Biochemical Pharmacology, Vol. 29, pp. 247–248.

0006-2952/80/0115–0247 $02.00/0

SHORT COMMUNICATIONS

Microsomal metabolism of diethyl ether

(*Received* 6 *July* 1979; *accepted* 6 *August* 1979)

Throughout much of its long history as a general anesthetic, diethyl ether was believed to be expired unchanged following administration. Recently, however, several investigators have shown that diethyl ether is not 100 per cent recoverable [1–4] and that as much as 5 per cent is metabolized [3]. The following pathway of diethyl ether metabolism has been proposed [3,4]:

$$CH_3CH_2\text{-}O\text{-}CH_2CH_3 \rightarrow CH_3CH_2OH + CH_3CHO \rightarrow$$

$$CH_3COOH \rightarrow CO_2, \text{ fatty acids, cholesterol, etc.}$$

Diethyl ether is cleaved to acetaldehyde and ethanol, which are rapidly oxidized to acetate by well-characterized enzymes [1]. The acetate subsequently enters the 2-carbon pool of intermediary metabolism. The initial step, ether cleavage, has not been well characterized despite its obvious importance in the above scheme.

Diethyl ether has been shown to be an inducer of the hepatic cytochrome P-450-containing mono-oxygenase enzyme system [5,6]. Diethyl ether also appears to inhibit both drug clearance *in vivo* [7] and microsomal drug metabolism *in vitro* [8]. Our own work has shown that diethyl ether also inhibits *p*-nitroanisole metabolism in isolated hepatocytes.* Microsomal preparations demethylate the ether methoxyflurane [9]. These observations prompted us to examine rate liver microsomes for the ability to metabolize diethyl ether.

Rats were treated with phenobarbital (80 mg/kg, i.p.) for 3 days prior to being killed (24 hr after final injection). Hepatic microsomes were prepared by the method of Kamataki and Kitagawa [10]. The incubations consisted of $NADP^+$ (0.15 mM), $MgCl_2$ (6.25 mM), glucose-6-phosphate (2.5 mM), EDTA (1.0 mM), glucose-6-phosphate dehydrogenase (5 I.U.), microsomal protein (2 mg) and diethyl ether (4.8 mM, added by microsyringe) in 50 mM potassium phosphate buffer, pH 7.6. The final volume was 2.0 ml, and incubations were conducted at 37° in stoppered 25 ml Erlenmeyer flasks equipped with a centerwell.

Acetaldehyde formation was determined as follows; the reactions were terminated by placing the flasks on ice and adding 0.1 ml of 46% perchloric acid. One ml of 0.2% 3-methyl-2-benzylthiazolinone hydrazone hydrochloride (Aldrich Chemical Co., Milwaukee, WI) in 50 mM glycine buffer, pH 3.5, was added to the centerwell, and the flasks were stoppered and left overnight. Next, 0.5 ml of the 3-methyl-2-benzylthiazolinone hydrazone solution was withdrawn and combined with 0.5 ml of 0.2% freshly prepared ferric chloride. After 5 min, 2.0 ml acetone were added and the color development was determined immediately at 663 nm. This is a slight modification of a method described elsewhere [11], is sensitive to 5 nmoles, and is linear through 200 nmoles acetaldehyde. In each experiment, 100 nmoles acetaldehyde were added to the reaction mixture described above, which was then immediately deproteinized and carried through the assay procedure as a recovery standard.

In experiments designed to characterize the reaction leading to the formation of acetaldehyde from diethyl ether, antibodies to rat cytochrome P-450 were prepared as described elsewhere [12] and added to the reaction mixture in a ratio (antibody protein/nmole of cytochrome P-450) of 5:1. Preimmune sera were added to separate reaction vessels as controls. In other experiments, the ability of carbon monoxide to inhibit microsomal diethyl ether metabolism was determined by performing the incubations under an atmosphere of oxygen and carbon monoxide. The CO/O_2 ratio (80/20) was achieved with the use of flow meters.

Preliminary experiments indicated that diethyl ether was metabolized to acetaldehyde by microsomes, and that this reaction was linear through 20 min. This reaction was characterized (Table 1) as requiring NADPH and was inhibited by both carbon monoxide and an antibody to rat liver cytochrome P-450. *B*-diethylaminoethyl diphenylpropylacetate (SKF 525-A) had no consistent effect on apparent ether metabolism, perhaps because as a diethylamine derivative, it also yields acetaldehyde upon microsomal oxidation.

These data suggest that the microsomal metabolism of diethyl ether is catalyzed by a cytochrome P-450-containing mono-oxygenase system in a reaction analogous to the *O*-dealkylation observed for other compounds, i.e. ethoxycoumarin [13]. Hence, it appears that simple aliphatic ethers should also be added to the list of microsomal mixed function oxidase substrates.

* Unpublished observation.

Table 1. Diethyl ether metabolism to acetaldehyde by phenobarbital-induced rat hepatic microsomes*

Condition	Rate (nmoles acetaldehyde/mg protein/10 min)	% Control
Complete system	37.3 ± 7.3	100
Without NADPH generating system	0	0
With preimmune serum	36.9 ± 7.4	99
With anti-P-450 antibody†	3.1 ± 0.6	8.3
With CO‡	14.3 ± 2.9	38.4

* Values are the means ± S.D. of 3–4 experiments. Reaction conditions are described in the text. Boiled microsomes or the complete system minus ether produced no acetaldehyde.

† Prepared as described [12].

‡ $CO/O_2 = 80/20$.

Acknowledgements—This work was supported by U.S.P.H.S. Grants ES 00075 and ES 00267. Training support provided by a National Research Service Award, Fellowship ES 05138, is also gratefully acknowledged.

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Biochemical Pharmacology, Vol. 29, pp. 248–250.

0006-2952/80/0115–0248 $02.00/0

The metabolism of alphaxalone by isolated rat hepatocytes

(*Received* 1 *August* 1979; *accepted* 17 *August* 1979)

Alphaxalone (3α-hydroxy-5α-pregane-11,20 dione) is a steroid anaesthetic agent which is used clinically both for the induction and maintenance of anaesthesia. Indirect evidence obtained from *in vivo* studies on rats [1] and rabbits [2] suggests that this drug is metabolized in the liver. No data relating to the rates of metabolism of alphaxalone by the liver *in vivo* or by liver preparations *in vitro* have been reported. Such data are of importance in assessing the ability of the liver to convert the drug to an inactive form under normal or pathological conditions.

Isolated hepatocytes in suspension have been used as a convenient model system to study many aspects of liver metabolism [3] including drug detoxification [4,5]. In this communication, a method is described for the measurement of the degradation of alphaxolone by isolated rat hepatocytes and the kinetic parameters of this process are reported.

Collagenase for the preparation of isolated hepatocytes was purchased from C.F. Boehringer und Soehne, Mannheim. Alphaxalone and 3β-hydroxy-5α-pregn-16-ene 11,20 dione were kindly donated by Glaxo–Allenbury's Research Ltd, Greenford, Middlesex, U.K. Bovine serum albumin was purchased from the Sigma Chemical Co., St Louis, MO, U.S.A.

Hepatocytes were prepared from normally fed male Wistar albino rats of weight 250–300 g by the method of Berry and Friend [6], as modified by Krebs *et al.* [7]. The viability of the cells was > 90%, as assessed by Trypan Blue exclusion. Cells were suspended at the appropriate concentration in Krebs–Henseleit bicarbonate buffer [8] at pH 7.4 and 37° under a gas phase of 95% O_2/5% CO_2. The incubation medium also contained 2% w/v dialysed bovine serum albumin (fraction V). The reaction was begun by the addition of the appropriate concentration of alphaxalone. Samples were withdrawn at various times and deproteinised by the addition of perchloric acid (5% w/v final concentration). Denatured protein was removed by centrifugation at 10,000 *g* for 30 sec in a bench centrifuge (Eppendorf Zentrifuge Model 3200). The supernatant was stored at −20° until assayed. Cell protein was determined by a biuret method [9] using bovine serum albumin as a standard. Alphaxalone was added to the cells as a concentrated solution in methanol. Control experiments showed that methanol at the concentrations used had no cytotoxic effects.

Alphaxalone was assayed by a gas chromatographic technique based on the method of Chambaz and Horning [10], as modified by Sear and Prys-Roberts [11] using 3β-hydroxy-5α-pregn-16-ene 11,20 dione as the internal standard. Extraction and derivatisation of alphaxalone from suspensions of isolated hepatocytes gave a recovery of 91 ± 5% (mean ± S.D. of five observations), and repeated assays of a single sample gave a coefficient of variance of < 10% (mean value 7.9%).

Figure 1 shows gas chromatograph tracings obtained from a typical experiment in which cells were incubated with 0.54 mM alphaxalone for 20 min at 37°. At the start of the incubation there is a single peak chromatographically identical with alphaxalone with an R_f value of 0.79 relative to the internal standard (Fig. 1a). After a 20 min incubation, the size of this peak was reduced and a single additional peak appeared with an R_f value of 1.11 (Fig. 1b). This peak represents the sole metabolic product of alphaxalone detected by this technique. The identity of this metabolite was not determined in the present investigation. However, other workers have shown that the primary metabolite of alphaxalone in the rat is 2α-hydroxy alphaxalone [12].

The time of course of alphaxalone disappearance from the incubation medium in a typical experiment is shown in Fig. 2. Alphaxalone was added at an initial concentration of 0.136 mM. Fifty per cent of the alphaxalone disappeared in 6.5 min and the appearance of the metabolic product followed a similar time course. In Fig. 2 the apparent concentration of the metabolite was calculated, assuming a similar chromatographic response factor to that of alphaxalone.

In order to use the isolated hepatocyte model to carry out quantitative studies on alphaxalone metabolism, it was necessary to establish that the initial rate of alphaxalone disappearance was proportional to cell protein concentration. In a series of experiments, alphaxalone was added at a near saturating concentration (136 μM) to suspensions of hepatocyte containing between 2 and 8 mg cell protein/ml. A linear relationship between initial rate of alphax-