British Journal of Pharmacology (1996) 119, 233-238

Evidence of differential effects produced by ethanol on specific phospholipid biosynthetic pathways in rat hepatocytes

María P. Carrasco, *María C. Sanchez-Amate, Carmen Marco & [1]Josefa L. Segovia

Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of Granada, Granada 18001, Spain and *Department of Clinical & Basic Psychobiology, University of Almería, Almería, Spain

1 The aim of the present study was to investigate the effects of ethanol *in vitro* on the phospholipid biosynthetic pathways in hepatocytes isolated from the rat. We have used [methyl-^{14}C]-choline, [1-^{3}H]-ethanolamine and L-[3-^{3}H]-serine as exogenous precursors of the corresponding phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS).

2 Incubation of hepatocytes in the presence of ethanol significantly alters the incorporation of radiolabel from [^{14}C]-choline and [^{3}H]-ethanolamine into the metabolic intermediates and the final products of the CDP-choline and CDP-ethanolamine pathways. Radioactivity in the metabolic intermediates of both pathways was significantly decreased and the amount of label in PE was reduced whilst that of PC was not modified.

3 In the presence of 4-methylpyrazole, an inhibitor of alcohol dehydrogenase (ADH) activity, ethanol produces a reduction in the label of choline phosphate, ethanolamine phosphate and a significant decrease in the amount of PC and PE radiolabel.

4 On the other hand, ethanol increases the incorporation of serine into phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine, although this effect is observed only in the absence of 4-methylpyrazole, indicating that this alteration is produced by some metabolite generated as a consequence of hepatic alcohol metabolism.

5 Ethanol also interferes with the methylation of phosphatidylethanolamine produced via the CDP-ethanolamine pathway but it does not alter phosphatidylethanolamine methylation when this phospholipid is produced by mitochondrial phosphatidylserine decarboxylation, suggesting the existence of different intramembrane pools of phosphatidylethanolamine, which may exhibit different sensitivity to alcohol.

6 Our results indicate that ethanol exerts two different effects on phospholipid metabolism in hepatocytes: a stimulatory effect on the incorporation of exogenous substrates into different phospholipids probably related to an alteration in the availability of lipogenic substrates as a consequence of ethanol metabolism, and another inhibitory effect produced by ethanol *per se*, which can be observed only when ethanol metabolism is inhibited by the presence of a specific inhibitor of alcohol dehydrogenase activity.

Keywords: Ethanol; rat hepatocytes; phospholipid biosynthesis

Introduction

It has been widely shown that chronic alcohol ingestion produces marked alterations in lipid metabolism both in hepatic and extrahepatic tissues (Forman, 1988; Sanchez-Amate *et al.*, 1991). The main pathway for ethanol metabolism involves hepatic alcohol dehydrogenase (ADH), a cytosolic enzyme that catalyzes the transformation of ethanol into acetaldehyde, which is in turn converted to acetate through acetaldehyde dehydrogenase activity. Both enzyme reactions generate an excess of reducing equivalents in the liver, primarily in the form of reduced nicotinamide adenine dinucleotide (NADH). Thus, after prolonged ethanol consumption NADH produces an increase in the concentration of α-glycerophosphate, which favours hepatic lipid accumulation by trapping fatty acids (Brindley, 1988). In addition, an excess of acetate and NADH may promote the synthesis of fatty acids (reviewed by Lieber, 1994). However, in spite of the fact that hepatic triacylglycerol accumulation has been consistently described in chronic alcoholism (for revision see Lieber, 1994), reports on the effects of ethanol administration on the biosynthesis of phospholipids are scarce and contradictory. Thus, Castro *et al.* (1987) reported that acute ethanol administration stimulates the synthesis of phospholipids from exogenous fatty acids in rat hepatocytes, whilst other authors have indicated a marked decrease in rat reticulocytes (Le Petit-Thevenin *et al.*, 1995).

The biosynthesis of different phospholipids takes place via specific pathways. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can be synthesized in microsomes via cytidine diphosphate-derivatives (CDP-derivatives) whilst both these phospholipids may also be produced from mitochondrial phosphatidylserine (PS) decarboxylation. To our knowledge there are few reports on the effects of ethanol on the biosynthetic pathways of individual phospholipids in spite of their importance as components of biological membranes and their involvement in processes such as membrane fusion, membrane asymmetry or interactions with specific proteins. Slomiany *et al.* (1994) have shown that in alcoholic rats, PC biosynthesis is reduced by 50% as compared to pair-fed controls. Also in our laboratory we have recently demonstrated that in rat hepatocytes, chronic alcohol treatment alters the incorporation of exogenous polar bases into PE and PC via CDP-derivatives and into PS via base-exchange reaction increasing PE and PS biosynthesis and decreasing synthesis of PC (Carrasco *et al.*, 1996).

Although *in vitro* studies cannot totally reflect *in vivo* mechanisms, they allow the possibility of eliminating many endogenous and exogenous factors, which could influence metabolism *in vivo*. To date, the studies on the effects of ethanol *in vitro* have been focused exclusively on the in-

[1]Author for correspondence at: Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, Granada 18001, Spain

corporation of exogenous fatty acids into phospholipids, but there are no reports on the effects of ethanol on individual phospholipid biosynthetic pathways. Thus in this work we have analysed the effects of ethanol *in vitro* on the biosynthesis of PC, PE and PS in rat isolated hepatocytes. To do this we have used serine, ethanolamine and choline as exogenous precursors to explore the possible interference of ethanol in the different steps of the phospholipid biosynthetic pathways. We have also studied the effects of ethanol in the presence of 4-methylpyrazole, a specific inhibitor of ADH activity, in order to analyze the effects of ethanol alone, with no relation to the action of different metabolites produced as a consequence of ethanol metabolism.

Methods

Animals

Male Sprague-Dawley rats (200–250 g) were fed *ad libitum* on a standard chow diet in a chamber with a light cycle from 09 h 00 min to 19 h 00 min and controlled temperature (25°C). The rats were deprived of food overnight when the hepatocytes were to be isolated.

Isolation of hepatocytes

Hepatocytes were isolated by the perfusion technique of Seglen (1976) using collagenase. After perfusion the liver was transferred to a Petri dish and gently dispersed. Aliquots of the suspension were tested for viability by Trypan blue exclusion and preparations with a viability lower than 70% were not used. The suspensions were filtered through a nylon mesh and the hepatocytes were placed on Percoll (1.08 mg ml^{-1}) in a centrifuge tube. After centrifugation for 30 min at 30000 *g*, the viable cells were collected and washed twice at 50 *g* for 2 min before being resuspended in buffered Krebs-Henseleit containing 1.5% bovine serum albumin (w/v) and 10 mM glucose. By this procedure a suspension of hepatocytes was obtained from the different hepatic zones. The cellular viability was higher than 90% in all the preparations used and it was maintained throughout all our experiments both in control and ethanol-treated cells.

Incubation of hepatocytes

Cells (5×10^6) were incubated at 37°C in a shaker water bath (80 oscillations min^{-1}) in 2.5 ml of Krebs-Henseleit solution, pH 7.4, containing 1.5% bovine-serum albumin (w/v) and 10 mM glucose both in the presence and absence of 100 mM ethanol, under an atmosphere of carbogen (95% O_2 : 5% CO_2). After 150 min incubation the reactions were initiated by the addition of 125 nmol of methionine and L-[3-^{3}H]-serine (125 nmol; 150968 d.p.m. $nmol^{-1}$) or [1-^{3}H]-ethanolamine (100 nmol; 119920 d.p.m. $nmol^{-1}$) or [methyl-^{14}C]-choline (125 nmol; 32667 d.p.m. $nmol^{-1}$). The reactions were continued for 30 min at 37°C and stopped by the addition of 7.5 ml of ice-cold Krebs-Henseleit. The cells were washed twice in Krebs-Henseleit medium at 50 *g* for 5 min and the pellet was collected and used for analysis of lipid and water-soluble metabolites. Incubations in the presence of 4-methylpyrazole were made as described above, except that the Krebs-Henseleit medium also contained 3 mM 4-methylpyrazole.

Extraction and analysis of lipids and water-soluble metabolites

Lipids were extracted from the cell pellet according to the procedure of Folch *et al.* (1957). The chloroform layer was dried by evaporation with a stream of nitrogen whilst the water-methanol phase was collected and used to determine water-soluble metabolites. The lipid residue was dissolved in chloroform and the different phospholipids were separated on silica-gel 60 G t.l.c. plates, as indicated by Higgins (1987). Phosphatidyl-N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine were isolated by t.l.c. on silica-gel H plates, using chloroform/methanol/acetic acid (70 : 30 : 4) as solvent, according to Marino *et al.* (1986).

The water-soluble intermediates, ethanolamine phosphate and CDP-ethanolamine, were separated from the aqueous fractions by the method of Wang and Moore (1991), using silica-gel G t.l.c. plates and a solvent system composed of 96% ethanol/3% NH_4OH in water (1 : 2).

The water-soluble PC precursors, choline phosphate and CDP-choline, were separated from the aqueous fractions according to Vance *et al.* (1981) using silica-gel G t.l.c. plates and methanol/0.6% ClNa/28% NH_4OH in water (50 : 50 : 5) as solvent.

The spots corresponding to each compound were visualized by exposing the plates to iodine vapours, then scraped and transferred to scintillation vials for measurement of radioactivity by a Beckman 6000-TA liquid scintillation counter.

The results are expressed as means ± s.e.mean as indicated in the legends to the tables. Data from ethanol-treated and control hepatocytes were compared by Student's *t* test, taking a value of $P < 0.05$ as significant.

Phospholipid head-group analysis

The amount of radioactivity from L-[3-^{3}H]-serine associated with the head-group of PC, PE and PS molecules was determined by phospholipase C digestion according to the method of Kuksis *et al.* (1991) with minor modifications. Phospholipids isolated by t.l.c. were dried under nitrogen. Each phospholipid was dissolved in 1 ml of diethyl ether and 5.5 ml of Tris-HCl buffer (13.3 mM, pH 7.4) containing 0.24% $CaCl_2$ and 1 unit of phospholipase C from *B. cereus* was added to the sample. The tubes were incubated at 30°C for 3 h in a shaker bath (250 oscillations min^{-1}). Digestion was stopped by the addition of 15 ml of chloroform/methanol (2 : 1) and 0.2 ml of 0.1 M HCl. The radioactivity associated with each fraction was determined after the isolation of diacylglycerides and phospholipids by t.l.c. in silica-gel G plates using hexane/ethyl ether/acetic acid (80 : 20 : 1) as solvent.

Chemicals

L-[3-^{3}H]-serine, [1-^{3}H]-ethanolamine and [methyl-^{14}C]-choline were bought from Amersham International (Amersham, Bucks, U.K.). Fatty-acid-free bovine-serum albumin and collagenase (Type A from *Clostridium histolyticum*) were supplied by Boehringer Mannheim. Choline, ethanolamine, L-serine and L-methionine were bought from Sigma. T.l.c. plates were from Sigma and Percoll was from Pharmacia. All other reagents used were of analytical grade.

Results

Incorporation of [methyl-^{14}C]-choline

The main pathway for PC biosynthesis in eukaryotic cells is the Kennedy pathway (Kennedy & Weiss, 1956), which involves the production of different water-soluble intermediates such as choline phosphate and CDP-choline. Results of the analysis in the incorporation of choline into these metabolic intermediates and into the final product, PC, are shown in Table 1. The incubation of control hepatocytes in the presence of [methyl-^{14}C]-choline produces a marked incorporation of this polar base to the corresponding phospholipid, PC. Choline phosphate also exhibits a significant radioactive label whilst the radioactivity in CDP-choline is markedly lower than that found in the other metabolites.

The effects produced by the incubation of hepatocytes in the presence of 100 mM ethanol are also shown in Table 1 and demonstrate a significant decrease in the radioactivity of both

choline phosphate and CDP-choline; the radioactive label of PC was not different in ethanol and control hepatocytes. It is noteworthy that when the cells were incubated in the presence of ethanol and 4-methylpryrazole, and consequently ethanol metabolism via ADH was inhibited, alcohol still produced a significant decrease in the label of choline phosphate. Also a marked reduction in the radioactive label of PC from choline was observed after incubation in the presence of ethanol and 4-methylpyrazole but no change in incorporation into CDP-choline.

Incorporation of [1-^{3}H]-ethanolamine

To study the influence of ethanol on the biosynthetic pathway of PE via CDP-ethanolamine we have determined the incorporation of [1-^{3}H]-ethanolamine into the different metabolic intermediates (results shown in Table 2). Similar to results obtained in the biosynthetic PC pathway in control hepatocytes, the highest radioactivity levels were observed in the final product, PE, although in this case ethanolamine phosphate also shows considerable label levels.

Ethanol produces significant alterations in the CDP-ethanolamine pathway. A significant reduction of radioactivity incorporated into ethanolamine phosphate and CDP-ethanolamine was observed in ethanol-treated hepatocytes, whilst the final product, PE, exhibited higher radioactivity than that of the control cells. Interestingly, when the metabolism of ethanol was inhibited by the addition of 4-methylpyrazole, the reduction in ethanolamine-phosphate synthesis persisted whilst a significant decrease in the incorporation of radioactivity into PE was observed with no change in CDP-ethanolamine incorporation.

Bearing in mind that PE can be successively methylated to give PC, we have also quantified the distribution of radioactivity between the different methylated intermediates (Table 2). It is noteworthy that in control hepatocytes only a small quantity of PE was methylated to PC. In fact, the value of the PC/PE_T radiolabel ratio is lower than 0.02 (PE_T is the total PE synthesized *de novo* and is calculated as the sum of the radioactivity found in the methyl-derivatives and PE produced from [1-^{3}H]-ethanolamine). As far as the effect of ethanol on the microsomal methylation of PE is concerned, our results demonstrate that in spite of the higher radioactivity in PE found in the presence of ethanol, both the methylated intermediates, phosphatidyl-N,N-dimethylethanolamine and phosphatidyl-N-monomethylethanolamine and the final product of this biosynthetic pathway, PC, exhibited a significant decrease in their radioactive label when compared to control hepatocytes. These effects were observed even when ethanol metabolism was suppressed by 4-methylpyrazole, although under these experimental conditions PE also showed a decrease in its radioactive label.

Incorporation of L-[3-^{3}H]-serine

It is well established that serine is incorporated into the head-group of PS by a microsomal base-exchange reaction (Bell & Coleman, 1980). The PS is further decarboxylated in the mitochondria to give PE by a PS decarboxylase activity and this PE can then be methylated to PC via three enzymatic steps, which take place in the endoplasmic reticulum.

In addition to the head-group, L-[3-^{3}H]-serine may also be incorporated into the fatty-acyl moiety of phospholipids (Samborski & Vance, 1993). Thus, we have quantified the distribution of radioactivity in both polar and apolar moieties by phospholipase C digestion of the intact phospholipids. The head-groups of PS, PE and PC contained 96%, 49% and 30% respectively of the total phospholipid label. Therefore, in the results shown in Table 3, the radioactivity of phospholipids labeled from serine was corrected for the percentage of label in the head-group moiety.

Table 3 shows the results obtained after incubation of hepatocytes with L-[3-^{3}H]-serine either in the presence or absence of ethanol. As might be expected, in control hepatocytes serine was mainly incorporated into PS, although a substantial amount of the radioactivity was distributed between PE and

Table 2 Effect of 100 mM ethanol in the absence and presence of 4-methylpyrazole on the incorporation of label from [1-^{3}H]-ethanolamine into metabolic intermediates of phosphatidylethanolamine biosynthesis and their methylated derivatives, (phosphatidyl-N-monomethylethanolamine (NPE), phosphatidyl-N,N-dimethylethanolamine (NNPE) and phosphatidylcholine) in rat hepatocytes

	Methyl pyrazole	*Control*	*+ Ethanol (100 mM)*
Ethanolamine phosphate	None	7.33±0.36	5.14±0.59[b]
	3 mM	7.29±0.18	4.99±0.25[c]
CDP-ethanolamine	None	2.29±0.23	1.55±0.18[a]
	3 mM	3.29±0.16	3.74±0.35
Phosphatidyl-ethanolamine	None	51.40±1.46	57.89±1.07[b]
	3 mM	47.86±0.75	38.39±1.05[c]
NNPE	None	0.27±0.01	0.26±0.03
	3 mM	0.27±0.03	0.17±0.02[a]
NPE	None	6.46±0.54	4.33±0.47[a]
	3 mM	7.04±0.66	6.07±0.46
Phosphatidylcholine	None	1.71±0.07	1.33±0.06[b]
	3 mM	1.61±0.02	0.71±0.04[c]

Data are expressed as pmol of [1-^{3}H]-ethanolamine incorporated min^{-1} per 10^6 cells. Values are expressed as means±s.e.mean for six different experiments. Statistical significance of difference from the controls are indicated by [a]$P \leqslant 0.035$; [b]$P \leqslant 0.01$; [c]$P \leqslant 0.0001$.

Table 1 Effect of 100 mM ethanol in the absence and presence of 4-methylpyrazole on the incorporation of label from [methyl-^{14}C]-choline into choline phosphate, CDP-choline and PC in rat hepatocytes

	Methyl pyrazole	*Control*	*+ Ethanol (100 mM)*
Cholinephosphate	None	43.61±1.08	37.47±1.96[a]
	3 mM	48.97±1.40	43.59±0.25[b]
CDP-choline	None	1.42±0.07	1.21±0.04[a]
	3 mM	1.91±0.06	1.96±0.07
Phosphatidylcholine	None	69.53±1.15	63.19±3.18
	3 mM	52.62±2.14	36.51±2.30[c]

Data are expressed as pmol of [methyl-^{14}C]-choline incorporated min^{-1} per 10^6 cells. Values are expressed as means±s.e.mean for six different experiments. Statistical significance of differences from the controls are indicated by: [a]$P \leqslant 0.03$; [b]$P \leqslant 0.005$; [c]$P \leqslant 0.001$.

Table 3 Effect of 100 mM ethanol in the absence and presence of 4-methylpyrazole on the incorporation of label from L-[3-^{3}H]-serine into the phospholipids PC, PE and PS in rat hepatocytes

	Methyl pyrazole	*Control*	*+ Ethanol (100 mM)*
Phosphatidylcholine	None	0.13±0.01	0.19±0.01[a]
	3 mM	0.14±0.01	0.12±0.01
Phosphatidylethanolamine	None	0.24±0.01	0.36±0.01[b]
	3 mM	0.19±0.01	0.19±0.01
Phosphatidylserine	None	1.83±0.01	2.27±0.01[b]
	3 mM	1.93±0.14	1.98±0.11

Data are expressed as pmol of L-[3-^{3}H]-serine incorporated min^{-1} per 10^6 cells. Values are expressed as means±s.e.mean for nine different experiments. Statistical significance of differences from the controls are indicated by: [a]$P \leqslant 0.001$; [b]$P \leqslant 0.0001$.

PC. In the presence of ethanol the hepatocytes showed higher radioactive levels in all the phospholipids compared to control cells. The increase was more marked in PS and PE than in PC and it was noticeable that when the ethanol metabolism was inhibited, alcohol did not produce any effect on the amount of radiolabel in the different phospholipids.

Discussion

We have made a comparative study in rat hepatocytes of the effect of ethanol *in vitro* on the incorporation of different polar bases into the metabolic intermediates and into the head-group moiety of phospholipids, PC, PE and PS. Hepatocytes were exposed to 100 mM ethanol concentration for 3 h. Although the ethanol amount was higher than clinically-relevant plasma concentrations it does not alter the cellular viability and this concentration is similar to that used by different authors in rat hepatocytes (Gutierrez-Ruiz *et al.*, 1995; Sergent *et al.*, 1995) and other cell types (Le Petit-Thevenin *et al.*, 1995).

Ethanol may exert its pharmacological effects by direct interaction with different structural components of biological membranes or by some metabolites produced during its hepatic oxidation. It must be borne in mind that in hepatocytes, ethanol is metabolized producing different lipogenic substrates, such as acetyl-CoA, α-glycerophosphate, diacylglycerol and phosphatidic acid (Brindley, 1988; Lieber, 1991), and thus it is difficult to distinguish between the effects produced by these metabolites and those caused by the interaction of ethanol with biological membranes. Thus, to obtain further insight into the mechanism of the effects of ethanol we have analyzed the alterations induced by alcohol on phospholipid biosynthesis, both in the absence and presence of 4-methylpyrazol, a specific inhibitor of ADH activity, and thus of ethanol metabolism in the liver.

Our results show that the incorporation of radiolabelled ethanolamine into PE is clearly increased after incubation of hepatocytes in the presence of ethanol whilst choline incorporation into PC was practically unaltered. In addition, radioactivity of the different metabolic intermediates of the CDP-choline and CDP-ethanolamine pathways was significantly decreased by ethanol. To the best of our knowledge, no experimental evidence concerning the effects of ethanol *in vitro* on the biosynthesis of the individual phospholipids exists, although Castro *et al.* (1987) have demonstrated that hepatocytes incubated in the presence of ethanol show an increase in the incorporation of radiolabelled palmitate in both triglycerides and total phospholipids. Also in our laboratory we have observed an increase in the incorporation of radioactive oleic acid into polar and neutral lipids after ethanol incubation of hepatocytes for 3 h (results not shown). This increase in the lipid biosynthesis induced by ethanol has been related to an enhancement in the hepatic α-glycerophosphate levels produced as a consequence of the shift in the redox state after ethanol metabolism (Castro *et al.*, 1987). The higher amount of radioactivity in PE found in the present study could also be the result of an increase in the levels of lipogenic substrates produced as a consequence of ethanol metabolism.

The mechanism of the effects of ethanol was further approached by use of the inhibitor of ADH activity, 4-methylpyrazole. The analysis of the PC and PE biosynthetic pathways when ethanol metabolism is inhibited, reveals that alcohol produces a reduction in the radioactive levels of both choline phosphate, ethanolamine phosphate and a significant decrease in the amount of PC and PE radiolabel. In the presence of 4-methylpyrazole, ethanol metabolism is inhibited and consequently there is no change in the availability of α-glycerophosphate or other lipogenic substrates and hence the effects observed are exclusively due to a direct action of alcohol on enzymes of the biosynthetic pathway. In this sense, the amount of radiolabel in the different metabolic intermediates in the presence of 4-methylpyrazole, suggests a specific inhibition induced by ethanol in the activity of the corresponding kinases and phosphotransferases that could explain the decrease in the label of choline phosphate, ethanolamine phosphate and the corresponding phospholipids PE and PC. Since the amount of radiolabel in CDP-derivatives is not modified, it could be indicative that the cytidylyltransferases, enzymes involved in the rate-limiting steps in PC and PE synthesis, are not altered after exposure to ethanol, in spite of the fact that it has been widely shown that these enzymes are sensitive to different hormonal and dietary factors (reviewed by Tronchere *et al.*, 1994).

All these results indicate that ethanol can exert two different effects on the CDP-derivatives biosynthetic pathways: a stimulatory effect depending on an increase in the availability of lipogenic substrates, as a consequence of alcohol metabolism, and another inhibitory effect on specific enzymes involved in the biosynthetic pathways. This last effect is produced by the presence of ethanol *per se* and can be observed only when ethanol metabolism is inhibited. Hence when cells are exposed to ethanol, the final effect on the individual phospholipid biosynthetic pathways is the result of two opposite effects. In this connection, we have recently demonstrated in our laboratory that hepatocytes isolated from alcoholic rats show opposite changes in the biosynthetic pathway of the major phospholipids, PE and PC (Carrasco *et al.*, 1996). Thus, whilst incorporation of ethanolamine into PE is significantly increased after chronic ethanol treatment, synthesis of PC from exogenous choline is markedly decreased.

Synthesis of PS is a complex process involving several cell compartments and some transport steps between different subcellular membranes. Our results demonstrated that when serine or choline are used as exogenous precursors, sphingomyelin does not show any isotopic label although both substrates are necessary precursors for the biosynthesis of sphingomyelin.

Ethanol markedly increases the incorporation of radioactivity from serine to PS, PE and, to a lesser extent, PC, although in the presence of 4-methylpyrazole these effects are not observed. These results show therefore that the effects of ethanol on phospholipid biosynthesis from serine are linked to the metabolism of ethanol. Other authors have demonstrated that free fatty acids exert a marked activator effect on phosphatidylserine synthase activity both in rat liver (Siddiqui & Exton, 1992) and brain (Kanfer & McCartney, 1991). Since the cytosolic fatty acids increase significantly as a consequence of ethanol metabolism (Lieber, 1994), they may be responsible for an enhancement in the synthase activity and thus in the synthesis of all the serine-derived phospholipids found in our study.

On the other hand, it is interesting to note that the high levels of radioactivity found in PS demonstrate that in control hepatocytes, PS is efficiently synthesized from exogenous serine and that approximately 17% of the total radioactivity initially incorporated into this phospholipid, appeared afterwards in PE plus PC, indicating that a substantial amount of the total PS synthesized in control hepatocytes is decarboxylated to PE. In addition, this PE, newly synthesized in the mitochondria, is actively methylated, producing PC. In fact, 38% of the PE labelled by this route is used in microsomes to give PC, as can be deduced from the PC/PE_T radioactivity ratio. This value is in marked contrast to the low production of PC from PE synthesized via the CDP-ethanolamine pathway and demonstrates that PE is more efficiently methylated to give PC when PE is produced in the mitochondria by PS decarboxylation than when it is synthesized in microsomes from ethanolamine. Other authors have also indicated that in liver the methylation of PE derived from PS apparently provides up to 30% of the cellular PC, the remainder being synthesized from the CDP-choline pathway (Vance & Vance, 1986a).

All this indicates that PE may be located to different extents in microsomal membranes depending upon the biosynthetic pathway which produces it. In this sense, Vance (1991) has suggested that there are pools of newly synthesized phospholipid different from the pools of older phospholipid pre-existing in the membranes and recently Ellingson & Seenaiah (1994)

have also indicated the existence of metabolic domains of membrane phospholipids, determined by the acyl-chains in the sn-1 and the sn-2 positions. In our study, however, we have found differences in the methylation of PE originated from two different biosynthetic pathways and thus our results are consistent with different pools in the membrane which differ in their accessibility to methyltransferases activity. In fact, two distinct methyltransferase activities have recently been described, probably involved in the methylation of serine- and ethanolamine-derived PE (Cui *et al.*, 1993). In addition there is evidence indicating that both pathways may be differently regulated (Vance & Vance, 1986b). In summary, it is possible that serine-labelled PC may be synthesized at a separate intramembrane location from ethanolamine-labelled PC, as a consequence of differences in the location of specific pools of microsomal PE.

With regard to the effects of ethanol on PE methylation, to date there are few and contradictory reports. It was reported in 1976 that chronic ethanol ingestion markedly increases hepatic phosphatidylethanolamine methyltransferase activity (Uthus *et al.*, 1976), although recently Lieber *et al.* (1994) have indicated that chronic alcohol consumption significantly decreases this activity. Our results demonstrate that the exposure of hepatocytes to ethanol induces a clear decrease in PE methylation synthesized via CDP-ethanolamine (Table 2), whilst alcohol does not affect the methylation of serine-derived PE (Table 3). This different response corroborates once again the existence of different pools of microsomal PE which are not similarly affected by ethanol. Since the above-mentioned inhibition in the methylation process can even be seen in the presence of 4-methylpyrazole, it may be the result of an interaction between ethanol and specific areas of the microsomal membranes although we cannot exclude a direct effect on the phosphatidylethanolamine methyltransferases. Bearing in mind that several authors have indicated (Wood & Schroeder, 1988) that alcohol alters specific domains of biological membranes, we think that it may well also modify the structural organization of areas where ethanolamine-derived PE is placed, affecting the methylation of this phospholipid, although other pools of PE located at different sites in the membrane may remain unaltered by exposure to ethanol.

This work was supported in part by grants from CICYT (PM90-144 and PB93-1117). We thank Dolores García for help in correction of the manuscript.

References

BELL, R.M. & COLEMAN, R.A. (1980). Enzymes of glycerolipid synthesis in eukaryotes. *Annu. Rev. Biochem.*, **49,** 459–487.

BRINDLEY, D.N. (1988). What factors control hepatic triacylglycerol accumulation in alcohol abuse? *Biochem. Soc. Trans.*, **16,** 251–253.

CARRASCO, M.P., SANCHEZ-AMATE, M.C., SEGOVIA, J.L. & MARCO, C. (1996). Studies on phospholipid biosynthesis in hepatocytes from alcoholic rats by using radiolabeled exogenous precursors. *Lipids*, **31,** 393–397.

CASTRO, J., MAQUEDANO, A. & GUZMAN, M. (1987). Short and long term effects of ethanol on glycerolipid synthesis from palmitate in fasted rat hepatocytes. *Int. J. Biochem.*, **7,** 595–599.

CUI, Z., VANCE, J.E., CHEN, M.H., VOELKER, D.R. & VANCE, D.E. (1993). Cloning and expression of a novel phosphatidylethanolamine N-methyltransferase. A specific biochemical and cytological marker for a unique membrane fraction in rat liver. *J. Biol. Chem.*, **268,** 16655–16663.

ELLINGSON, J.S. & SEENAIAH, B. (1994). The selective use of stearoyl-polyunsaturated molecular species of phosphatidylcholine and phosphatidylethanolamine for the synthesis of phosphatidylserine. *Biochim. Biophys. Acta*, **1213,** 113–117.

FOLCH, J., LESS, B. & SLOANE-STANLEY, G. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **226,** 497–509.

FORMAN, D.T. (1988). The effect of ethanol and its metabolites on carbohydrates, protein, and lipid metabolism. *Ann. Clin. Lab. Sci.*, **18,** 181–189.

GUTIERREZ-RUIZ, M.C., GOMEZ, J.L. & BUCIO, L. (1995). Chronic and acute ethanol treatment modifies fluidity and composition in plasma membranes of a human hepatic cell line (WRL-68). *Cell. Biol. Toxicol.*, **11,** 69–78.

HIGGINS, J.A. (1987). Separation and analysis of membrane lipid components. In *Biological Membranes. A Practical Approach*, ed. Evan, W.H. pp. 103–137. Findlay J.B.C.

KANFER, J.N. & MCCARTNEY, D. (1991). Sphingosine and unsaturated fatty acids modulate the base exchange enzyme activities of rat brain membranes. *FEBS Lett.*, **291,** 63–66.

KENNEDY, E.P. & WEISS, S.B. (1956). The function of cytidine coenzymes in the biosynthesis of phospholipids. *J. Biol. Chem.*, **222,** 193–213.

KUKSIS, A., MYHER, J.J., GREHER, K., BRECKENRIDGE, W.C., JONES, G.J.L. & LITTLE, J.A. (1981). Lipid class and molecular species interrelationship among plasma lipoproteins of normolipemic subjects. *J. Chromatogr.*, **224,** 1–23.

LE PETIT-THEVENIN, J., NOBILI, O., VERINE, A. & BOYER, J. (1995). Differential *in vitro* effects of ethanol on glycerolipid acylation and biosynthesis in rat reticulocytes. *Biochim. Biophys. Acta*, **1257,** 103–110.

LIEBER, C.S. (1991). Alcohol, liver, and nutrition. *J. Am. Coll. Nutr.*, **10,** 602–632.

LIEBER, C.S. (1994). Alcohol and the liver: 1994 update. *Gastroenterology*, **106,** 1085–1105.

LIEBER, C.S., ROBINS, S.J. & LEO, M.A. (1994). Hepatic phosphatidylethanolamine methyltransferase activity is decreased by ethanol and increased by phosphatidylcholine. *Alcohol Clin. Exp. Res.*, **18,** 592–595.

MARINO, A., SALGADO, E., TRUEBA, M. & MACARULLA, J.M. (1986). Phosphatidylethanolamine methyltransferase activity in chick liver microsomes. *Comp. Biochem. Physiol.*, **85B,** 795–803.

SAMBORSKI, R.W. & VANCE, D.E. (1993). Phosphatidylethanolamine derived from phosphatidylserine is deacylated and reacylated in rat hepatocytes. *Biochim. Biophys. Acta*, **1167,** 15–21.

SANCHEZ-AMATE, M.C., ZURERA, J.M., CARRASCO, M.P., SEGOVIA, J.L. & MARCO, C. (1991). Ethanol and lipid metabolism. Differential effects on liver and brain microsomes. *FEBS Lett.*, **293,** 215–218.

SEGLEN, P.O. (1976). Preparation of isolated rat liver cells. *Methods Cell Biol.*, **13,** 29–83.

SERGENT, O., MOREL, I., CHEVANNE, M., CILLARD, P. & CILLARD, J. (1995). Oxidative stress induced by ethanol in rat hepatocyte cultures. *Biochem. Mol. Biol. Int.*, **35,** 575–583.

SIDDIQUI, R.A. & EXTON, J.H. (1992). Phospholipid base exchange activity in rat liver plasma membrane. Evidence for regulation by G-protein and P_{2Y}-purinergic receptor. *J. Biol. Chem.*, **267,** 5755–5761.

SLOMIANY, A., GRABSKA, M., PIOTROWSKI, E., SENGUPTA, S., MORITA, M., KASINATHAN, C. & SLOMIANY, B.L. (1994). Intracellular processes associated with vesicular transport from endoplasmic reticulum to Golgi and exocytosis: ethanol-induced changes in membrane biogenesis. *Arch. Biochem. Biophys.*, **310,** 247–255.

TRONCHERE, H., RECORD, M., TERCE, F. & CHAP, H. (1994). Phosphatidylcholine cycle and regulation of phosphatidylcholine biosynthesis by enzyme translocation. *Biochim. Biophys. Acta*, **1212,** 137–151.

UTHUS, E.O., SKURDAL, D.N. & CORNATZER, W.E. (1976). Effect of ethanol ingestion on choline phosphotransferase and phosphatidylethanolamine methyltransferase activities in liver microsomes. *Lipids*, **11,** 641–644.

VANCE, J.E. (1991). Newly made phosphatidylserine and phosphatidylethanolamine are preferentially translocated between rat liver mitochondria and endoplasmic reticulum. *J. Biol. Chem.*, **266,** 89–97.

VANCE, D.E., PELECH, S.D. & CHOY, P.C. (1981). CTP:phosphocholine cytidylyltransferase from rat liver. In *Methods in Enzymology*, Vol 71, pp. 576–581. ed. Lowenstein, J.M. New York: Academic Press Inc.

VANCE, J.E. & VANCE, D.E. (1986a). Specific pools of phospholipids are used for lipoprotein secretion by cultured rat hepatocytes. *J. Biol. Chem.*, **261,** 4486–4491.

VANCE, J.E. & VANCE, D.E. (1986b). A deazaadenosine-insensitive methylation of phosphatidylethanolamine is involved in lipoprotein secretion. *FEBS Lett.*, **204,** 243–246.

WANG, X. & MOORE, T.S. (1991). Phosphatidylethanolamine synthesis by castor bean endosperm. Intracellular distribution and characteristics of CTP:ethanolaminephosphate cytidylyltransferase. *J. Biol. Chem.*, **266,** 19981–19987.

WOOD, V.G. & SCHROEDER, F. (1988). Membrane effects of ethanol: bulk lipid versus lipid domains. *Life Sci.*, **43,** 467–475.

(Received February 16, 1996
Revised June 4, 1996
Accepted June 7, 1996)