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ENHANCED FORMATION OF ETHANE AND *n*-PENTANE BY RAT HEPATOCYTES IN THE PRESENCE OF DIMETHYL SULFOXIDE

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Abstract—Incubation of rat hepatocytes with 14 mM dimethyl sulfoxide (DMSO) produced an increase in the formation of ethane, measured by capillary column gas chromatography, to 18.0 pmoles/hr/10^7 cells from 11.2 pmoles/hr/10^7 cells in control hepatocytes, and an increase in the formation of *n*-pentane to 17.5 pmoles/hr/10^7 cells from 5.6 pmoles/hr/10^7 cells in control hepatocytes. This was about one-third the stimulation of ethane and *n*-pentane formation produced by incubation of hepatocytes with 13 mM carbon tetrachloride. DMSO-stimulated ethane and *n*-pentane formation was inhibited up to 63% by 0.1 μM α-tocopherol and up to 89% by N_2. Formation of dimethylsulfide from DMSO by hepatocytes was the same in air and N_2. DMSO increased methane production by hepatocytes to 31.3 pmoles/hr/10^7 cells from 6.9 pmoles/hr/10^7 cells in control hepatocytes. Although DMSO apparently stimulated lipid peroxidation by hepatocytes, as measured by ethane and *n*-pentane formation, there was no increase in the formation of thiobarbituric acid reactive material. DMSO was not toxic to hepatocytes, measured by release of cytosolic lactate dehydrogenase, over a 2-hr incubation. Possible mechanisms for the increase in alkane formation by DMSO are discussed.

The formation of ethane and *n*-pentane by subcellular fractions, by isolated cells, and by the whole animal provides an index of lipid peroxidation and is generally preferred to other measures of lipid peroxidation such as thiobarbituric acid (TBA†) reacting substances or conjugated diene formation [1–3]. During the course of a study of lipid peroxidation by rat isolated hepatocytes, we observed that dimethyl sulfoxide (DMSO), which was being used as a drug vehicle, stimulated the formation of ethane and *n*-pentane.

DMSO is a hygroscopic, dipolar solvent frequently used as a drug vehicle in *in vitro* studies. It is used in pharmaceutical products for topical application because of its solvent properties and its ability to enhance the absorption of many compounds through the skin [4, 5]. DMSO has a wide range of pharmacological effects [6] and has been used clinically to treat musculoskeletal disorders, interstitial cystitis and central nervous system injury [6, 7]. DMSO is also used as a cryoprotectant during freezing of cells and tissues [6]. In this paper we report studies on the effects of DMSO upon lipid peroxidation, measured by the formation of ethane and *n*-pentane by rat isolated hepatocytes and have compared its effects to those of the well known stimulator of hepatic lipid peroxidation carbon tetrachloride (CCl_4).

MATERIALS AND METHODS

Male Sprague–Dawley rats (Sprague–Dawley, Madison, WI) weighing 180–250 g were used for all studies. The animals were allowed free access to food and water. Hepatocytes were prepared by a modification of the method of Berry and Friend [8] as previously described [9]. Hepatocyte viability was determined by trypan blue exclusion and was routinely greater than 90%. All values are expressed per number of viable cells.

Hepatocytes were incubated at a concentration of 1.0 to 1.5 × 10^7 cells/ml in Eagle's minimum essential medium containing 10 mM HEPES, pH 7.4, and 2% bovine serum albumin. The medium was saturated with 95% air, 5% CO_2 prior to use. It was not necessary to use special hydrocarbon free air as the background contamination of ethane and *n*-pentane in the 95% air, 5% CO_2 gas mixture was minimal. Incubations were performed in sealed 22-ml vials (Pierce Chemical Co., Rockford, IL) with 4 ml of hepatocyte suspension. CCl_4 (Mallinckrodt, St. Louis, MO), 1.25 μl/ml, and DMSO (Burdick & Jackson, Muskegon, MI), 1 μl/ml, were added directly to the vials. Control vials received no addition. Following treatment additions, vials were tightly sealed with metal caps and thick silicone septa and placed in a 37° shaking water bath set at 85 reciprocations/min. Some vials, after sealing, but before addition of DMSO, were placed on ice and flushed with 95% N_2, 5% CO_2 with gentle shaking for 30 min to remove oxygen from the incubation medium. Other vials, prior to addition of cells and

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† Abbreviations: TBA, thiobarbituric acid; DMSO, dimethyl sulfoxide; CCl_4, carbon tetrachloride; LDH, lactate dehydrogenase; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

media, received an aliquot of ethanol containing 440 pmoles of *d*,α-tocopherol (Sigma Chemical Co., St. Louis, MO). The ethanol was evaporated under a stream of N_2 before adding medium and hepatocytes.

At the end of the incubation period, 5 ml of gas phase was removed from each vial using a gas-tight syringe (Precision Sampling Co., Baton Rouge, LA). The gas was compressed to 1.5 ml volume and injected onto a Hewlett–Packard 5880 gas chromatograph with a Chrompack 50 meter fused silica, porous layer open tubular (PLOT) capillary column coated with Al_2O_3/KCl (Chrompack Inc., Bridgewater, NJ). Chromatographic conditions were: split ratio, 9:1; carrier gas, nitrogen; make-up gas, helium; injector temperature, 100°; oven temperature, 130°; and flame ionization detector temperature, 150°. The concentrations of methane, ethane and *n*-pentane in the gas phase were quantified by comparison with known concentrations of the appropriate reference gases (Scott Specialty Gases, Plumstead, PA). Rates of alkane formation, unless stated otherwise, are expressed over the first 2 hr of incubation.

TBA reactive material in hepatocyte incubations was determined by a modification of the procedure of Gutteridge [10]. Incubation vials were opened, and a 1-ml aliquot of cell suspension was removed. To this was added 1 ml of 0.1 M potassium hydrogen phthalate buffer, pH 3.5, and the mixture was vortexed. One millilitre of 1% (w/v) TBA in 50 mM NaOH was added, and the mixture was again vortexed and heated for 15 min at 100°. The sample was centrifuged, and the resulting supernatant fraction was measured for TBA reactive species by its absorbance at 532 nm. Results are expressed as the change in absorbance at 532 nm. Leakage of lactate dehydrogenase (LDH) from hepatocytes into the incubation medium was measured using a commercial test kit for LDH (Sigma Chemical Co.). LDH in the cell-free incubation medium is expressed as a percentage of the total LDH in the hepatocyte preparation at zero time released by repeatedly freezing and thawing an aliquot of hepatocyte suspension [11]. Dissolved oxygen concentrations in the hepatocyte incubation media were measured using a Clark type polarographic electrode (Yellow Springs Instrument Co., Yellow Springs, OH) inserted through the silicone seal of the flask. Gas phase oxygen concentrations were measured using an oxygen analyzer (model 0200, Salter Instruments, Arvin, CA) with the probe inserted through the silicone seal of the flask. Groups of data were analyzed for statistical significance using Student's *t*-test [12].

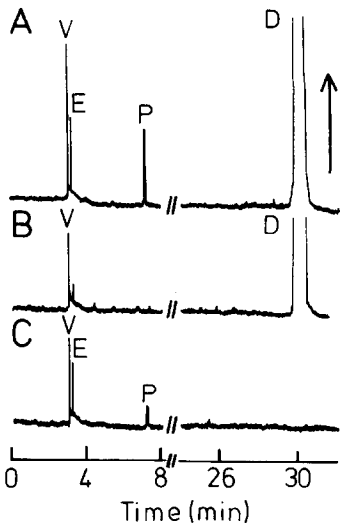

Fig. 1. Chromatograms of alkane formation by isolated hepatocytes. (A) Hepatocytes with 14 mM DMSO after a 2-hr incubation in air; (B) hepatocytes with 14 mM DMSO after a 2-hr incubation in N_2; (C) control hepatocytes after a 2-hr incubation in air. V = void peak (including methane), E = ethane, P = *n*-pentane, and D = dimethylsulfide. The arrow indicates detector response.

RESULTS

The PLOT Al_2O_3/KCl capillary column gave excellent resolution of low molecular weight alkanes with a limit of detection for ethane of 10 pmoles injected and for *n*-pentane of 4.5 pmoles injected. Methane was not separated from the void peak under the chromatographic conditions employed. A typical chromatogram showing the formation of ethane and *n*-pentane by hepatocytes is given in Fig. 1. There

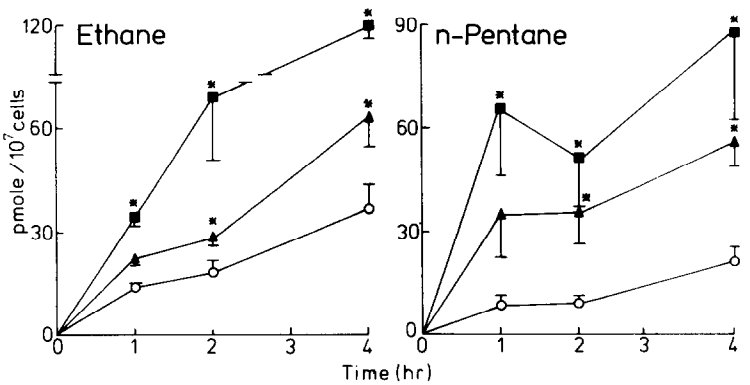

Fig. 2. Ethane and *n*-pentane formation by isolated hepatocytes. Key: (○) control hepatocytes, N = 8; (▲) hepatocytes in the presence of 14 mM DMSO, N = 8; and (■) hepatocytes in the presence of 13 mM CCl_4, N = 8. N = number of preparations. Bars are S.E. of mean. (*) Indicates $P < 0.05$ compared to control values.

Table 1. Alkane production by isolated hepatocyte suspensions

Treatment	Ethane (pmoles/10^7 hepatocytes)		*n*-Pentane (pmoles/10^7 hepatocytes)	
	2 hr	4 hr	2 hr	4 hr
Control	18.0 ± 3.6	36.6 ± 7.5	8.4 ± 2.4	21.3 ± 4.2
DMSO, 14 mM	34.8 ± 3.9*	90.9 ± 4.2*	36.6 ± 5.4*	59.1 ± 13.2*
DMSO, 14 mM/Vit. E, 0.1 μM	21.9 ± 3.3†	37.5 ± 8.1†	19.2 ± 1.5*,†	21.9 ± 0.9†
DMSO 14 mM/N_2	6.6 ± 2.4*,†	16.2 ± 4.5*,†	4.2 ± 1.5*,†	6.6 ± 1.5*,†

Values are mean ± S.E.M. of six preparations.
* $p < 0.05$ compared to control values.
† $p < 0.05$ compared to DMSO alone.

was a progressive increase in the amount of ethane and *n*-pentane formed by control hepatocytes over 4 hr of incubation (Fig. 2) with rates over the first 2 hr of 11.2 pmoles ethane/hr/10^7 cells and 5.6 pmoles *n*-pentane/hr/10^7 cells. Formation of ethane and *n*-pentane was inhibited completely if incubations were carried out under N_2 (these results are not shown).

Addition of DMSO to the incubation medium at a concentration of 14 mM produced an increase in ethane and *n*-pentane formation by hepatocytes (Figs. 1 and 2) to 18.0 pmoles ethane/hr/10^7 cells and 17.5 pmoles *n*-pentane/hr/10^7 cells. The identity of the alkanes formed on addition of DMSO to hepatocytes as ethane and *n*-pentane was confirmed by mass spectrometry on a LKB 2091 gas chromatograph-mass spectrometer. There was no detectable alkane formation when DMSO was incubated with medium in the absence of hepatocytes (results not shown). In the presence of DMSO, hepatocyte incubations also gave a chromatographic peak which co-eluted with dimethylsulfide (Fig. 1). The area of this peak was the same with either air or N_2 in the flask. No attempt was made to quantitate the amount of dimethysulfide formed. The effect of CCl_4 at a concentration of 13 mM on alkane formation by hepatocytes is shown in Fig. 2. In the presence of CCl_4, ethane formation was 34.5 pmoles/hr/10^7 cells and *n*-pentane formation 32.0 pmoles/hr/10^7 cells. DMSO-stimulated ethane and *n*-pentane formation was inhibited up to 63% by 0.1 μM α-tocopherol and up to 89% by N_2 (Table 1).

DMSO reacts with hydroxyl radicals to form methane [13]. Although we were not specifically studying methane formation, we observed an increase in the area of the void peak where methane eluted when hepatocytes were incubated with DMSO. This suggested an increase in methane formation. After subtracting the zero time void peak, methane formation (±S.E.M.) was, in the presence of DMSO, at 1 hr 31.3 ± 6.7 compared to 6.9 ± 3.6 pmoles/10^7 cells in control hepatocytes, and at 2 hr 27.8 ± 4.6 compared to 14.8 ± 4.9 pmoles/10^7 cells in control hepatocytes ($P < 0.05$ in both cases). There was no change in the area of the void peak over 2 hr of incubations conducted under anaerobic conditions, with or without DMSO. CCl_4 did not produce a significant increase in methane formation (results not shown).

The effects of DMSO and CCl_4 on lipid peroxidation by isolated hepatocytes were also measured by formation of TBA reactive material. The results are shown in Fig. 3. Unlike CCl_4, which produced a significant increase in TBA reactive material, DMSO did not increase TBA reactive material. In fact, DMSO produced a decrease in the formation of TBA reactive material, although the effect was not significant.

Because hepatocyte incubations were conducted in sealed flasks, measurements were made of dissolved oxygen concentrations in the incubation medium. Dissolved oxygen concentrations fell rapidly over the first 10 min but then remained in the range of 3–5 μM (Fig. 4). A similar pattern of change in dissolved oxygen concentrations was seen with hepatocyte incubations where a mixture of humidified 95% air, 5% CO_2 was continually passed through the flasks. The results suggest that the transfer of oxygen from the gas phase to the incubation medium was a limiting factor in determining dissolved oxygen concentrations. The gas mixture in the sealed flask, which initially contained 20% oxygen, at the end of the 4-hr incubation period still contained 17% oxygen. When CCl_4 or DMSO was added to sealed flasks 10 min after the start of incubation, enhanced ethane and *n*-pentane formation was seen as before, indicating that lipid peroxidation occurred not only in the first few minutes of incubation when the oxygen concentrations were relatively high, but

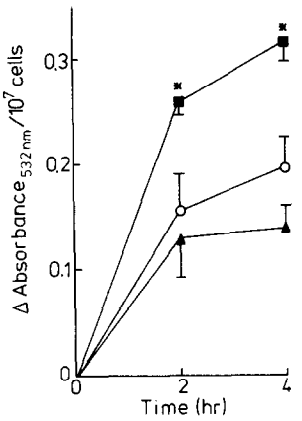

Fig. 3. Formation of thiobarbituric acid reactive material by isolated hepatocytes. Key: (○) control hepatocytes, (▲) hepatocytes in the presence of 14 mM DMSO, and (■) hepatocytes in the presence of 13 mM CCl_4. Each value is the mean of three determinations; bars are S.E. of mean. (*) Indicates $P < 0.05$ compared to control values.

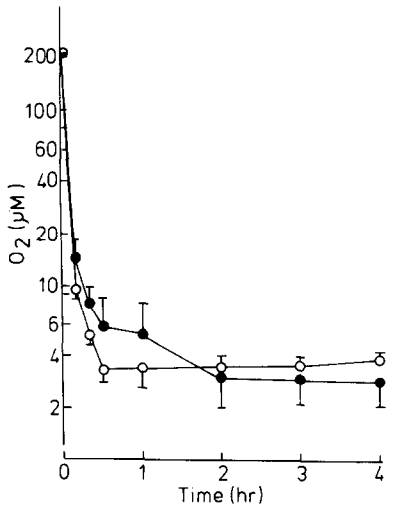

Fig. 4. Dissolved oxygen concentration in hepatocyte incubations. Incubation conditions are described in the text and employed 10^7 cells/ml; incubations were conducted (●) in a sealed vessel, and (○) in a vessel continually gassed with air, 5% CO_2. Each value is the mean of three determinations; bars are S.E. of mean.

throughout the incubation period (these results are not shown).

The effects of DMSO and CCl_4 on release of lactate dehydrogenase from hepatocytes are shown in Table 2. CCl_4 but not DMSO produced a significant increase in lactate dehydrogenase release at 2 hr. By 4 hr LDH release was the same for all preparations and indicated extensive cell damage. The loss of cell viability over time with hepatocytes maintained in sealed vials has been reported previously by Smith *et al.* [14] and limits the amount of useful toxicity information that can be gained to the early time points only.

DISCUSSION

Previous studies have shown that isolated hepatocytes form ethane and *n*-pentane [14–16]. The values for unstimulated ethane and *n*-pentane formation by hepatocytes, obtained in this study, of 11.2 and 5.6 pmoles/hr/10^7 cells, respectively, are similar to values reported by other workers. Ethane is formed during breakdown of ω3-unsaturated fatty acid hydroperoxides and *n*-pentane during breakdown of ω6-unsaturated fatty acid hydroperoxides [1–3]. Ethane or *n*-pentane formation is generally considered to provide a better method of measuring lipid peroxidation than other available methods [1–3, 14–16]. Hepatocytes incubated with DMSO exhibited an increase in ethane and *n*-pentane production over control hepatocytes without an increase in the formation of TBA reactive products. TBA reactive products of lipid peroxidation such as malondialdehyde can be further metabolized by intact hepatocytes and the formation of ethane and *n*-pentane is a more sensitive measure of lipid peroxidation than TBA reactive material [17]. Smith *et al.* [14] have shown in CCl_4-treated hepatocytes that ethane formation precedes detectable TBA reactive material by 90 min.

Table 2. Lactate dehydrogenase release by hepatocytes

	2 hr Percent total	4 hr Percent total
Control	28.4 ± 0.9	57.2 ± 7.5
DMSO, 14 mM	25.5 ± 2.7	66.8 ± 5.1
CCl_4, 13 mM	53.7 ± 11.6*	67.0 ± 4.0

The average total releasable lactate dehydrogenase was 4000 ± 175 units/liter/10^7 cells.

* $P < 0.05$ compared to control values.

DMSO produced an increase in the formation of ethane and *n*-pentane by isolated hepatocytes to about one-third the values seen with CCl_4. CCl_4 is thought to stimulate ethane and *n*-pentane formation by hepatocytes through a mechanism involving metabolism of CCl_4 by cytochrome P-450 to a free radical species [14, 15]. The mechanism for the increased ethane and *n*-pentane formation in the presence of DMSO is not clear. DMSO might undergo metabolism to a free radical species that initiates lipid peroxidation, increasing ethane and *n*-pentane formation in a manner similar to that for CCl_4. There are, however, no reports that DMSO is metabolized to a free radical species. In the intact animal, DMSO is metabolized primarily to dimethyl sulfone which is excreted in the urine [18] and in lesser amounts to dimethylsulfide which is excreted with the expired air [19]. A sulfoxide reductase, probably involving thioredoxin, has been reported in rat liver cytosol [20, 21]. Sulfoxide reductase activity was not inhibited by N_2. DMSO appears to be a substrate, albeit a poor one, for the sulfoxide reductase as shown by its competitive inhibition of the metabolism of other sulfoxides with a K_i around 0.2 mM [21]. Sulfoxide reductase is probably responsible for reduction of DMSO to dimethylsulfide by isolated hepatocytes.

Another explanation for the increased ethane and *n*-pentane formation by hepatocytes in the presence of DMSO is that DMSO in some way facilitates ethane and *n*-pentane production without increasing the rate of endogenous lipid peroxidation. Ethane and *n*-pentane can be further metabolized *in vivo*, and agents that inhibit alkane metabolism can produce an increase in the accumulation of ethane and *n*-pentane in the expired air of an animal in a closed system without stimulating lipid peroxidation [22, 23]. However, in a closed *in vitro* system containing hepatocytes, de Ruiter *et al.* [15] have shown that *n*-pentane is not appreciably metabolized. Since alkane metabolism is a function of molecular mass [22], it is even more unlikely that ethane is appreciably metabolized by hepatocytes. It does not appear likely that DMSO increases the formation of ethane and *n*-pentane by hepatocytes by inhibiting their metabolism. It is also unlikely that DMSO increases the efficiency of release of ethane and *n*-pentane from the incubation medium into the gas phase because de Ruiter *et al.* [15] have shown that the transfer is almost complete under normal conditions.

DMSO reacts with hydroxyl radicals to give the methyl radical [13, 24]. Abstraction of a hydrogen atom by the methyl radical gives methane while ethane can be formed by dimerization of the methyl radical [25]. Only a fraction of the hydroxyl radicals captured by DMSO react to give methane [13]; despite this, the production of methane from DMSO has been used to detect hydroxyl radicals in biological systems [25]. Although we were not specifically studying methane formation by hepatocytes, our results suggest that there was an increased formation of methane in the presence of DMSO indicating, perhaps, the endogenous production of hydroxyl radicals by hepatocytes. The formation of ethane by reaction of hydroxyl radicals with DMSO [25] is a possible explanation for the increase in ethane formation by hepatocytes in the presence of DMSO. It does not, however, explain the increase in the formation of *n*-pentane. Raleigh and Kremers [26] have reported that, in a model system, DMSO does not protect lipid membranes against hydroxyl radical induced lipid peroxidation and that at high concentrations (>0.3 M) DMSO promotes lipid peroxidation. This promotion was suggested to be due to a change in the structure of the lipid micelles. The concentration of DMSO found to stimulate ethane and *n*-pentane formation by hepatocytes (14 mM) was considerably lower than the concentration found by Raleigh and Kremers [26] to promote lipid peroxidation. It is possible, however, that DMSO alters the structure of lipid membranes in the cell in a way that favors lipid peroxidation.

Some workers have expressed concern that incubations in sealed vessels for measuring ethane and *n*-pentane production by hepatocytes might lead to the hepatocytes becoming anoxic [14]. The continual increase in ethane and *n*-pentane over extended periods has been taken as evidence that this is not the case [15]. In the present study, measurement of dissolved oxygen concentrations in the hepatocyte incubation medium showed that there was a rapid depletion of oxygen in the first few minutes of incubation but relatively constant levels of oxygen, of 3–5 μM, over the next several hours. Oxygen concentrations in the hepatocyte incubation medium were not appreciably affected by continually flushing the flask with air. Thus, the limiting factor in determining the dissolved oxygen concentration appears to be the slow diffusion of oxygen from the gas phase into the medium. Although low, the steady-state dissolved oxygen concentrations in the hepatocyte incubation medium appear to be in the physiological range for liver *in vivo*. Reported values for oxygen concentrations of rat liver *in vivo* range from 1 to 60 μM [27, 28]. Low oxygen concentrations between 1 and 11 μM O_2 have been found to stimulate CCl_4-induced microsomal lipid peroxidation [29], probably because oxygen inhibits the cytochrome P-450 dependent formation of free radicals from CCl_4, while sufficient oxygen is required to allow propagation of lipid peroxidation. We do not know whether low oxygen concentrations facilitate DSMO-enhanced ethane and *n*-pentane formation since only one initial oxygen concentration was used in our study.

The role of lipid peroxidation in cell injury has not been resolved. The onset of lipid peroxidation is an important event in CCl_4 hepatotoxicity but may be only a secondary event for other hepatotoxic compounds [30]. DMSO is not a hepatotoxic agent [5, 31] and has been used at relatively high concentrations to protect hepatocytes during freezing and thawing [32]. In the present study, DMSO did not cause hepatotoxicity as measured by release of cytosolic lactate dehydrogenase. DMSO appears, therefore, to show a separation between an enhancement of lipid peroxidation, measured by ethane and *n*-pentane production, and hepatotoxicity.

In summary, we have shown that DMSO produced an increase in ethane and *n*-pentane formation by rat isolated hepatocytes, suggesting an increase in lipid peroxidation. There was, however, not a concomitant increase in TBA reactive materials. DMSO did not exhibit toxicity towards the hepatocytes.

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