Int. J. Dev. Biol. 33: 239-244 (1989)



*Original Article*

# Effect of prenatal exposure to alcohol on membrane-bound enzymes during astrocyte development *in vivo* and in primary culture

CONSUELO GUERRI*, AMPARO MARQUÉS, MARÍA SANCHO-TELLO and JAIME RENAU-PIQUERAS

*Instituto de Investigaciones Citológicas (Centro Asociado del CSIC), Valencia, Spain*

ABSTRACT. In the present work we have analyzed the effect of prenatal ethanol exposure on the activity of several glial marker and functional enzymes during the development of astrocytes isolated from rat brain as well as in primary culture. The activity of marker enzymes glutamine synthetase and butylcholinesterase showed no differences between isolated astrocytes from 15 and 70 day old control rats. However, the activity of the membrane-bound enzymes (Na+K)ATPase and 5'-nucleotidase was higher in astrocytes from 70 day old control rats than in those from 15 day old animals. Although the pattern found in astrocytes from alcohol-exposed rats was similar to that of controls, the levels of activity of the enzymes were lower in alcoholic than in control animals. When control astrocytes in primary culture were used, the activity of (Na+K)ATPase and 5'-nucleotidase increased throughout the entire culture period. In contrast, the maximal activity of glutamine synthetase was found at 7 days of culture. Ethanol also induced a decrease in the activity of all enzymes, which was more evident at the end of the culture period. These results indicate that the activity of the enzyme markers analyzed increased mainly during the first weeks of life and remained constant after this period. By contrast, the membrane-bound enzymes studied showed a progressive increase with age. In conclusion, since these astrocyte enzymes are important in the regulation of several neuronal functions through the control of the composition of extracellular fluid, the effect of ethanol on their activities could explain some of the neuronal alterations reported in children and animals exposed to ethanol during development.

KEY WORDS: *ethanol, astrocytes, primary culture, (Na+K)ATPase, glutamine synthetase, 5'-nucleotidase*

## Introduction

Central nervous sytem (CNS) dysfunction in children is one of the most striking consequences of maternal alcohol consumption during pregnancy and can occur independently of the craneofacial malformations which are characteristic of the fetal alcohol syndrom (FAS) (Lemoine *et al.*, 1968; Streissguth and Martin, 1983). A variety of CNS anomalies have also been observed in experimental animals following *in utero* alcohol exposure (Streissguth and Martin, 1983; Sanchis *et al.*, 1984). Thus, it has been demonstrated both in children with FAS and in experimental animals that ethanol alters glial migration as well as neuronal proliferation, migration, formation of processes and production of neurotrophic factors (Clarren *et al.*, 1978; Dow and Riopelle, 1985; Miller, 1986). In spite of these data, the pathogenesis of the CNS dysfunction remains unknown. However, it is possible that, since astrocytes play an important role in maintaining neuronal functions, the effects observed in neurons could be the consequence of a primary effect of ethanol on these cells during development. We have recently reported that ethanol alters not only the maturation of astrocytes in primary culture, but also the organization of their cytoskeleton (Renau-Piqueras *et al.*, 1989).

Primary culture of astrocytes provides a useful model system for studying the molecular aspects of the differentiation and function of these cells (Hertz *et al.*, 1982; Hansson, 1986). At the same time, this system could provide a good tool for analyzing the direct effect of ethanol avoiding other secondary related factors (Mandel *et al.*, 1980). It must be emphasized, however, that a correlation between *in vitro* and *in vivo* results in these types of studies should be established.

On the other hand, it is known that ethanol interacts with the plasma membrane, modifying the properties of several proteins of this cell component (Guerri and Grisolía, 1983). Since the astrocyte plasma membrane plays an important role in the control of the composition of extracellular fluid in brain (Hertz, 1982), the aim of the present work has been to study the effect of ethanol exposure on the activity of several membrane-bound enzymes of astrocytes during their development both *in vivo* and *in vitro*. The first approach was achieved by using Ficoll gradients to isolate astrocytes from rats pre+postnatally exposed to ethanol; the second was accomplished using primary cultures of astrocytes derived from brain cortex of control rats and those prenatally exposed to alcohol.

## Results

### *Ficoll isolated astrocytes*

Cell suspensions from brains of 15 and 70 day old rats

* Address for reprints: Instituto de Investigaciones Citológicas (Centro Asociado del CSIC), Amadeo de Saboya, 4, 46010 Valencia, Spain

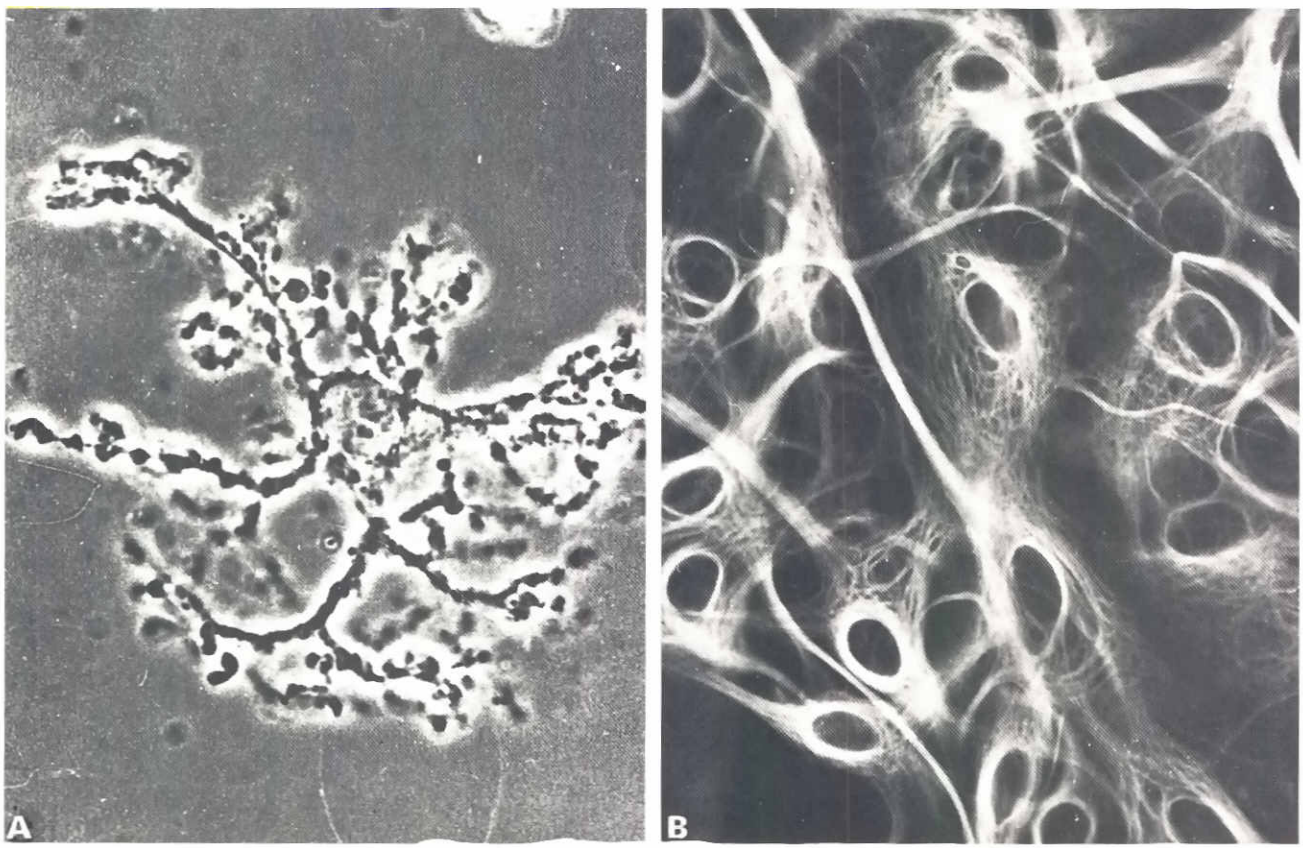


**Fig. 1.** *(A) Phase contrast micrograph of a glial cell of astrocyte-rich fraction. (B) Indirect immunofluorescence of 21 day old cultured astrocytes using an anti-GFAP antibody. Binding of IgG was revealed using FITC-conjugated goat anti-mouse IgG.*

were separated by gradient centrifugation on discontinuous Ficoll gradients, and three astrocyte-rich fractions were obtained. These fractions were similar to the fractions $P_1/22$, $P_2/22$ and $P_3/22$ described by Farooq and Norton (1978). The appearance of astrocytes in these fractions corresponded to a mixture of fibrous and protoplasmic types. In Fig. 1a a typical fibrous astrocyte is illustrated. Using phase-contrast microscopy and the criteria of Farooq and Norton (1978), we determined that the astrocyte purity in these fractions was ca. 73%. The major contaminants were certain free processes, capillary fragments and debris. Neuronal bodies were scarce.

In Table 1, the activities of the different enzymes measured in the isolated astrocytes are summarized. Both glial enzyme markers, glutamine synthetase (Martínez-Hernández *et al.*, 1977) and butylcholinesterase (Giacobini, 1964) were essentially the same in astrocytes derived from 15 and 70 day old control rats. The same pattern was also found in astrocytes from alcoholic rats. But while butylcholinesterase activity showed a significant decrease after ethanol exposure in both periods, only 15 day old rats showed significant differences for glutamine synthetase activity.

In contrast to the glial enzyme markers, both membrane-bound enzymes, (Na+K)ATPase and 5'-nucleotidase, showed an important increment in their activity from 15 to 70 days, indicating that these activities increased with the age of the rat. When these enzymes were analyzed in astrocytes from 15 and 70 day old alcoholic rats, a significant decrease in the activity of (Na+K)ATPase was found with respect to control cells.

### *Primary culture of astrocytes*

Astrocytes in primary culture grew rapidly for 7-10 days, after which the cell number increased slowly, corresponding to a typical logarithmic growth pattern. The purity of cultures was determined using a monoclonal anti-GFAP antibody. The GFAP positive cells ranged from 85-90%, indicating that almost all the cells in the cultures were astrocytes (Fig. 1B).

The activity of the enzyme marker GS increased rapidly during the first week of culture or proliferation period, and then decreased until the end of the culture (Fig 2A). Alcohol decreased this activity as shown in Fig. 2A. Although the curve pattern was similar to that of controls, when

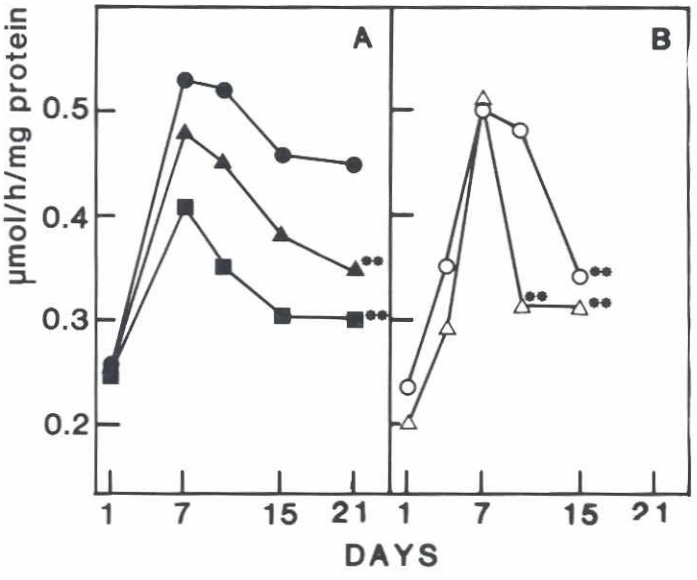


**Fig. 2.** *Activity of the enzyme marker glutamine synthetase in astrocytes at different periods of primary culture. Cells were obtained from fetuses derived from control (A) and alcoholic (B) rats. Control cells (●) were cultured in presence of 25 (▲) or 100mM (■) ethanol. Prenatally alcohol-exposed cells were cultured in absence (O) or presence (Δ) of ethanol (25 mM). Each point represents the average of 3-4 different cultures per duplicate. Significant differences are expressed to respect control values (*p < 0.05, **p < 0.01).*

astrocytes were from fetuses obtained from alcoholic rats, there was an important decrease in enzymatic activity after 15 days of culture (Fig. 2B).

To evaluate the enzymatic activity in plasma membrane during astrocyte development, as well as the effect of ethanol, we measured the activity of (Na+K)ATPase and 5'-nucleotidase. As shown in Figs. 3A and 4A, the activity of both enzymes in control cultures showed a slow increase during the proliferation period (1-7 days) and then a striking increase during the period of differentiation (7-28 days). When ethanol was added to the culture medium (25 and 100 mM), there was a decrease in both enzyme activities. This effect was also found to be dose-dependent. Figs. 2-4 show that astrocytes derived from fetuses prenatally exposed to alcohol and cultured in the absence of this substance had decreased activities of the above-mentioned enzymes when compared with control cells. This decrease was even more evident when these cells, obtained from alcoholic fetuses, were incubated in medium containing 25 mM ethanol.

## Discussion

In this work we have used astrocytes in primary culture as a model to study certain aspects of the development of these cells. The results obtained with this model have been compared with those obtained from glial cells *in vivo*. In addition, the effect of ethanol has also been evaluated.

The analysis of the two enzymes used as glial enzyme markers, GS and BuCh (Giacobini, 1964; Martínez-Hernández et al., 1977), indicate that both enzymes reached maximum activity in vivo after 15 days since no significant differences were found compared with astrocytes isolated from 70 day old rats. These results for GS agree with data obtained with astrocytes in primary culture, indicating that this cytoplasmic enzyme activity increased during the proliferation period showing a maximum activity at 7-20 days. Although there was then a small decrease, the activity remained constant until the end of the culture period.

By contrast, the two membrane-bound enzymes measured ((Na+K)ATPase and 5'-nucleotidase) showed in both isolated and cultured cells a progressive increase mainly during glial differentiation. The results concerning (Na+K)ATPase agree with previous results in the tissue (Guerri *et al.*, 1984) and with the concept that the activity of this enzyme in astrocytes increases as a response to enhanced potassium concentrations which occur during glial cell development (Hertz, 1982). This potassium effect on (Na+K)ATPase activity is consistent with the potassium-induced stimulation of oxygen uptake which might also be the result of a stimulation of other energy requiring processes in astrocytes (Hertz, 1982).

We have also demonstrated that ethanol exposure alters the activity of all the enzymes measured. However, since the enzymes studied have different functions and cell localizations, the specific alterations induced by ethanol on each enzyme activity could reflect different aspects of ethanol-induced toxicity on astrocytes.

GS is an enzyme implicated in ammonia detoxification

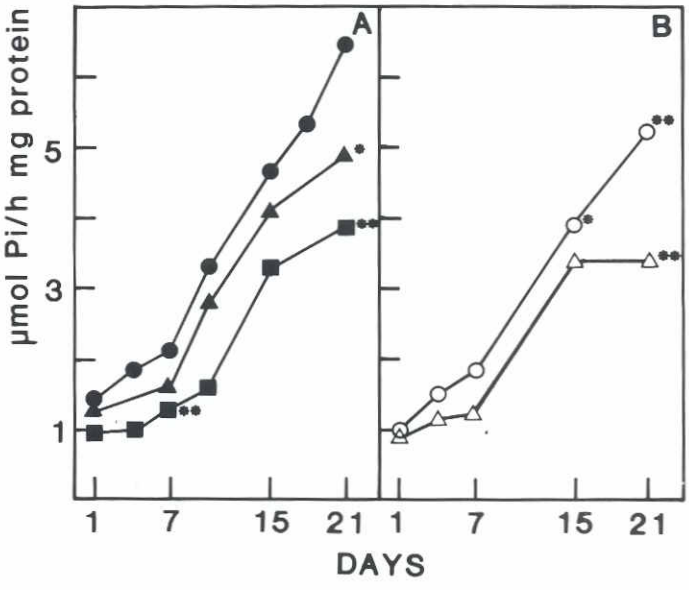


**Fig. 3.** *Activity of the membrane-bound enzyme (Na+K)ATPase in astrocytes at different periods of primary culture. Conditions and symbols as in Fig. 2.*

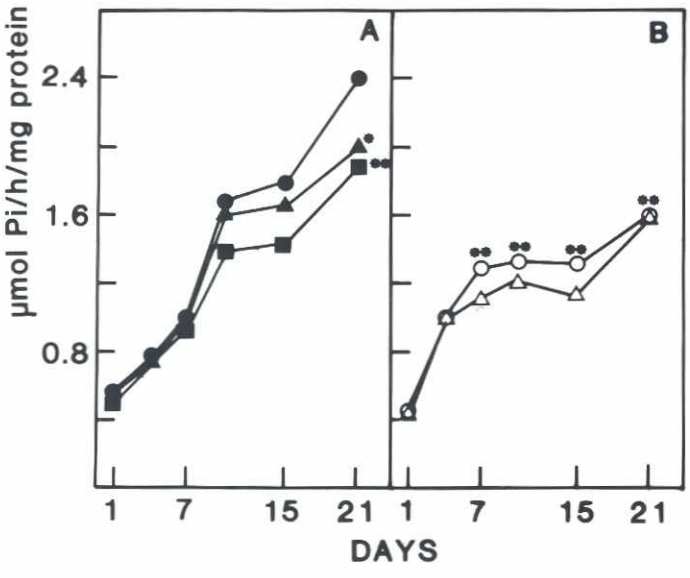


**Fig. 4.** *Graph showing the activity of the enzyme 5'-nucleotidase in cultured astrocytes. Conditions and symbols as in Fig. 2.*

as well as in the metabolism of the neurotransmitters α-amino-butyric acid (GABA) and glutamic acid. Thus, glial cells appear to play an important role in the intercellular exchanges of glutamate and glutamine (Patel, 1982). A decrease in the activity of GS induced by ethanol, which has also been reported by Davis and Vernadakis (1984), could alter the glutamine cycle between glia and nerve endings, thereby changing the regulation of glutamate homeostasis.

It has been demonstrated that in isolated cells the activity of (Na+K)ATPase is higher in astrocytes than in neurons (Hertz, 1965), due to the role of the glia in the uptake and clearance of extracellular potassium after a volley at the nerve ending. The effect of ethanol on the decrease of activity has already been reported *in vivo* after *in utero* alcohol exposure (Guerri *et al.*, 1984). However, it has been observed that chronic ethanol consumption increases the activity of this enzyme in adult animals (Guerri and Grisolía, 1983; Ledig *et al.*, 1985). Although this enzyme is inhibited by alcohol *in vitro*, in previous work we have suggested that the decrease in its activity *in vivo* is due to an alteration in the lipid membrane composition (Guerri and Grisolía, 1983; Marqués and Guerri, 1988). Furthermore, since this protein seems to be located mainly in the processes, a delay in the formation of these structures in astrocytes exposed to ethanol, as reported by Renau-Piqueras *et al.* (1989), could also produce a decrease in the level of this activity.

5'-Nucleotidase is an enzyme that is synthesized in the rough endoplasmic reticulum, glycosylated in the Golgi apparatus and then transported to the plasma membrane. In the CNS this enzyme is of particular interest due to its highly specific regional distribution (Schubert *et al.*, 1979). At cellular levels it has been associated with glial plasma membranes, synaptic structures or intracellular neuronal compartments (Heymann *et al.*, 1984). This enzyme catalyzes the final step in the production of adenosine, which has a potent modulatory effect on synaptic transmission (Sulakhe and Phillis, 1975). The decrease in the 5'-nucleotidase activity induced by ethanol could be explained by several mechanisms: 1) one possibility is that ethanol could decrease the glycosylation and/or transport of this glycoprotein to the plasma membrane, as occurs in the liver; or 2) ethanol could change the composition of plasma membranes, altering the activity of this enzyme. In fact, it is known that ethanol modifies the physico-chemical properties of plasma membranes (Guerri and Grisolía, 1983).

Finally, since astrocytes play an important role in maintaining neuronal development and function, the results described here could be related with the neuronal alterations observed in children and animals prenatally exposed to ethanol (Clarren *et al.*, 1978; Miller, 1986) and could be

TABLE 1

**SPECIFIC ACTIVITIES OF SEVERAL ENZYMES IN ASTROCYTE-RICH FRACTIONS**

| | 15 day old rats | | 70 day old rats | |
|---|---|---|---|---|
| Activity | **Control** | **Ethanol** | **Control** | **Ethanol** |
| Butylcholinesterase | 0.16 ± 0.01 | 0.11 ± 0.01* | 0.16 ± 0.05 | 0.10 ± 0.02* |
| Glutamine synthetase | 1.98 ± 0.54 | 1.71 ± 0.64* | 1.82 ± 0.25 | 1.67 ± 0.22 |
| (Na+K)ATPase | 6.48 ± 1.9 | 2.68 ± 1.28** | 11.43 ± 0.89 | 6.27 ± 0.13* |
| 5'-nucleotidase | 0.55 ± 0.02 | 0.33 ± 0.05* | 0.86 ± 0.12 | 0.80 ± 0.13 |

All the activities are expressed as µmols/h/mg prot. except that of butylcholinesterase, which is expressed as Δ412/min/mg prot. Each value represents the average of 3-4 different experiments. Significant differences *$P \leq 0.05$, **$P \leq 0.01$.

one of the mechanisms underlying mental retardation and motor dysfunctions, which are characteristic of the fetal alcohol syndrome.

## Materials and Methods

### *Animal treatment*

Female Wistar rats weighing 150-200 g were used. All rats were maintained under controlled conditions of light (12/12), temperature (23° C) and humidity (60%). Rats were fed the Lieber-DeCarli (1976) liquid diet containing either 5% (w/v) ethanol or an isocalorically balanced diet with maltose-dextrin for pair-fed controls. Female rats were maintained on the ethanol or control liquid diet for a minimum of 30 days prior to exposure to male rats. After mating (as determined by vaginal smears), the rats were maintained during gestation in separate cages on the ethanol or control diet.

After birth, the pups remained with their mothers until day 25, after which all rats were put on laboratory rat chow and tap water *ad libitum*.

### *Isolation of astrocytes*

The isolation of astrocyte-enriched fractions from rat brain tissue was carried out using the method of Farooq and Norton (1978) with some modifications. Briefly, brains without cerebellum were sectioned in several slices and incubated in medium containing trypsin. The tissue was then dissociated and the resultant cell suspension was centrifuged. The pellet was resuspended in medium with 7% Ficoll and centrifuged to obtain the neuron-enriched pellet. The supernatant was centrifuged again to obtain a pellet containing neurons and astrocytes. The last supernatant was diluted in medium and centrifuged again to obtain the astrocyte-enriched pellet. Each pellet was then suspended in 7% Ficoll and layered on a discontinuous Ficoll gradient. The tubes were centrifuged in a Spinco SW-27 rotor at 8500 g for 5 min. The layers at each interface were removed and cell composition was determined by microscopy. For biochemical analysis the different astrocyte-rich fractions were pooled, washed with 10 mM phosphate buffer (pH 6.0), resuspended in 10 mM Tris-HCl (pH 7.4) and stored at -70° C.

### *Primary astrocyte cultures*

Primary monolayer cultures of astrocytes were prepared from the cerebral hemispheres of 21 day old rat fetuses (Renau-Piqueras *et al.*, 1989). The fetuses were decapitated and their brains removed and placed in plastic petri dishes containing Dulbecco's modified Eagle's medium. The cerebral hemispheres were dissected free of meninges and dissociated by gentle aspiration through a 10 ml pipette. The resulting cell suspension was vortexed at maximum speed for 1 min and passed through a sterile nylon sieve (90 μ pore size). Cells ($1.5 \times 10^6$) were plated on 35 mm plastic dishes and maintained in the same medium supplemented with 20% fetal calf serum, 30 mM glucose and 1% antibiotics. The cultures were incubated at 37° C in a humidified 5% $CO_2$-95% air atmosphere. After one week, serum and glucose were reduced to 10% and 5.5 mM, respectively. Medium was changed every 3 days. Some cultures contained 25 or 100 mM ethanol in the medium, which was added at the beginning of the culture. The ethanol concentration in the medium was checked every day.

### *Indirect immunofluorescence staining*

The presence of glial fibrillary acidic protein (GFAP) was determined to evaluate the purity of cultures, as previously described (Renau-Piqueras *et al.*, 1989).

### *Enzymatic analyses*

Protein content and enzymatic activities were measured in Ficoll isolated astrocytes as well as in astrocytes in primary culture. For astrocytes in primary culture, monolayers at different culture times were washed with PBS, resuspended in 10 mM Tris-HCl buffer (pH 7.0) and stored at -70° C overnight. Cells were later thawed, homogenized in the same buffer and used for enzymatic analysis. As glial enzyme markers, the activities of glutamine synthetase (GS) and butylcholinesterase (BuCh) were assayed according to Ellman *et al.* (1961) and Rowe *et al.* (1970), respectively. (Na+K)ATPase and 5'-nucleotidase were measured as previously described (Guerri *et al.*, 1984). Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

## Acknowledgments

This research was partially supported by the DGICYT (PB87-0505) and the FISss (89/0002). We thank M. March for expert technical assistance.

## References

CLARREN, S.K., ALVORD, E.C., SUMI, S.M., STREISSGUTH, A.P. and SMITH, D.W. (1978). Brain malformations related to prenatal exposure to ethanol. *J. Pediatr. 92:* 64-67.

DAVIS, L.D. and VERNADAKIS, A. (1984). Responses in astrocytic C6 glioma cells to ethanol and dibutyryl cyclic AMP. *Dev. Brain Res. 16:* 27-35.

DOW, K.E. and RIOPELLE, R.J. (1985). Ethanol neurotoxicity: effects on neurite formation and neurotrophic factor production *in vitro*. *Science 228:* 591-593.

ELLMAN, G.L., COURTNEY, K.D., ANDRES, V. and FEATHERSTONE, E.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol. 7:* 88-95.

FAROOQ, M. and NORTON, W.T. (1978). A modified procedure for isolation of astrocyte- and neuron-enriched fractions from rat brain. *J. Neurochem. 31:* 887-894.

GIACOBINI, E. (1964). *Morphological and Biochemical Correlation of Neural Activity* (Eds. M.M. Cohen and R.S. Snider). Harper and Row, New York, pp. 15-38.

GUERRI, C. and GRISOLÍA, S. (1983). Chronic ethanol treatment affects synaptosomal membrane-bound enzymes. *Pharmacol. Biochem. Behav. 18:* 45-50.

GUERRI, C., ESQUIFINO, A., SANCHIS, R. and GRISOLÍA, S. (1984). Growth, enzymes and hormonal changes in offspring of alcohol-fed rats. In *Mechanism of Alcohol Damage* In Utero. Ciba Foundation, Symposium 105, Pittman, London, pp. 85-102.

HANSSON, E. (1986). Primary astroglial cultures. A biochemical and functional evaluation. *Neurochem. Res. 11:* 759-766.

HERTZ, L. (1965). Possible role of neuroglia: a potassium-mediated neuronal-neuroglia-neuronal impulse transmission system. *Nature 206:* 1091-1094.

HERTZ, L. (1982). Astrocytes. In *Hand-Book of Neurochemistry, Vol. 1* (Ed. A. Lajtha). Plenum Press, New York, London, pp. 319-355.

HERTZ, L., JUURLINK, B.H.J., FOSMARK, H. and SCHOUSBOE, A. (1982). Astrocytes in primary cultures. In *Neuroscience Approached Through Cell Culture* (Ed. J. Pfeiffer). CRC Press, Florida, p. 175.

HEYMANN, D., REDDINGTON, M. and KREUTZBERG, G.W. (1984). Subcellular localization of 5'-nucleotidase in rat brain. *J. Neurochem. 43:* 971-978.

LEDIG, M., KOPP, P. and MANDEL, P. (1985). Effect of ethanol on adenosine triphosphatase and enolase activities in rat brain and in cultured nerve cells. *Neurochem. Res. 10:* 1311-1324.

LEMOINE, P., HARROUSSEAU, M., BORTEYRU, J.P. and MENUET, J.C. (1968). Les enfants de parents alcooliques. Anomalies observées. A propos de 127 cas. *Quest. med. 21:* 476-482.

LIEBER, C.S. and DE CARLI, L.M. (1976). Animal models of ethanol dependence and liver injury in rats and baboons. *Fed. Proc. 35:* 1232-1236.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193:* 265-275.

MANDEL, P., LEDIG, M. and M'PARIA, J.R. (1980). Ethanol and neuronal metabolism. *Pharmacol. Biochem. Behav. (Supp. 1) 13:* 175-182.

MARQUES, A. and GUERRI, C. (1988). Effect of ethanol on rat brain (Na+K)ATPase from native and delipidized synaptic membranes. *Biochem. Pharmacol. 37:* 601-606.

MARTÍNEZ-HERNÁNDEZ, A., BELL, K.P. and NORENBERG, M.D. (1977). Glutamine synthetase: glial localization in brain. *Science 195:* 1356-1358.

MILLER, M.W. (1986). Effects of alcohol on the generation and migration of cerebral cortical neurons. *Science 233:* 1308-1311.

PATEL, A.J. (1982). The distribution and regulation in nerve cells and astrocytes of certain enzymes associated with the metabolic compartmentation of glutamate. In *Neurotransmitter: Interactions and Compartmentation* (Ed. H.F. Ford). Plenum Press, New York, pp. 411-430.

RENAU-PIQUERAS, J., ZARAGOZA, R., DE PAZ, P., BAGUENA, E., MEGÍAS, L. and GUERRI, C. (1989). Effects of prolonged ethanol exposure on the glial fibrillary acidic protein-containing intermediate filaments of astrocytes in primary culture: a quantitative immunofluorescence and immunogold electron microscopic study. *J. Histochem. Cytochem. 37:* 229-240.

ROWE, W.B., RONZIO, R.A., WELLNER, V.P. and MEISTER, A. (1970). Glutamine synthetase. In *Methods in Enzymology, Vol. XVIIA* (Eds. H. Tabor and C.W. Tabor). Academic Press, New York, pp. 900-910.

SANCHIS, R., GUERRI, C., RENAU-PIQUERAS, J. and GRISOLÍA, S. (1984). Effects of prenatal and postnatal alcohol intake on brain development. In *Developmental Neuroscience: Physiological, Pharmacological and Clinical Aspects* (Eds. E. Caciagly, E. Giacobini, R. Paleotti). Elsevier, Amsterdam, pp. 217-223.

SCHUBERT, P., KOMP, W. and KRENTZBERG, W. (1979). Correlation of 5'-nucleotidase activity and selective trans-neuronal transfer of adenosine in hippocampus. *Brain Res. 168:* 419-424.

SULAKHE, P.W. and PHILLIS, J.W. (1975). The release of $^{3}$H-adenosine and its derivatives from rat sensorimotor cortex. *Life Sci. 17:* 551-556.

STREISSGUTH, A.P. and MARTIN, J.C. (1983). Prenatal effects of alcohol abuse in humans and laboraory animals. In *The Pathogenesis of Alcoholism, Vol. 7* (Eds. B. Kissin, H. Begleiter). Plenum Press, New York, pp. 539-589.