Ethanol treatment increases triacylglycerol and cholesteryl ester content of cultured hepatoma cells

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Abstract Well-differentiated Reuber H35 rat hepatoma cells in culture maintain a variety of biochemical functions characteristic of hepatocytes [Deschatrette, J., and M. C. Weiss. 1974. *Biochimie.* **56:** 1603–1611]. To demonstrate the suitability of this system as a model for exploring mechanisms of ethanol hepatotoxicity, the following were investigated: *1*) ethanol metabolism in whole cells and cell extracts and *2*) effects of ethanol exposure on cellular lipid content. Cultures of H35 cells exposed to 10 mM ethanol metabolized the ethanol at rates similar to those reported in rat liver. Under these conditions, soluble alcohol dehydrogenase activity accounted for greater than 87% of total ethanol metabolism. H35 cells exposed to 240 mM ethanol for 3 days contained four times more triacylglycerol and cholesteryl ester than control cells. Total phospholipid and unesterified cholesterol levels were unaffected by ethanol. Neutral lipid content of Chinese hamster ovary cells was unchanged after ethanol exposure. The increased triacylglycerol content of ethanol-treated H35 cells appeared to result from an accelerated rate of conversion of long chain fatty acids into triacylglycerol. Several lines of evidence indicated that alcohol dehydrogenase-mediated ethanol oxidation was critical in promoting increased triacylglycerol content of cultured cells. Since 240 mM ethanol blocked cellular proliferation, long term effects of ethanol were studied at a level of 10 mM, which allowed a nearly normal growth rate. After 7 weeks of continuous exposure, 10 mM ethanol-treated H35 cells contained five times more triacylglycerol than paired controls. The well-differentiated H35 cell appears to be an excellent in vitro model system for studying both short-term and long-term effects of ethanol on liver cells.—**Polokoff, M. A., M. Iwahashi, and F. R. Simon.** Ethanol treatment increases triacylglycerol and cholesteryl ester content of cultured hepatoma cells. *J. Lipid Res.* 1983. **24:** 1030–1038.

Supplementary key words Reuber H35 cells • lipid metabolism • ethanol toxicity • alcohol dehydrogenase activity • fatty liver

The chronic abuse of alcohol is a major factor contributing to the development of liver cirrhosis, a leading cause of death among adults (1). The studies of Lieber and his coworkers have demonstrated that ethanol itself is a hepatotoxin, even in the presence of a nutritionally adequate diet (2). Nevertheless, only a small fraction of chronic alcoholics ever develop cirrhosis (3). Recently, it has become apparent that as yet unknown hereditary factors may predispose certain individuals to the development of serious ethanol-related liver damage (4).

Progress in understanding the molecular basis of ethanol hepatotoxicity, and identifying genetic factors influencing it, has been hampered by the lack of suitable in vitro model systems. The extensive biochemical and genetic studies of the effects of ethanol on microorganisms (5, 6) or cultured fibroblasts (5, 7), may be of limited utility in this regard. These cells are structurally and functionally distinct from liver cells, particularly with respect to ethanol metabolic capacity. On the other hand, isolated hepatocytes or the perfused liver are not suited to long-term biochemical and genetic studies.

We have chosen the Reuber H35 cell, adapted to growth in culture by Pitot et al. (8) from a bile-secreting rat hepatoma (9), as a model to investigate the molecular basis of ethanol hepatotoxicity because *1*) it can be propagated indefinitely in culture; *2*) it maintains a number of "liver-specific" functions, including alcohol dehydrogenase (ADH) activity (EC 1.1.1.1) (10); and *3*) "dedifferentiated" variants lacking liver-specific functions are available (11). The suitability of this model system was evaluated by the following criteria: *1*) ability to metabolize ethanol in whole cells and cell extracts, *2*) effects of ethanol on cell growth rate and viability, and *3*) ability of ethanol to alter neutral lipid storage and metabolism. The latter factor was chosen because the most characteristic response of hepatocytes in vivo to acute or chronic ethanol exposure is a dramatic increase in the content of triacylglycerol (TG) and cholesteryl ester (CE) (12). Dedifferentiated H35 cells and non-liver-derived Chinese hamster ovary (CHO) cells were employed to help distinguish responses common to all mammalian cells from those specific to liver cells.

Abbreviations: ADH, alcohol dehydrogenase; TG, triacylglycerol; CE, cholesteryl ester; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium.

EXPERIMENTAL PROCEDURES

Materials

Trypsin, Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (F12), were obtained from Gibco, Grand Island, NY. Calf serum was the product of Biocell Laboratories. [1-^{14}C]Acetate, [1,3-^{3}H]glycerol, and [9,10-^{3}H]palmitic acid came from New England Nuclear. [1-^{14}C]Ethanol was obtained from Amersham. Silica gel G, 250 μm thin-layer plates were obtained from Analtech. Purified yeast alcohol dehydrogenase was a product of Boehringer Mannheim. Crystalline 4-methyl pyrazole (Labkemie, Sweden) was a gift from the University of Colorado Alcohol Research Center.

Gas–liquid chromatography was performed on a Perkin-Elmer model 3920B equipped with a flame ionization detector. All columns and column packings, triheptadecanoin, cholesteryl heptadecanoate, and fatty acid methyl ester standards were obtained from Supelco. Applied Sciences Laboratory was the source of coprostanol.

Chloroform, methanol, benzene, and 95% ethanol were reagent grade and were glass distilled before use.

Cell lines and culture

The following Reuber H35 hepatoma-derived cell lines were employed. ACL-1 is a "well-differentiated" subclone[1] of H4 EII-C3, obtained from Dr. Wesley Wicks, University of Pittsburgh. UD5L, a stably "de-differentiated" variant (13), was provided by Dr. Emma Moore, Department of Pathology, University of Colorado Health Sciences Center. All H35 cells were grown in DMEM supplemented with 5% (v/v) each of calf serum and fetal calf serum in an atmosphere of 10% CO_2/90% air.

The fibroblast line CHO-K1 was obtained from The American Type Culture collection and grown in F12 supplemented with 8% (v/v) fetal calf serum in an atmosphere of 5% CO_2/95% air.

All cells were grown at 37°C as monolayers in plastic flasks or dishes as indicated in the table and figure legends. Stock cultures and long-term control and ethanol-treated cultures were maintained in logarithmic phase growth by subculturing every 3–4 days into fresh media after detachment with trypsin. All lines were judged free of mycoplasma by a fluorescence dye binding technique (14). Incubations involving volatile compounds were performed in capped flasks using growth media pre-equilibrated in the appropriate CO_2/air atmosphere.

Lipid isolation and quantitation

Cell monolayers were washed twice with calcium- and magnesium-free phosphate-buffered saline (PBS), and detached by incubation for 30 min at 4°C in PBS containing 0.2 mg/ml disodium EDTA. Cells were pelleted at 400 *g* for 10 min, washed twice, and finally taken up in PBS. A portion of the cell suspension was extracted by the method of Bligh and Dyer (15) after addition of triheptadecanoin, cholesteryl heptadecanoate, and coprostanol as internal standards. A portion of the total lipid extract was analyzed for phospholipid by the method of Ames and Dubin (16). Unesterified cholesterol in the total lipid extract was separated by gas–liquid chromatography on a 3 ft × 2 mm column of 3% SP-2250 on 100/120 mesh Supelcoport. The column temperature was 280°C and carrier helium flow rate was 30 cc/min. Quantitation was achieved by comparison of the peak height of cholesterol to that of the coprostanol internal standard.

Triacylglycerol and cholesteryl ester fractions were isolated from a portion of the total lipid extract by thin-layer chromatography on silica gel G developed in hexane–diethyl ether–glacial acetic acid 80:20:1 (v/v/v). The appropriate zones of the plate were scraped into plugged Pasteur pipets, and the lipids were eluted with cholorform. Triacylglycerol fatty acids were converted to their methyl esters (17) and separated by gas–liquid chromatography on a 6 ft × 2 mm column of 10% SP2330 on 100/120 mesh Chromosorb WAW. Following injection, the column was run at 175°C for 10 min, followed by a 32°/min gradient to 225°C, which was then maintained 4 min. Helium carrier flow was 30 cc/min. Individual peaks were cut out and weighed. Quantitation was achieved by reference to the mass of the standard methyl heptadecanoate peak.

Cholesteryl esters were separated on the basis of fatty acid chain length by gas–liquid chromatography on 1% dexsil 300 on 100/120 mesh Supelcoport. A continuous gradient of 2°C/min from 280°C to 310°C was employed. Helium carrier flow was 30 cc/min. Individual peaks were cut out and weighed. Quantitation was achieved by comparison to the mass of the standard cholesteryl heptadecanoate peak.

Individual phosopholipid polar headgroup species were separated by two-dimensional thin-layer chromatography as described by Esko and Raetz (18).

Preparation of cell extracts for enzyme assay

Monolayers were washed twice with PBS. Cells were detached from the flasks and simultaneously lysed by

[1] Simon, F. R., and M. Iwahashi. Unpublished observations.

incubation in 1 mM sodium bicarbonate for 15 min at 4°C. Total particulate and soluble fractions were separated by centrifugation of the lysate, following adjustment to 0.25 M sucrose, 5 mM Tris-Cl, pH 7.4, at 100,000 *g* for 1 hr.

Alcohol dehydrogenase assays

Alcohol dehydrogenase (EC 1.1.1.1) (ADH) activity was assayed in the direction of ethanol oxidation at 23°C at pH 9.6 according to the method of Cederbaum et al. (19). The reaction was proportional to time for up to 3 min and to the amount of protein up to 100 μg.

Measurement of ethanol in growth media

A 40-μl aliquot of growth medium was quickly transferred to 2 ml of ice-cold 2% perchloric acid. Portions of this solution were incubated in 0.5 M Tris-HCl, pH 8.8, containing 1.5 mM nicotinamide adenine dinucleotide and 2.2 units/ml of purified yeast alcohol dehydrogenase (total volume 2.0 ml) at 30°C for 15 min. The absorbance at 340 nm was taken and ethanol concentration was determined by reference to a standard curve.

Estimation of rate of ethanol metabolism by cell monolayers

A series of parallel confluent monolayer cultures of H35-ACL-1 (1×10^7 cells per 75-cm^2 flask) were incubated in growth media (10 ml per flask) to which 10 mM ethanol had been added. Over a period of 24 hr flasks were pulled at various times and the ethanol concentrations in the growth media were determined as described above. Control experiments employing capped flasks with cells demonstrated that no significant loss of ethanol due to evaporation occurred during 24 hr.

RESULTS

Ethanol metabolism

Two different cell lines derived from the Reuber H35 hepatoma, and the non-liver-derived CHO-K1 fibroblast, were tested for the ability to oxidize ethanol. In cell extracts, the "well-differentiated" H35-ACL-1 cell showed considerable soluble ADH activity (**Fig. 1A**). The velocity versus ethanol concentration curve appeared to be bimodal. The first component had an apparent half-maximal concentration of about 0.6 mM ethanol, while the second component was not saturated with ethanol at 400 mM. 4-Methyl pyrazole completely abolished ADH activity in the soluble fraction when 10 mM ethanol was employed (Fig. 1B). When 400 mM ethanol was employed, only about 40% inhibition of the activity occurred. No ADH activity was observed in the total particulate fraction (data not shown).

The "de-differentiated" (13) H35-UD5L line had no detectable soluble ADH activity below 20 mM ethanol

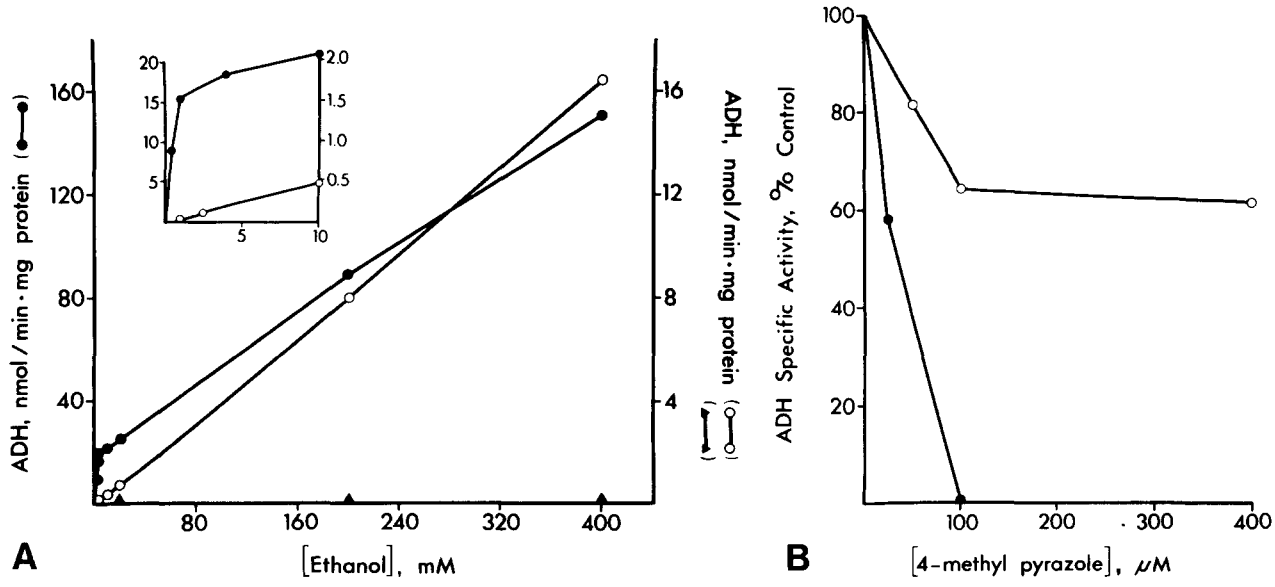

Fig. 1. Alcohol dehydrogenase activity in cultured cells. Soluble extracts were prepared from logarithmically-growing cultures. A. Activity of ADH at 23°C was determined at pH 9.6 in the direction of ethanol oxidation as described in Experimental Procedures. Assays employed 50 μg of soluble protein. Inset: ADH activity in the range 0–10 mM ethanol. (●), H34-ACL-1; (○), H35-UD5L; (▲), CHO-K1. B. ADH activity of H35-ACL-1 in the presence of 4-methyl pyrazole. Assays employed 50 μg of protein. (●), Activity determined with 10 mM ethanol; (○), activity determined with 400 mM ethanol.

(Fig. 1A). Above this concentration, activity increased and, similar to ACL-1, was not saturated at 400 mM ethanol. CHO-K1 cells had no detectable ADH activity at any ethanol concentration up to 400 mM (Fig. 1A).

When H35-ACL-1 monolayers were supplemented with [1-^{14}C]ethanol in the growth media, over 60% conversion of the labeled ethanol into nonvolatile material occurred within 24 hr. Most of this material was recovered in the growth medium in a non-lipid-extractable form. Of the total nonvolatile radioactivity, 3% was lipid-extractable (**Table 1**). Upon thin-layer chromatography, greater than 95% of this material was found to consist of phospholipid, triacylglycerol, and unesterified cholesterol (data not shown). The conversion of [^{14}C]ethanol into nonvolatile products by H35-ACL-1 cells was inhibited 88% by addition of 200 μM 4-methyl pyrazole to the incubation (Table 1), although cell viability was unaffected (data not shown). CHO-K1 monolayers had very little capacity to metabolize [^{14}C]ethanol, being about 100 times less active than H35-ACL-1 (Table 1).

When H35-ACL-1 monolayers were incubated in media containing 10 mM ethanol, an approximately linear rate of disappearance of ethanol occurred over a 24-hr period (data not shown). Under these conditions a rate of ethanol metabolism of about 28 nmol/min per 10^7 cells can be calculated.

Effect of ethanol on cellular lipid content

Logarithmically growing cultures of H35-ACL-1 cells were exposed for 3 days to various ethanol concentrations. Cellular TG content increased with increasing ethanol concentrations, reaching a maximum value fourfold higher than control when 240 mM ethanol was employed (**Fig. 2**). Higher ethanol levels did not further increase cellular TG content. When 240 mM ethanol was added, TG was increased 60% above control levels after 6 hr, and reached a maximum value within 3 days. Ethanol did not alter the fatty acid composition of TG, which consisted of 60% 18:1 and about 10% each of 16:0, 18:0, and 18:2 (data not shown).

TABLE 1. Metabolism of [^{14}C]ethanol by cultured cells

Cells	4-Methyl Pyrazole	Lipids	Nonlipids	Total
	mM	*% of radioactivity recovered in nonvolatile compounds*		
H35-ACL-1	0			61.0
Cells		1.7	0.2	
Media		0.3	58.5	
H35-ACL-1	0.2			7.4
Cells		0.3	0.0	
Media		0.1	7.0	
CHO-K1	0			0.6
Cells		0.2	0.0	
Media		0.0	0.4	

Confluent monolayer cultures (2×10^6 cells/25-cm^2 flask) were incubated for 24 hr in 3 ml of media containing 1 μCi/ml of [1-^{14}C]ethanol, 56 Ci/mol, in the presence or absence of 4-methyl pyrazole. Media was removed and cells were harvested by scraping with a rubber policeman into PBS. Lipids and nonlipids were separated by Bligh-Dyer extraction (15), and the isolated fractions were incubated under a stream of nitrogen to remove volatile compounds.

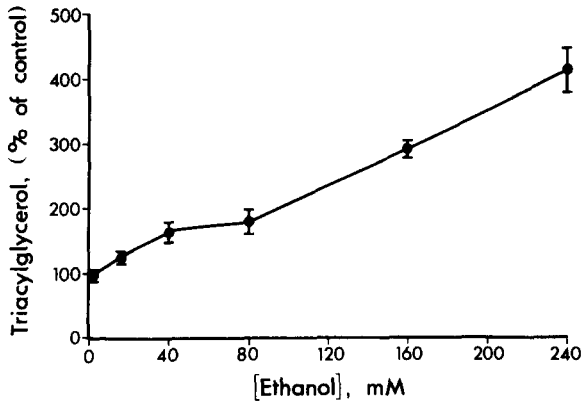

Fig. 2. Effect of 3-day ethanol exposure to TG content of hepatoma cells. Cultures (1×10^6 cells/75-cm^2 flask) of logarithmically-growing H35-ACl-1 cells were incubated for 3 days in the presence of various ethanol concentrations. Media (10 ml/flask) and ethanol were changed daily. Cells were then harvested and protein and TG content were determined as described under Experimental Procedures. Results are expressed as mean ± standard deviation for three separate experiments at each ethanol level. TG content of control cells was 23.6 nmol per mg cell protein.

To determine whether the effect of ethanol was generalized to other lipid classes or other cultured cell lines, parallel cultures of H35-ACL-1 and CHO-K1 were incubated for 3 days in the presence or absence of ethanol (**Table 2**). In H35-ACL-1 cells, both TG and CE content were dramatically increased by ethanol. In contrast, no effect of ethanol on content of these lipids in CHO-K1 cells was observed. In both cell lines, ethanol had no effect on the content of phospholipid or unesterified cholesterol, or on the ratio of DNA to protein (Table 2). Ethanol treatment did not alter the total cellular phospholipid polar headgroup distribution in H35-ACL-1 cells (data not shown).

The possibility was tested that the observed increases in cellular TG and CE content were a secondary consequence of an ethanol-induced inhibition of cellular proliferation. Employing confluent H35-ACL-1 monolayers, whose cell density remains constant, cellular lipid content was altered by ethanol exactly as had been seen in the experiments with logarithmically growing cells.

TG metabolism

The effect of 240 mM ethanol exposure on the metabolism of TG in H35-ACL-1 cells was investigated.

TABLE 2. Effect of ethanol exposure on cellular constituents

Cell	[Ethanol]	No. of Determinations	Total Cellular Content mg Protein				
			DNA	Phospholipid	Cholesterol	CE	TG
	mM		*μg*	*nmol*	*nmol*	*nmol*	*nmol*
H35-ACL-1	0 (Control)	13	126 ± 30[a]	184 ± 34	38.6 ± 6.2	4.0 ± 1.9	19.8 ± 7.9
	240	12	141 ± 50	213 ± 64	35.2 ± 8.0	16.0 ± 8.0[b]	93.1 ± 2.5[b]
CHO-K1	0 (Control)	3	100 ± 60	121 ± 12	41.1 ± 0.3	24.2 ± 1.6	6.8 ± 2.3
	240	3	109 ± 30	133 ± 10	43.5 ± 4.7	25.8 ± 3.2	8.7 ± 1.9
H35-UD5L	0 (Control)	4	—[d]	—	—	4.0 ± 1.4	7.4 ± 4.3
	40	4	—	—	—	3.4 ± 1.1	8.1 ± 2.8
	240	4	—	—	—	3.2 ± 1.0	14.7 ± 3.5[c]

Logarithmically growing H35-ACL-1, CHO-K1, or H35-UD5L (initial cell densities 2×10^6, 1×10^6, and 2×10^6 cells per 75-cm^2 flask, respectively), were incubated for 72 hr in the presence or absence of ethanol in 10 ml of growth media. Media and ethanol were changed daily. Cells were harvested and cellular constituents were determined as described in Experimental Procedures.

[a] Values are expressed as mean ± standard deviation.

[b] $P < 0.001$ versus control cells.

[c] $P < 0.05$ versus control cells.

[d] Not determined.

The rate of TG turnover was estimated by following the loss of radioactivity from this lipid after cells prelabeled with [^{3}H]glycerol were incubated in media containing unlabeled glycerol. When prelabeled control cells were incubated in the presence or absence of 240 mM ethanol, no difference was seen in the turnover rate of TG (**Fig. 3A**). Cells prelabeled in the presence of 240 mM ethanol, as expected, initially had more labeled TG than control cells (Fig. 3B). The rate of turnover of this labeled TG in the continuing presence of ethanol was greater than or equal to that observed in untreated cells throughout the chase period (Fig. 3B).

Rates of lipid biosynthesis in H35-ACL-1 cells were estimated by pulse-labeling with radioactive lipid precursors. Cells were labeled with [^{3}H]palmitic acid (**Table 3**). Incorporation of label into total lipid was increased 45% in cells pre-treated with 240 mM ethanol for 20 hr, compared to control cells. The majority of the labeled palmitate was incorporated into TG. The rate of labeling of TG was increased 60% by ethanol pre-treatment. In contrast to these findings, similar rates of incorporation of [^{14}C]acetate into TG were observed in control cells and 240 mM ethanol-treated cells (data not shown).

Role of ethanol oxidation in neutral lipid accumulation

Total cellular TG and CE were not increased by ethanol in CHO-K1 cells, which lack ADH activity (Table 2 and Fig. 1A). Therefore the hypothesis was tested that neutral lipid accumulation was dependent on ADH-mediated ethanol oxidation. Incubation of H35-ACL-1 cells with 200 μM 4-methyl pyrazole had no significant effect on TG or CE levels (**Table 4**). Ethanol alone, at either 40 mM or 240 mM, significantly increased the cellular content of both lipids. At 40 mM ethanol, 4-methyl pyrazole prevented the increase in TG but not in CE. When 240 mM ethanol was employed, 4-methyl pyrazole was unable to prevent the ethanol-mediated increase in TG.

"De-differentiated" H35-UD5L cells were exposed to various ethanol levels for 3 days (Table 2). At 40 mM ethanol, cellular TG and CE were unchanged from control. At 240 mM ethanol, TG was significantly increased over control levels but CE was unchanged. The magnitude of the effect of 240 mM ethanol on TG content was smaller in UD5L than in ACL-1.

Effects of ethanol on growth rate and viability of H35-ACL-1

Exposure of logarithmically growing cultures to ethanol decreased the rate of growth in a concentration-dependent fashion (**Fig. 4**). Cells exposed to 240 mM ethanol did not proliferate. Cell viability, as judged by the ability of single cells to form colonies following removal of ethanol from the growth medium, remained high after exposure to 240 mM ethanol (Fig. 4). Viability was reduced by 50% at 400 mM ethanol and by 100% at 800 mM ethanol.

Effects of long-term growth in 10 mM ethanol or H35-ACL-1

H35-ACL-1 cells were grown and serially passaged for 7 weeks in the continuous presence or absence of 10 mM ethanol. The ethanol-treated cells proliferated about as rapidly as control cells, and by light and electron microscopy no morphological differences were observed between the two groups (data not shown). The

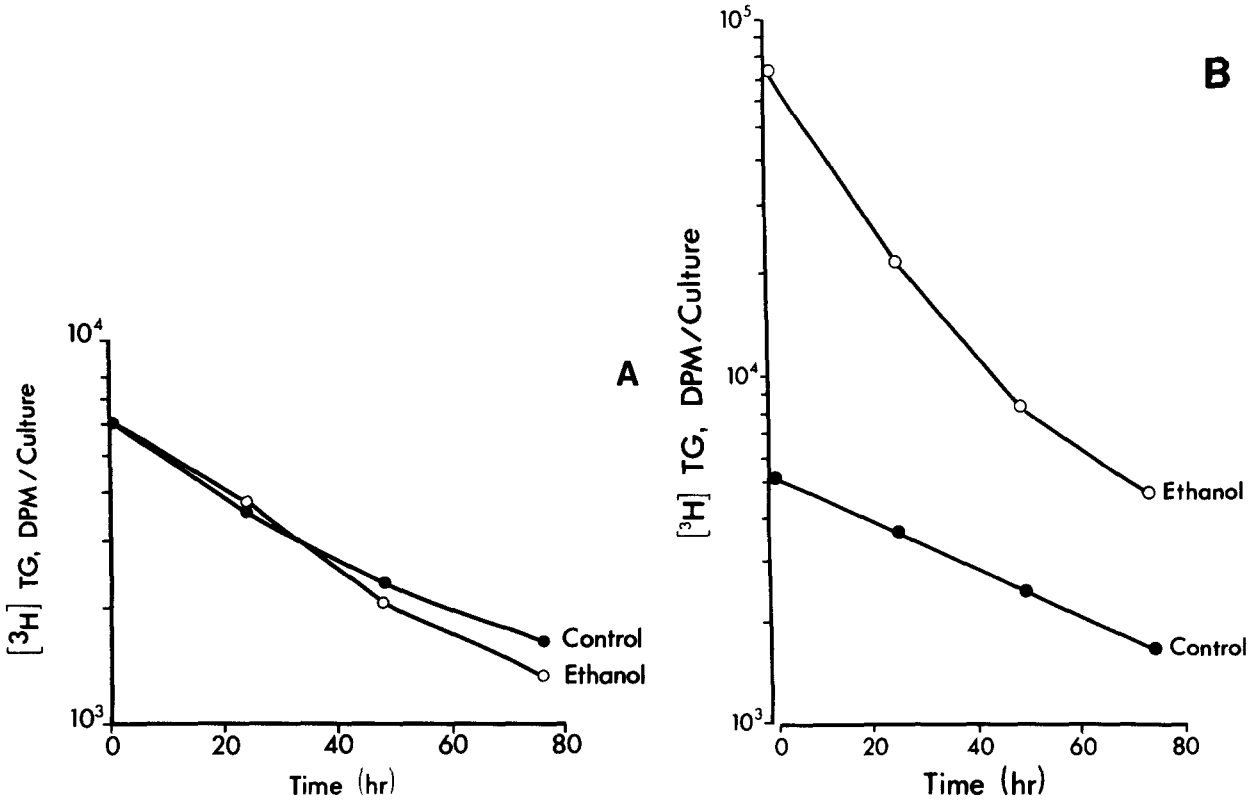

Fig. 3. Effect of ethanol on the rate of turnover of cellular TG. A. H35-ACL-1 cells were seeded at a density of 5×10^5 cells per 75-cm^2 flask and incubated for 1 week in the presence of 50 μM [1,3-^{3}H]glycerol, 10 Ci/mol, in 10 ml of growth media. Media was changed once. These cells were seeded into a series of 100-mm dishes at 1.3×10^6 cells per dish in 10 ml of the same [^{3}H]glycerol-containing media. After 48 hr all dishes were changed to media containing 50 μM unlabeled glycerol, in the absence (●) or presence (○) of 240 mM ethanol. See Experimental Procedures for details. B. The experimental design in part A was modified as follows. After 1 week of growth in the presence of [^{3}H]glycerol without added ethanol, cells were seeded into 100-mm dishes in [^{3}H]glycerol-containing media, and half the dishes also received 240 mM ethanol. After 48 hr, all dishes were changed to media with unlabeled glycerol, and those that had previously received ethanol were again given 240 mM ethanol.

TABLE 3. Effect of 240 mM ethanol on rate of [^{3}H]palmitate incorporation into lipids

Lipid Class	[Ethanol]	$10^3 \times$ DPM/mg Protein	% Increase with Ethanol
	mM		
Total	0	248	45
	240	359	
TG	0	135	60
	240	216	
Phospholipid	0	64.9	26
	240	81.7	
Other	0	48.1	27
	240	61.3	

H35-ACL-1 monolayers (2×10^6 cells/100 mm dish) in logarithmic phase growth were pre-incubated for 20 hr in the presence or absence of 240 mM ethanol. Cultures were then pulse-labeled for 2 hr in 3 ml of the same media containing 10 μM [9,10-^{3}H]palmitate, 100 Ci/mol. This media was prepared by adding the labeled palmitate in a small volume of ethanol (1 μl per ml of media), followed by incubation overnight at 37°C in an unstoppered container in a 10% CO_2/90% air atmosphere.

ethanol-treated cells contained about 5 times more TG than the control cells, while total phospholipid in the two groups was similar (**Table 5**).

DISCUSSION

Ethanol oxidation in differentiated H35 cells is similar in several respects to that reported in rat liver. The rate of oxidation in the hepatoma cells, exposed to 10 mM ethanol is of the same order of magnitude as that reported in rat liver.[2] In cell extracts, the presence of an ADH activity with a low apparent K_m for ethanol, reported to correspond to a liver-specific isozyme (10), was confirmed in this study (Fig. 1A). This low K_m ac-

[2] Assuming 1×10^7 cells contain 2 mg of total protein, and H35 cells metabolized 14 nmol ethanol min^{-1} mg protein^{-1}. The rate of ethanol oxidation in rat liver has been estimated to be 3 μmol min^{-1} g wet weight^{-1} (20). Assuming 1 g wet weight contains 170 mg of liver protein, this rate is equivalent to 17.6 nmol min^{-1} mg protein^{-1}.

TABLE 4. Effect of 4-methyl pyrazole on lipid content of control and ethanol-treated H35-ACL-1 cells

Additions to Growth Media	No. of Determinations	nmol TG / mg Protein	nmol CE / mg Protein
0 (Control)	4	26.4 ± 2.0[a]	3.8 ± 2.2
0.2 mM 4-MP	4	24.4 ± 0.8	4.2 ± 1.8
40 mM Ethanol	4	49.4 ± 4.4[b]	7.7 ± 2.2[c]
0.2 mM 4-MP + 40 mM Ethanol	4	31.2 ± 7.4	7.4 ± 1.4[c]
240 mM Ethanol	3	70.6 ± 11.4[b]	17.6 ± 3.4[b]
0.2 mM 4-MP + 240 mM Ethanol	3	75.5 ± 10.0[b]	20.0 ± 5.6[b]

Monolayer cultures were incubated for 72 hr under the conditions described in the table. The culture conditions employed and the analysis of cellular constituents were exactly as described in Table 2.

[a] Mean ± standard deviation.

[b] $P < 0.001$ versus control cells.

[c] $P < 0.05$ versus control cells.

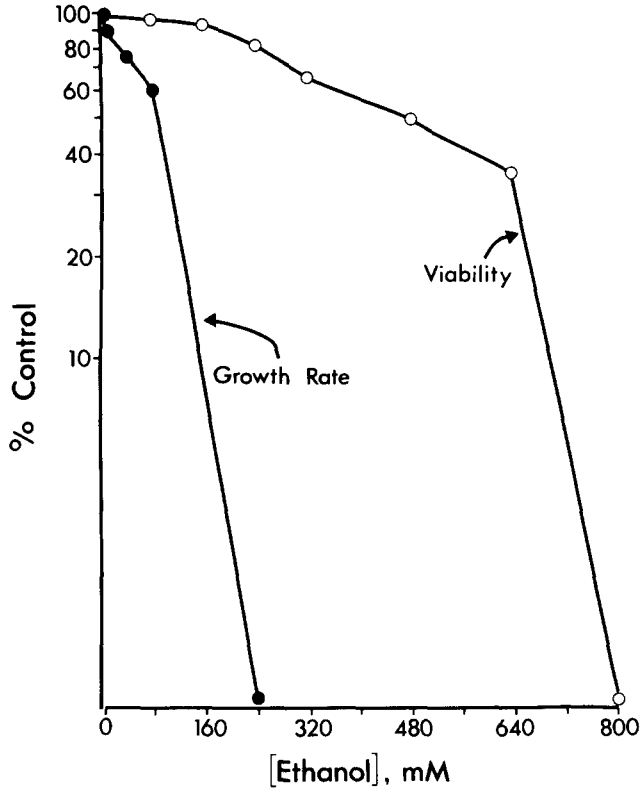

Fig. 4. Effects of ethanol or growth rate and viability of H35-ACL-1 cells. Growth rate was determined as follows. A series of 75-cm^2 flasks was seeded with 2 × 10^5 H35-ACL-1 cells per flask, in 10 ml of growth media. After 24 hr ethanol was added as indicated, and at various times during the next 72 hr, flasks were harvested with trypsin and cells were counted with a hemocytometer. For each level of ethanol, an approximate generation time was determined from a semi-log plot of the data. The generation time of cells grown in the absence of ethanol was 24 hr. Viability was determined as follows. A series of 100-mm dishes was seeded with 500 H35-ACL-1 cells per dish in 10 ml of regular growth media. After 4 hr, ethanol was added to the indicated levels. After 48 hr, all dishes were changed to media lacking ethanol and the dishes were incubated for 14 days. Colonies were fixed with 2% trichloroacetic acid, stained with Coomassie blue, and counted. Viability was expressed as colonies formed relative to non-ethanol-treated control cells, which had an absolute colony forming ability of 83%.

tivity was sensitive to inhibition by 4-methylpyrazole in whole cells (Table 1) and in soluble extracts (Fig. 1B). Since this inhibition reduced ethanol metabolism by 88% in whole cells incubated with low ethanol concentrations (Table 1), ADH-mediated oxidation appears to be the predominant metabolic fate of ethanol in H35-ACL-1. A similar argument has been made for liver cells (21).

In contrast to hepatocytes, H35 hepatoma cells also possess a high apparent K_m ADH activity (Fig. 1A), which likely corresponds to the "stomach" isozyme described by Cederbaum et al. (19) in hepatoma 252, and by Bertolotti and Weiss (10) in H35 cells. In agreement with Moore and Weiss (13), the high apparent K_m ADH is still expressed in UD5L, a de-differentiated H35 cell variant that does not express the low apparent K_m activity (Fig. 1A).

Well-differentiated H35-ACL-1 cells exposed to ethanol underwent a response characteristic of hepatocytes in vivo, the accumulation of excess TG and CE. When 240 mM ethanol was employed in a 3-day incubation, the magnitude of this effect was similar to or greater than that observed in the livers of animals given

TABLE 5. Effect of 7-week exposure to 10 mM ethanol on H35-ACL-1 cells

Additions to Growth Media	nmol TG / mg protein	nmol Phospholipid / mg Protein	μg DNA / mg Protein
0 (Control)	10.2 ± 2.6[a]	186 ± 6.6	104 ± 5.0
10 mM ethanol	49.2 ± 12.1[b]	204 ± 12.4	83 ± 4.6

H35-ACL-1 cells were grown and subcultured in the continuous presence or absence of 10 mM ethanol for 7 weeks. Cells were seeded at a density of 3 × 10^5 per 75-cm^2 flask in 20 ml of growth media. Media was changed every 3 days and cells passaged once per week. Cellular TG, phospholipid, DNA, and protein were determined as described under Experimental Procedures.

[a] Mean ± standard deviation for three separate cultures in each group.

[b] $P < 0.01$ versus control cells.

a large single dose of ethanol, or fed an ethanol-containing diet (12, 22).

The biochemical mechanism(s) of ethanol-induced TG accumulation in the liver remain controversial. Ethanol or its metabolism has been proposed to *1*) decrease the rate of fatty acid oxidation (23); *2*) raise the intracellular concentrations of fatty acyl-CoA or *sn*-glycerol 3-phosphate available to the enzymes of TG synthesis (23, 24); *3*) activate one or more of these TG biosynthetic activities (22, 25, 26); *4*) decrease the rate of TG export (27); or *5*) lead to synthesis of a slowly turning over pool of TG in the liver (28). The data in Fig. 3 argue against the latter two mechanisms in H35 cell ethanol-induced TG accumulation. Thus, an effect of ethanol on the rate of triacylglycerol synthesis is implicated, and the data in Table 3 indicate that the rate of incorporation of exogenous fatty acid into TG is modestly increased. If ethanol treatment raises the intracellular pool of free fatty acid of fatty acyl-CoA, then the magnitude of the ethanol-induced increase in TG synthesis rate may be an underestimate due to isotope dilution. One unresolved question raised by our studies is whether a 60% increase in the rate of TG synthesis could account for a fourfold greater intracellular TG level. The steady-state concentration of cellular TG represents a balance between the rates of synthesis, turnover, and export. At present, little is known about the regulation of these processes in H35 cells, or indeed in the liver.

The role of ADH-mediated oxidation in the process of ethanol-induced hepatic TG accumulation is controversial. It has been shown that oxidation of ethanol via ADH alters the $NAD^+/NADH$ ratio in favor of the reduced pyridine nucleotide (29). In this more reductive environment, it has been argued, generation of *sn*-glycerol 3-phosphate and de novo synthesis of fatty acids would be promoted, and oxidation of fatty acids would be inhibited (23, 24, 29). Such changes would be expected to lead to increased TG synthesis, if the overall rate of the pathway is substrate limited. Direct evidence relating to the importance of ADH-mediated oxidation in hepatic TG accumulation has been sought by treating animals with ADH inhibitors together with ethanol. Some workers found that ADH inhibitors prevented increased hepatic TG levels (30), while others found that the inhibitors actually potentiated ethanol's effect (31).

In our studies, three lines of evidence suggest that ethanol oxidation via ADH is required to produce the increased TG levels seen in ethanol-treated H35 cells. First, in H35-ACL-1 cells exposed to 40 mM ethanol, the presence of 4-methyl pyrazole in the incubation, under conditions expected to inhibit ADH activity (Fig. 1B and Table 1), prevented the increase in cellular TG seen with ethanol alone (Table 4). However, when 240 mM ethanol was employed, under conditions where 4-methyl pyrazole is unable to completely suppress ADH activity (Fig. 1B), the inhibitor failed to prevent the ethanol-mediated increase in TG (Table 4). Second, ethanol had no significant effect on TG content of CHO-K1 cells (Table 2), which lack ADH activity (Fig. 1A). Third, in H35-UD5L cells where the low apparent K_m ADH activity has been lost, (Fig. 1A and reference 13), TG levels were elevated only after exposure to high (240 mM) ethanol concentrations (Table 2).

One unexpected finding in our studies was that unphysiologically high concentrations of ethanol were required to produce maximum increases in cellular TG levels in H35-ACL-1 cells exposed to ethanol for 3 days. The importance of ethanol oxidation in the alcohol-mediated TG accumulation suggests a possible explanation for this unusual observation. The well-differentiated H35 cell contains a high apparent K_m ADH activity that is lacking in the liver parenchymal cell. Assuming that this activity is functional in whole cells as well as in cell extracts, increasing the intracellular ethanol concentration well beyond the level necessary to saturate the low apparent K_m, "liver-specific" ADH activity would lead to an accelerated rate of ethanol oxidation in hepatoma cells, but not in hepatic parenchymal cells. Thus the concentration dependences of phenomena dependent on ADH-mediated ethanol oxidation might be very different in these two types of cells.

Increased hepatic CE content has been reported following chronic ethanol feeding of rats (32). The mechanism underlying this effect of ethanol has received little attention. Well-differentiated H35 hepatoma cells in culture respond to ethanol exposure in a similar fashion (Tables 2 and 4). The ethanol-induced increase in cellular CE is not a generalized response of all cultured mammalian cells, since it did not occur in CHO-K1 cells (Table 2). In contrast to TG, ethanol-dependent CE accumulation was not prevented by 4-methyl pyrazole in H35 ACL-1 cells (Table 4), nor did it occur in H35-UD5L cells exposed to 240 mM ethanol (Table 2). Thus it appears that the increased CE content may be provoked by ethanol itself, rather than as a consequence of ethanol metabolism.

The well-differentiated Reuber H35 cell in culture appears to be an excellent model for exploring the molecular events underlying ethanol hepatotoxicity. Exposure of H35 cells to ethanol leads rapidly to alterations of lipid metabolism characteristic of liver parenchymal cells. The importance of ADH activity in ethanol-related phenomena can be assessed either by employing ADH inhibitors like 4-methyl pyrazole or by comparing the responses of cells that do not express

ADH activity. Finally, long-term effects of ethanol can be observed since cells can be cultured indefinitely in the presence of physiological ethanol concentrations, which do not inhibit growth, but provoke major alterations in lipid metabolism (Table 5).

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