Biochemical Pharmacology, Vol. 36, No. 15, pp. 2443–2448, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

ROLE OF ACETALDEHYDE IN ETHANOL-INDUCED INCREASE IN THE ACTIVITY OF PHOSPHATIDATE PHOSPHATASE IN RAT LIVER

HAYAO IDE* and YASUO NAKAZAWA

Medical Research Institute, Tokyo Medical and Dental University, No. 3–10, Kandasurugadai 2-Chome, Chiyoda-ku, Tokyo 101, Japan

(*Received* 19 *August* 1986; *accepted* 22 *January* 1987)

Abstract—The effect of ethanol on the activity of phosphatidate phosphatase was studied in rat liver using membrane-bound phosphatidate and phosphatidate emulsion as substrate. A single large dose of ethanol (5 g/kg body wt) caused an increase in the enzyme activity measured with membrane-bound phosphatidate after an approximate 2-hr lag period in both cytosolic and microsomal fraction and the increase was approximately 2.2- and 1.8-fold that in control rats at 5 hr in cytosol and microsomes, respectively. A similar time-course of the increase was obtained with phosphatidate emulsion as substrate. These ethanol-induced increases in the activity of cytosolic and microsomal phosphatidate phosphatase were blocked by the pretreatment of rats with actinomycin D.

The ethanol-induced rise in the activity of cytosolic and microsomal phosphatidate phosphatase measured with membrane-bound phosphatidate was abolished when rats were injected with pyrazole prior to ethanol administration. On the other hand, pretreatment with cyanamide enhanced the increase in cytosolic activity produced by a suboptimal dose of ethanol (1 g/kg), while microsomal activity was not affected by the same treatment, suggesting that acetaldehyde may be selectively involved in the ethanol-induced increase in the activity of cytosolic phosphatidate phosphatase. This hypothesis was supported by a finding that administration of paraldehyde, a cyclic trimer of acetaldehyde, produced an increase (35%) in cytosolic activity, but not in microsomal activity.

Acute ethanol ingestion leads to an accumulation of triacylglycerol in the liver of man and experimental animals. This ethanol-induced rise in triacylglycerol content is generally attributed to an increased capacity of hepatic triacylglycerol formation. The increase in the substrate level may explain the enhanced synthesis of triacylglycerol provided that their normal content is below that required for maximum enzyme activity. Ethanol intake has been known to increase the hepatic *sn*-glycerol 3-phosphate concentration [1, 2]. Availability of the other substrate, fatty acid, also increases in the liver after ethanol intake [3, 4], which could result from an enhanced synthesis of fatty acids [5], decreased oxidation of fatty acids [6, 7] and/or enhanced mobilization of fatty acids from depot fat [8].

Alternatively, ethanol increases the formation of triacylglycerol by elevating the enzymatic capacity of glycerolipid biosynthesis. Of the enzymes involved in the triacylglycerol synthesis, phosphatidate phosphatase (EC 3.1.3.4) is the only enzyme the activity of which increases shortly after treatment with a single dose of ethanol [9–11]. The activity of this enzyme has been found to increase by metabolic stress such as starvation, subtotal hepatectomy and diabetes, which are often accompanied by increases in the rate of triacylglycerol synthesis (for a review, see [12]), and therefore it has been proposed that this enzyme plays a regulatory role in hepatic neutral lipid biosynthesis [12]. The activity of this enzyme has been reported to occur in the particulate and soluble fractions of mammalian cells. When emulsion of phosphatidate is used as substrate most of the activity is found in particulate fractions, while the highest proportion is associated with soluble fraction when membrane-bound phosphatidate is utilized [13–16]. The relationship between the activities detectable with the two different forms of the substrate is not clear at present.

In the present study, we investigated the effect of ethanol administration on the activity of cytosolic and microsomal phosphatidate phosphatase in rat liver using either phosphatidate emulsion or membrane-bound phosphatidate as substrate. The effect of the inhibitors of ethanol metabolizing enzymes was also examined in order to determine whether metabolites of ethanol are involved in the ethanol-dependent elevation of phosphatidate phosphatase activity.

MATERIALS AND METHODS

Materials. All chemicals were of reagent grade and ethanol was distilled before use. Radioactive orthophosphate, $H_3[^{32}P]O_4$ was purchased from Japan Atomic Energy Institute. Glycerol 3-[^{32}P]phosphate was synthesized enzymatically from [γ-^{32}P]ATP, which had been prepared from [^{32}P]orthophosphate according to the method of Walseth and Johnson [17], and glycerol with glycerol kinase as previously described [18].

Treatment of rats. Male Wistar rats weighing 140–170 g were used in all experiments. Unless otherwise stated, they were allowed free access to Clea CF-2

* To whom all correspondence should be addressed.

Laboratory Chow and water prior to experiments. Ethanol was administered p.o. as a 50% (w/v) solution in water. The dose of ethanol was 5 g per kg body wt except in the experiment shown in Fig. 3 where 1 g/kg was used. Control animals received the equivalent volumes of water instead. Paraldehyde was given orally at a dose of 250 mg/kg.

Preparation of rat liver cytosol and microsomes. Rat liver cytosol and microsomes was prepared as described previously [18]. In order to check the extent of cross-contamination between the two fractions, the activities of the marker enzymes for the two subcellular fractions were measured. The activities of lactate dehydrogenase (EC 1.1.1.27) determined according to the method of Nisselbaum and Bodansky [19] were 4.37 and 0.34 μmol/min/mg protein in cytosol and microsomes, respectively, and those of glucose 6-phosphate (EC 3.1.3.9) determined according to the method of Swanson [20] were 2.20 and 27.2 nmol/min/mg protein in cytosol and microsomes, respectively. These values indicate that the separation of these two subcellular fractions is satisfactory.

Assay of phosphatidate phosphatase. Either ^{32}P-phosphatidate bound to microsomal membrane (membrane-bound phosphatidate) or ^{32}P-phosphatidate dispersed with microsomal lipids (phosphatidate emulsion) was used as substrate [18]. The activity of cytosolic and microsomal phosphatidate phosphatase was determined essentially as previously described [18]. The standard incubation mixture contained 50 mM Tris–HCl (pH 7.4), 0.25 M sucrose and 0.4 mM either form of phosphatidate in a final volume of 0.15 ml. In some experiments (Figs 2–4) 5 mM $MgCl_2$ was added to the incubation mixture, which caused an approximately 1.5–2-fold increase in both cytosolic and microsomal activities. After incubation for 20 min at 37° the reaction was stopped by adding 3 ml of 0.1 N HCl in chloroform–methanol (1:1) followed by the addition of 1 ml of chloroform and 2 ml of 2 M KCl. The mixture was centrifuged briefly and radioactivity of [^{32}P]Pi released into water layer was measured with 10 ml of ACS scintillation fluid.

It has been reported that phosphatidate can be hydrolyzed by phospholipases of the A type to yield glycerol 3-phosphate, which can further be broken down to phosphate, causing overestimation of the phosphatidate phosphatase when it is determined by measuring the release of water-soluble ^{32}P from phosphatidate. Therefore, we analyzed the reaction products by incubating membrane-bound ^{14}C-phosphatidate or ^{14}C-phosphatidate emulsion, which had been prepared similarly to ^{32}P-phosphatidate, with cytosolic or microsomal protein. It was found that water-soluble radioactivity produced through deacylation of phosphatidate was less than 3% of the amount of phosphatidate hydrolyzed in all cases tested.

Other procedure. Protein was determined according to the method of Lowry *et al.* [22] using bovine serum albumin as standard.

RESULTS

A single oral administration of ethanol at a dose of 5 g/kg body wt caused an increase in the activity of cytosolic and microsomal phosphatidate phosphatase in rat liver (Fig. 1). When assayed with membrane-bound phosphatidate as substrate (Fig. 1, left), both cytosolic and microsomal activities started to increase at 3 hr after ethanol administration, and at 5 hr the activity was approximately 2.2- and 1.8-fold that in control rats given water, in cytosol and microsomes, respectively. An isocaloric replacement of ethanol by glucose (8.7 g/kg) did not cause a significant increase in the phosphatidate phosphatase activity at 5 hr (not shown) as has been reported by other workers [10, 23], indicating that the increase in the enzyme activity was not simply the consequence of calories given as ethanol but resulted from ethanol *per se*.

Similar profiles were observed using phosphatidate emulsion as substrate (Fig. 1, right) except the degree of the increase was somewhat smaller. Dose-dependency of ethanol-induced increases in phosphatidate phosphatase is shown in the insets of Fig. 1. In all cases, maximal increase was obtained by the administration of 3–5 g/kg ethanol. In the experiment described above, rats were fasted overnight before the administration of ethanol. It has been known that the capacity of ethanol to produce fatty liver is often affected by the nutritional conditions. Therefore, we also tested the effect of ethanol in fed rats. However, there was no significant difference in the level of hepatic phosphatidate phosphatase activity between fasted and fed groups either before or 5 hr after administration of ethanol (not shown). Since we occasionally experienced the death of fasted rats after giving high doses of ethanol, all rats were fed in the following experiments.

To investigate whether the increase in the activity of phosphatidate phosphatase is due to an activation of available enzyme or due to the synthesis of new enzyme protein, the effect of actinomycin D on the rise in the phosphatidate phosphatase activity 5 hr after administration of ethanol was studied. Administration of actinomycin D abolished the ethanol-dependent increase in cytosolic activity regardless of the form of the substrate used, while actinomycin D *per se* had little effect on the basal activity of cytosolic phosphatidate phosphatase (Table 1). Actinomycin D itself caused a slight increase in the microsomal phosphatidate phosphatase activity in the livers of control rats for unknown reasons. Pretreatment of rats with the antibiotic also reduced the ethanol-dependent increase in microsomal phosphatidate phosphatase activity to the level in the liver of rats treated only with actinomycin D.

Figure 2 shows the effect of pyrazole, a potent competitive inhibitor [24] of alcohol dehydrogenase (EC 1.1.1.1), on the ethanol-dependent rise in the hepatic phosphatidate phosphatase activity. Pretreatment of rats with pyrazole reduced the rise in the enzyme acticity at 5 hr after ethanol administration by approximately 59 and 72% in cytosol and microsomes, respectively, indicating that the oxidation of ethanol by alcohol dehydrogenase is essential for the stimulation of the enzyme.

Acetaldehyde formed from ethanol is next converted to acetate by aldehyde dehydrogenase (EC

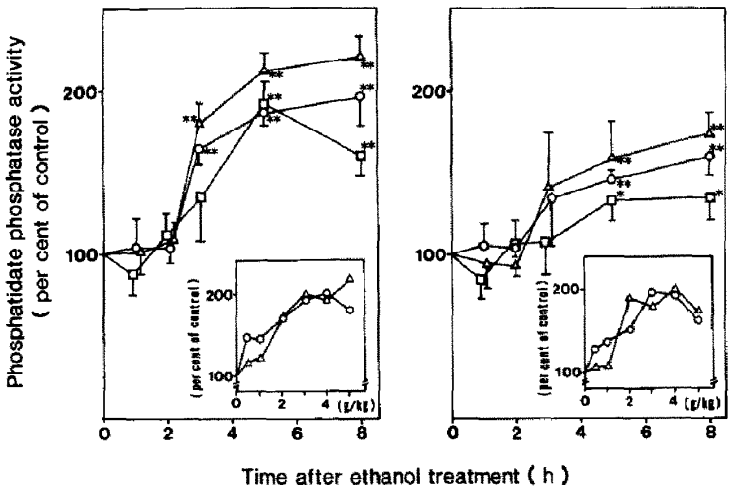

Fig. 1. Effect of administration of ethanol on the activity of phosphatidate phosphatase. Ethanol (5 g/kg) or equivalent amount of water (control) was administered p.o. at 0 time. The enzyme was measured with either membrane-bound phosphatidate (left) or phosphatidate emulsion (right) as substrate. The results were expressed as per cent of the average activity obtained from control rats killed at the corresponding time point. The activities in control rats at 0 time were: homogenate, 0.519 ± 0.035; cytosol, 0.813 ± 0.057; microsomes, 0.291 ± 0.052 (nmol/min/mg protein) with membrane-bound phosphatidate, and homogenate, 0.258 ± 0.036; cytosol, 0.307 ± 0.032; microsomes, 0.185 ± 0.025 (nmol/min/protein) with phosphatidate emulsion. Control values at other time points did not deviate significantly from those at 0 time. The results are means ± SEM from 4–6 rats. * $P < 0.05$ level of significance from control (water) group, ** $P < 0.01$ level of significance from control (water) group. Insets: Dose-dependency of the effect of ethanol on phosphatidate phosphatase activity. Five hours after giving various doses of ethanol, rats were killed and cytosol and microsomes were prepared. The results are averages of two experiments. Symbols: O—O, homogenate; △—△, cytosol; □—□ microsomes.

1.2.1.3), the second step of ethanol oxidation. Cyanamide is known as a potent *in vivo* inhibitor of liver aldehyde dehydrogenase [25]. In order to test whether acetaldehyde is involved in the ethanol-induced rise in phosphatidate phosphatase activity, rats were pretreated with cyanamide. At an ethanol dose of 5 g/kg, which produced maximum increase in the phosphatidate phosphatase activity, pretreatment with cyanamide did not affect the ethanol-dependent increase in the enzyme activity (data not shown). However, when the dose of ethanol was reduced to 1 g/kg, which increased the basal activity of cytosolic and microsomal phosphatidate phosphatase by approximately 42 and 21%, respectively,

Table 1. Effect of actinomycin D on the ethanol-induced increase in the activity of phosphatidate phosphatase

	Cytosol		Microsomes	
	nmol/min/mg protein	% control	nmol/min/mg protein	% control
Membrane-bound phosphatidate				
A. Control	0.884 ± 0.063	100	0.312 ± 0.032	100
B. Ethanol	1.567 ± 0.184*	177	0.606 ± 0.061*	194
C. Actinomycin D	0.848 ± 0.152	96	0.412 ± 0.062	132
D. Actinomycin D plus ethanol	1.056 ± 0.066†	119	0.425 ± 0.047†	136
Phosphatidate emulsion				
A. Control	0.271 ± 0.045	100	0.161 ± 0.013	100
B. Ethanol	0.387 ± 0.028*	142	0.227 ± 0.015*	141
C. Actinomycin D	0.218 ± 0.041	80	0.218 ± 0.024	135
D. Actinomycin D plus ethanol	0.266 ± 0.031†	98	0.219 ± 0.031	136

Actinomycin D at a dose of 1 mg/kg (C, D) or equivalent amount of saline (A, B) was injected i.p. 1 hr before oral administration of ethanol (5 g/kg) or equivalent amount of water (A, C). Rats were killed 5 hr after the administration of ethanol (water). The results are means ± SEM from five rats. * $P < 0.01$ level of significance from control (water) group; † $P < 0.01$ level of significance from ethanol group.

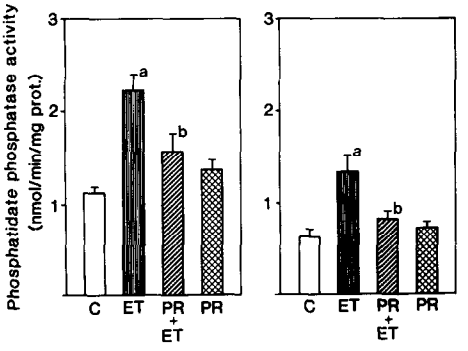

Fig. 2. Effect of pyrazole pretreatment on ethanol-induced increase in the activity of cytosolic (left) and microsomal (right) phosphatidate phosphatase in rat liver. Rats were divided into four groups. Groups 1 (C) and 2 (ET) were administered i.p. with saline (6 ml/kg) and groups 3 (PR + ET) and 4 (PR) were injected i.p. with pyrazole (300 mg/kg) as a 5% solution in saline. Fifteen minutes later, ethanol (5 g/kg) was given p.o. to groups 2 and 3, and groups 1 and 4 received equivalent amount of water instead. All rats were killed 5 hr after second treatment and phosphatidate phosphatase activity was measured with membrane-bound phosphatidate. Results are expressed as mean ± SEM from 6–8 rats. a, $P < 0.01$ level of significance from control (water) group; b, $P < 0.01$ level of significance from ethanol group.

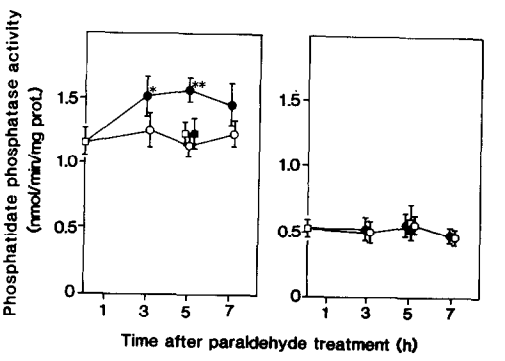

Fig. 4. Effect of the administration of paraldehyde on the activity of cytosolic (left) and microsomal (right) phosphatidate phosphatase in rat liver. Rats were injected i.p. with cyanamide (25 mg/kg) or equivalent volumes of saline. Fifteen minutes later paraldehyde (250 mg/kg) or water was given p.o. The results are means ± SEM from 3–5 rats. Symbols: ○—○, vehicle only; ●—●, cyanamide + paraldehyde; □—□, cyanamide; ■—■, paraldehyde. * $P < 0.05$ level of significance from control (water) group, ** $P < 0.01$ level of significance from control (water) group.

the pretreatment with cyanamide potentiated the effect of ethanol (Fig. 3) suggesting that acetaldehyde may be involved in the ethanol-induced increase in the cytosolic enzyme activity. It is not clear at present why cyanamide failed to enhance the ethanol-induced increase in cytosolic phosphatidate phosphatase activity, but a possible explanation would be that acetaldehyde concentration produced by 5 g/kg ethanol could be sufficient to cause maximal effect. In contrast, cyanamide exhibited no effect on ethanol-induced increase in the enzyme activity in microsomal fraction. These results prompted us to the experiment in which the effect of acetaldehyde on hepatic phosphatidate phosphatase can be seen more directly. Since acetaldehyde is extremely toxic to the respiratory system and other organs, we employed paraldehyde, a trimer of acetaldehyde, to see the effect of acetaldehyde. The latter compound is much less toxic than the former and is known to be decomposed slowly to acetaldehyde in the body [26]. Paraldehyde was administered p.o. to the rats in combination with cyanamide because the decomposition rate of paraldehyde is known not to exceed the rate of acetaldehyde oxidation so that acetaldehyde does not accumulate [26]. As shown in Fig. 4, administration of paraldehyde caused an approximately 35% increase in the activity of cytosolic phosphatidate phosphatase. On the other hand, no significant change was observed in the activity of microsomal phosphatidate phosphatase.

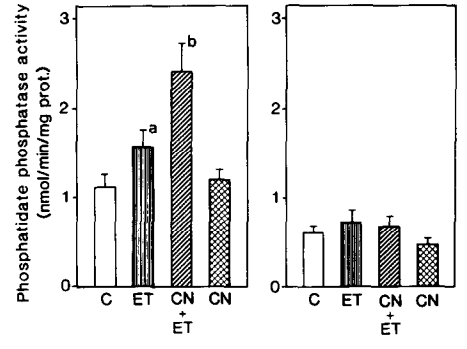

Fig. 3. Effect of cyanamide pretreatment on ethanol-induced increase in the activity of cytosolic (left) and microsomal (right) phosphatidate phosphatase in the liver. Groups 1 (C) and 2 (ET) were administered i.p. with saline (5 ml/kg) and groups 3 (CN + ET) and 4 (CN) were injected with cyanamide (25 mg/kg) as a 5 mg/ml (w/v) solution. Thirty minutes later ethanol (1 g/kg) was given p.o. to groups 2 and 3, and groups 1 and 4 received equivalent amount of water. The other conditions are the same as Fig. 2. a, $P < 0.01$ level of significance from control (water) group; b, $P < 0.01$ level of significance from ethanol group.

DISCUSSION

Following an acute dose of ethanol phosphatidate phosphatase activity increased in cytosolic and microsomal fractions of rat liver (Fig. 1) in agreement with the results obtained by other workers [9–11]. Time-course of the changes was similar between the activity measured with membrane-bound phosphatidate and that with phosphatidate emulsion (Fig. 1). The relationship between these activities detectable with different forms of the substrate is not clear at present. As already mentioned, subcellular distribution of phosphatidate phosphatase activity with membrane-bound phosphatidate is different from that with phosphatidate emulsion. It has been reported that emulsion of phosphatidate from which Ca^{2+} ions are carefully removed can be used suc-

cessfully to assay for the soluble phosphatidate in the presence of phosphatidylcholine [12, 16]. However, when phosphatidate phosphatase activities in rat liver cytosol were separated on Bio-Gel A-5m, a profile obtainable with phosphatidate emulsion, which was prepared by sonicating with microsomal lipids and was essentially Ca^{2+}-free, was different from that obtained with membrane-bound phosphatidate [18]. Separation of the bulk of the two activities by gel filtration has also been reported in rat lung [27, 28]. Furthermore, enzymological properties such as metal ion requirement and heat stability are different between the two activities. However, these observations do not necessarily indicate the existence of two independent activities. We have shown in the previous report that an association of phosphatidate phosphatase with the membrane could modify the properties and apparent molecular size of the enzyme [18]. The membrane-associated form of the enzyme seems to prefer phosphatidate emulsion as substrate, while membrane-bound phosphatidate seems to be a better substrate for the solubilized form. The parallel response to ethanol treatment of the activities with membrane-bound phosphatidate and that with phosphatidate emulsion observed in the present study may further suggest that these activities are not independent, although much more work will be required to establish this point.

The ethanol-induced increase in the enzyme activity in each fraction was preceded by an approximately 2-hr lag period and was largely prevented by the treatment of rats with actinomycin D (Table 1), indicating that the increase is, at least in part, due to an induction of enzyme protein synthesis. The induction could be mediated through increased concentration of blood corticosteroids, since adrenalectomy greatly reduces the ethanol-dependent rise in phosphatidate phosphatase activity [9, 10]. It has also been reported that circulating corticosterone concentration increases shortly after ethanol ingestion [9] and that the administration of corticosterone or dexamethasone causes a rise in cytosolic phosphatidate phosphatase activity [29]. However, the hormonal regulation does not seem, totally, to explain the ethanol-induced rise in phosphatidate phosphatase activity since ethanol can cause a significant rise in the enzyme activity even in the liver of adrenalectomized rats [10] or in cultured rat hepatocytes [30]. Furthermore, the increase in phosphatidate phosphatase activity observed after the feeding of sorbitol, fructose or glucose, all of which increase circulating glucocorticoids to the level seen after ethanol feeding, is much smaller than that brought about by ethanol. Therefore, it is likely that non-hormonal factors are also involved in ethanol-induced stimulation of phosphatidate phosphatase. Metabolites of ethanol such as acetaldehyde are among the candidates. In fact, it was shown in the present study that pretreatment of rats with pyrazole greatly reduced the ethanol-dependent rise in the activity of phosphatidate phosphatase in both cytosolic and microsomal fractions of rat liver (Fig. 2). Savolainen and Hassinen [11] have reported that pyrazole pretreatment abolishes the ethanol-induced increase in soluble phosphatidate phosphatase activity, although the increase in microsomal activity was not affected in their experimental condition. Treatment with pyrazole has also been shown to prevent the ethanol-induced rise in the phosphatase activity in cultured hepatocytes [30]. These results suggest that oxidation of ethanol to acetaldehyde is an essential step for the ethanol-induced increase in phosphatidate phosphatase activity. Moreover, we found that pretreatment with cyanamide, an inhibitor of aldehyde dehydrogenase, enhanced the increase in the activity of cytosolic phosphatidate phosphatase caused by a suboptimal dose of ethanol (Fig. 3). On the other hand, no significant change was observed in the activity of microsomal fraction (Fig. 3). From these results, it was suggested that acetaldehyde may be selectively involved in the stimulation of cytosolic phosphatidate phosphatase activity. This hypothesis was supported by the finding that paraldehyde causes an increase in the activity of phosphatidate phosphatase activity in cytosol, but not in microsomes (Fig. 4).

Acetaldehyde is known as a highly reactive compound and could complex with a variety of naturally-occurring compounds such as cysteine, thiamine, glutathione and coenzyme A by way of free sulfhydryl groups, or with amino acids through amino groups, in the liver. When acetaldehyde is metabolized by aldehyde oxidase and xanthine oxidase, a minor pathway for hepatic acetaldehyde oxidation, oxygen radicals are formed and promote lipid peroxidation. These reactions could produce functional alterations in biological systems in the liver. Unfortunately, it cannot be concluded at the moment whether the effect of acetaldehyde on phosphatidate phosphatase is exerted directly, or indirectly through other intracellular components.

Non-hormonal factor(s) other than acetaldehyde could also be involved in the ethanol-induced rise in the activity of phosphatidate phosphatase. Oxidation of ethanol is known to cause a rapid change in the redox state in the liver [31, 32] which results in the increase of intermediary metabolites such as glycerol 3-phosphate [1, 2]. A positive correlation has been observed between glycerol 3-phosphate concentration and phosphatidate phosphatase activity in the liver of rats given various oxidizable substrates including ethanol, although the perfusion of rat liver with ethanol does not cause a significant increase in the enzyme activity [11].

Recently, Brindley *et al.* [33, 34] have demonstrated that long-chain fatty acids and their CoA esters are able to promote a translocation of phosphatidate phosphatase from cytosol to microsomes either in hepatocytes or in a cell-free system. They have proposed that cytosolic phosphatidate phosphatase which they assume is an inactive reserve of the enzyme becomes physiologically active when it is transferred to microsomes (endoplasmic reticulum), the tentative site of action of the enzyme [35]. If this is true, ethanol may increase both the active form and the storage form of the enzyme and acetaldehyde formed from ethanol may have a role in increasing the content of the enzyme in a cytosolic reservoir.

Acknowledgement—We greatly appreciate the technical assistance of Mr. Masayasu Takase.

REFERENCES

1. E. A. Nikkil, *Proc. Soc. exp. Biol. Med.* **113**, 814 (1963).
2. C.-J. Estler, *Res. exp. Med.* **163**, 95 (1974).
3. C. S. Lieber, N. Spritz and L. M. DeCarli, *J. Lipid Res.* **10** 283 (1969).
4. J. A. Ontko, *J. Lipid Res.* **14**, 78 (1973).
5. S. Mallov and J. L. Bloch, *Am. J. Physiol.* **184**, 29 (1956).
6. C. S. Lieber and R. Schmid, *J. clin. Invest.* **40**, 394 (1961).
7. M. Poggi and N. R. DiLuzio, *J. Lipid Res.* **5**, 437 (1964).
8. M. G. Horning, E. A. Williams, H. Maling and B. B. Brodie, *Biochem. biophys. Res. Commun.* **3**, 635 (1960).
9. D. N. Brindley, J. Cooling, S. L. Burditt, P. H. Pritchard, S. Pawson and R. G. Sturton, *Biochem. J.* **180**, 195 (1979).
10. M. J. Savolainen, *Biochem. biophys. Res. Commun.* **75**, 511 (1977).
11. M. J. Savolainen and I. E. Hassinen, *Archs Biochem. Biophys.* **201**, 640 (1980).
12. D. N. Brindley, in *Phospholipids* (Eds. J. N. Hawthorne and G. B. Ansell), p. 179. Elsevier Biomedical Press, Amsterdam (1982).
13. R. Coleman and G. Hübscher, *Biochim. biophys. Acta* **56**, 479 (1965).
14. B. Sedgwick and G. Hübscher, *Biochim. biophys. Acta* **106**, 63 (1965).
15. R. G. Lamb and H. J. Fallon, *Biochim. biophys. Acta* **348**, 166 (1974).
16. K. Hosaka, Y. Yamashita and S. Numa, *J. Biochem. (Tokyo)* **77**, 501 (1975).
17. T. F. Walseth and R. G. Johnson, *Biochim. biophys. Acta* **256**, 11 (1979).
18. H. Ide and Y. Nakazawa, *J. Biochem. (Tokyo)* **97**, 45 (1985).
19. J. S. Nisselbaum and O. Bodansky, *J. biol. Chem.* **234**, 3276 (1959).
20. M. A. Swanson, *J. biol. Chem.* **184**, 647 (1950).
21. R. G. Sturton and D. N. Brindley, *Biochem. J.* **171**, 263 (1978).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
23. R. G. Sturton, P. H. Pritchard, L.-Y. Han and D. N. Brindley, *Biochem. J.* **174**, 667 (1978).
24. H. Theorell and T. Yonetani, *Biochem. Z.* **338**, 537 (1963).
25. J. K. W. Ferguson, *Can. Med. Ass. J.* **74**, 793 (1956).
26. R. H. Dreisbach, *Industrial hazards.* In *Handbook of Poisoning*, 11th Edn., p. 151. Lange Medical Publications, Canada (1983).
27. P. G. Casola and F. Possmayer, *Biochim. biophys. Acta* **664**, 298 (1981).
28. P. G. Casola and F. Possmayer, *Biochim. biophys. Acta* **665**, 177 (1981).
29. R. J. Jenning, N. Lawson, R. Fears and D. N. Brindley, *FEBS Lett.* **133**, 119 (1981).
30. C. K. Wood and R. G. Lamb, *Biochim. biophys. Acta* **572**, 121 (1979).
31. C. Cascales, E. H. Mangiapane and D. N. Brindley, *Biochem. J.* **219**, 911 (1984).
32. T. F. Slater, B. C. Sawyer and V. D. Strauli, *Biochem. J.* **93**, 267 (1964).
33. G. R. Cherrick and C. M. Leevy, *Biochim. biophys. Acta* **107**, 29 (1965).
34. P. Martin-Sanz, R. Hopewell and D. N. Brindley, *FEBS Lett.* **175**, 284 (1984).
35. D. N. Brindley, *Prog. Lipid. Res.* **23**, 115 (1985).