg protein/min. Given that the mouse liver, which constitutes 6.5% of body weight [24], contains, on average, 63.1 mg microsomal protein/g [25], it can be shown that the rate of acetaldehyde formation from paraldehyde would be 3.64 mg/kg/min. The relatively close agreement between the values obtained supports the hypothesis that the two processes correspond and that the first step in the *in vivo* metabolism of paraldehyde is the formation of acetaldehyde.

The generation of AcH is probably a limiting step in the overall metabolism of paraldehyde *in vivo*. The rate constant for the first step in the *in vivo* metabolism of paraldehyde is much lower than those of other processes controlling its *in vivo* disposition [11]. It is generally recognized that *in vivo* any micromolar concentrations of AcH formed can be rapidly and efficiently removed by aldehyde dehydrogenase-catalyzed oxidation to acetate and then to carbon dioxide [26, 27]. This enzyme is most likely responsible for blood levels of AcH remaining negligible during metabolism of ethanol as long as liver concentrations of AcH do not exceed 0.1 mM and aldehyde dehydrogenase inhibitors are not coadministered [28, 29]. The absence of AcH from the blood and breath of animals [10, 11, 19, 30] and humans [31] administered paraldehyde may be ascribed to the effectiveness of this enzyme system in removing AcH generated from paraldehyde.

Evidence for the role of cytochrome P-450 mixed function oxygenase in the generation of AcH from paraldehyde accrued from (1) the requirements of the reaction, (2) the effect of inhibitors, and (3) inducers of the monooxygenase system. Thus, the reaction was observed to have an absolute requirement for molecular oxygen and a strong NADPH dependence. Although NADH could also act as the source of reducing equivalents, the resulting reaction rate was much lower. In the presence of both reduced pyridine nucleotides, the rate of AcH formation was significantly higher than the arithmetic sum of the rates seen with each nucleotide singly. This finding is not unique; there are several reports that the simultaneous presence of NADPH and NADH result in a higher than additive rate of some mixed function oxidation reactions [32–34]. The phenomenon has been attributed to the interaction of cytochrome b_5 with the electron transport chain containing cytochrome P-450 [35, 36].

Second, carbon monoxide, an agent which combines with cytochrome P-450 and is a potent inhibitor of cytochrome P-450-catalyzed reactions [37], inhibited AcH formation, as did pretreatment of mice with SKF-525A, another cytochrome P-450-dependent monooxygenase inhibitor [38, 39]. The latter result is fully consistent with our previous finding *in vivo* [10, 11]. It should be mentioned that the *in vitro* addition of SKF-525A, at a concentration (0.5 mM) known to inhibit the metabolism of other xenobiotics [34, 40], did not affect the formation of AcH from paraldehyde (results not shown). Zera and Nagasawa [14] in a similar experiment with rat liver microsomes reported *in vitro* addition of SKF-525A to lead to stimulation rather than inhibition of AcH generation.

Finally, in experiments employing microsomes from mice pretreated with phenobarbital or 3-methylcholanthrene, potent inducers of the cytochrome P-450 microsomal monooxygenase system known to increase microsomal content of different forms of cytochrome P-450 with different but overlapping substrate specificities [41–43], AcH formation was found to be stimulated significantly (Fig. 3). Pretreatment with phenobarbital resulted, however, in an almost three times greater stimulation than did pretreatment with 3-methylcholanthrene, suggesting that the conversion of paraldehyde to AcH may be catalyzed predominantly by the phenobarbital-inducible form of cytochrome P-450. Further, only phenobarbital-induced microsomes showed a high susceptibility to inhibition by low concentration of metopirone (Fig. 4). Metopirone is a differential inhibitor, with a higher binding affinity for the phenobarbital-inducible variant of cytochrome P-450 than for that induced by polycyclic hydrocarbons [39].

Metopirone has been shown to inhibit the cytochrome P-450-mediated O-dealkylation of 7-ethoxycoumarin [16, 44], a reaction which like the formation of acetaldehyde from paraldehyde is stimulated several times more by phenobarbital than 3-methylcholanthrene pretreatment of mice [44]. More recently, it has been reported that diethyl ether is cleaved to AcH by a cytochrome P-450-containing monooxygenase system in a reaction analogous to O-dealkylation [45] and that the process is inhibited by metopirone [46]. Our results suggest that paraldehyde, which is a cyclic ether, is also cleaved by a process analogous to O-dealkylation.

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