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Comparative metabolism of bis(2-methoxyethyl)ether in isolated rat hepatocytes and in the intact rat: effects of ethanol on in vitro metabolism

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Abstract. The metabolism of the reproductive and developmental toxicant bis(2-methoxyethyl)ether (diglyme) was studied in isolated rat hepatocytes and in the intact rat. Male Sprague-Dawley rats (190–220 g) were used in both studies. Hepatocytes, isolated by a two-step in situ collagenase perfusion of the liver, were cultured as monolayers and incubated with [^{14}C]diglyme at 1, 10, 30, and 50 μM for up to 48 h. For the in vivo study, rats were given single oral doses of [^{14}C]diglyme at 5.1 mmol/kg body wt, and urine was collected for up to 96 h. Radioactive compounds in the culture medium or in the urine were separated by high performance liquid chromatography and quantified with an in-line radioactivity monitor. Metabolites were identified by comparison of their chromatographic retention times and their mass spectra with those of authentic compounds. The principal metabolite from hepatocytes and in the urine was (2-methoxyethoxy)acetic acid (MEAA). This metabolite accounted for approximately 36% of the radioactivity in the 48-h culture medium and about 67% of the administered dose in the 48-h urine. Other prominent metabolites common to both systems included 2-(2-methoxyethoxy)ethanol, methoxyacetic acid (MAA), 2-methoxyethanol, and diglycolic acid. The diglyme metabolite profiles from urine and from hepatocytes were qualitatively similar, demonstrating that, in the rat, hepatocytes serve as a good model system for predicting the urinary metabolites of diglyme. Moreover, MEAA was shown to be the metabolite best suited for use as a short-term biological marker of exposure to diglyme. Pretreatment of rats with ethanol resulted in a marked increase in the overall in vitro metabolism of diglyme. The major metabolic pathways for diglyme involve O-demethylation and cleavage of the central ether bond, and it is the latter pathway that leads to the formation of MAA, the metabolite associated with the reproductive and developmental toxicity of diglyme. The amounts of MAA formed in hepatocytes from ethanol-pretreated rats ranged from two to four times those formed in hepatocytes from untreated rats.

Key words: bis(2-Methoxyethyl)ether – Diglyme – Metabolism – Methoxyacetic acid – (2-Methoxyethoxy)acetic acid

Introduction

bis(2-Methoxyethyl)ether (diglyme; CAS Registry No. 111-96-6) belongs to a family of compounds classified as glycol ethers. Diglyme is used as an aprotic solvent in several industries, and potential occupational exposure would occur by inhalation and dermal contact. Diglyme has been shown to be a reproductive toxicant in male rats (McGregor et al. 1983; Cheever et al. 1989b; Lee et al. 1989) and male mice (McGregor et al. 1983) and a developmental toxicant in pregnant mice (Schuler et al. 1984; Plasterer et al. 1985; Hardin and Eisenmann 1987; Price et al. 1987; Daniel et al. 1991) and in rabbits (Schwetz et al. 1992). Such toxicity has been attributed to a metabolite, methoxyacetic acid (MAA), formed by the cleavage of the interior ether bond of diglyme and the subsequent oxidation of the resulting primary alcohol (McGregor et al. 1983; Cheever et al. 1988). This metabolite has been identified in the urine of rats given single po doses of diglyme (Cheever et al. 1988) and in the blood and urine of pregnant mice given embryotoxic oral doses of diglyme as well as in embryonic tissue taken from the exposed dams (Daniel et al. 1991). The metabolic pathway involving the cleavage of the central ether bond is thought to be mediated by certain cytochrome P-450 isozymes, as first evidenced by the finding that MAA is formed in

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$$CH_3-O-{}^{14}CH_2-{}^{14}CH_2-O-CH_2-CH_2-O-CH_3$$

Fig. 1. Chemical structure of [^{14}C]diglyme

significantly greater amounts in rats pretreated with phenobarbital than in rats given no such pretreatment (Cheever et al. 1989a). More recently, it was reported that the metabolism of diglyme to 2ME, a precursor of MAA, by rat liver microsomes was NADPH-dependent and that microsomes from rats pretreated with ethanol produced an increased amount of 2ME over that formed in microsomes from untreated rats (Tirmenstein 1992). In the male rat, diglyme undergoes nearly complete metabolism with greater than 83% of the dose excreted as urinary metabolites within 96 h of administration (Cheever et al. 1988). The urinary MAA accounted for only 6.2% of the administered dose, whereas the principal metabolite, identified as (2-methoxyethoxy)acetic acid (MEAA), represented 67.9% of the dose.

The objective of the present study was to determine the effect of ethanol-induced hepatic enzymes on the production of the toxic metabolite of diglyme. Since consumption of alcohol is prevalent in society, such use may result in enhanced toxicity in workers exposed to diglyme. To minimize the use of animals, isolated rat hepatocytes were evaluated as a model system for the in vivo metabolism of diglyme in this study. Previous studies have shown that hepatocytes isolated from animals of different species metabolize particular xenobiotics in a manner similar to that which occurs in the corresponding intact animal (Billings et al. 1977; Green et al. 1986; Jatoe and Gorrod 1987; Fry 1988; Jajoo et al. 1990). In the present investigation, in vitro metabolism data derived from isolated hepatocytes were demonstrated to be in qualitative agreement with previously unreported data collected during the in vivo metabolism study (Cheever et al. 1988). Using this in vitro model system, the effect of the pretreatment of rats with ethanol on the metabolism of diglyme in isolated hepatocytes was evaluated.

Materials and methods

Chemicals and solutions. The test compound bis(2-methoxyethyl)ether, having a chemical purity of >99%, was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). (1,2-ethylene-^{14}C)bis(2-methoxyethyl)ether, referred hereafter as [^{14}C]diglyme, was synthesized by Pathfinder Laboratories, Inc. (St Louis, MO) and by Chemsyn Science Laboratories (Lenexa, KS) with specific activities of 0.88 and 29.8 mCi/mmol, respectively. The chemical structure of this radioisotope is shown in Fig. 1. Commercially obtainable reference compounds corresponding to diglyme metabolites were obtained at the highest available purity. 2-(2-Methoxyethoxy)ethanol (MEE) (99%), 2-methoxyethanol (2ME) (99%), methoxyacetic acid (MAA) (99%), and diglycolic acid (DGA) (98%) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). (2-Methoxyethoxy)acetic acid was synthesized by reaction of the sodium salt of 2-methoxyethanol with monochloroacetic acid as described previously by Cheever et al. (1988). Williams' Medium E (without L-glutamine) (WME), L-glutamine, heat-inactivated fetal bovine serum (FBS), insulin (bovine), and gentamicin were obtained from GIBCO BRL (Grand Island, NY). Hanks' balanced salt solution (without calcium and magnesium) (HBSS), ethylene glycol-bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA), [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)] (HEPES), and dexamethasone were purchased from Sigma Chemical Company (St Louis, MO). Collagenase (CLS2) was obtained from Worthington Biochemical Corporation (Freehold, NJ).

A dosing sulution for the in vivo study was prepared by dissolving appropriate amounts of [^{14}C]diglyme and non-radiolabeled diglyme in purified water (resistance greater than 17 megohm-cm) to give a final concentration of 1.02 mmol/ml (sp act 29 μCi/mmol). Solutions of diglyme for the in vitro study were prepared in incubation medium consisting of WME supplemented with L-glutamine at 2 mM, insulin at 1 μM, dexamethasone at 1 μM, and gentamicin at 50 mg/ml. [^{14}C]Diglyme was dissolved in medium to give final concentrations of 1 and 10 μM, each with a specific activity of 29.8 mCi/mmol, or combined with non-radioactive diglyme to give final concentrations of 30 and 50 μM with specific activities of 9.9 and 6.0 mCi/mmol, respectively.

Animals. Weanling male Sprague-Dawley [Crl:CD (SD) BR outbred], rats were obtained from Charles River Breeding Laboratories, Inc., (Wilmington, MA). Immediately upon receipt by the AAALAC-accredited NIOSH animal facility, the rats were placed in quarantine for 7 days. At all times, the rats were housed three per cage and provided Formulab Chow No. 5008 (Ralston Purina Co., St Louis, MO) and water ad libitum. Rats used in the induction study were given ethanol (15% v/v) in their drinking water for 5 days immediately before hepatocyte isolation. At the time of use in the experiments, all rats ranged in weight from 190 to 220 g.

In vivo metabolism. For the in vivo studies, five rats were placed in individual Roth-type glass metabolism cages for the separate collection of urine and feces. The rats had free access to food except for the 18-h period immediately prior to treatment, when food was withheld. Distilled water was available at all times. Each rat received a single oral dose of [^{14}C]diglyme at 5.1 mmol (148 μCi)/kg body wt. The urine was collected at 6, 12, 24, 48, 72, and 96 h following the administration of the [^{14}C]diglyme for analysis for radioactive components. Each urine sample was diluted to a specific volume and immediately passed through an Acrodisc filter having a 0.45-μm pore size (Gelman Sciences, Ann Arbor, MI). Urine samples were stored at –20° C until analyzed by liquid scintillation counting (LSC) and by high performance liquid chromatography (HPLC).

In vitro metabolism. Hepatocytes were obtained from the livers of rats by the two-step in situ collagenase perfusion technique described by Williams et al. (1977) with only minor modifications. The principal variation of this procedure involved the introduction of the perfusion solutions through a cannula (20-gauge Angiocath, Deseret Medical Inc., Becton Dickinson and Company, Sandy, UT) in the subhepatic inferior vena cava. Isolated hepatocytes were suspended in attachment medium consisting of WME supplemented with L-glutamine at 2 mM, 10% FBS, insulin at 1 μM, dexamethasone at 1 μM, and gentamicin at 50 mg/ml. The proportion of viable cells was assessed by the trypan blue exclusion technique. At a minimum, 85% of the cells were viable in all preparations. The hepatocytes were diluted with attachment medium to approximately 4×10^5 viable cells/ml, and primary cultures were initiated by adding approximately 2×10^6 viable cells in a final volume of 5 ml to separate 60-mm plastic culture dishes. The cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2: 95% air for 2 h to allow the viable hepatocytes to attach to the plates. The attachment efficiency was then determined by dividing the DNA content of the cells attached to each of five randomly selected plates by that in 5-ml aliquots of the initial cell suspension. The DNA content was measured according to the method of Labarca and Paigen (1980).

Immediately following the 2-h attachment period, the medium was replaced with 5-ml volumes of warmed, sterile maintenance medium containing [^{14}C]diglyme at 1 μM (0.15 μCi) or at 10, 30, or 50 μM (1.5 μCi), and the cultures were again incubated under the same conditions as before. Control plates without cells were incubated with medium containing [^{14}C]diglyme at the same concentrations as those in parallel metabolism experiments to ascertain any transformation of the diglyme

Table 1. Radioactive compounds excreted in the urine of rats following oral administration of [^{14}C]diglyme[a]

[^{14}C] Urinary component	Percentage of administered dose (cumulative)					
	6 h	12 h	24 h	48 h	72 h	96 h
Metabolite I (unidentified)	0.0±0.0[b]	0.0±0.0	0.1±0.0	0.3±0.1	0.3±0.1	0.3±0.1
N-(Methoxyacetyl)glycine	0.0±0.0	0.0±0.0	0.1±0.1	0.2±0.1	0.2±0.1	0.3±0.2
Diglycolic acid (DGA)	0.1±0.0	0.7±0.1	2.8±0.5	3.5±0.7	3.8±0.9	3.9±1.0
Metabolite IV (unidentified)	0.0±0.0	0.2±0.0	0.8±0.2	0.8±0.3	0.9±0.3	1.0±0.3
Methoxyacetic acid (MAA)	0.2±0.1	0.7±0.1	2.5±0.2	5.1±0.5	6.0±0.8	6.2±0.8
2-Methoxyethanol (2ME)	0.0±0.0	0.2±0.0	0.8±0.2	0.8±0.2	0.8±0.2	0.8±0.3
(2-Methoxyethoxy)acetic acid (MEAA)	12.6±2.2	37.0±2.9	65.3±4.0	67.2±3.5	67.7±3.3	67.9±3.3
2-(2-Methoxyethoxy)ethanol (MEE)	0.2±0.1	0.6±0.2	1.1±0.3	1.2±0.3	1.2±0.3	1.2±0.3
Diglyme	0.8±0.2	1.5±0.2	1.8±0.3	1.8±0.3	1.8±0.3	1.8±0.3
Total urinary recovery	13.9±2.2	40.9±3.0	75.3±4.1	80.9±3.3	82.7±3.2	83.4±3.3

[a] Male Sprague-Dawley rats (190–220 g) were given single po doses of [^{14}C]diglyme at 5.1 mmol (148 μCi)/kg body weight
[b] Each value represents the mean ± SEM of 5 rats

unrelated to cell-mediated metabolism. After 12, 18, 24, or 48 h, the medium was removed from triplicate cell cultures and from a single control plate at each diglyme concentration. The medium from each incubation was acidified to pH 2 with concentrated H_3PO_4 and was passed through a separate Centrifree micropartition tube (Amicon Division, W. R. Grace & Co., Danvers, MA) by centrifugation for 10 min at 1000 *g* to remove cellular debris and protein. The filtrates were stored at –20° C until analyzed for radioactive components by HPLC.

Lactate dehydrogenase assay. The cytotoxicity of diglyme to rat hepatocytes in primary culture was assessed prior to the initiation of in vitro metabolism studies. Leakage of intracellular lactate dehydrogenase (LDH) into the culture medium was used as an indicator of toxicity. Hepatocytes were exposed to diglyme at 100 μM for 12, 18, 24, and 48 h. Control cultures of hepatocytes were incubated without diglyme concurrently with the test cultures. After each incubation period, the medium was removed from the cells in each culture by aspiration and was centrifuged at 1000 *g* for 10 min. The hepatocytes from corresponding cultures were lysed by alternately freezing (–20° C) and thawing for three cycles, and the cell debris was sedimented by centrifugation. The LDH activity was measured separately in each medium sample and corresponding lysate using a commercial enzyme assay kit (Sigma Diagnostic Kit LD-L; Sigma Chemical Company). The LDH in medium from each culture exposed to diglyme, expressed as a percentage of the total LDH in the culture (medium and cells), was compared with that from cultures of unexposed cells, incubated for corresponding time periods.

Liquid scintillation spectrometry. Total radioactivity in urine and in culture medium was quantified using a Model LS8100 liquid scintillation spectrometer (Beckman Instrument Co., Fullerton, CA). Counting efficiencies were determined by the external method of Horrocks (1974).

High performance liquid chromatography. [^{14}C]Diglyme and its radioactive metabolites in urine and in culture medium were separated using a high performance chromatograph system consisting of two Model 510 solvent delivery systems, a Model 380 system controller, a 715 Ultra WISP sample processor, and a column heater module with an associated temperature control module (Waters, Division of Millipore, Milford, MA). The HPLC column system consisted of a 50 cm × 9.4 mm i.d. stainless steel column packed with Partisil 10 ODS-2 (Whatman Inc., Clifton, NJ) in series with a 15 cm × 3.9 mm i.d. stainless steel column packed with 5-μm spherical Nova-Pak C_{18} (Waters, Division of Millipore), maintained at 30° C. Aliquots of membrane-filtered urine or medium samples were introduced onto the column system with the 715 Ultra WISP sample processor. Separations of radioactive components were achieved by a stepwise gradient elution with a 1% acetic acid and methanol solvent system: 1) 1% acetic acid for 10 min, 2) a linear increase of methanol from 10% to 80% over a 30-min period, 3) methanol: 1% acetic acid (80:20) for 20 min, and 4) methanol for 5 min. The column was then re-equilibrated to the initial condition with 1% acetic acid for 10 min. The solvent flow rate was maintained at 1 ml/min. The radioactive components in the effluent were quantified with an in-line TRACE II radioactivity flow monitor (Packard Instruments, Downers Grove, IL), and radioactive fractions were collected separately for characterization by combined gas chromatography-mass spectrometry (GC-MS).

Gas chromatography-mass spectrometry. Mass spectra were obtained with a Model 5890A gas chromatograph fitted with a 50-m × 0.2-mm i.d. Ultra 1 cross-linked methyl silicone (d_f = 0.33 μm) fused silica capillary column (Hewlett-Packard Co., Avondale, PA) coupled by direct capillary interface with a Model 5970B quadrapole mass spectrometer (Hewlett-Packard Co., Palo Alto, CA). Helium was used as the carrier gas at a linear velocity of 38 cm/s at 200° C. Splitless injections were made with the column at 10° C, and the oven temperature was then programmed to 200° C at 8° C/min. Electron impact spectra were acquired at an electron energy of 70 eV with an ion source temperature of 200° C.

Data analysis. Statistical evaluations of differences between group means were tested by Student's *t*-test with $p < 0.05$ set as the level of significance. Linear regression lines were plotted using SigmaPlot, Version 5.0 (Jandel Scientific, Corte Madera, CA).

Results

In vivo metabolism

The time course for the excretion of each of the radioactive urinary components by rats after receiving single po doses of [^{14}C]diglyme at 5.1 mmol/kg is shown in Table 1. These urinary components had been characterized in a previous study (Cheever et al. 1988). The predominant metabolite MEAA was excreted rapidly during the first 24 h to account for 65.3% of the administered dose and then plateaued at 67–68% of the dose between 48 and 96 h. The second most abundant metabolite MAA was excreted more slowly, representing only 2.5 ± 0.2% of the dose after 24 h. However, the cumulative level of MAA more than doubled to 5.1 ± 0.5% during the 24- to 48-h period before reaching 6.2 ± 0.8% at the end of the 96-h metabolism period. Other metabolites included MEE, the precursor of MEAA; 2ME, the precursor of MAA; *N*-(methoxyacetyl)glycine; and

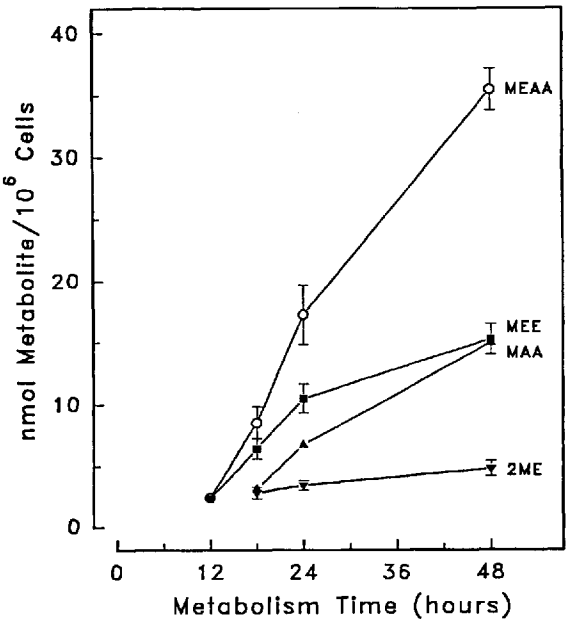

Fig. 2. Time course for the formation of principal diglyme metabolites in isolated rat hepatocytes. Cultures of hepatocytes, plated at 2×10^6 cells/60-mm culture dish, were incubated at 37° C with 30 μM [^{14}C]diglyme (1.5 μCi) for 12, 18, 24, or 48 h. Metabolites in the incubation medium were separated by HPLC and quantified with an in-line radioactivity monitor. Each point represents the mean ± SEM of incubations with hepatocytes (three replicate cultures) isolated from five rats. Points without visible error bars had SEM ranges covered by the corresponding symbols

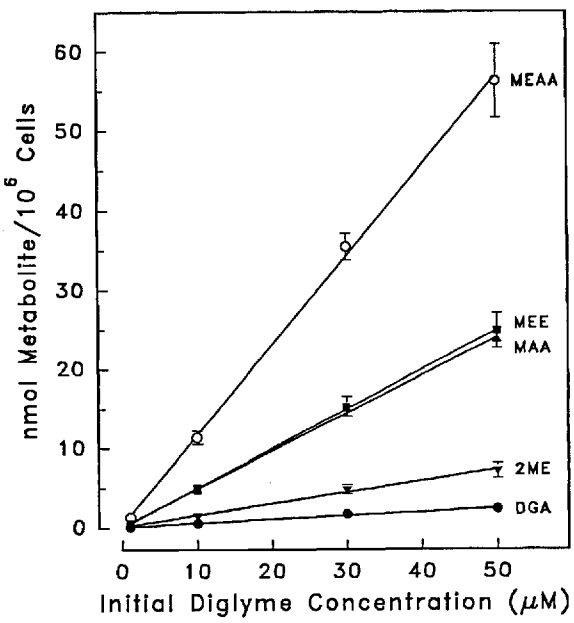

Fig. 3. Effect of diglyme concentration on the formation of principal metabolites in isolated rat hepatocytes. Monolayers of hepatocytes, plated at 2×10^6 cells/60-mm culture dish, were incubated at 37° C with [^{14}C]diglyme at 1 μM (0.15 μCi) at or 10, 30, or 50 μM (1.5 μCi) for 48 h. Metabolites in the incubation medium were separated by HPLC and quantified with an in-line radioactivity monitor. Each point represents the mean ± SEM of incubations with hepatocytes (three replicate cultures) isolated from five rats. Points without visible error bars had SEM ranges covered by the corresponding symbols

diglycolic acid. Metabolites I and IV, representing less then 2% of the administered dose, were not identified. Unchanged diglyme, representing 1.8% of the administered dose, was excreted within 24 h.

Cytotoxicity of diglyme

Before initiating the in vitro metabolism studies, the toxicity of diglyme to isolated rat hepatocytes in primary culture was evaluated. The release of intracellular LDH from exposed cells into the culture medium was used as the indicator of cytotoxicity (Anuforo et al. 1978). Exposure of hepatocytes to 100 μM diglyme for up to 48 h resulted in no net release of LDH when compared with that of unexposed control cells at corresponding incubation periods (data not shown). Based on LDH release as the criterion for toxicity, diglyme is not toxic to hepatocytes at twice the highest concentration used in the in vitro metabolism study.

In vitro metabolism of diglyme

Isolated rat hepatocytes in monolayer culture metabolized [^{14}C]diglyme and released radioactive metabolites into the incubation in a time-dependent manner (Fig. 2). In an initial experiment, greater than 97% of the administered radioactivity was recovered from the culture medium after each incubation period, and medium from control incubations of [^{14}C]diglyme without cells showed no detectable radioactive metabolites (data not shown). Routinely, five metabolites and unmetabolized diglyme were found in medium recovered from 48-h cultures. During the first 12-h metabolism period, however, only MEAA and MEE were detected in the incubation medium, with each of these metabolites representing less than 2.5% of the total radioactivity added to the cultures (Fig. 2). The detection limit using the radioactivity monitor was approximately 1300 dpm, which corresponded to 2.7 ng or 1% of the added parent compound. In addition to MEAA and MEE, 2ME and MAA were present in 18-h medium. The concentrations of the 2ME and MAA in 18-h medium were similar, but proportionally greater amounts of MAA were present in the medium after 24- and 48-h metabolism periods. Diglycolic acid (DGA) was detected only in 48-h medium, and consequently, this metabolite is not included in Fig. 2. After a 48-h incubation period, the formation of all in vitro metabolites was linear with the initial diglyme concentration over the 1- to 50-μM range (Fig. 3). The r values for the regression curves were: 0.9994 for MEAA, 0.9991 for MEE, 0.9998 for MAA, 0.9975 for 2ME, and 0.9954 for DGA. A comparison of 48-h patterns of the five in vitro metabolites of diglyme and the five corresponding urinary metabolites show that these profiles are qualitatively similar, with MEAA being by far the most predominant metabolite in both systems (Fig. 4).

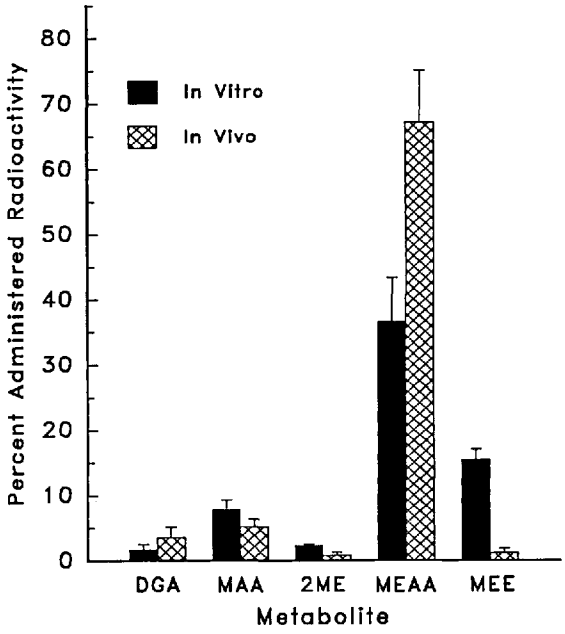

Fig. 4. Comparison of diglyme metabolites formed in isolated rat hepatocytes with major urinary metabolites excreted by rats. Rat hepatocytes, plated at 2×10^6 cells/60-mm culture dish, were incubated at 37° C with [^{14}C]diglyme at 30 μM (1.5 μCi) for 48 h. Rats were given single oral doses of [^{14}C]diglyme at 5.1 mmol (148 μCi)/kg body wt. In vivo values represent the cumulative amounts of metabolites as percent of administered radioactivity appearing in the urine during the first 48 h following dosing. Results are expressed as means ± SEM ($n = 5$)

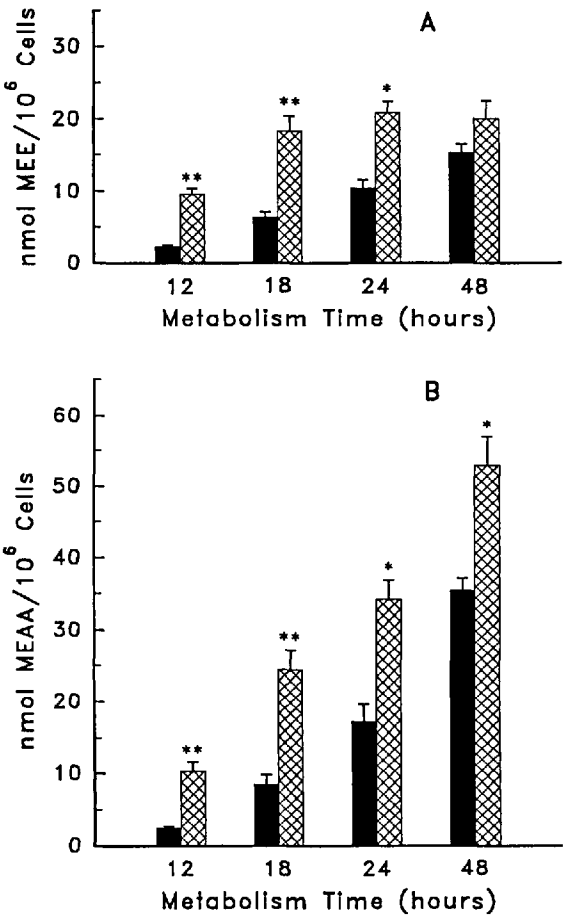

Fig. 5 A, B. The production of diglyme metabolites MEE (**A**) and MEAA (**B**) by the primary pathway. Hepatocytes isolated from ethanol-pretreated (▨) or untreated (■) rats were plated at 2×10^6 cells/60-mm culture dish and were incubated at 37°C with [^{14}C]diglyme at 30 μM (1.5 μCi) for 12, 18, 24, or 48 h. Metabolites in the incubation medium were separated by HPLC and quantified by an in-line radioactivity monitor. Amounts of the indicated metabolite are cumulated over time. Results are expressed as means ± SEM ($n = 4$ pretreated rats; $n = 5$ untreated rats). *Asterisks* denote values significantly different from those obtained from hepatocytes isolated from untreated rats. Significance levels by Student's *t*-test: ** $p < 0.001$; * $p < 0.05$

Effect of ethanol pretreatment on the metabolism of diglyme

The metabolism of diglyme in hepatocytes isolated from rats pretreated with ethanol was studied at a single intermediate diglyme concentration of 30 μM. For the most part, the amounts of metabolites formed in hepatocytes isolated from ethanol-pretreated rats were significantly greater than those formed in hepatocytes from untreated rats at all corresponding time periods. A comparison of the amounts of the major metabolites (MEE and MEAA) formed by the primary pathway is shown in Fig. 5. With a single exception, significantly ($p < 0.05$) greater amounts of both metabolites were produced in cultures of hepatocytes from ethanol-pretreated rats. The exception noted was that no significant difference occurred in the relative amounts of MEE formed after 48 h. The greatest differences occurred at the 12-h time point where more than 4 times the amount of MEE or MEAA was formed in hepatocytes from ethanol-pretreated rats. The amount of MEE present in the medium from cultures of cells from pretreated rats increased nearly two-fold from 12 to 18 h but then plateaued between 18 and 48 h. The MEE in medium from cells from untreated rats increased steadily with time and at 48 h reached a level not significantly different from that present in medium from cells from pretreated rats. In contrast, the levels of MEAA in both cell systems increased sharply throughout the metabolism period.

The major metabolites (2ME and MAA) produced via the secondary pathway in hepatocytes from ethanol-pretreated and untreated rats are compared in Fig. 6. At all time periods, significantly ($p < 0.05$) greater amounts of the metabolites were formed in hepatocytes from ethanol-pretreated rats. Both 2ME and MAA were present in the 12-h medium from cultures of hepatocytes from ethanol-pretreated rats, whereas neither metabolite was detected in 12-h medium from cultures of hepatocytes from untreated rats. At 18 h, more than three times the amount of 2ME and four times the amount of MAA were formed in hepatocytes from ethanol-pretreated rats. However, these differences in amounts of metabolites decreased progressively to a factor of only two at 48 h. The time course for the production of metabolites via the secondary pathway paralleled that of metabolites formed by the primary pathway. As in the case of MEE, the amount of 2ME produced in cells from pretreated rats increased from 12 to 18 h but plateaued between 18 and 48 h, and as in the case of MEAA, the amounts of MAA produced in both systems increased

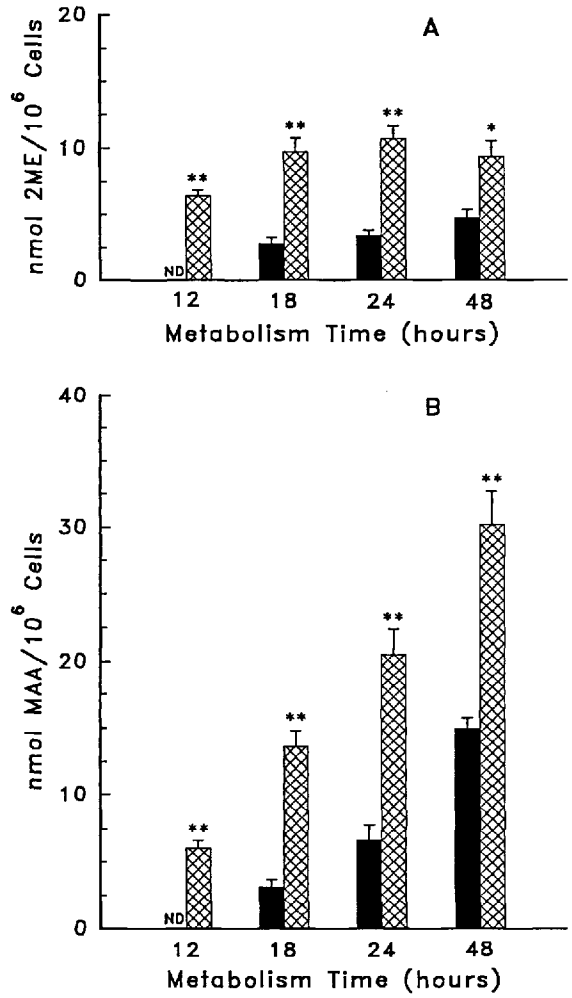

Fig. 6 A, B. The production of diglyme metabolites 2ME (**A**) and MAA (**B**) by the secondary pathway. Hepatocytes isolated from ethanol-pretreated (▩) or untreated (■) rats were plated at 2×10^6 cells/60-mm culture dish and were incubated at 37° C with [^{14}C]diglyme at 30 μM (1.5 μCi) for 12, 18, 24, or 48 h. Metabolites in the incubation medium were separated by HPLC and quantified with an in-line radioactivity monitor. Amounts of the indicated metabolite are cumulated over time. Results are expressed as means ± SEM (n = 4 pretreated rats; n = 5 untreated rats). *Asterisks* denote values significantly different from those obtained from hepatocytes isolated from untreated rats. Significance levels by Student's t-test: ** p <0.001; * p <0.05. ND indicates not detected. Limit of detection: 0.1 nmol metabolite/10^6 cells

sharply with time. The primary difference in the production of metabolites between the two metabolic pathways was in the formation of the respective alcohols. The amount of MEE produced in hepatocytes from untreated rats increased steadily with time; in contrast, the amount of 2ME formed in hepatocytes from untreated rats at 24 h was not significantly greater than that formed at 18 h but was significantly less than that formed at 48 h.

Discussion

In a previous study from this laboratory, it was demonstrated that diglyme is metabolized extensively in the rat with the metabolites being eliminated principally in the urine (Cheever et al. 1988). A metabolic scheme involving two separate pathways was proposed. The primary pathway involves a single O-demethylation step to form MEE, which is then oxidized first to (2-methoxyethoxy)acetaldehyde by alcohol dehydrogenase and subsequently to MEAA by aldehyde dehydrogenase. A secondary pathway proceeds by cleavage of the interior ether bond to form 2ME, which, in part, undergoes similar enzymatic oxidation to form the reproductive and developmental toxicant MAA.

In the present comparative metabolism study, isolated rat hepatocytes were evaluated as an in vitro model system for the in vivo metabolism of diglyme. Over a 48-h incubation period with diglyme, hepatocytes produced five metabolites, which corresponded to the five most prominent metabolites identified in urine excreted by rats within 48 h of receiving a single oral dose of diglyme. The linear formation of the in vitro metabolites by both major pathways with increasing diglyme concentration indicated that the primary metabolic pathway was not saturated and that metabolism of diglyme proceeded concurrently by both pathways. The major metabolite was MEAA both in vitro and in vivo. After incubation of hepatocytes with diglyme for 18 h, the toxic metabolite MAA and its precursor 2ME were present in the culture medium in nearly equivalent amounts; however, at all later time points, MAA was present in significantly greater amounts. This trend paralleled the in vivo results. These findings demonstrate that hepatocytes can be used to predict the urinary metabolites of diglyme and that urinary MEAA would be useful as a short-term biological indicator of exposure to diglyme.

In the induction study, there was a marked increase in amounts of diglyme metabolites formed by both major pathways in hepatocytes isolated from rats pretreated with ethanol over those formed in hepatocytes from untreated rats. Ethanol has been shown to induce cytochrome P-450IIE1 in the rat (Ryan et al. 1985) and in man (Wrighton et al. 1986). This isozyme of cytochrome P-450 has been reported to catalyze the N-demethylation of *N*-nitrosodimethylamine (Tu and Yang 1985; Levin et al. 1986; Lasker et al. 1987), and this ethanol-inducible cytochrome P-450 may be involved in the O-demethylation of diglyme as well. In a previous study, rats pretreated with phenobarbital or with diglyme itself excreted significantly greater amounts of MAA from a subsequent single dose of [^{14}C]diglyme than did rats given no such pretreatment (Cheever et al. 1989a). Phenobarbital is a known inducer of cytochromes P-450IIB1 (P-450$_b$; Ryan et al. 1979), P-450IIB2 (P-450$_e$; Ryan et al. 1979, 1982), and P-450IIIA1 (P-450$_{PCN}$; Heuman et al. 1982) in live rats, but P-450IIE1 (P-450$_j$) has been reported not to be induced in microsomes from rats pretreated with diglyme (Ballow et al. 1991). More recently, the cleavage of the central ether bond was determined to be NADPH-dependent in rat liver microsomes. Furthermore, it was shown that cytochrome P-450 and P-450 reductase levels were significantly increased in liver microsomes from rats pretreated with diglyme in their drinking water over those of untreated controls (Tirmenstein 1992). The results of the present in vitro induction study in rat hepatocytes suggest that an ethanol-inducible cytochrome P-450 may be involved in the cleavage of the central ether bond of diglyme. Although diglyme

may be considered a condensation product of two molecules of 2ME (McGregor et al. 1983), oxidative cleavage of the central ether bond of this molecule mediated by cytochrome(s) P-450 would yield only one molecule of 2ME along with one molecule of methoxyacetaldehyde. However, methoxyacetaldehyde was not reported as a urinary metabolite of diglyme in previous studies and was not identified as a metabolic product in either rat urine or culture medium in the present studies. It is most likely that this reactive aldehyde was rapidly oxidized to MAA. The amounts of MAA resulting from this conversion together with those formed from the metabolism of 2ME may account for the fact that, in the present in vivo study, MAA was detected in rat urine earlier (6 h) than 2ME (12 h) and was excreted in greater amounts after all time intervals following the administration of diglyme (Table 1).

In summary, the results of the in vitro/in vivo comparative metabolism study demonstrate that isolated rat hepatocytes in monolayer culture can be used to predict the urinary metabolites of diglyme. In addition, the urinary metabolite MEAA would best serve as a short-term indicator of exposure to diglyme. The in vitro metabolism of diglyme in hepatocytes isolated from rats pretreated with ethanol was increased over that in hepatocytes from untreated rats. Of particular significance, the induction of hepatic enzymes by ethanol resulted in the conversion of increased amounts of diglyme to MAA, the metabolite associated with the reproductive and developmental toxicity of diglyme.

References

Anuforo DC, Acosta D, Smith RV (1978) Hepatotoxicity studies with primary cultures of rat liver cells. In Vitro 14: 981–988

Ballow E, Bounds PL, Adams A, Many C, Winston GW (1991) Induction of the ethanol-inducible cytochrome P450 (P450j) in rat liver by ethylene glycol methylethers. Alcohol Clin Exp Res 15: 353 (Abstract)

Billings RE, McMahon RE, Ashmore J, Wagle SR (1977) The metabolism of drugs in isolated rat hepatocytes. A comparison with in vivo drug metabolism and drug metabolism in subcellular liver fractions. Drug Metab Dispos 5: 518–526

Cheever KL, Richards DE, Weigel WW, Lal JB, Dinsmore AM, Daniel FB (1988) Metabolism of bis(2-methoxyethyl)ether in the adult male rat: evaluation of the principal metabolite as a testicular toxicant. Toxicol Appl Pharmacol 94: 150–159

Cheever KL, Richards DE, Weigel WW, Begley KB (1989a) The role of enzyme induction on metabolite formation of bis(2-methoxyethyl)ether in the rat. Toxicol Ind Health 5: 601–607

Cheever KL, Weigel WW, Richards DE, Lal JB, Plotnick HB (1989b) Testicular effects of bis(2-methoxyethyl)ether in the adult male rat. Toxicol Ind Health 5: 1099–1109

Daniel FB, Cheever KL, Begley KB, Richards DE, Weigel WW, Eisenmann CJ (1991) Bis(2-methoxyethyl)ether: metabolism and embryonic disposition of a developmental toxicant in the pregnant CD-1 mouse. Fundam Appl Toxicol 16: 567–575

Fry JR (1988) Metabolism of xenobiotics in isolated cells: comparison with other in vitro systems and in vivo. In: Gorrod JW, Oelschläger H, Caldwell J (eds) Metabolism of xenobiotics. Taylor & Francis, London New York Philadelphia, pp 303–309

Green CE, LeValley SE, Tyson CA (1986) Comparison of amphetamine metabolism using isolated hepatocytes from five species including human. J Pharmacol Exp Ther 237: 931–936

Hardin BD, Eisenmann CJ (1987) Relative potency of four ethylene glycol ethers for induction of paw malformations in the CD-1 mouse. Teratology 35: 321–328

Heuman DM, Gallagher EJ, Barwick JL, Elshourbagy NA, Guzelian PS (1982) Immunochemical evidence for induction of a common form of hepatic cytochrome P-450 in rats treated with pregnenolone-16α-carbonitrile or other steroidal or non-steroidal agents. Mol Pharmacol 21: 753–760

Horrocks DL (1974) Applications of liquid scintillation counting. Academic Press, New York, pp 72–74

Jajoo HK, Blair IA, Klunk LJ (1990) In vitro metabolism of the anti-anxiety drug buspirone as a predictor of its metabolism in vivo. Xenobiotica 20: 779–786

Jatoe SD, Gorrod JW (1987) The in vitro/in vivo comparative metabolism of 4-aminobiphenyl using isolated hepatocytes. Arch Toxicol 60: 65–68

Labarca C, Paigen K (1980) A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102: 344–352

Lasker JM, Raucy J, Kubota S, Bloswick BP, Black M, Lieber CS (1987) Purification and characterization of human liver cytochrome P-450-ALC. Biochem Biophys Res Commun 148: 232–238

Lee KP, Kinney LA, Valentine R (1989) Comparative testicular toxicity of bis(2-methoxyethyl) ether and 2-methoxyethanol in rats. Toxicology 59: 239–258

Levin W, Thomas PE, Oldfield N, Ryan DE (1986) N-Demethylation of *N*-nitrosodimethylamine catalyzed by purified rat hepatic microsomal cytochrome P-450: Isozyme specificity and role of cytochrome b_5. Arch Biochem Biophys 248: 158–165

McGregor DB, Willins MJ, McDonald P, Holmström M, McDonald D, Niemeier RW (1983) Genetic effects of 2-methoxyethanol and bis(2-methoxyethyl) ether. Toxicol Appl Pharmacol 70: 303–316

Plasterer MR, Bradshaw WS, Booth GM, Carter MW, Schuler RL, Hardin BD (1985) Developmental toxicity of nine selected compounds following prenatal exposure in the mouse: naphthalene, *p*-nitrophenol, sodium selenite, dimethyl phthalate, ethylenethiourea, and four glycol ether derivatives. J Toxicol Environ Health 15: 25–38

Price CJ, Kimmel CA, George JD, Marr MC (1987) The developmental toxicity of diethylene glycol dimethyl ether in mice. Fundam Appl Toxicol 8: 115–126

Ryan DE, Thomas PE, Korzeniowski D, Levin W (1979) Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. J Biol Chem 254: 1365–1374

Ryan DE, Thomas PE, Levin W (1982) Purification and characterization of a minor form of hepatic microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls. Arch Biochem Biophys 216: 272–288

Ryan DE, Ramanathan L, Iida S, Thomas PE, Haniu M, Shively JE, Lieber CS, Levin W (1985) Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. J Biol Chem 260: 6385–6393

Schuler RL, Hardin BD, Niemeier RW, Booth G, Hazelden K, Piccirillo V, Smith K (1984) Results of testing fifteen glycol ethers in a short-term in vivo reproductive toxicity assay. Environ Health Perspect 57: 141–146

Schwetz BA, Price CJ, George JD, Kimmel CA, Morrissey RE, Marr MC (1992) The developmental toxicity of diethylene and triethylene glycol dimethyl ethers in rabbits. Fundam Appl Toxicol 19: 238–245

Tirmenstein MA (1992) Metabolism of bis(2-methoxyethyl)ether by rat and human liver microsomes. Toxicologist 12: 59 (Abstract)

Tu YY, Yang CS (1985) Demethylation and denitrosation of nitrosamines by cytochrome P-450 isozymes. Arch Biochem Biophys 242: 32–40

Williams GM, Bermudez E, Scaramuzzino D (1977) Rat hepatocyte primary cultures. III. Improved dissociation and attachment techniques and the enhancement of survival by culture medium. In Vitro 13: 809–817

Wrighton SA, Thomas PE, Molowa DT, Haniu M, Shively JE, Maines SL, Watkins PB, Parker G, Mendez-Picon G, Levin W, Guzelian PS (1986) Characterization of ethanol-inducible human liver *N*-nitrosodimethylamine demethylase. Biochemistry 25: 6731–6735