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Alterations in the cytochemical activity of several phosphatases in hepatocytes from rats exposed prenatally to ethanol

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Summary. The activities of acid phosphatase, alkaline phosphatase, glucose-6-phosphatase, uridine diphosphatase, inosine diphosphatase, thiamine pyrophosphatase and 5′-nucleotidase have been investigated cytochemically in hepatocytes of the offspring of alcohol-fed rats, using cerium ions as a capturing agent and qualitative and quantitative electron microscopy. All these enzyme activities were decreased in the experimental animals compared with controls not exposed to ethanol. The pattern of deposition of the product of glucose-6-phosphatase activity in the cisternae of the endoplasmic reticulum was also different in the two groups. The phosphatases analyzed are functional markers of different cell components, and the results suggest that prenatal exposure of rats to ethanol causes functional alterations in the endoplasmic reticulum, Golgi apparatus, lysosomes and plasma membrane of hepatocytes.

Key words: Ethanol – Prenatal exposure – Cytochemistry – Hepatocytes – Rat

Introduction

Clinical, epidemiological and behavioral studies in humans have demonstrated abnormalities in the offspring of mothers consuming ethanol during pregnancy (Streissguth et al. 1980; Abel 1982; Colangelo and Jones 1982), and controlled laboratory experiments have shown teratogenic effects of alcohol in animals (Streissguth et al. 1980). Despite extensive studies of the fetal alcohol syndrome, few reports have focused on the effect of ethanol on the ultrastructure of organs and tissues in humans and animals following prenatal exposure. The liver, which is the main site of alcohol metabolism in adults, undergoes marked pathological and ultrastructural changes as

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a consequence of alcohol intake (Lieber 1983). Little is known, however, of the possible changes induced in the liver by prenatal exposure to alcohol (Rømert and Matthiessen 1983, 1984). Fetal liver differs from adult liver in many aspects such as the stage of differentiation, functional activity, the support of nutrients and other substances and the response to drugs, and a study of these potential changes appeared of interest. We have recently demonstrated marked qualitative and quantitative ultrastructural alterations in the hepatocytes of newborn rats exposed prenatally to alcohol (Renau-Piqueras et al. 1985). Using cytochemical methods we have now extended that study by comparing the localization and activity of several phosphatases known to be markers of different cell components (Novikoff 1976), in the hepatocytes of the offspring of control rats with those fed ethanol during pregnancy. To maximize the quantitative nature of the data, stereologic methods of analysis have been used.

Material and methods

Treatment of rats. Female Wistar rats (200–250 g) were treated as described previously (Guerri and Grisolía 1982; Renau-Piqueras et al. 1985). All rats were maintained on a 12 h light-dark cycle in plastic cages. They received the Lieber-DeCarli liquid diet (Lieber and DeCarli 1976, 1982) either containing 5% (v/v) ethanol or isocalorically balanced with maltose-dextrine for the pairfed controls. Female rats were maintained on the ethanol liquid diet for a minimum of 30 days prior to exposure to male rats. Male and female pairs were housed separately, the male animals being removed once pregnancy was confirmed. One-day-old pups (three per group) were randomly chosen and sacrificed by decapitation between 8.30 and 9.00 am.

Cytochemistry. Control and experimental animals were processed in parallel. The left lobe of liver was excised and immersed in ice-cold 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 5% sucrose. After 15 min, a thin slide from the central region was cut and then divided into 30 cubes. From these, 12 were chosen at random and fixed in the glutaraldehyde solution for 45 additional min. Blocks were then washed repeately in the buffer and immersed in the same buffer containing 10% DMSO. After 1 h they were frozen in liquid nitrogen-cooled isopentane, and 20 μm thick frozen sections were prepared in a cryostat. The use of cryostat sections minimizes variations in the rate of penetration of reagents into the cells. Acid phosphatase (AcPase), alkaline phosphatase (AlkPase), glucose-6-phosphatase (G6Pase), uridine diphosphatase (UDPase), inosine diphosphatase (IDPase), thiamine pyrophosphatase (TPPase) and 5′-nucleotidase activities were assayed by immersing sections in the appropiate solutions, which also contained cerium ions as a capturing agent (Robinson and Karnovsky 1983a). Incubation was at 37° C for 90 min with shaking (the medium was changed at 45 min) and was terminated by washing briefly in cacodylate buffer followed by refixing in glutaraldehyde for 60 min, washing overnight in the same buffer, and postfixing for 3 h in 2% OsO_4–0.8% $K_4Fe(CN)_6$ (Hulstaert et al. 1983; Renau-Piqueras et al. 1985). After washing, the samples were stained in block with uranyl acetate, dehydrated in acetone and embedded in Poly/Bed-812. Sections of equal thickness (usually 80 nm, occasionally 200 nm) were observed, without further staining, in a Philips 300 EM at 60 kV.

Cytochemical controls were sections incubated without substrate (Robinson and Karnovsky 1983a; Hulstaert et al. 1983). For assay of 5′-nucleotidase, specimens were processed as described by Robinson and Karnovsky (1983b).

Stereology. From the 12 blocks per case and animal, one was chosen at random and one representative section was selected. Micrographs of the periportal zone at the same magnification (×47,880) were developed identically and analyzed using a Leitz ASM system for semi-automatic image analysis (Renau-Piqueras et al. 1985). The stereologic procedure was always

applied to deposits of reaction product of those cytochemical activities contained in cytoplasmic organelles (G6Pase, TPPase, UDPase, IDPase, and AcPase). In some cases, these deposits completely filled a given organelle while in others they were filled only partially (see below). The values obtained, therefore, do not necessarily represent the volume density of organelles containing deposits but rather the volume density of the deposits. In this procedure, all deposits were considered irrespective of their intensity.

The minimal sample sizes was determined in all cases by the progressive mean technique (Williams 1977; confidence limit $\pm 5\%$). Since no statistical differences by analysis of variance were found between animals within each group, the results are expressed as the mean $\pm$ sd of each group (control and prenatally exposed to alcohol (PEA)). Statistical comparison of the stereologic data was by Student's t-test ($P \leq 0.05$).

Results

In confirmation of our earlier findings (Renau-Piqueras et al. 1985), the Golgi apparatus of hepatocytes from newborn rats exposed prenatally to alcohol was frequently disorganized (in about 40% of cells) and composed mainly of small-size vesicles (Fig. 2) which lacked phosphatase activity with any of the substrates tested (e.g. Fig. 9). Therefore, the cytochemical observations reported here on the Golgi apparatus of cells from PEA animals refer only to the morphologically "normal" Golgi apparatus present in about 60% of the hepatocytes (Fig. 1).

Phosphate produced in the reactions yielded a very fine electron-dense precipitate of cerium phosphate. The localization of the different phosphatases investigated here was similar to that described in the adult rat liver (Novikoff 1976; Robinson and Karnovsky 1983a; Hulstaert et al. 1983). Some examples of these localizations are illustrated in Figs. 3–14. Nonspecific deposits were rare in both control and treated hepatocytes and no phosphatase activity was detected in the cytochemical controls.

G6Pase activity was detected in all cisternae of the rough and smooth endoplasmic reticulum (rER and sER, respectively) and in the nuclear envelope of control hepatocytes. In these cells, the deposits filled the cisternae homogeneously (Fig. 3). In constrast, in most fo the hepatocytes of PEA rats, deposits were distributed in the ER as spots of different size and electron-density (Figs. 4, 5). In some treated cells the deposits were less electron-dense than in control cells or were virtually absent.

Activities of the three NDPases investigated (UDPase, IDPase, and TPPase) were detected in all hepatocytes, both from controls and PEA rats. With UDP and IDP as substrates the deposit was found between the cisternae of the ER and in the trans-side of the Golgi apparatus (Figs. 6–9). TPPase, which is used commonly as a cytochemical marker of the trans-face of the Golgi apparatus, was consistently present between the inner one or two (trans) cisternae of this organelle. This activity, although less intense, was also detected in GERL (Figs. 10, 11).

AlkPase and 5′-nucleotidase, which are markers of the plasma membrane, were found primarily in the bile canaliculi of both control and experimental animals. With 5′-nucleotidase there was also deposits along the microvilli protruding into the space of Disse. The electron-density of deposits

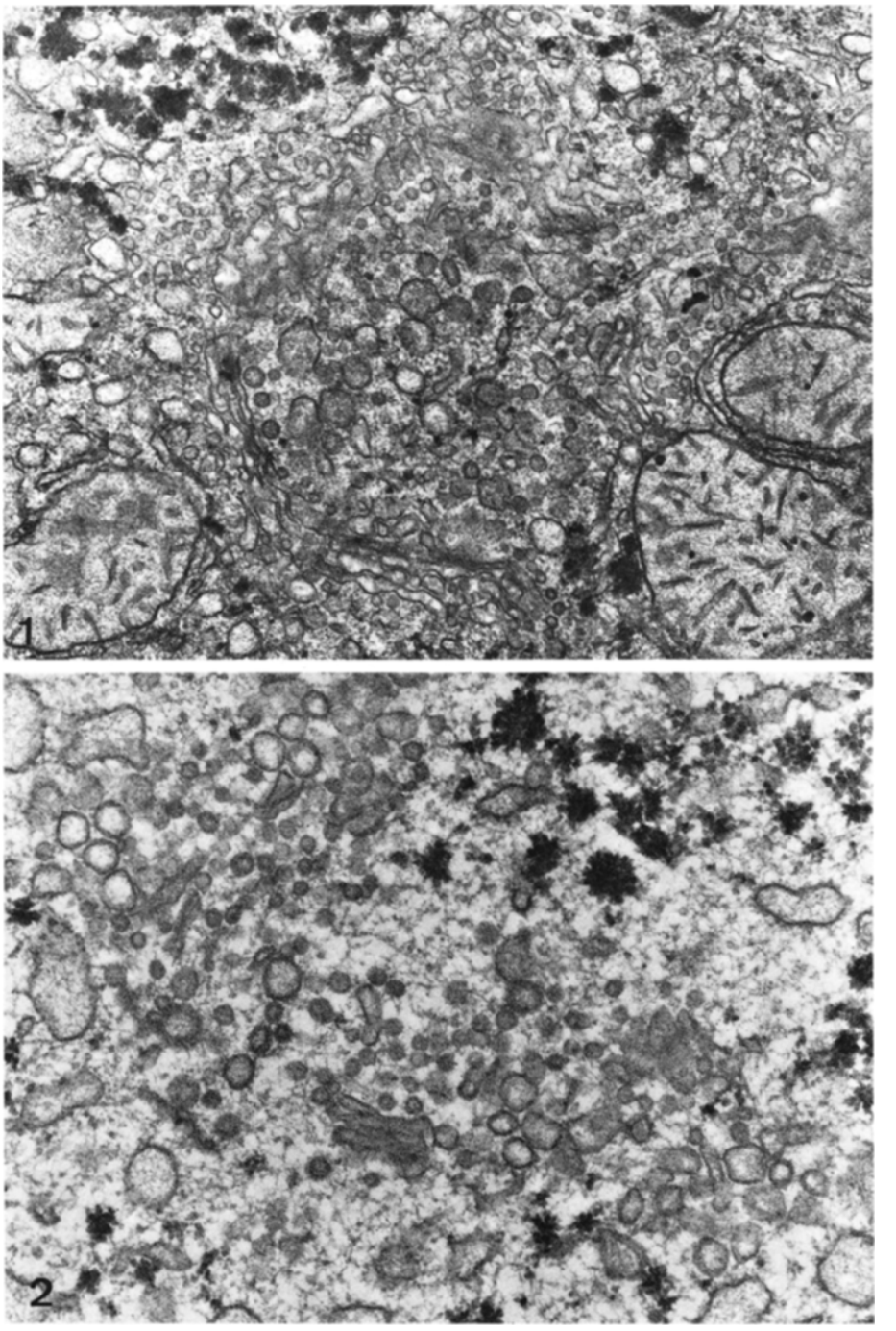


Fig. 1. Micrograph showing the Golgi apparatus of a control rat hepatocyte (× 30,800)

Fig. 2. The Golgi apparatus in many PEA rat hepatocytes appears composed mainly of small-size vesicles. Uranyl acetate and lead citrate (× 46,900)

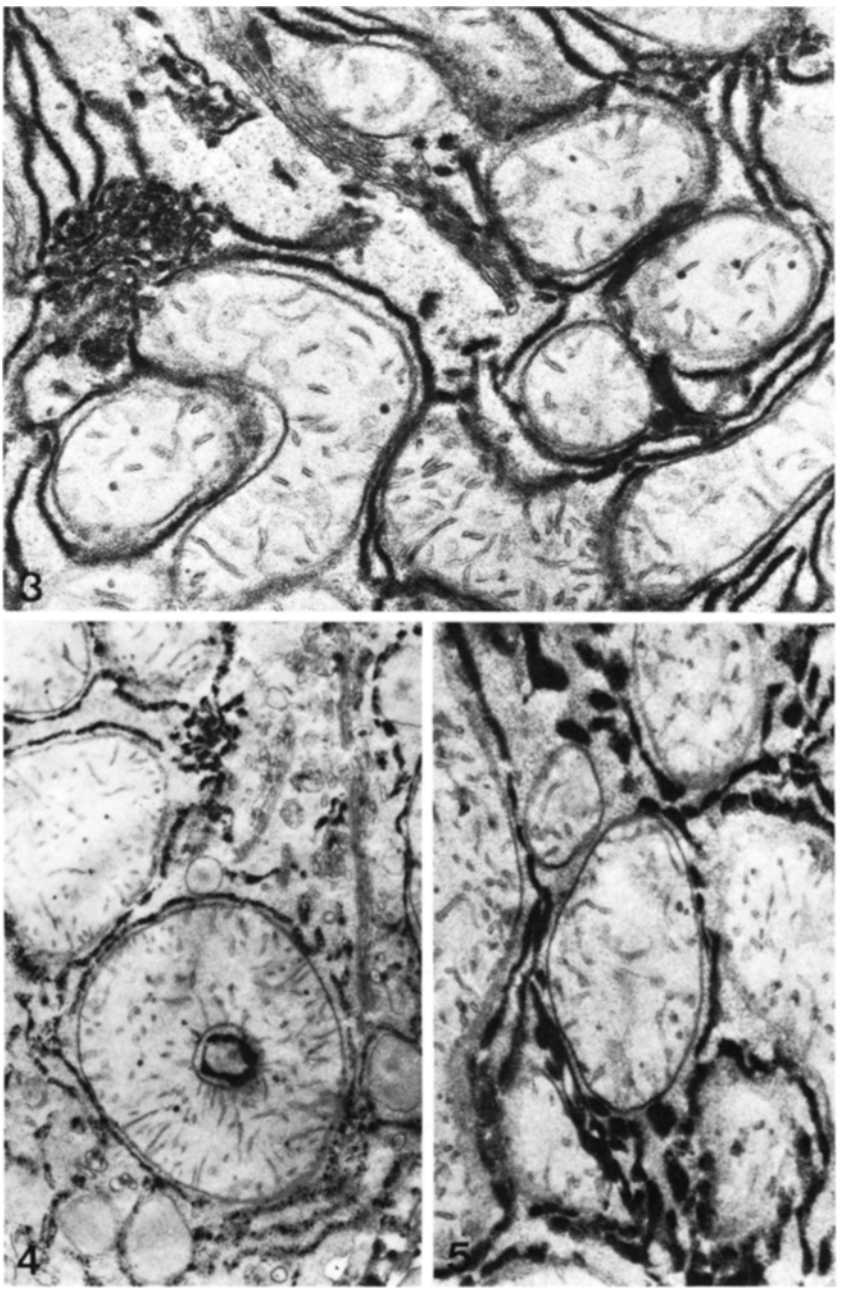
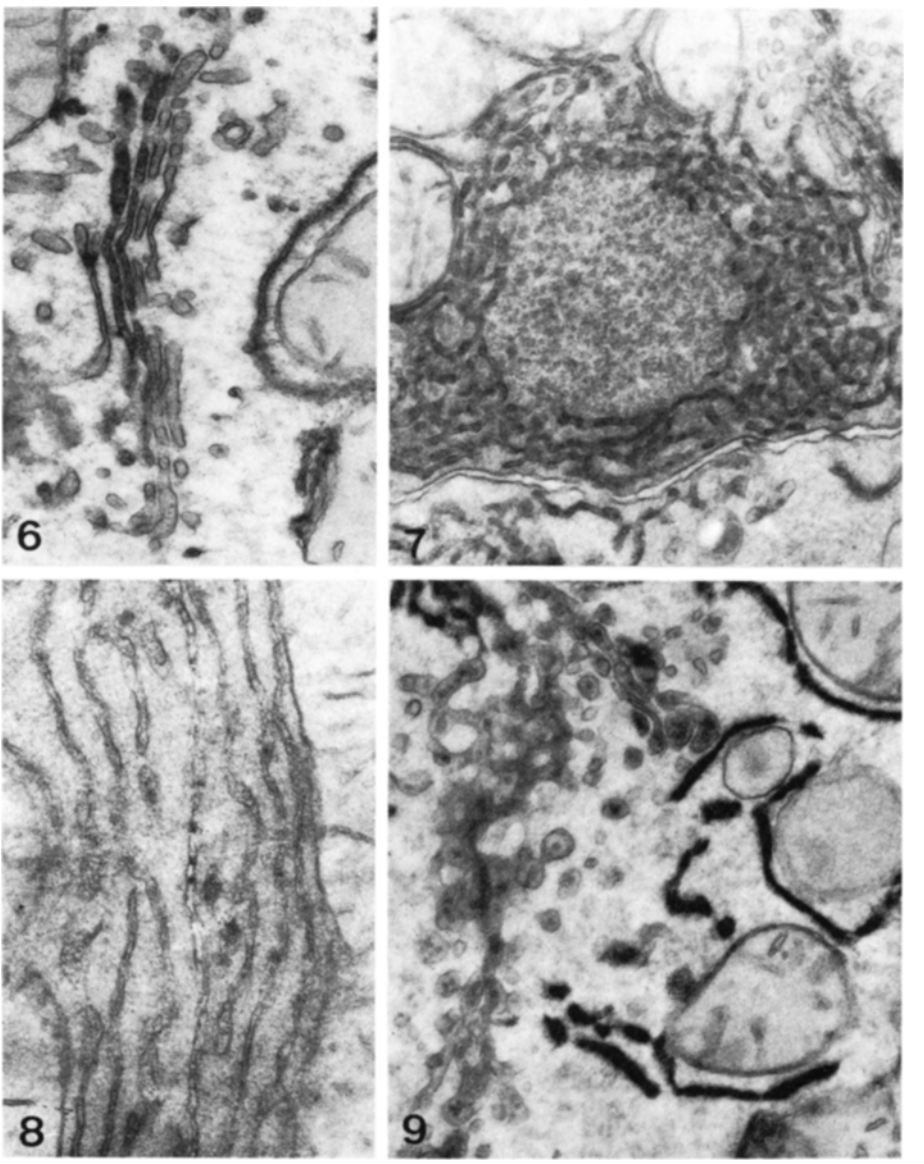
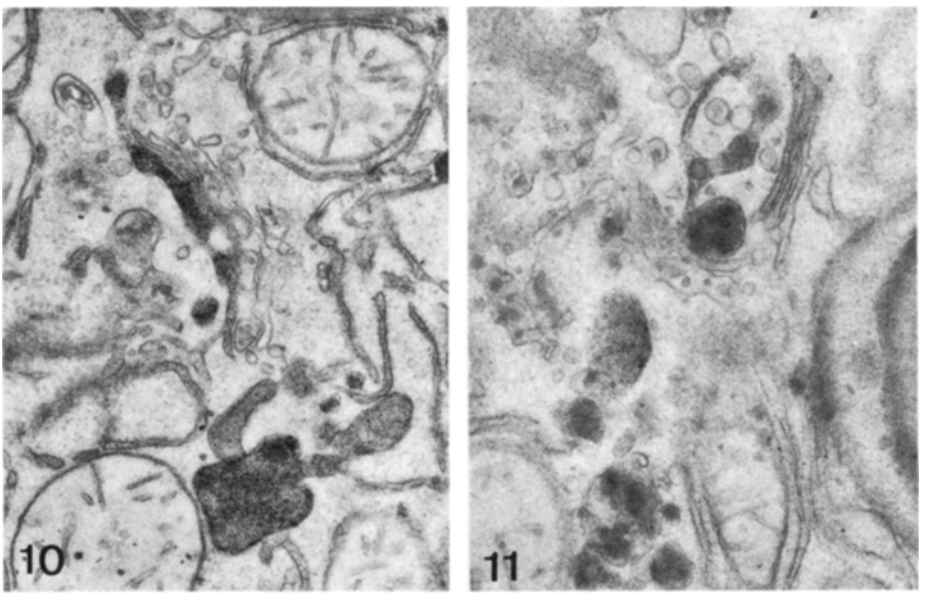


Fig. 3. Control hepatocytes. The cytochemical activity of G6Pase appears localized in the sER and rER. Note the continuity between both types of ER (× 46,100)

Figs. 4–5. Ultrastructural localization of G6Pase in PEA rat hepatocytes. Figure 4 shows a poor and discontinuous deposition of reaction product in the ER cisternae. This pattern is the most characteristic in treated cells (× 19,200). Figure 5 is a detail of this pattern in a 200 nm section (× 31,900)



Figs. 6–9. Ultrastructural localization of IDPase and UDPase in control and treated hepatocytes. Figure 6 shows the pattern of IDPase in control cells. A similar distribution was found for UDPase. These activities are localized in the trans-portion of the Golgi apparatus as well as in the ER cisternae. Note in Fig. 7 a portion of ER of a control hepatocyte surrounding an accumulation of glycogen (× 31,900 and × 30,500, respectively). Figure 8 illustrates an aspect of UDPase activity in a treated hepatocyte. UDPase activity in treated cells is less pronounced than in control cells (× 38,000). Figure 9 shows a 200 nm section of partially vesiculated Golgi apparatus in a treated hepatocyte processed for UDPase localization. Note the absence of activity in this cell component (× 38,100)



Figs. 10–11. Figures 10 and 11 correspond to TPPase activity in the Golgi apparatus of control and treated hepatocytes, respecitively. The cytochemical activity of TPPase is more intense in control cells than in treated hepatocytes (× 38,100 and × 38,100, respectively)

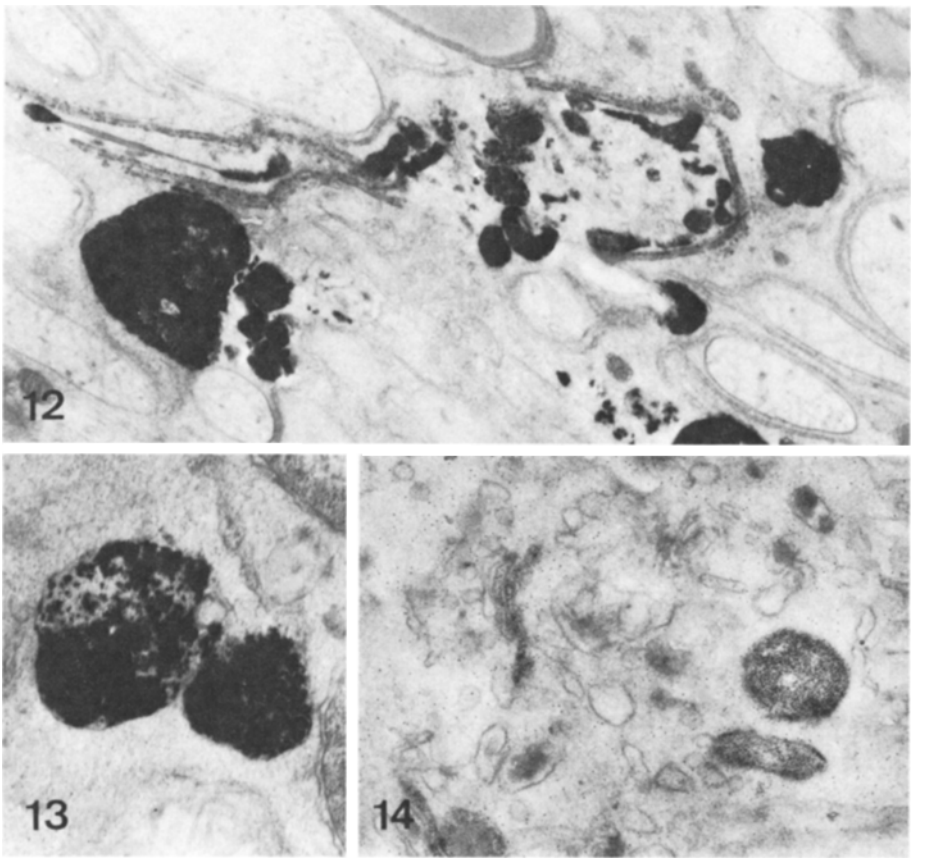
from these two enzymes was less in cells from treated animals, and some hepatocytes from alcohol-exposed rats lacked 5′-nucleotidase activity.

In both control and PEA rat hepatocytes the AcPAse activity produced deposition of product in cisternae and vesicles of the transportion of the Golgi apparatus and in tubuli and vesicles throughout the GERL region. The deposit over the several consecutive Golgi cisternae was faint and increased from cis- to trans-side. An intense deposit was observed in lysosomes. In PEA rat cells the reaction product was less heavy than in control hepatocytes (Figs. 12–14).

The stereological data obtained for G6Pase, NDPase (UDPase, IDPase and TPPase) and AcPase are summarized in Table 1. They show a significant decrease of relative volume of deposits of reaction product of these phosphatases after treatment.

Discussion

Cytochemical techniques are useful in the functional characterization of cell components, making it possible to correlate their biochemical function with their morphology. However, it is wellknown that these methods present some difficulties. Non-specific deposits and variations in the penetration of reagents are problems commonly found with cytochemical techniques. To minimize these problems we have used cryostat sections for incubation and cerium as a phosphate trapping agent. The advantages of cerium over lead, in terms of exactitude and reproducibility, are well documented (Ro-



Figs. 12–14. Micrographs showing the AcPase activity in control (Figs. 12 and 13) and in treated cells (Fig. 14). The difference in the amount of deposited reaction product in the two cell types is evident (× 15,300, × 30,600, and × 37,600, respectively)

binson and Karnovsky 1983a, b; Hulstaert et al. 1983). Moreover, with cerium as a capturing agent, the amount of reaction product formed is directly proportional to the amount of enzyme present in a cell-free system (Robinson and Karnovsky 1983a, b).

Although stereologic methods are useful for approximate quantification of cytochemical reactions, they do not take into account changes in intensity of the electron-dense precipitates (Weibel 1979). Since we observed greater precipitate intensities in control than in alcohol-treated animals, our stereological results certainly underestimate any differences. We have shown that prenatal exposure to ethanol causes marked qualitative and quantitative changes in the ultrastructure of rat hepatocytes (Renau-Piqueras et al. 1985). Notably in cell size, mitochondrial volume and shape, size and number of peroxisomes, and particularly in the organization of the Golgi apparatus. These changes, which are confirmed in the present study, suggest that the functions of hepatocytes would also be altered in PEA rats. The decreased

Table 1. Volume density of deposits of reaction product of phosphatases in hepatocytes from control and PEA rats (mean ± s.d.)

Enzyme activity	Control	PEA	Ratio (Vv_c/Vv_t)
G6Pase	16.499 – 1.456	13.496 – 1.351[a]	1.240 – 0.217
TPPase	1.798 – 0.228	0.899 – 0.087	2.452 – 0.701
UDPase			
(total ER)	14.700 – 4.740	7.253 – 2.105	2.027 – 0.215
(Golgi)	1.534 – 0.153	0.722 – 0.073	2.160 – 0.233
IDPase	11.588 – 2.211	6.711 – 0.475	1.754 – 0.432
AcPase	5.767 – 0.763	2.674 – 0.088	2.171 – 0.362

Vv_c, Volume density of deposits in control cells; Vv_t, Volume density of deposits in treated cells; Vv values in percent of cytoplasmic volume

[a] Significant differences, using Student's t test ($P \leq 0.05$) were found in all cases

activities of a number of phosphatases tested cytochemically in cells from treated rats would certainly support this view. In addition, the deposits of reaction product of some of these enzymes were distributed differently in the PEA rat cells than in cells from control animals. Glucose-6-phosphatase, for example, belongs to group c of ER enzymes which are usually located in the lumen of this organelle with heterogeneous lateral distribution (Cardell 1977; Amar-Costessec and Beaufay 1981; Dallner and DePierre 1982). Other enzymes of this group such as esterase or NDPase occur either free in the lumen or loosely bound to the ER membrane (Amar-Costessec and Beaufay 1981). Glucose-6-phosphatase, however, is an integral membrane protein (Amar-Costessec and Beaufay 1981); wheather its intrinsic location is significant is not known, but it is apparently related to the regulation of the conversion of G6Phase to glucose (Dallner and DePierre 1982). A diminution in its cytochemical activity and a change in its pattern of distribution in PEA rat cells would therefore suggest that its function had been altered by alcohol treatment. The decrease in its cytochemical activity could not be due simply to a diminution in the relative volume of the ER, since we have demonstrated that this parameter is unaffected by prenatal alcohol exposure (Renau-Piqueras et al. 1985). However, it might contribute to the decrease in plasma glucose level which has been described in fetal and newborn rats exposed prenatally to ethanol (Tanaka et al. 1982; Marquis et al. 1984). Other group c ER enzymes such as NDPase (UDPase and IDPase) also show decreased cytochemical activity after prenatal alcohol treatment. These enzymes appear to be related with the glycosylation of proteins in the ER and a decrease in their activity could indicate an alteration of this process.

The Golgi apparatus, which is responsible for terminal glycosylation of proteins (Farquhar and Palade 1981), is markedly altered by prenatal exposure to ethanol. It has been demonstrated that ethanol inhibits glycosy-

lation of secretory proteins in the liver (Baraona and Lieber 1982), and decreases the activity of UDP-galactose: N-acetylglucosamine galactosyltransferase (Nanni et al. 1978). Uridine diphosphate, one of the products of galactosylation in the Golgi apparatus (Fleischer 1983) is highly inhibitory for galactosyltransferase but is rapidly converted to UMP by UDPase (Khatra et al. 1984; Kuhn and White 1977; Fleischer 1983). The marked decrease in UDPase cytochemical activity, which we have demonstrated, suggests that UDP might accumulate in treated cells to levels sufficient to inhibit glycosylation of secreted products. The decrease in the Golgi apparatus NDPase activity has been observed with UDP as well as with less specific substrates (IDP and TPP), supporting the conclusion that in treated animals NDPase is either decreased or less active. The finding that vesicles in the disorganized Golgi apparatus of PEA rat hepatocytes do not stain with any of the phosphatase reactions tested, suggest that they are more closely related to the cis-component, which is also unstained, than to the trans-component which does stain with some of these reactions (Robinson and Karnovsky 1983a; Farquhar and Palade 1981).

Two enzymes, AlkPase and 5′-nucleotidase, which are bound to the plasma membrane were also decreased by alcohol treatment, as expected from the decreased activity reported for these and other plasma membrane-bound enzymes of brain after prenatal alcohol exposure (Guerri and Grisolía 1982; Sanchis et al. 1984; Guerri et al. 1984). Finally, from the decrease in cytochemical activity of AcPase, ethanol would also appear to affect the function of the lysosomes.

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References

Abel EL (1982) Consumption of alcohol during pregnancy: A review of effects on growth and development of offsprings. Human Biol 54:421–453

Amar-Cortessec A, Beaufay H (1981) A structural basis of enzymatic heterogeneity within liver endoplasmic reticulum. J Theor Biol 89:217–130

Baraona E, Lieber CS (1982) Effects of alcohol on hepatic transport of proteins. Ann Rev Med 33:281–292

Cardell RR (1977) Smooth endoplasmic reticulum in the rat hepatocyte during glycogen deposition and depletion. Int Rev Cytol 48:221–279

Colangelo W, Jones DG (1982) The fetal alcohol syndrome: A review and assessement of the syndrome and its neurological sequelae. Prog Neurobiol 19:271–314

Dallner G, DePierre JW (1982) Endoplasmic reticulum. In: Arias I, Popper H, Schachter D, Shafritz DA (eds) The liver: Biology and pathobiology. Raven Press, New York, p 41

Farquhar MG, Palade GE (1981) The Golgi apparatus (complex) – (1954–1981) – from artifact to center stage. J Cell Biol 91:77s–103s

Fleischer B (1983) Mecanism of glycosylation in the Golgi apparatus. J Histochem Cytochem 31:1033–1040

Guerri C, Grisolía S (1982) Effects of prenatal and postnatal exposure of rats to alcohol: Changes in (Na^+, K^+)ATPase. Pharmacol Biochem Behav 17:927–932

Guerri C, Esquifino A, Sanchis R, Grisolía S (1984) Growth enzymes and hormonal changes

in offsprings of alcohol-fed rats. In: Mechanism of alcohol damage in utero. Pitman, London (Ciba Foundation Symposium 105) p 85

Hulstaert CE, Kalicharan D, Hardonk MJ (1983) Cytochemical demonstration of phosphatases in the rat liver by a cerium-based method in combination with osmium tetroxide and potassium ferrocyanide postfixation. Histochemistry 78:71–79

Khatra BS, Herries DG, Brew K (1974) Some kinetic properties of human-milk galactosyltransferase. Eur J Biochem 44:537–401

Kuhn NJ, White A (1977) The role of nucleoside diphosphatase in a uridine nucleotide cycle associated with lactose synthesis in rat mammary-gland Golgi apparatus. Biochem J 168:423–426

Lieber CS (1983) Alcohol and the liver. In: Arias IM, Frenkes M, Wilson JHP (eds) The liver annual. Elsevier, Amsterdam, New York, Oxford, p 106

Lieber CS, DeCarli LM (1976) Animal models of ethanol dependence and liver injury in rats and banboons. Fed Proc 35:1232–1236

Lieber CS, DeCarli LM (1982) The feeding of alcohol in liquid diets: Two decades of applications and 1982 update. Alcohol Clin Exp Res 6:523–531

Marquis SM, Leichter J, Lee M (1984) Plasma amino acids and glucose levels in the rat fetus and dam after chronic maternal alcohol consumption. Biol Neonate 46:36–43

Nanni G, Pronzato MA, Averame MM, Gambella GR, Cottalaso D, Marinari UM (1978) Influence of acute ethanol intoxication on rat liver Golgi apparatus glycosylation activities. FEBS Lett 93:242–246

Novikoff AB (1976) The endoplasmic reticulum: A cytochemist's view (A review). Proc Natl Acad Sci [USA] 73:2781–2787

Renau-Piqueras J, Gómez-Perretta C, Guerri C, Sanchis R (1985) Qualitative and quantitative ultrastructural alterations in hepatocytes of rats prenatally exposed to ethanol with special reference to mitochondria, Golgi apparatus and peroxisomes. Virchows Arch [Pathol Anat] 405:237–251

Robinson JM, Karnovsky MJ (1983a) Ultrastructural localization of several phosphatases with cerium. J Histochem Cytochem 31:1197–1208

Robinson JM, Karnovsky MJ (1983b) Ultrastructural localization of 5′-nucleotidase in guinea pig neutrophils based upon the use of cerium as capturing agent. J Histochem Cytochem 31:1190–1196

Rømert P, Matthiessen ME (1983) Alcohol-induced injury of mitochondria in hepatocytes of mini-pig fetuses. Virchows Arch [Pathol Anat] 399:299–305

Rømert P, Matthiessen ME (1984) Fine structure of hepatocytes from mini-pigs and mini-pig fetuses exposed to alcohol (ethanol) in vivo. Acta Anat 120:190–195

Sanchis R, Guerri C, Renau-Piqueras J, Grisolía S (1984) Effects of prenatal and postnatal alcohol intake on brain development. In: Caciagli F, Giacobini E, Paoletti R (eds) Developmental neuroscience: Physiological, pharmacological and clinical aspects. Elsevier Sci Publ B.V., Amsterdam, p 217

Streissguth AP, Ladesman-Dwyer S, Martin JC, Smith DW (1980) Teratogenic effects of alcohol in humans and laboratory animals. Science 209:353–361

Tanaka H, Suzuki N, Arima M (1982) Hypoglycemia in the fetal alcohol syndrome in rat. Brain Dev 4:97–103

Weibel ER (1979) Practical methods for biological morphometry, vol 1. Academic Press, New York, pp 228–229

Williams M (1977) Stereological techniques. In: Glauert AM (ed) Practical methods in electron microscopy. Vol 6, part 2, chap 2. North Holland/American Elsevier, Amsterdam, pp 1–84

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