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PRODUCTION OF ACETALDEHYDE AND ETHANOL BY ISOLATED RAT LIVER PARENCHYMAL CELLS IN THE PRESENCE OF DIETHYL ETHER

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Abstract—Isolated rat liver parenchymal cells incubated with anaesthetic concentrations of diethyl ether were shown to produce acetaldehyde and ethanol in a dose dependent manner. The acetaldehyde and ethanol production from ether was stimulated in hepatocytes derived from phenobarbital treated rats and could be only partially inhibited by 4-methyl pyrazole (250 μM). The present results support previous suggestions that diethyl ether is metabolized by an inducible microsomal enzyme system which cleaves diethyl ether in a reaction analogous to the well known *O*-dealkylation reactions.

Although it is now well known that diethyl ether is metabolized by mammalian tissues [1–4], the mechanism of its breakdown and the first intermediates formed during this process are as yet undefined. Several authors have suggested that ethanol and acetaldehyde may be the first products of diethyl ether metabolism by microsomal enzyme systems [1–4], but this idea has been supported mainly by studies which have demonstrated enzymic cleavage of aromatic and halogenated ethers to produce alcohol and aldehyde products [5–7]. However, the results of some recent studies have provided more direct evidence for the formation of acetaldehyde during diethyl ether metabolism. Aune *et al.* [8] were able to measure acetaldehyde in the blood of diethyl ether-anaesthetized humans, and Chengelis and Neal [9] demonstrated what appeared to be acetaldehyde production by rat hepatic microsomes *in vitro* in the presence of diethyl ether. Unfortunately, the analytical method employed for the *in vitro* study was not specific for acetaldehyde [10] and it is very difficult to interpret human blood acetaldehyde data when large corrections must be made for the spontaneous formation of acetaldehyde occurring during treatment of human blood samples prior to assay [8, 11].

In this report, we describe the use of an isolated rat hepatocyte system combined with sensitive and specific gas chromatographic analysis which enabled us to observe the production of both acetaldehyde and ethanol by hepatocytes exposed to anaesthetic concentrations of diethyl ether.

MATERIALS AND METHODS

Chemicals. Collagenase (Type I), bovine serum albumin (Fraction V, defatted), HEPES (N-2-hydroxyethylpiperazine N'-2-ethane sulfonic acid) and cyanamide (carbodiimide) were obtained from the Sigma Chemical Company (St. Louis, MO). Sodium phenobarbital was obtained from the Norwegian Drug Monopoly (Oslo, Norway) and 4-methyl pyrazole was purchased from Polysciences, Inc. (Warrington, PA). Diethyl ether and acetaldehyde were obtained from Merck (Darmstadt, West Germany) and were analytical grade. Acetaldehyde was redistilled prior to use.

Animals. Male Wistar rats (220–340 g) from Möllegaards Avlslaboratorium (Skensved, Denmark) were kept on a 12 hr light–dark cycle. All animals were fasted for 18–20 hr prior to sacrifice but had free access to rat chow from Felleskjøpet A/S, Oslo, Norway, and tap water before the fasting period.

To obtain hepatocytes with barbiturate induced enzyme systems, some rats were given intraperitoneal doses of sodium phenobarbital (80 mg/kg) in isotonic NaCl solution, approximately 44 and 20 hr prior to sacrifice. For this study, control animals were injected with isotonic NaCl only.

Liver cell preparation and incubation. Isolated rat liver parenchymal cell suspensions (1.5–5.3 g wet wt/100 ml) were prepared by collagenase perfusion of rat livers as described previously [12, 13]. Aliquots of these suspensions (5, 10 or 20 ml) were incubated at 37° under air in sealed flasks (0.5 or 1.0) l. with constant gentle shaking in a medium containing 140 mM NaCl, 5.4 mM KCl, 0.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 0.34 mM Na_2HPO_4, 0.35 mM KH_2PO_4, 20 mM HEPES, 5.5 mM glucose and 2 per cent bovine serum albumin (pH = 7.5). All incubations lasted for 65 min, inhibitor addition (see below) occurring at 5 min and ether and ethanol addition at 35 min. Dead cells were prepared by freezing and thawing cells suspended in the above medium.

For each experiment involving phenobarbital induced hepatocytes, cells from a phenobarbital induced rat liver and a control liver were incubated simultaneously after matching the test and control suspensions with respect to cell concentration.

Inhibitor, ether and ethanol treatment. The enzyme inhibitors cyanamide and 4-methyl pyrazole were

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added to cell suspensions as 200 × concentrated solutions in isotonic NaCl to give final concentrations of 400 and 250 μM respectively. Pure diethyl ether was added to cell suspensions to give the desired final concentrations. Ethanol was added as concentrated solutions ranging from 10 mM to 4 M, in isotonic NaCl. None of the above additions alone or in combination increased the suspension volumes by more than 2 per cent.

Sampling and analysis of ether, ethanol and acetaldehyde. After ether or ethanol addition, cell suspension samples (3 or 2 ml) were taken at intervals via a syringe attached to a Venflon® 3-way stopcock (Viggo AB, Helsingborg, Sweden) fitted to an 18 gauge syringe needle penetrating a rubber stopper sealing the incubation flask. The samples were immediately injected into 10 ml Venoject® tubes (Terumo Corp., Haasrode, Belgium) containing one vol. of ice-cold 1M $HClO_4$, centrifuged at 4° and aliquots (2 ml) of the resulting protein-free supernatants were analyzed for acetaldehyde, ethanol and ether by head-space gas chromatography. Samples were kept sealed at 4° and stored for no longer than 8 hr.

A Perkin–Elmer F42 head space gas chromatograph equipped with a 1 m × 2 mm i.d. stainless steel column packed with Porapak-Q, 100/120 mesh (Supelco, Inc., Bellefonte, PA) was used. Operating conditions were as follows; sample thermostat: 65°, injector temperature: 200°, column temperature: 150°, detector temperature: 200°, and carrier gas (N_2) flow rate: 19 ml/min.

Aqueous external standards were used for ethanol, acetaldehyde and ether and recoveries of these compounds when added to $HClO_4$ treated cell suspensions were identical to recoveries from aqueous solutions. Retention times for acetaldehyde, ethanol and ether were 1.7 min, 3.1 and 5.2 min respectively. Acetone and methanol had retention times of 1.5 and 4.6 min respectively but of these compounds, only acetone was seen in cell samples. Sensitivity limits for acetaldehyde and ethanol were 0.5 and 5 μM respectively, corresponding to 1 and 10 μM in the undiluted cell suspensions.

When liver cell suspensions containing either ethanol or ether were treated with $HClO_4$ some acetaldehyde was generated. Such spontaneous formation of acetaldehyde from ethanol and ether during deproteinization of biological samples has been observed previously [8, 11]. In this study, acetaldehyde formed as a result of deproteinization gave concentrations no greater than 1.5 μM in the $HClO_4$ supernatants and were determined essentially by the method described by Stowell *et al.* [14]. All acetaldehyde data presented have been corrected for this artifact.

Other analyses. The percentage of viable cells existing before and after the 65 min incubation periods was estimated by the trypan-blue exclusion method [15]. Cells were counted in a Bürker Chamber. Periodic determinations of cell ATP content were also carried out using the enzymic method described by Lamprecht and Trautschold [16], employing the lower glucose concentration as suggested by Lund *et al.* [17]. The total protein concentration of each cell suspension was determined using a Biuret method [18], packed cells being homogenized prior to protein analysis.

RESULTS

Cell viability

For all experiments, the percentage of viable cells in the freshly prepared washed parenchymal cell suspensions was 95 ± 4 ($\bar{x}$ ± S.D., N = 27). For control 65 min incubations with cyanamide only, the percentage of viable cells remaining after the incubation period was 90 ± 7 ($\bar{x}$ ± S.D., N = 27). None of the different treatments used resulted in cell viabilities which were significantly different from those measured for cells treated with cyanamide only.

Freshly prepared cells were found to have ATP concentrations close to those found *in vivo*, i.e. 2.5–3.6 μmoles/g wet wt and incubation of cells for 65 min without additions or with cyanamide resulted in no more than a 10 per cent decrease in cell ATP content. Addition of 20 mM ethanol and 30 mM ether to the cell suspensions containing cyanamide resulted in mean decreases in cell ATP concentrations of 4 and 16 per cent respectively compared to control cells treated with cyanamide only. The effects of ethanol and ether on cell ATP content were additive.

Acetaldehyde accumulation in ether treated hepatocyte suspensions in the absence of enzyme inhibitors

In the absence of enzyme inhibitors, low concentrations of acetaldehyde (2–5 μM) could be detected in cell suspensions incubated with 30 mM ether while no acetaldehyde could be detected after incubation of cells in medium without ether, or during incubation of cell-free medium in the presence or absence of 30 mM ether.

As a positive control, cells were also incubated in the presence of ethanol (20 mM), a compound known to be metabolized via acetaldehyde, but under the above conditions no acetaldehyde could be detected in the ethanol treated cell suspensions.

Accumulation of acetaldehyde and ethanol in ether treated hepatocyte suspensions in the presence of cyanamide

In view of the above results, an attempt was made to optimize conditions for acetaldehyde detection by treating the cells with an aldehyde dehydrogenase (E.C. 1.2.1.3) inhibitor, cyanamide [19]. In preliminary experiments with cell suspensions of 2.3 g wet wt/100 ml, cyanamide (400 μM) increased the half life of added acetaldehyde (200 μM) from 3 to 18 min and in positive control experiments with 20 mM ethanol added to cyanamide treated cell suspensions, acetaldehyde was found to accumulate throughout the 30 min incubation period to concentrations ranging from 227 to 399 μM.

When cyanamide treated cell suspensions (2.3 g wet wt/100 ml) were incubated in the presence of varying concentrations of ether, a concentration dependent production of acetaldehyde was clearly observed (Fig. 1a). In addition, a simultaneous accumulation of ethanol was seen (Fig. 1b).

Quantitatively, this accumulation of acetaldehyde

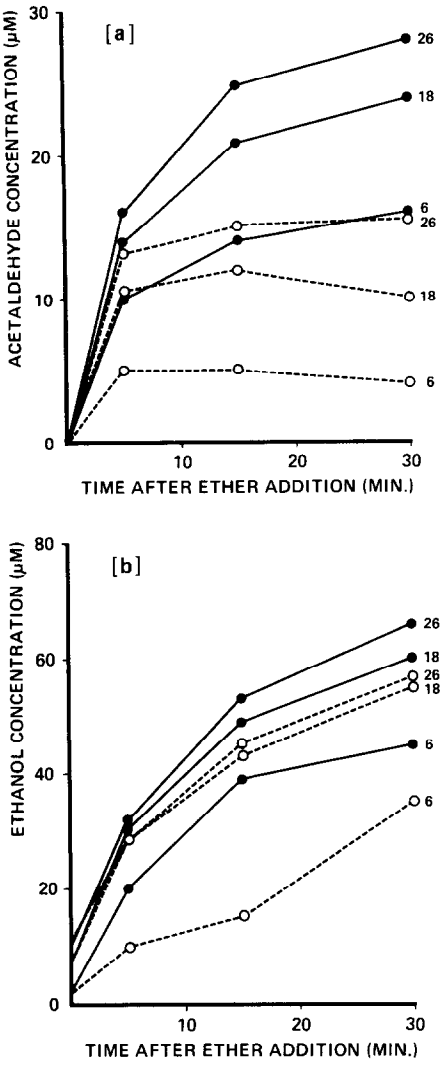

Fig. 1. Accumulation of acetaldehyde (a) and ethanol (b) in ether treated hepatocyte suspensions in the presence of cyanamide. The results were obtained from a single experiment in which six identical aliquots from a cell suspension obtained from a single rat liver were incubated in the presence of 400 μM cyanamide plus diethyl ether in the absence (●——●) or presence (○-----○) of 250 μM 4-methyl pyrazole. The ether concentration (mM) used for each incubation is shown near the last point on each experimental curve.

and ethanol varied from experiment to experiment, maximum acetaldehyde concentrations in the presence of 26 mM ether, ranging from 10 to 43 μM ($\bar{x}$ = 22 μM, N = 5), with maximum ethanol concentrations ranging from 30 to 100 μM ($\bar{x}$ = 60 μM, N = 5).

The results of only a single experiment are presented in Fig. 1, but the results of other identical experiments were qualitatively similar. In every case, the addition of 4-methyl pyrazole caused some inhibition of both acetaldehyde and ethanol accumulation.

In seven experiments with more concentrated cell suspensions containing 3.5–5.3 g wet wt cells/100 ml in the presence of cyanamide and 30 mM ether, maximum acetaldehyde concentrations ranged from 16 to 61 μM ($\bar{x}$ = 31 μM) and maximum ethanol concentrations ranged from 50 to 125 μM ($\bar{x}$ = 85 μM).

Control experiments

The ether used for the present study was found to contain contaminating ethanol which was sufficient to give initial ethanol concentrations in the cell suspensions of approximately 10 μM when 30 mM ether was used. Therefore, in experiments with cells incubated in the absence of the alcohol dehydrogenase (E.C.1.1.1.1) inhibitor 4-methyl pyrazole [20], some of the accumulated acetaldehyde (maximum 10 μM) could have resulted from the oxidation of this contaminating ethanol. However, in six control experiments in which ether free cells were treated with cyanamide and ethanol (50 μM), acetaldehyde was undetectable in all cases after 30 min incubation. In addition, when ether free cells were treated with cyanamide, ethanol and 4-methyl pyrazole, no acetaldehyde could be detected in the presence of ethanol concentrations up to 1 mM. No acetaldehyde or ethanol could be detected in cell suspensions treated with 4-methyl pyrazole or cyanamide alone or in combination.

Suspensions of dead cells (no cells excluding trypan blue) treated with cyanamide and ether produced no detectable concentrations of ethanol or acetaldehyde.

Production of acetaldehyde and ethanol from diethyl ether by phenobarbital induced hepatocytes

Hepatocytes prepared from phenobarbital induced livers produced markedly greater concentrations of ethanol and acetaldehyde from ether (17 mM) than did the non-induced cells (Fig. 2). This difference occurred despite the fact that the two types of cell suspension were found not to differ significantly from each other with respect to mean cell viability, mean number and wet wt of cells per unit volume of medium or protein concentration. There was also no significant difference between the mean weights of the control and induced rats from which the hepatocytes were obtained.

As observed in the previous experiments, 4-methyl pyrazole markedly reduced the cyanamide enhanced acetaldehyde accumulation, but its effect on ethanol accumulation was not statistically significant in the four experiments conducted with induced and non-induced cells in the presence of 17 mM ether.

Control incubations of both types of cells with no additions or with cyanamide resulted in no detectable production of acetaldehyde or ethanol.

Ether concentrations

Throughout the 30 min incubation periods, ether concentrations in all cell suspensions were found to decrease slowly, initial concentrations being reduced by up to 15 per cent. The mean decrease was only about 5 per cent, however, and no significant differences existed between mean ether concentrations in any of the experimental groups represented in Fig. 2.

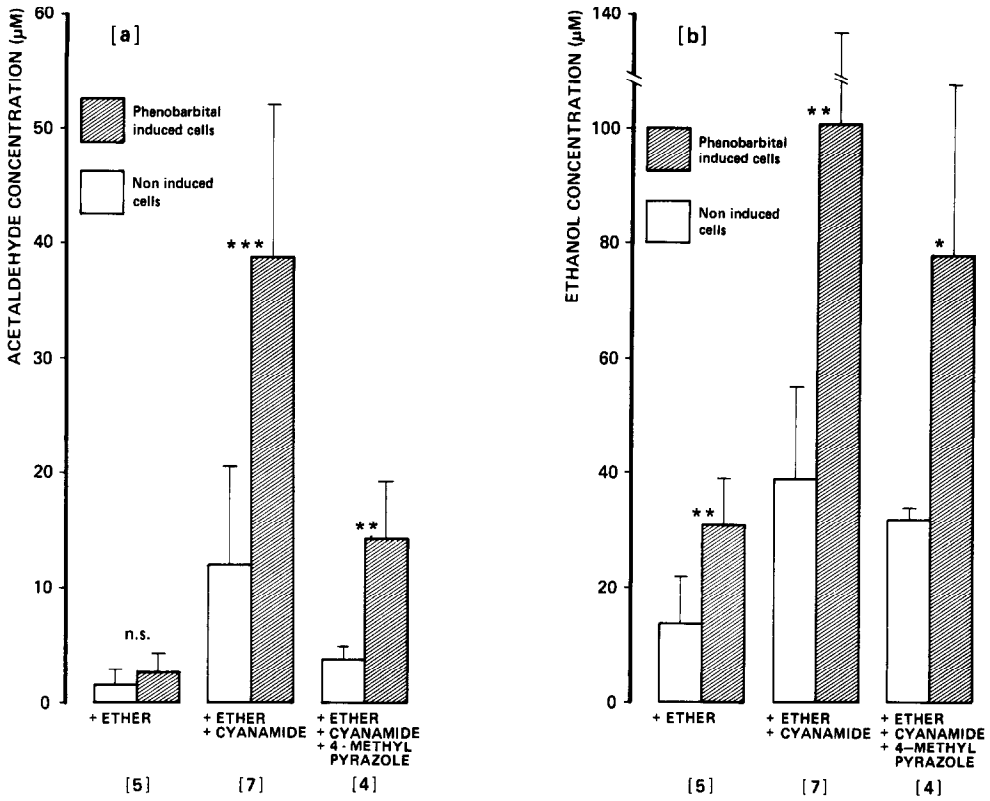

Fig. 2. Mean acetaldehyde (a) and ethanol (b) concentrations measured in suspensions of phenobarbital induced and non-induced hepatocytes after 30 min incubation in the presence of 17 mM diethyl ether. Hepatocytes were prepared and incubated as described in Methods. The significances of differences between phenobarbital induced and non-induced cells were determined using Students' *t*-test for independent variables and are represented as follows: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, and n.s. = not significant. Numbers in parentheses at the bottom of the figure refer to number of experiments.

Ether losses from cell free medium were of a similar magnitude to those observed in incubations containing cells.

DISCUSSION

The present results constitute what to our knowledge is the first evidence for a concentration dependent production of ethanol and acetaldehyde from diethyl ether by hepatocytes. The high percentage of hepatocytes excluding trypan blue and the close to normal cell ATP concentrations were assumed to indicate that most of the cells used for the present study were metabolically active throughout the 65 min incubation period employed. If this assumption is correct, then the fact that dead cells produced no measurable acetaldehyde or ethanol in the presence of ether, may be regarded as good evidence for a metabolic, rather than a purely physico-chemical conversion of ether to acetaldehyde and ethanol.

Neither the acetaldehyde nor the ethanol measured in hepatocyte suspensions after 30 min incubation with cyanamide and ether could have been derived completely from the small amount of ethanol found as a contaminant of the ether. The sum of the concentrations of accumulated acetaldehyde and ethanol was most often more than an order of magnitude higher than the maximum concentration of contaminating ethanol (about 10 μM). Furthermore, incubation of ether treated cells with 4-methyl pyrazole did not abolish the accumulation of acetaldehyde whereas 4-methyl pyrazole completely inhibited acetaldehyde accumulation in hepatocyte suspensions containing added ethanol at a concentration 100 fold higher than the maximum concentration of contaminating ethanol. In this study, 4-methyl pyrazole would be expected to inhibit ethanol metabolism by at least 92 per cent [21].

The lack of detectable acetaldehyde in cell suspensions treated with ethanol only (20 mM) was not unexpected as other workers have reported similar findings [21, 22]. It is therefore difficult to explain why, in the absence of cyanamide, low but detectable acetaldehyde concentrations were found when hepatocytes were incubated with ether. This result is especially surprising when it is considered that the acetaldehyde concentrations resulting from incubation of cyanamide treated cells with ethanol (20 mM) were about ten fold higher than those produced in similar cells treated with ether (30 mM). This discrepancy might be explained if ether has a weak inhibitory effect on mitochondrial acetaldehyde

metabolism. This could then represent some general inhibitory effect on mitochondrial function, also demonstrated by the effect of ether on hepatocyte ATP content and the inhibition of mitochondrial respiration [23].

It has previously been suggested that diethyl ether metabolism in mammals may be accomplished by a hepatic cytochrome P-450 mono-oxygenase system in a reaction analogous to the *O*-dealkylation observed for compounds such as ethoxycoumarin [9]. If this were so, acetaldehyde and ethanol would be the expected first products of the oxidative cleavage of diethyl ether. The fact that phenobarbital is a potent inducer of this mono-oxygenase system [6, 24] and that hepatocytes prepared from phenobarbital induced rats produced greater quantities of both ethanol and acetaldehyde from ether than did control cells, supports the above hypothesis. Since the inhibitory effect of cyanamide on aldehyde dehydrogenase is not dependent on its metabolism [19], it is unlikely that the observed differences between control and phenobarbital induced cells were the results of faster metabolism of cyanamide in the induced cells. Further support for the involvement of a cytochrome P-450 mediated *O*-dealkylation reaction was obtained in preliminary experiments with phenobarbital induced hepatocytes and metyrapone* (250 μM), an inhibitor of cytochrome P-450 mediated *O*-dealkylation reactions [6]. The build up of both ethanol and acetaldehyde in hepatocyte suspensions treated with cyanamide and ether was inhibited approximately 40 per cent by metyrapone (results not shown).

The possibility also exists that diethyl ether may be converted to two molecules of ethanol, with acetaldehyde being formed as a secondary intermediate only. However, this seems unlikely because 4-methyl pyrazole would then have been expected to completely eliminate acetaldehyde build up. The 4-methyl pyrazole mediated inhibition of acetaldehyde accumulation (Figs. 1 and 2) may have been due to inhibition of the postulated ether cleaving cytochrome P-450 system rather than alcohol dehydrogenase. It has been shown that pyrazole inhibits some types of cytochrome P-450 enzyme systems [25]. However, our results do not exclude the possibility that alcohol dehydrogenase might be involved either directly or indirectly in the formation of some acetaldehyde from ether.

If ether cleavage results in the production of equal numbers of ethanol and acetaldehyde molecules as expected for a dealkylation reaction then equal concentrations of acetaldehyde and ethanol might be expected to accumulate. However, the absence of equal concentrations of accumulated ethanol and acetaldehyde in this study is most likely due to differences between the rates of ethanol and acetaldehyde metabolism. When ethanol and acetaldehyde were added to cell suspensions to give concentrations similar to those found in the presence of ether, acetaldehyde was always seen to decay at a faster rate than ethanol, both in the absence and presence of cyanamide and especially when 4-methyl pyrazole was present (result not shown).

* 2-methyl-1,2-di-pyridyl-1-propanone.

In conclusion, our results provide support for those previous studies which indicated that diethyl ether may be metabolized to acetaldehyde [6, 8] and in addition, it seems that ethanol is another important breakdown product. At present it may only be speculated whether these findings are related to the sympathomimetic effect of ether in man [26], an effect which is also induced by acetaldehyde [27, 28].

The metabolism of ether at anaesthetic concentrations [29] to ethanol and acetaldehyde may also have important consequences in studies of ethanol effects and metabolism performed *in vivo* with experimental animals under ether anaesthesia. The possibility that ether might mimic some of the adverse effects of ethanol should not be ruled out, especially when the toxicity of acetaldehyde is considered [30].

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