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Review

Effects of prenatal exposure to ethanol on rat liver development

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The effects of ethanol on development: the fetal alcohol syndrome (FAS)

It has been established that alcohol is a teratogen in the human (Jones and Smith, 1973). When ingested during pregnancy it readily crosses the placenta (Guerri and Sanchis, 1985), enters the fetal circulatory system and has been associated with a range of adverse effects of the offspring which span the continuum from death to subtle growth and central nervous system (CNS) effects, depending on the dose, the total amount of the drug, the pattern of the drug use and the stage of cell differentiation at the time of exposure. The extreme of these effects is the fetal alcohol syndrome (FAS), the name given to a pattern of anomalies observed in children born to alcoholic mothers. The main features of this pattern are pre- and postnatal growth deficiency, characteristic facial features, CNS dysfunction, and variable major organ system malformations (Jones and Smith, 1973). FAS is at present one of the most frequent recognizable causes of mental and growth retardation in human beings (Abel and Sokol, 1986).

Since antenatal effects of ethanol in the etiology of mental deficiency are so important, we need to consider the mechanism(s) by which alcohol induces alterations in fetal development. How are these mechanisms likely to be established? There seem to be two main approaches: clinical studies and the use of animal models. As stated by Pratt (1984), in clinical studies the mechanism itself is usually inaccessible, but by looking not only at the affected children but also at their siblings and other relatives one can expect to detect (a) individuals with abnormal reactions to ethanol and (b) evidence of other kinds of prenatal damage from ethanol, not necessarily FAS. This damage in relatives is evidently widespread although it may be less severe and less easily detected than in the primary patient. Beyond these considerations, there are certain well-known problems associated with the use of humans as research subjects, including the physical and psychological complexity of the human being, ethical considerations, etc. Experimental models thus provide a valuable means of examining those mechanisms that are difficult to study in humans. No attempt will be made here, however, to analyze the advantages of animal models since several reviews on this subject exist (Boggan, 1982).

The animal model

The choice of the appropriate experimental animal as model to study the effect of ethanol on fetal development depends upon two main conditions: (a) the model should reproduce most of the alterations observed in children with FAS, and (b) it should eliminate a number of risk factors which are frequently associated with heavy alcohol intake (greater maternal age, poor nutritional status, smoking) so as to analyze the direct effect of ethanol. Since it has been reported that children with FAS are born from chronic alcoholic mothers, we have used as our experimental model chronic alcoholic female rats. We used an alcohol-containing liquid diet which, in the amount consumed, meets nutritional requirements for the mother and provides high blood alcohol levels, and which in addition facilitates the use of pair-fed isocaloric controls (Lieber and DeCarli, 1976). We have also assessed the nutritional status of the alcoholic dams during gestation and lactation, because the decreased food intake and malnutrition often associated with high alcohol consumption could be crucial in studying the direct effect of alcohol *in utero* (Sanchis *et al.*, 1986).

Abbreviations used in this paper: AcPase: acid phosphatase; ADH: alcohol dehydrogenase; ALAT: alanine amino transferase; ALDH: aldehyde dehydrogenase; AlkPase: alkaline phosphatase; CNS: central nervous system; ER: endoplasmic reticulum; FAS: fetal alcohol syndrome; GA: Golgi apparatus; GERL: Golgi-endoplasmic reticulum-lysosomes; GJ: gap junction; G6Pase: glucose-6-phosphatase; IDPase: inosine diphosphatase; IMP: intramembranous particles; NDPase: nucleoside diphosphatase; PEA: prenatal exposure to alcohol; PP: periportal; PV: perivenous; RER: rough endoplasmic reticulum; SER: smooth endoplasmic reticulum; TJ: tight junction; TPPase: thiamine pyrophosphatase; UDPase: uridine diphosphatase; UMP: uridine monophosphate; VLDL: very low density lipoprotein; Vv: volume density.

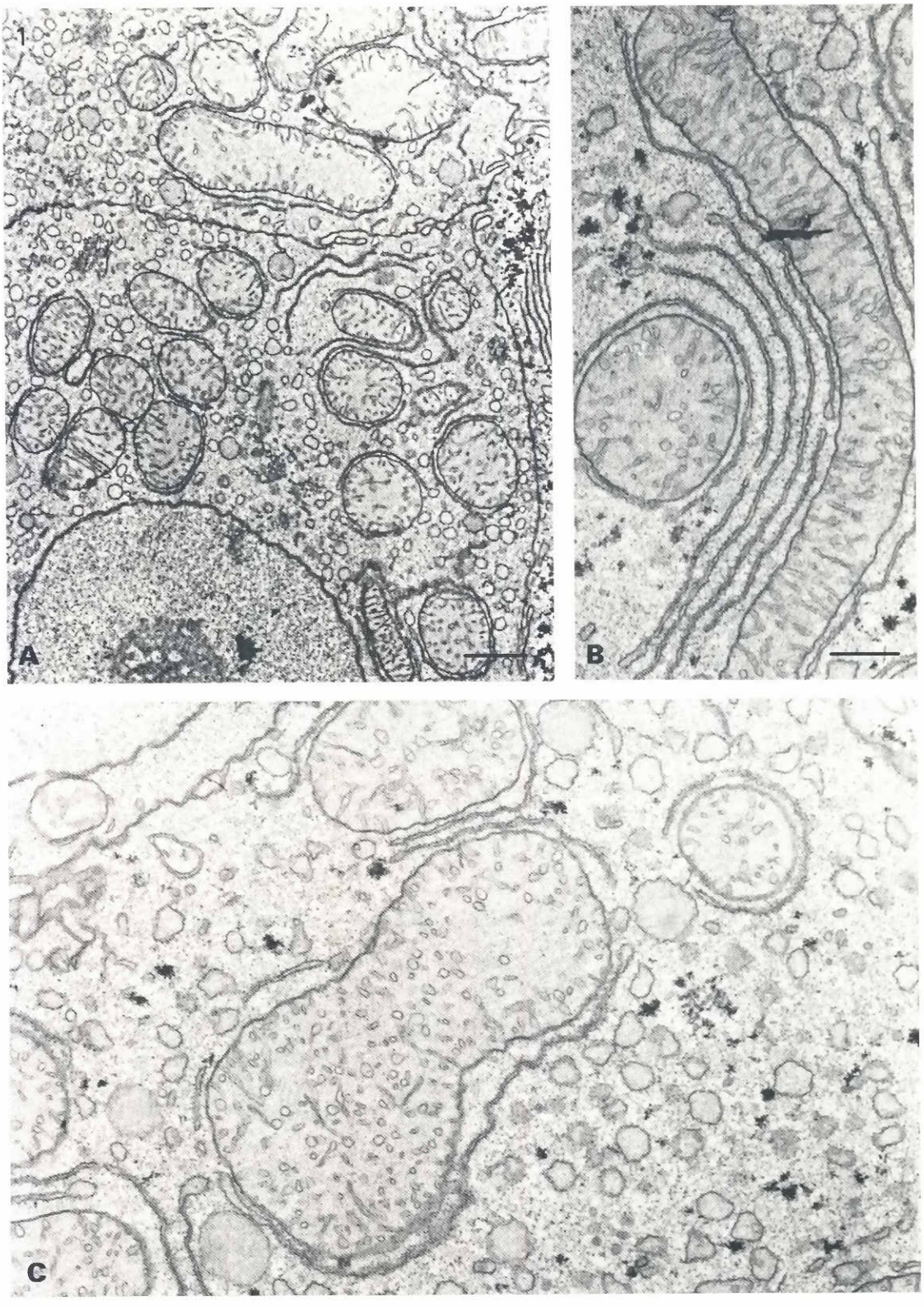


Fig. 1. Micrographs of hepatocytes, prenatally exposed to ethanol, showing several abnormally large mitochondria. *Mitochondria in these cells are 1.5 times larger (absolute volume) than those of controls (***A:** *x11,170, Bar=1μm;* **B:** *x24,280, Bar=0.5μm;* **C:** *x24,280, Bar=0.5μm) See also mitochondria in Figs. 3A and 3B.*

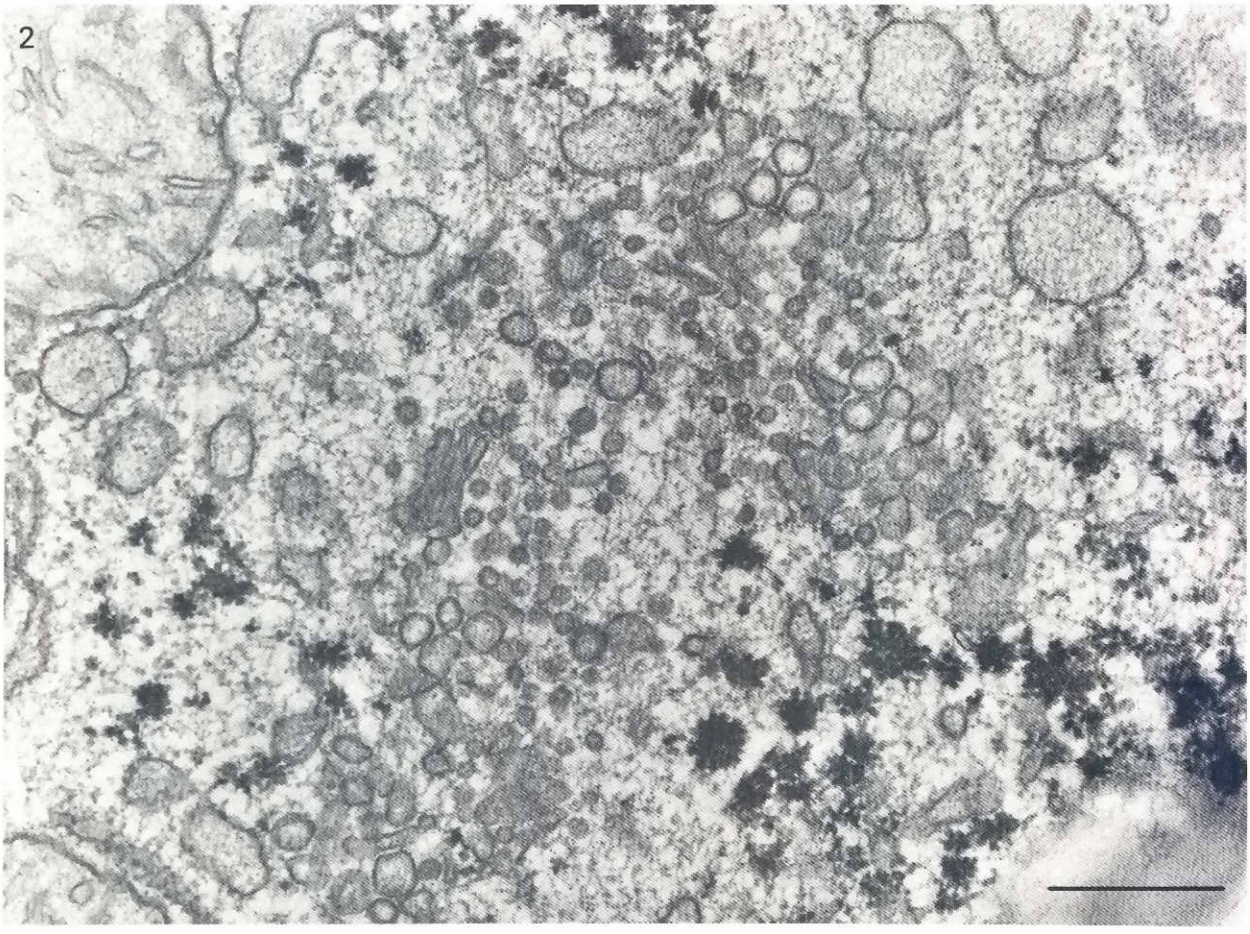


Fig. 2. Micrograph showing detail of Golgi apparatus of a hepatocyte from a newborn rat prenatally exposed to alcohol. *About 30% of Golgi apparatus in these hepatocytes is composed of numerous vesicles measuring 60 nm in diameter (x48,000; Bar= 0.5μm).*

Using this model we have demonstrated that chronic ethanol consumption during pregnancy is embryotoxic and embryolethal as well as toxic to the mother (Sanchis *et al.*, 1986, 1987). We have found in the offspring of alcoholic rats a number of alterations including malformations in the phenotype, decreases in fetal viability, and diminution of the weights of the fetal liver and brain (Guerri *et al.*, 1984; Sanchis *et al.*, 1986). The reduced body and organ weights continued for the first two to three months of the postnatal period but was most apparent during lactation. Moreover, the delay in brain development (pre- and postnatal) was accompanied by the decrease in activity of several membrane-bound synaptic enzymes as well as alterations in the levels of several hormones (Guerri *et al.*, 1984; Sanchis *et al.*, 1984). The decrease in activities of neuronal enzymes and changes in astrocyte development could produce profound alterations in brain functions. These adverse effects cannot be attributed to a primary alcohol malnutrition, because they were not observed in the pair-fed controls.

Alcoholic liver disease

On the basis of numerous experimental and epidemiological studies it is clear that ethanol itself must play a major role in the pathogenesis of adult alcohol liver disease, independently of malnutrition. The multiple and often complex nutrient abnormalities seen in human alcoholics, however, may contribute to the liver disease and to many of its complications (see reviews in Lieber, 1985).

The liver, which is the main organ of alcohol metabolism in adults, undergoes marked physiological, biochemical and ultrastructural changes as a consequence of alcohol intake (Lieber, 1985). Little is known, however, of the possible injury induced in the liver by prenatal exposure to alcohol. 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 response to drugs. Therefore, during the past several years we have been interested

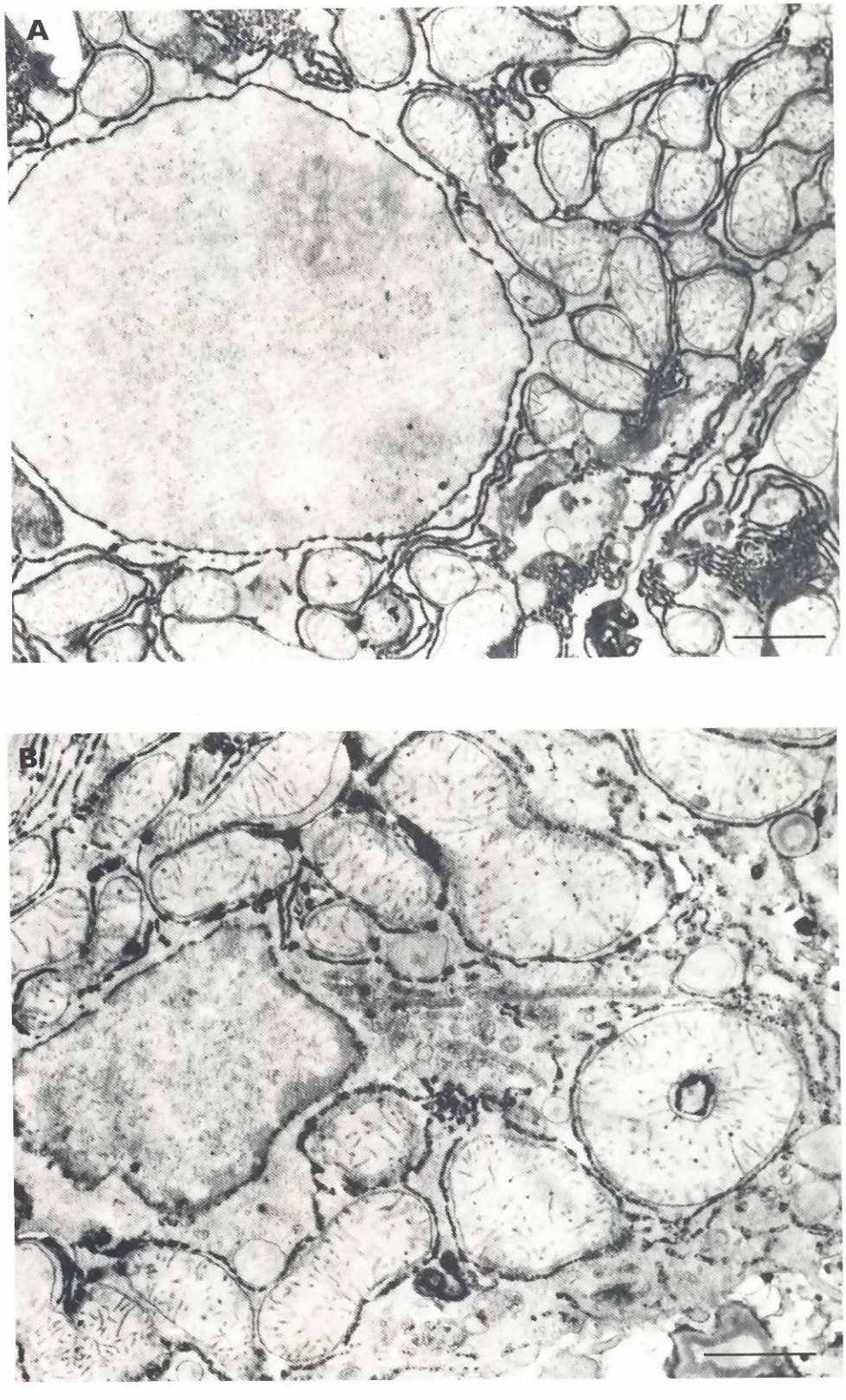


Fig. 3. Cytochemical activity of glucose 6-phosphatase. (A) *In hepatocytes from controls, staining reveals G6Pase in the SER, RER and nuclear envelope.* **(B)** *In hepatocytes prenatally exposed to ethanol, the deposits of reaction product (cerium phosphate) are scarce and distributed in the endoplasmic reticulum (ER) as spots of different size and electron-density (Bar=1μm).*

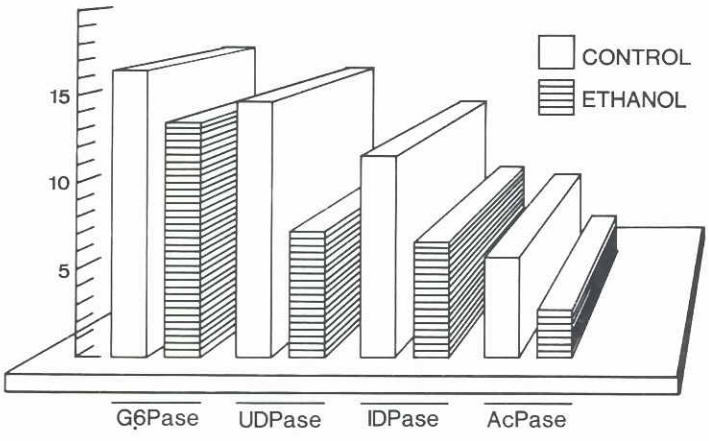


Fig. 4. Stereological analysis of the cythochemical activity of marker enzymes. *Graph showing the volume density of reaction product deposits in control and ethanol-treated hepatocytes processed for the cytochemical demonstration of glucose 6-phosphatase (G6Pase), uridine diphosphatase (UDPase), inosine diphosphatase (IDPase) and acid phosphatase (AcPase). Volume density values are expressed as percent of cytoplasmic volume. Significant differences, using Student's t test ($P \leq 0.05$) were found in all cases.*

in analyzing the possible effects of prenatal alcohol exposure on the development of the liver. In this paper we summarize the main findings obtained in our laboratory in this field.

Effect of prenatal exposure to ethanol on liver development

Qualitative and quantitative ultrastructural alterations

When livers from rat fetuses prenatally exposed to ethanol were analyzed using both qualitative and quantitative electron microscopy, we found that mitochondria of these animals were abnormally shaped and larger than those of pair-fed controls (Fig. 1). At the same time, the Golgi apparatus (GA) of many treated hepatocytes appeared to be composed mainly of vesicles. Cisternae and VLDL particles were rarely found (Fig. 2). Glycogen was not uniformly distributed among these cells, and its distribution within the cell was diffuse. Although lysosomes were scarce in both treated and control cells, no qualitative differences were observed. Finally, in some treated cells, myelinic figures indicating residues of membrane lipid metabolism were found (Renau-Piqueras *et al.*, 1985a).

Stereological and morphometric analysis showed that hepatocytes prenatally exposed to alcohol (PEA) and control cells differ in the absolute volume of the whole cell, nucleus, cytoplasm, and single mitochondrion, the volume of surface density of mitochondria and surface density of peroxisomes, the total number of peroxisomes and of lipid droplets and in the numerical density of peroxisomes, which are greater in alcohol-treated than in control hepatocytes. However, other stereological parameters, such as the surface density of the nucleus and of the smooth endoplasmic reticulum (SER) and the absolute volume of a single peroxisome, are smaller in treated cells than in control hepatocytes (Renau-Piqueras *et al.*, 1985a). Stereological analysis of the GA in treated cells shows that in about 30% of the cells this component is composed of vesicles with a mean diameter of 6.29×10^{-2} μm and a mean individual volume of 1.88×10^{-4} μm^3. They represent 14.67% of total GA volume and their number per μm^3 of GA is 78.3 (Renau-Piqueras *et al.*, 1985a).

The significant increase in hepatocyte volume described in chronic alcoholism has been postulated to be due mainly to an accumulation of proteins, fatty acids and water (Israel *et al.*, 1982). This increment in size has been determined to be 1.5 times that of the control cells (Israel *et al.*, 1982), and is similar to the cell volume increase (1.4 times) we have observed in PEA hepatocytes. On the other hand, several studies have clearly demonstrated that chronic and acute exposure to alcohol of adult rats and humans induces changes in both size and shape of hepatocytic mitochondria (Oudea *et al.*, 1973a, b). Qualitative electron microscopy has shown that exposure to ethanol also causes similar changes in hepatocytes of half-term mini-pig fetuses and pups (Romert and Matthiessem, 1983). Our work extends these results with data showing that the variation in the mitochondrial volume density in PEA rats is due to an increase in the size of these organelles and not to an increment in their number. The mechanism for ethanol-induced swelling of mitochondria remains controversial (Gordon, 1984; Rottenberg *et al.*, 1984). On the other hand, an increase in the peroxisome compartment, as described here, has also been reported in both humans and rats consuming ethanol (Porta *et al.*, 1965; Rubin and Lieber, 1967). Further, an increment in ethanol peroxidation in rats chronically exposed to alcohol has been described (Khanna and Israel, 1980). Our results demonstrated a significant variation in the number and size of peroxisomes of PEA hepatocytes, indicating a proliferation of these organelles after ethanol exposure. Peroxidatic oxidation of ethanol by catalase might, therefore, be an alternative mechanism for alcohol clearance in PEA rats as it is in chronic ethanol-fed rats (Lazarow, 1982).

One of the most striking qualitative morphological changes observed in PEA rats is the morphologic disorganization of the GA, suggesting an alteration in its function and will be discussed at length further on in this review.

Hepatocyte phosphatases

To extend the ultrastructural data and to learn whether the ultrastructural changes were correlated with functional alterations, we analyzed, using cytochemistry,

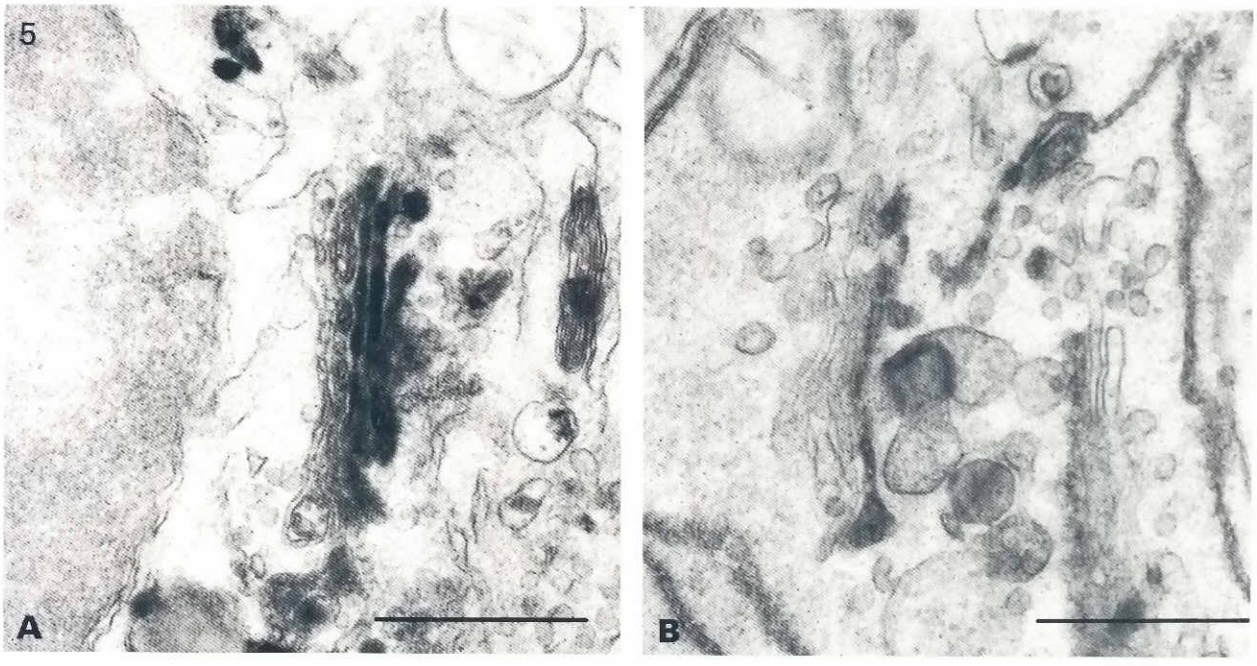


Fig. 5. Micrographs showing the UDPase cytochemical activity in the Golgi apparatus of a control (A) and an alcohol-treated (B) newborn hepatocytes. *The activity appears located in the trans Golgi apparatus, but in the treated cell the deposits of reaction product are less prominent than in the control. Similar cytochemical patterns were found for IDPase and TPPase activities (A: x58,000, Bar= 0.5μm; B: x59,850, Bar= 0.5μm).*

the localization and activity of several phosphatases known to be markers of different cell components (Novikoff, 1976; Renau-Piqueras *et al.*, 1985b) in the hepatocytes of the offspring of control and PEA rats. The cytochemical activity of these phosphatases was studied in frozen sections, using cerium as capturing agent (Robinson and Karnovsky, 1983).

Glucose-6-phosphatase (G6Pase) was detected in all cisternae and in 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. In contrast, in the hepatocytes of PEA rats, deposits were distributed in ER as spots of different size and electron density (Fig. 3). In some treated cells the deposits were less electron-dense than in control cells or were virtually absent.

The activity of two nucleoside-diphosphatases (NDPase) – (uridine diphosphatase (UDPase) and inosine diphosphatase (IDPase) – were detected in all hepatocytes, from both 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 GA.

Alkaline phosphatase (AlkPase) and 5'-nucleotidase, which are markers of the plasma membrane, were found primarily in the bili canaliculi of both control and experimental animals. With 5'-nucleotidase there were also deposits along the microvilli protruding into the space of Disse. The electron-density of deposits 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 treated cells the acid phosphatase (AcPase) activity produced deposition of product in cisternae and vesicles of the trans-portion of the GA and in the tubuli and vesicles throughout the GERL region. An intense deposit was observed in lysosomes. In PEA rat hepatocytes, the reaction product was less heavy than in control cells.

The stereological data obtained for G6Pase, UPDase, IDPase and AcPase are summarized in Fig. 4. They show a significant decrease in relative volume of deposits of reaction product after prenatal alcohol exposure.

These cytochemical results confirm the ultrastructural changes observed in PEA hepatocytes, suggesting that the functions of these cells are altered in PEA rats. In addition to the decreased cytochemical activities of a number of phosphatases, the deposits of reaction product of some of these enzymes were distributed differently in PEA rat cells than in hepatocytes from control rats. G6Pase, for example, belongs to group c of ER enzymes which are usually located in the lumen of this

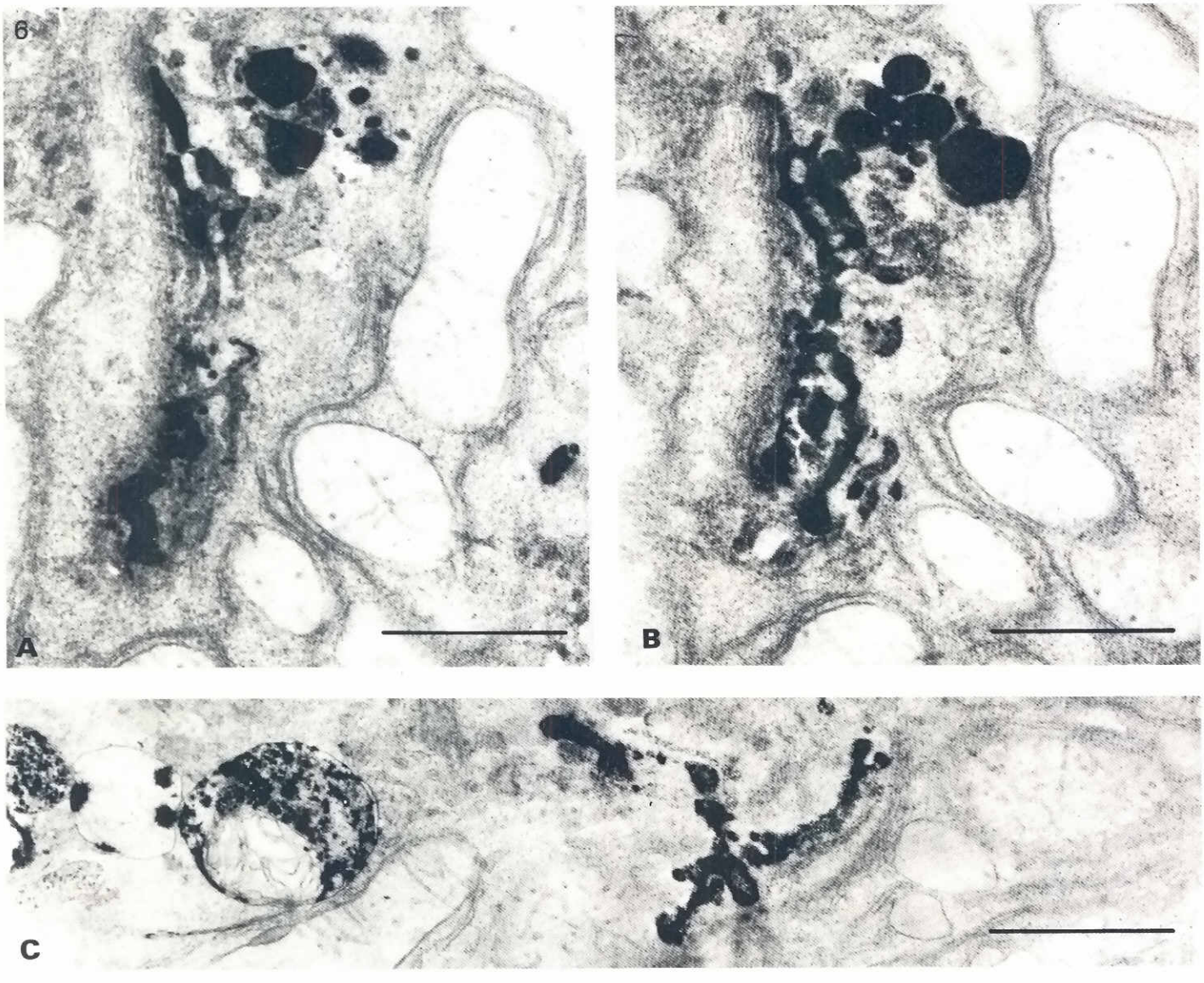


Fig. 6. Micrographs illustrating the cytochemical activity of AcPase in the Golgi apparatus. (A) *and* **(B)** *are two serial sections of a control hepatocyte.* **(C)** *corresponds to an alcohol-treated cell (A,B and C:x55,000; Bar=0.5μm).*

organelle, and is apparently related to the regulation of the conversion of G6 phosphate to glucose. The diminution in its cytochemical activity as well as in its distribution may contribute to the decrease in the plasma glucose level which has been described in fetal and newborn rats exposed prenatally to ethanol (Marquis *et al.*, 1984). Other group c ER enzymes such as UDPase and IDPase also appeared to have decreased after prenatal alcohol treatment. These enzymes are related with glycosylation of proteins in the ER and a decrease in their activity could result in an alteration in this process, as discussed below.

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 *et al.*, 1984). Finally, from the decrease in cytochemical activity of AcPase, ethanol would also appear to affect the function of the lysosomes.

Golgi apparatus

As indicated above, GA is one of the cell components most affected by prenatal alcohol exposure. Since this organelle plays a key role in several cell functions, including glycosylation of proteins and lipids, processing of lysosomal enzymes, and synthesis of plasma membrane moieties (Farquhar and Palade, 1981), we extend-

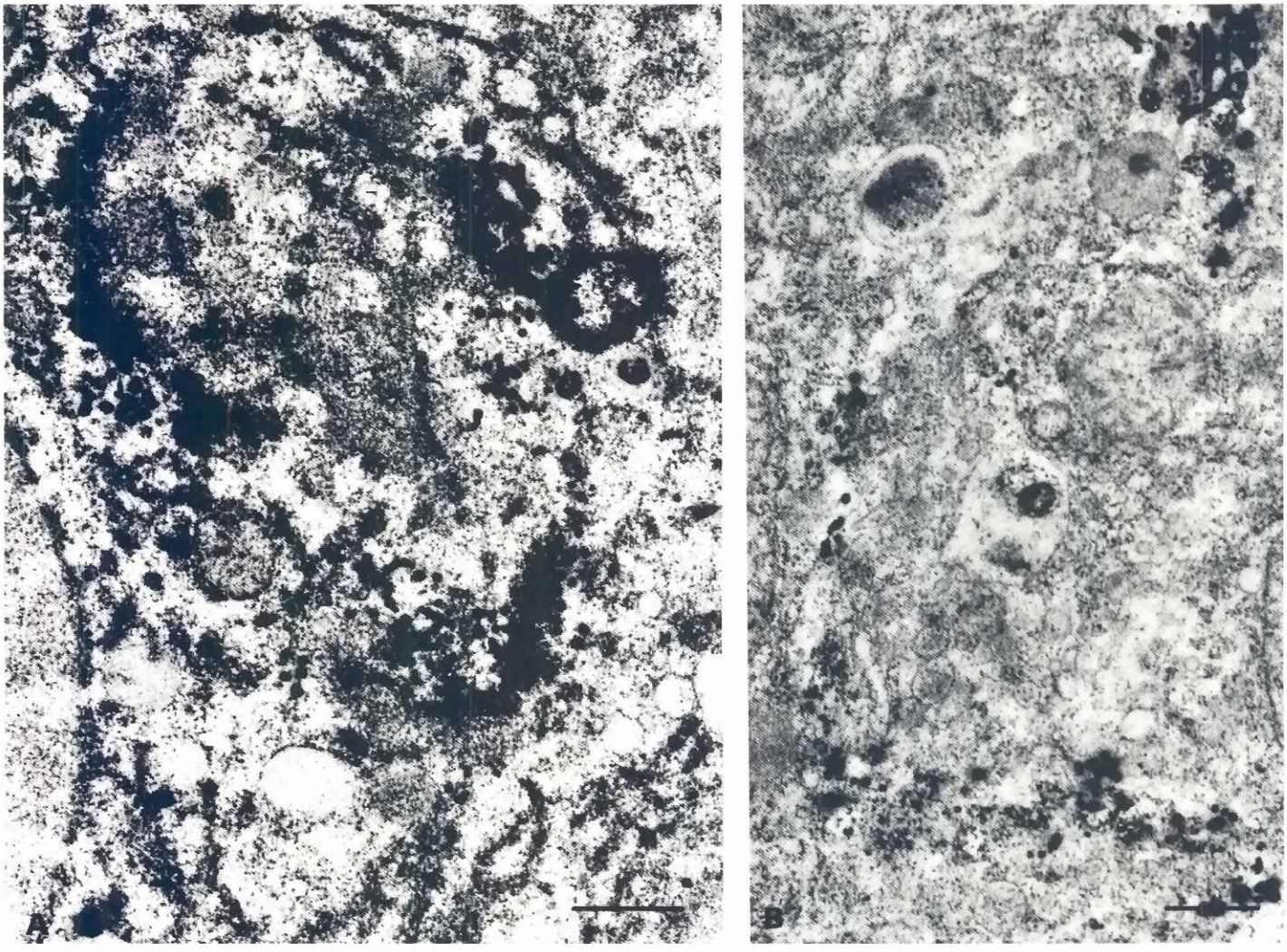


Fig. 7. Ultrastructural localization of reduced osmium compounds in the Golgi apparatus of control (A) and alcohol-treated (B) newborn hepatocytes. *Whereas in control cells all the components of the Golgi apparatus appear strongly labeled, in hepatocytes prenatally exposed to ethanol the deposits are mainly seen in small vesicles located in the cytoplasm adjacent to the Golgi apparatus. (A: x30,500, Bar= 0.5μm; B: x27,000, Bar= 0.5μm).*

ed our previous ultrastructural studies analyzing the effect of prenatal alcohol exposure on the cytochemical characteristics of the GA of newborn rat hepatocytes (Renau-Piqueras *et al.*, 1987a).

As markers of the trans-side GA we used the cytochemical activities of AcPase, thiamine pyrophosphatase (TPPase), IDPase, UDPase and 5'-nucleotidase (Farquhar and Palade, 1981; Farquhar, 1985), whereas the cis-portion was stained by the OsO4-impregnation procedure (Friend, 1969).

In treated rats, as already reported, the GA of about 40% of the hepatocytes lacked cisternae and was composed of small vesicles. The absence of cisternae in these GAs was confirmed by examining consecutive serial sections of several cells. Stereological analysis showed that the volume density (Vv) of the GA was not altered after prenatal alcohol exposure when only morphologically normal GAs were considered in treated rats. On the other hand, since the vesiculated GAs lacked cytochemical activity, only results from GAs with unaltered morphology were considered.

In hepatocytes of both control and treated animals, the reaction product for all the NDPases investigated (TPPase, UDPase, and IDPase) was localized in the last trans-GA cisternae and in some nearby vacuoles. The main difference between treated and control cells was that the deposits of reaction product in the latter cells were more electron-dense than in the former (Fig. 5).

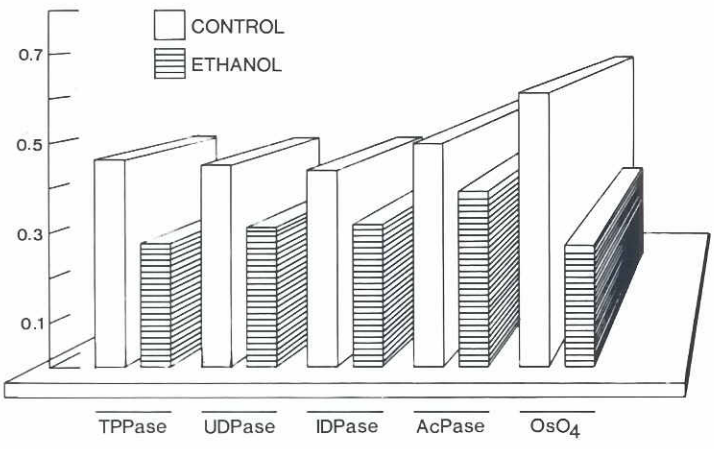


Fig. 8. Stereological analysis of the cytochemical activity of several GA enzymes. *Graph showing the results of the stereological analysis of reaction product deposits in the Golgi apparatus of control and alcohol-treated hepatocytes processed for the cytochemical demonstration of TTPase, UDPase, IDPase and OsO4 impregnation. The data refer to the ratio volume density of deposits/volume density of Golgi apparatus. Significant differences were found in all cases, by Student's t test (P≤0.005).*

When 5'-nucleotidase was investigated, cerium phosphate deposits were consistently found in two to three trans-GA cisternae of control hepatocytes. In hepatocytes from treated animals, cisternae of the GA lacked this activity and deposits were found only in vesicles near the trans-side (GERL). The vesicles were connected by small bridges also showing positive cytochemical activity.

In control cells, the product of AcPase reaction was found in the GERL, in some vacuoles and in many cases in the trans-cisternae of the GA (Fig. 6). In treated hepatocytes the pattern of reaction product was similar to that in control cells, but less marked.

With respect to the OsO4-impregnation technique, most GA cisternae in many hepatocytes from control rats contained the reduced osmium compounds, whereas in other hepatocytes this activity appeared in only one or two cis-cisternae. In most control cells, many small positive vesicles were seen in the cytoplasm adjacent to the GA. After alcohol exposure, the activity in the GA cisternae was considerabily reduced, but the deposits in vesicles usually remained (Fig. 7).

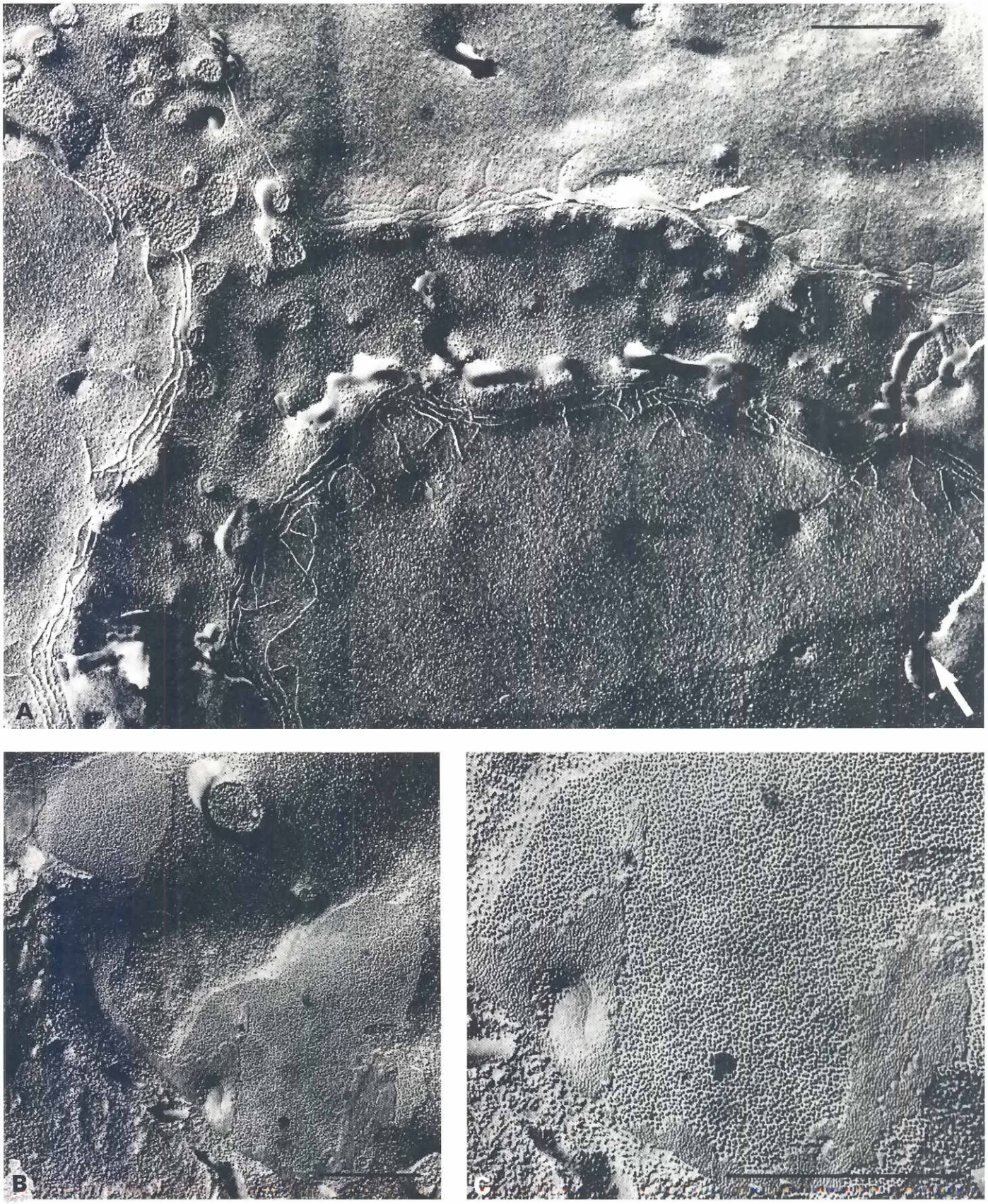
Data obtained from the stereological analysis show a significant decrease in relative volume of deposits of reaction product of TPPase, UDPase, IDPase, and AcPase as well as in the amount of reduced osmium compounds (Fig. 8). It has been demonstrated that acute ethanol intoxication of adult rats induces morphological alterations in the GA of hepatocytes (Ehrenreich *et al.*, 1973; Farquhar *et al.*, 1974). Moreover, it has been reported that acute and chronic ethanol treatment impairs the final steps of hepatic glycoprotein secretion and results in an ethanol-induced retention of secretory glycoproteins (Nani *et al.*, 1978; Baraona and Lieber, 1982). We have found that prenatal exposure to alcohol also induces important morphological alterations in the GA of 40% of the hepatocytes of newborn rats, as mentioned above. This gives rise to the question whether GAs with unaltered morphology retain their normal physiological functions. Our results clearly show that prenatal exposure to alcohol reduces the cytochemical activity of all the phosphatases investigated. Some of these cytochemical alterations could be explained in functional terms. Thus, the decrease in the UDPase activity suggests an accumulation of UDP in the alcohol-treated hepatocytes, which could inhibit glycosylation of secreted proteins (Tartakoff, 1980; Fleischer, 1983). This agrees with previous work with adult liver showing that ethanol decreases the glycosylation process of hepatic proteins (Tuma *et al.*, 1980; Lieber, 1985). The results obtained with the 5'-nucleotidase, which is responsible for the breakdown of UMP formed by UDPase, confirm and extend the above mechanism.

In conclusion, these results indicate that prenatal exposure to alcohol induces alterations in the cytochemical properties of both the cis- and trans-GA of rat hepatocytes, which are probably related to the functional impairment of this organelle. Although the mechanisms leading to these effects are not clear, two interpretations could explain these alcohol-induced alterations: 1) ethanol could directly affect enzyme activities of the GA, and/or 2) ethanol may have a direct or indirect effect on protein synthesis or on such factors as hormones, which could modulate this process. The activity of some of the enzymes analyzed here has been shown to depend on the functional state of the cells (Farquhar, 1985), and indeed we have demonstrated significant alterations in the levels of several hormones in rats prenatally exposed to ethanol (Esquifino *et al.*, 1986).

Protein synthesis and glycosylation

As we have commented, one of the most striking alterations observed in hepatocytes from PEA rats is a disorganization of GA and a decrease in the cytochemical activity of several enzyme markers. Since GA is res-

Fig. 9. Freeze-fracture replicas of control newborn hepatocytes. *In* **(A)** *the appearance of the bile canaliculus and the disposition of tight junctions in both P- and E- fracture faces is shown. No differences between the zonula occludens of controls and treated cells were found.* **(B)** *Gap junctions of a control hepatocyte. In* **(C)** *the distribution of particles in the gap junction can be assessed. The direction of shadowing (arrow) is from the bottom (right corner) of the micrographs to the top (left corner). (A: x42,600, Bar= 0.5μm; B: x37,600, Bar= 0.5μm; C: x88,000, Bar= 0.5μm).*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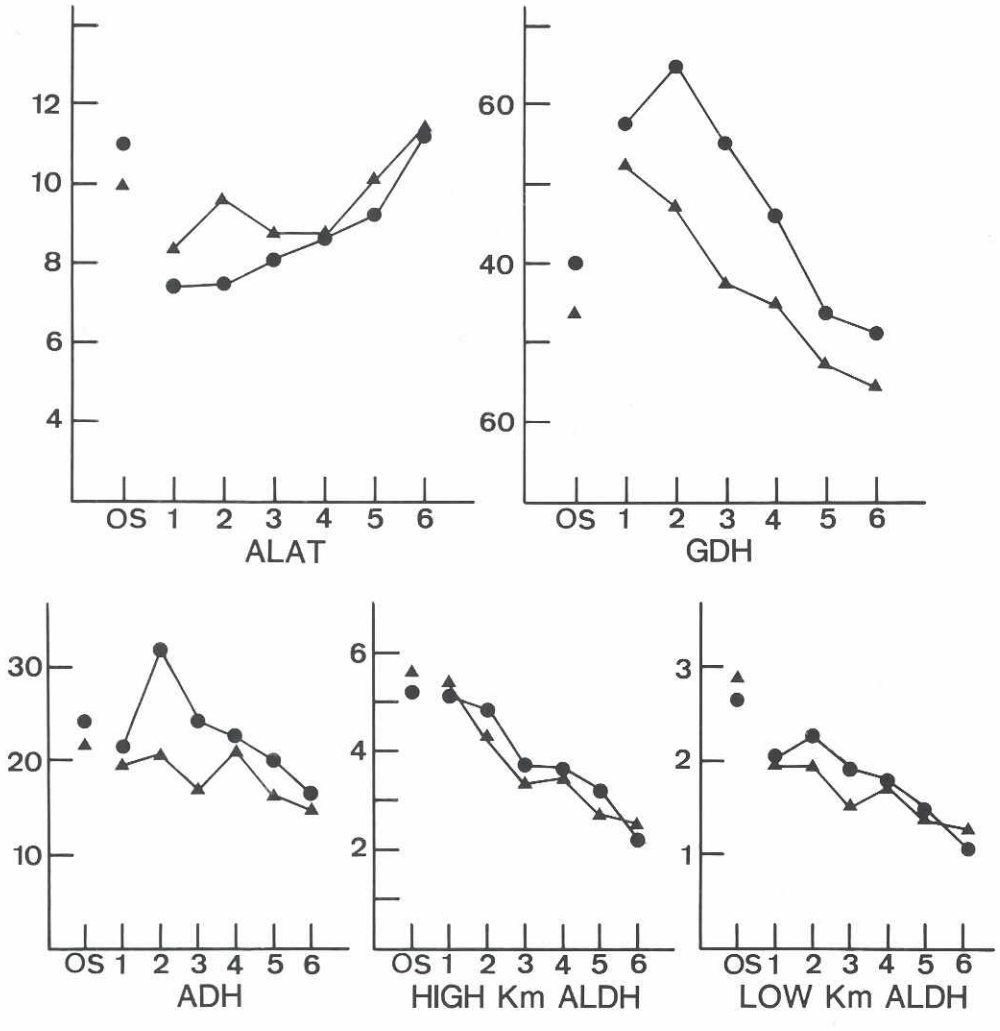

Fig. 10. Activity of zone marker and alcohol-metabolizing enzymes in hepatocyte subpopulations. *Alanine aminotransferase (ALAT), glutamate dehydrogenase (GDH), alcohol dehydrogenase (ADH), and aldehyde dehydrogenase (high and low Km, ALDH) activities of the original hepatocyte suspension (OS) and hepatocyte subpopulations (1-6, abscissas) isolated and subfractionated on Percoll density gradients, from control (●) and pre+postnatally ethanol-exposed (▲) 12-day-old rats. Values are means from 5-6 different experiments. Enzyme activities (ordinates) are expressed as nmoles/min/mg protein.*

ponsible for glycosylation of hepatic secretory proteins we have extended these studies analyzing the possible alcohol-induced changes in this process. Two systems were used: a) incorporation of methionine in the liver tissue and b) incubation of isolated hepatocytes from control and newborn PEA rats with ^{3}H-leucine, ^{3}H-mannose or ^{3}H-galactose for a 5-min pulse period, and chases with unlabeled medium for 0-3 h and then assessed biochemically or by radioautography (Whur *et al.*, 1969).

Mannose was used to test for the addition of sugars to the oligosaccharide "core" of glycoproteins in the RER, whereas galactose was used to study the glycosylation in the trans-portion of GA (Dunphy and Rothman, 1985).

Incubation of liver homogenate with methionine showed that prenatal exposure to ethanol reduced protein synthesis by about 30%. Moreover, these results were confirmed when ^{3}H-leucine was incorporated into isolated hepatocytes (Renau-Piqueras *et al.*, 1989). Analysis of radioautographic data indicates in all cases that there was a significant retention of label in the GA after 1-3 h chase periods, suggesting an important delay in the glycosylation or secretion process. In contrast, plasma membrane of treated cells appeared less labeled than that of control hepatocytes. In fact (Tuma *et al.*, 1980) reported that ethanol decreased glycoprotein secretion in adult rat liver slices.

Although conflicting results have been published on the effect of acute or chronic ethanol consumption on protein synthesis in adult liver (Zern *et al.*, 1982), our results agree with reports indicating that prolonged maternal ethanol consumption resulted in a significant inhibition of the rate of ^{14}C-leucine incorporation into ribosomes from fetal and neonatal liver (Rawat, 1976). The mechanism of this inhibitory effect is unclear (Zern *et al.*, 1982). In adult liver it has been postulated that the

effect of ethanol on protein synthesis is mediated through changes in the hepatic NADH/NAD^+ ratio through alcohol dehydrogenase (Zern *et al.,* 1982). However, in fetal liver the activity of this enzyme is very low (Sanchis and Guerri, 1985), making this mechanism unlikely in this situation. Other mechanisms which have been proposed are, a) ethanol alters energy levels which may also affect protein synthesis, b) ethanol could indirectly alter protein synthesis through an effect on hormones, or c) the reduction in protein synthesis may be secondary to effects on protein secretion, as we have observed (Zern *et al.,* 1982).

In addition to the inhibitory effect of ethanol on protein synthesis in the fetal hepatocyte, we have found, as already mentioned, an accumulation of mannose and galactose labeled glycoproteins in the GA. However, plasma membranes of alcohol-exposed hepatocytes showed a decrease in the labeled glycoproteins after the chase periods (Renau-Piqueras *et al.,* 1989). This is in agreement with previous findings that acute ethanol exposure produces an accumulation of VLDL in the GA of hepatocytes (Ehrenreich *et al.,* 1973), suggesting that both prenatal and acute exposure to ethanol alter the transport of glycoprotein between the GA and the plasma membrane, and indeed recent work (Tuma and Sorrell, 1988) confirms this interpretation. It has been suggested that this alteration in glycoprotein transport is due to a disorganization of microtubule structure by ethanol or acetaldehyde (French *et al.,* 1987). The integrity of microtubule organization appears to be necessary for the intracellular transport and secretion of proteins as well as for maintaining the GA structure (Thyberg and Moskalewski, 1985). This mechanism however remains under discussion (Virtanen and Vartio, 1986; Goldstein, 1987).

Plasma membrane and cell junctions

It is recognized that alcohol alters the physico-chemical properties of plasma membrane lipids, expanding them and increasing the membrane fluidity (Virtanen and Vartio, 1986; Goldstein, 1987). Prolonged exposure to ethanol seems to result in compensatory changes in the membrane to restore the original state of fluidity. These changes may involve increases in the longer-chain-length fatty acids and in cholesterol (Goldstein, 1987). The lipid composition and thus physical properties of membranes have a direct influence on membrane proteins (Guerri and Grisolía, 1983). Moreover, we have demonstrated that prenatal exposure to ethanol produces a decrease in the cytochemical activity of 5'-nucleotidase and alkaline phosphatase, two enzymes located in the plasma membrane. All these studies indicate that alcohol induces changes in the plasma membrane of newborn rat hepatocytes.

This gives rise to the question whether specialized areas of the hepatocyte plasma membrane might be affected by alcohol. Cell junctions, in particular tight and gap junctions (TJ and GJ, respectively) are plasma membrane specializations which play important roles in hepatocyte physiology. Using freeze-fracture, we have investigated the fine structure of the lateral (contiguous) plasma membrane of hepatocytes from newborn control and PEA rats, with special emphasis on TJ and GJ (Fig. 9) (Renau-Piqueras *et al.,* 1987b). Whereas in PEA rats, TJ were similar in morphology to those of controls, the GJ of these animals showed qualitative alterations which included the presence of particle-free areas within some of them, an apparent increase in their size and, in some cases, a very irregular profile. Quantitative analyses demonstrate that prenatal exposure to ethanol is associated with a significant reduction in the intramembranous particle (IMP) density of both P- and E-faces of lateral plasma membranes. No differences were found in the diameters of IMP from membranes of control and treated-animals, nor did alcohol exposure alter the depth, length or the number of strands of TJ. The mean size of individual GJ, on the other hand, was increased three-fold, and the total area occupied by these junctions on the alcohol-exposed membrane was also found to be significantly increased.

In contrast, the total and relative numbers of GJ in these cells decreased. Further, the interparticle distance in GJ measured directly on the micrographs was 8.95 and 8.87 nm, respectively, for control versus treated cells. The increase in the size of GJ, therefore, seems to be due to an increment in the number of GJ particles, which is supported by the fact that the mean interparticle distance, the particle size and particle density were similar in both control and alcohol-treated animals.

This effect could be explained by several mechanisms: a) ethanol could affect the fluidity of plasma membrane (Goldstein, 1987) inducing the addition of preexisting IMP to the GJ. This could be related, in part, to the diminution of IMP density in the lateral plasma membranes of treated cells as well as to the decreased number of GJ, suggesting a redistribution of gap IMP; b) ethanol may have a direct or indirect effect on glycoprotein synthesis, transport and plasma membrane assembly, as discussed above, or on factors such as hormones which might modulate the size of GJ.

The reduction in the IMP density confirms previous results from our laboratory on presynaptic membranes from chronic alcoholic rats (Renau-Piqueras *et al.,* 1987c). This effect could be due to partial translocation of IMP from lateral membranes to GJ, as stated above. Changes in the physico-chemical properties of membrane lipids might also affect the conformation of some IMP or cause a disassembling and dispersion of small IMP subunits. In either case, the resulting particles would not be seen in freeze-fracture replicas.

Finally, although it is difficult to explain these findings in physiological terms from our still limited knowledge of GJ, it is clear that ethanol has a specific effect, whether direct or indirect, on these structures and probably on their functions.

Hepatocyte subpopulation

Various studies have demonstrated ultrastructural, biochemical and functional heterogeneity of hepatocytes within the liver acinus (Gumucio and Miller, 1982). Thus, the liver acinus has been divided into three functional areas (Rappaport *et al.*, 1954): zone 1 or periportal (PP), zone 2 or intermediate, and zone 3 or perivenous (PV). Although some reports indicate that early alterations by ethanol occur in the PV region of adult liver, only a few studies have considered this heterogeneity. While hepatocyte subpopulations have been clearly identified *in situ* using quantitative electron microscopy (Loud, 1968), there are technical problems in showing biochemical and functional heterogeneity of the hepatocytes in the tissue. Therefore, methods have been developed to study this heterogeneity in adult animals, including isolation and subfractionation of hepatocytes on density gradients after collagenase perfusion of liver (Lindros *et al.*, 1986). Due to the difficulty in perfusing small livers, new procedures have been developed to purify rat hepatocytes during development (Radford and Bhathal, 1985).

Recently we have begun to assess the effect of pre- and postnatal exposure to ethanol on rat hepatocyte subpopulations. Since metabolic zonation of the liver acinus does not occur until the 2nd week of life (Katz *et al.*, 1976), we have isolated and subfractionated hepatocytes from 12-day-old control and alcohol-exposed rats, which were analyzed using qualitative and quantitative biochemical and ultrastructural procedures (Guerri *et al.*, 1987; Sancho-Tello *et al.*, 1987).

The isolated hepatocytes, after separation by discontinuous Percoll density gradients, were distributed in all experiments in a similar seven-layer pattern. The first, at the top of the tube, was composed of non-sedimented material, mainly cell debris, dead cells and small clumps of hepatocytes. The activity of two enzymes was measured as markers to identify the PV and PP origen of cells in fractions. The specific activity of alanine aminotransferase (ALAT), considered a PP marker enzyme (Gumucio and Miller, 1982) increased from F_2 to F_6 (Fig. 10). In contrast, glutamate dehydrogenase (GDH) used as a PV marker enzyme (Gumucio and Miller, 1982), showed the highest activity in F_2 (Fig. 10). The same distribution pattern of ALAT and GDH activities was obtained for cells from alcohol-treated rats, although a lower specific activity for GDH was found in all fractions (Fig. 10). The distribution of alcohol-metabolizing enzymes, alcohol dehydrogenase (ADH) and high and low km aldehyde dehydrogenase (ALDH), was similar to that of GDH. In alcohol-treated hepatocytes the distribution was similar, but with a decrease in specific activity of ADH in all fractions and some small variations in that of ALDH (Fig. 10).

Stereologic data obtained in the analyses of the control hepatocytes of the different fractions were similar to those described by Loud (1986) for PP and PV cells in adult animals. Thus, a progressive increase in the mean absolute cell volume was observed from F_2 to F_6 fractions, with the largest cells in the F_6 fraction. This distribution was confirmed by cell sorting analysis. With respect to the cytoplasmic components of control hepatocytes, it was found that the volume density of mitochondria, GA, glycogen and RER was higher in F_6 than in F_2 hepatocytes. Also, mitochondria of F_6 hepatocytes were twice as large as those of F_2 cells, although the total number of these organelles per cell was similar in both cell populations. The numerical density of mitochondria and lysosomes and the volume density of SER and lysosomes were greater in F_2 than in F_6 hepatocytes.

In F_3, F_4, F_5 and F_6 fractions of alcohol-exposed hepatocytes, there was a variable proportion of cells whose morphology was clearly different from that of the rest of the cell population. The cytoplasm of these cells, which we have called "dense cells", was almost completely filled by mitochondria, free ribosomes and RER. The stereological analysis of subfractionated hepatocytes derived from alcoholic rats was carried out on the two morphologically different types of cells - "dense cells" and the normal (non-dense) cells. The proportion of dense cells in the different fractions was: 0%(F_2), 45.5%(F_3), 36%(F_4), 28%(F_5) and 9%(F_6). These cells were smaller than normal cells. Although the variation in the different stereological parameters in all fractions of alcohol-treated hepatocytes was similar to that for control cells, there were some differences between the two groups. Thus, the volume density of mitochondria, RER and SER, the numerical density of mitochondria and the total number of mitochondria per cell were greater in all fractions in treated cells vs. controls. In contrast the mean volume of a single mitochondria and the volume density of the GA and glycogen appeared greater in all the fractions of control hepatocytes. The results regarding the mitochondrial size are due to the contribution of "dense cells" which show small mitochondria. Also, exposure to ethanol alters the size of F_2 and F_6 hepatocytes. Mitochondria and GA appeared to be more altered in F_2 cells, whereas glycogen, RER and probably SER were more altered in F_6 cells.

The analysis of the results presented here indicate there are significant biochemical and ultrastructural differences between F_2 and F_6 hepatocytes, and suggest that these cells do indeed constitute two different populations of hepatocytes. These subpopulations could correspond to PV and PP hepatocytes, respectively.

The biochemical data show that the activities of both GDH and ALAT marker enzymes were altered after alcohol exposure, mainly in the PV hepatocytes; whereas ALAT activity showed an increase, GDH activity diminished significantly. The alteration in ALAT activity may reflect an adaptative response to PV hypoxia due to an increase in oxygen uptake. On the other hand, the decrease in GDH activity may reflect mitochondrial damage, which might be enhanced in the PV zone as seen in the stereological results. ADH activity was also decreased in the PV hepatocytes.

The stereological results have indicated, therefore, that pre- and postnatal exposure to ethanol could have a selective effect on specific components of the liver cell depending on the acinar zone, and that the PV hepatocytes appear to be more altered under these conditions.

KEY WORDS: *hepatocytes, prenatal exposure, ethanol, rat, electron microscopy*

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