ABSTRACT

Douglas Casey Lee. THE EFFECTS OF ETHANOL ON ADENYLATE CYCLASE ACTIVITY IN CULTURE CHICK NEURAL RETINA CELLS (Under the supervision of Dr. Sam N. Pennington and Dr. Gerhard Kalmus) Department of Biology, August 1985.

Fetal Alcohol Syndrome (FAS) is a collective term encompassing the many detrimental fetal effects resulting from maternal ethanol consumption. Some of these effects include: microcephaly, cleft palate and reductions in overall size of the neonate. This thesis addresses the hypothesis that ethanol reduces growth in an organism via a mechanism involving cyclic 3',5' adenosine monophosphate (cAMP). In this study, growth and cAMP concentrations were measured in response to varying ethanol dosages in cultured chick neural retina cells. It was demonstrated that ethanol alone (50 and 200 mg/dl) did not significantly reduce the total protein content of the cells, while a 50 mg/dl dose of ethanol did not significantly affect DNA content of the cells. However, when a phosphodiesterase inhibitor, 11.5 ug/ml of 1 methyl,3 isobutylxanthine (MIX), was added at low concentrations to the ethanol-treated cells (50 and 200 mg/dl), protein and DNA content was significantly reduced. Total protein content in the ethanol-dosed cells treated with MIX (11.5 ug/ml) was reduced 23% ($p < 0.001$) for the 200 mg/dl ethanol-dosed samples and 19% ($p < 0.001$) for 50 mg/dl ethanol-dosed samples compared to the vehicle dosed cells. DNA content for the 50 mg/dl ethanol plus MIX (11.5 ug/ml)

treated cells although not statistically significantly different was 20% lower than the vehicle treated cells. Cyclic AMP concentrations for the ethanol-treated cells (50 mg/dl) samples were 20% ($p < 0.01$) higher than the control cells. Cyclic Amp concentrations for 50 mg/dl ethanol plus 11.5 ug/ml MIX were 24% ($p < 0.01$) higher than the vehicle values. These data suggest that although ethanol treatment does increase cAMP levels, a phosphodiesterase inhibitor is necessary to maintain the cAMP concentrations and thus elicit a cAMP-mediated suppression of growth.

THE EFFECTS OF ETHANOL ON ADENYLATE CYCLASE ACTIVITY OF CULTURED CHICK NEURAL RETINA CELLS

A Thesis

Presented to

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In Partial Fulfillment

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Master of Science in Biology

by

Douglas Casey Lee

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ADENYLATE CYCLASE ACTIVITY

OF CULTURED CHICK NEURAL RETINA CELLS

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DEDICATION

This thesis is dedicated to my grandmother, Ms. Elizabeth H. Casey, a lady who taught me to appreciate the value of a good education. I will love her forever.

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INTRODUCTION

The effects of maternal ethanol consumption have been recognized as far back as the Roman empire. The Romans believed that intoxication at the time of procreation resulted in the birth of a damaged child. Vulcan, the blacksmith of the gods, was said to have been disfigured as a result of maternal alcohol intoxication (Green, 1974). However, it was not until 1973 that this condition was termed Fetal Alcohol Syndrome (FAS) by Jones and Smith. They described a pattern of abnormalities in 11 newborn children. These aberrations include the following: pre- and post-natal growth deficiencies, microcephaly, cleft palate, various joint disorders, and cardiac anomalies. In addition to these disorders, Ferrier and co-workers (1976) observed reductions in birth weight and overall size. Mulvihill and co-workers (1976) reported mental deficiencies associated with reduced head circumference in their patients.

Early observations by Streissguth and co-workers (1981) suggested that FAS is only a problem in alcoholic women (i.e. chronic alcohol consumption of greater than 30 g of absolute alcohol per day). In a later study, Streissguth and co-workers (1982) extended their concepts to include the lighter, social drinking women. Therefore, it is clear that women that who are not addicted, but drink moderately large amounts of alcohol during pregnancy have an increased risk

of bearing infants with some fetal alcohol effects (Poskitt, 1984).

Ethanol studies in laboratory animals have produced offspring with certain characteristics of FAS. Pennington and co-workers (1984) observed the effects of ethanol on the early development of rat embryos. They observed that 8 hours after mating, a single dose of ethanol (via inhalation or stomach tube) resulted in a dose-dependent retardation of cell division in the zygote. They also noted that the growth inhibition was sustained for up to 48 hours after the dose. In the same study, animals with blood-alcohol levels above 150 mg/dl demonstrated significant increases in abnormal embryo development. Fernandez and co-workers (1983) reported that maternal ethanol consumption in rats produced offspring with reduced body weights as well as increasing mortality within the litters. Abel and co-workers (1983) demonstrated that rats exposed to ethanol in utero, did not perform as well as vehicle-dosed animals in a two-way shock avoidance test. In the same study, histological work revealed significant deficits in dendritic structure in the area of the hippocampus in the fetal brain. Sorette and co-workers (1980) observed significant growth reductions in fetal rat organs (e.g. heart, liver, kidney, and brain) in response to maternal ethanol diets. They attributed this decrease in size to a significant reduction in total protein content observed in the organs being studied.

Related to this lowered protein content, it has been shown that ethanol appears to inhibit protein synthesis. Several studies have demonstrated that ethanol consumption inhibits incorporation of radioactively labelled leucine and valine in: (1) cerebral and cerebellar ribosomes of mice (Noble and Tewari, 1975 and 1979); (2) placental ribosomes in rats (Wunderlich, et al., 1979); (3) rat fetal hepatic and cerebral ribosomes (Rawat, 1979, 1976, and 1980); and (4) the livers of rats (Smith-Kielland, et al., 1983).

In conjunction with observed growth reductions, studies have also shown that maternal ethanol consumption reduces the deoxyribonucleic acid (DNA) content in fetal tissues. Woodson and Richey (1979) demonstrated that chronic maternal ethanol consumption reduced DNA content and total cell number in the brain of fetal rats. They showed that although cell number is significantly decreased, cell size does not appear to be affected. Dreosti and co-workers (1981) observed that acute maternal ethanol intoxication reduced (^{3}H)-thymidine incorporation in the fetal DNA of rats, especially in the central nervous system. Utilizing a dye-binding method of DNA analysis, Pennington and co-workers (1983) observed an increase in cell division time in response to ethanol exposure in the developing chick brain.

It is proposed that the growth suppression associated with FAS may be related to alcohol-induced increases in cyclic 3'-5' adenosine monophosphate (cAMP) concentrations.

Adenylate cyclase, a membrane-bound enzyme, produces cAMP from adenosine triphosphate (ATP) and it has been demonstrated that increased cAMP concentrations inhibits growth in several types of tissue grown in culture including fibroblast, melanoma, hepatoma, neuroblastoma, astrocytoma, and glial cells. Kalmus and co-workers (1982) also observed significant growth retardation in cultured chick neural retina cells when the cells were exposed to cAMP and cAMP analogs. They observed significant reductions in (^{3}H)-thymidine and (^{14}C)-leucine incorporation in these cultured cells.

High cAMP levels as a result of ethanol exposure have been reported in several tissues. For example, cAMP levels in cerebral homogenates from ethanol-dosed mice showed a 64%, 49%, and 14% increase after 1, 2, and 3 weeks, respectively, of chronic ethanol ingestion. In the same study, acute ethanol consumption indicated no significant change in cAMP levels (Kuriyama and Israel, 1973).

Within a broad range of ethanol doses (80-40,000 mg/dl) human lymphocytes exposed to acute and chronic ethanol doses, *in vitro*, showed a significant increase in cAMP levels (Atkinson *et al.*, 1977). In the same study, kinetic analysis revealed that adenylate cyclase activity showed an approximate 60% increase in cAMP production during the initial 10 minutes of ethanol exposure and a return to control levels after 60 minutes.

In rat myocardium, cAMP levels rose significantly as a result of ethanol dose *in vitro* (Vesely *et al.*, 1978). After a 3 minute incubation period, cAMP in the ethanol group increased approximately 65%. However, the authors pointed out that the 2000 mg/dl ethanol concentration used in this study would not be obtainable *in vivo*.

In homogenates of mouse striatum, adenylate cyclase activity increased when exposed to chronic ethanol dosages (Rabin and Mollinoff, 1981). One dose of ethanol (either 200 or 1400 mg/dl) increased adenylate cyclase activity in a linear fashion during a 12 minute incubation period. Lucchi and co-workers (1983) reported similar results in rat straitum. They observed that after 21 days of chronic ethanol ingestion, striatal cAMP increased over calorically equal, sucrose-fed controls.

In rat jejunal, adenylate cyclase activity was shown to increase with chronic ethanol treatments (Greene, *et al.*, 1971). Experiments utilizing 160 mg/dl, 1950 mg/dl, 9000 mg/dl, and 27,000 mg/dl ethanol concentrations *in vitro*, produced linear increases in cAMP levels.

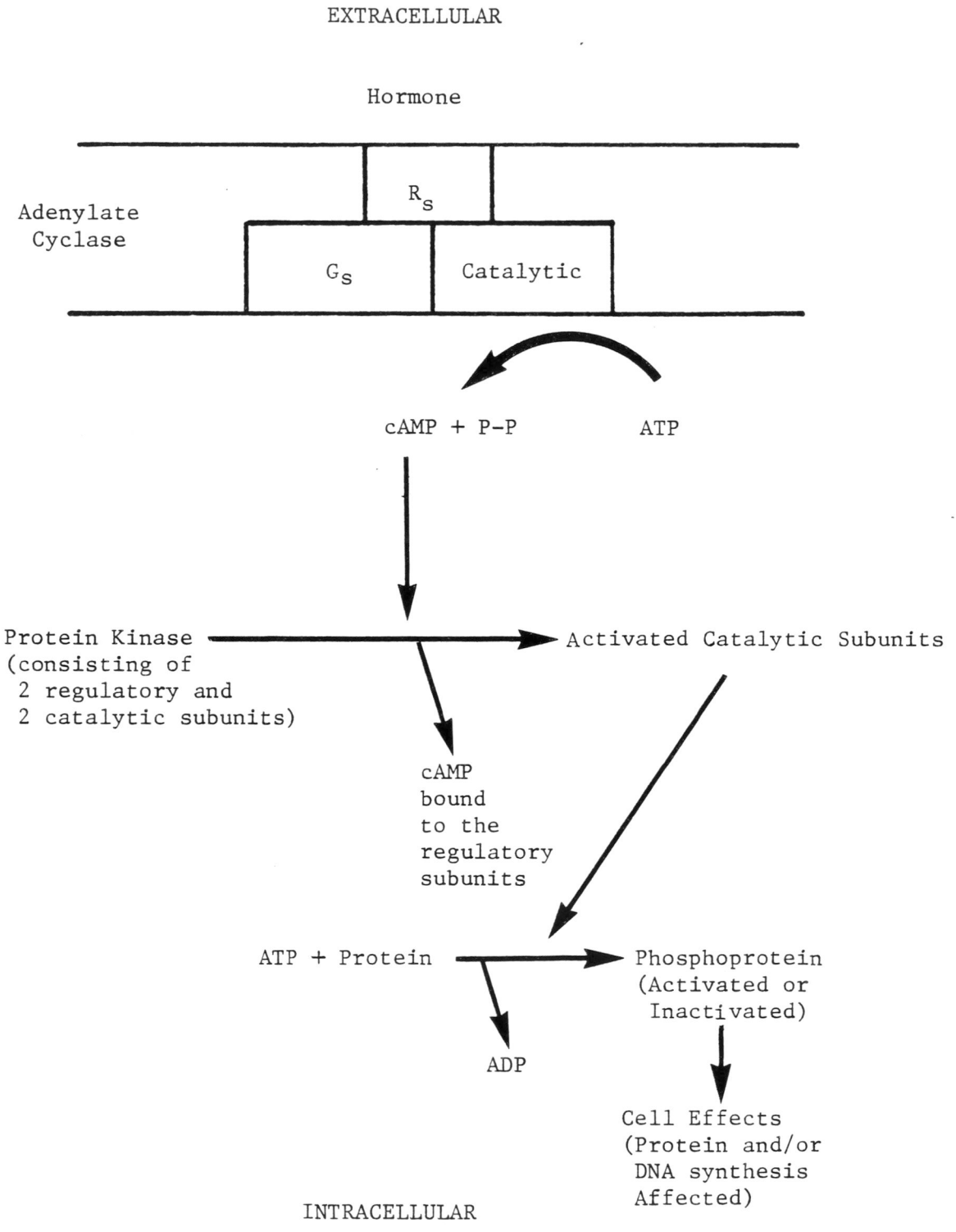
Utilizing hamster renal tubules *in vitro*, it was demonstrated that ethanol plus parathyroid hormone significantly increased cAMP levels over ethanol and saline doses (Biddulph *et al.*, 1983). Maximal potentiation of the parathyroid hormone response occurred at 2400 mg/dl ethanol concentrations while the minimal response observed occurred at 250 mg/dl ethanol. The removal of the ethanol from the

renal tubules prior to the addition of parathyroid hormone, resulted in a basal cAMP levels indicating that ethanol is required for maximal stimulation.

Rotrosen and co-workers (1980) observed that ethanol (100-1000 mg/dl) enhanced prostaglandin (PG) E_1-stimulated cAMP accumulation in slices of rat corpus striatum (up to 171%) and cortex (up to 124%). In the same study, (^{3}H)-cAMP accumulation in human platelets was markedly increased above PGE_1 stimulated cells when the cells are preincubated with ethanol.

A possible pathway through which these high cAMP levels resulting from ethanol exposure may affect growth is as follows: (1) An extracellular messenger binds to adenylate cyclase, thus activating the enzyme complex. Adenylate cyclase catalyzes the synthesis of intracellular cAMP (Zalin and Montague, 1975). (2) The cAMP produced activates a protein kinase by binding to the kinase's regulatory dimers and dissociating them from the kinase's catalytic dimers (Robison et al., 1968; Ogreid and Doskeland, 1981). (3) The activated catalytic dimers, utilizing ATP, phosphorylate a variety of proteins, thus activating them or inactivating them (Rudolph and Krueger, 1979). Although not completely understood, these phosphorylated proteins may affect protein and DNA content by altering one or more of the enzymes responsible for protein and/or DNA synthesis (Pennington et al., 1983) (Fig. 1).

Fig. 1. Proposed mechanism for how an extracellular hormone can affect cell growth (protein/DNA synthesis) via a cAMP mechanism. See text for explanation of abbreviations.


EXTRACELLULAR
Hormone
Adenylate Cyclase
R_s
G_s
Catalytic
cAMP + P-P
ATP
Protein Kinase (consisting of 2 regulatory and 2 catalytic subunits)
Activated Catalytic Subunits
cAMP bound to the regulatory subunits
ATP + Protein
Phosphoprotein (Activated or Inactivated)
ADP
Cell Effects (Protein and/or DNA synthesis Affected)
INTRACELLULAR

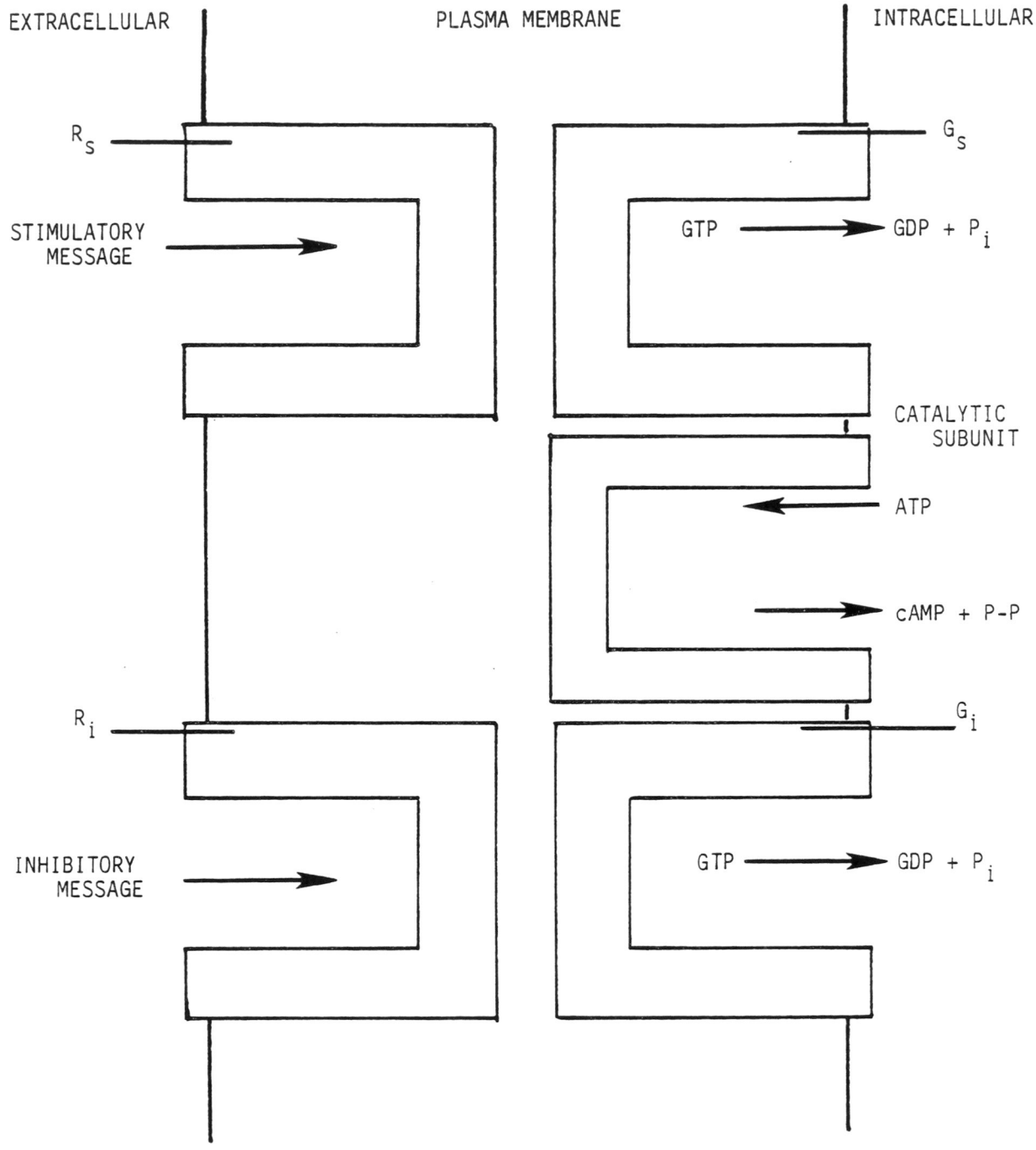
The membrane-bound enzyme, adenylate cyclase consists of at least three subunits: a stimulatory receptor, guanine nucleotide-binding protein, and the catalytic subunit (Moss and Vaughn, 1979). The stimulatory receptor subunit (R_s) has its receptor site located on the outer margins of the cellular membrane. The R_s binds an extracellular messenger (e.g. epinephrine or PGE_1) that initiates a chain of events that ultimately activates the adenylate cyclase catalytic subunit. Closely associated with the R_s is a guaine nucleotide-binding protein (G_s). The G_s protein has been purified from several cell types and is known to be composed of two major polypeptides with molecular weights of 35K and 45K (Gilman, 1984). The G_s is believed to reside on the inner membrane and plays an important role in coupling the R_s with the catalytic subunit. The G_s binds guanidine triphosphate (GTP) which is gradually hydrolyzed to guanidine diphosphate (GDP) and inorganic phosphate via a GTP'ase mechanism (Cassel and Selinger, 1976 and 1977). Adenylate cyclase is only active as long as GTP is bound to the G_s (Hanski and Gilman, 1980). The catalytic subunit of adenylate cyclase is also located on the inner margins of the cell's membrane. This unit catalyzes the conversion of ATP to cAMP and pyrrophosphate (Rawn, 1983).

Recently, two other proteins that are associated with adenylate cyclase have been isolated. They are the inhibitory receptor (R_i) (Hsia, et al., 1984) that is associated with another guanine nucleotide-binding protein

(G_i) (Gilman, 1984). The R_i binds an extracellular messenger (e.g. opiate or somatostatin) and inhibits adenylate cyclase catalytic subunit when coupled via the G_i (Hsia, et al., 1984). The G_i is also a heterodimer (like G_s) consisting of at least two polypeptides with identical molecular weights as found in the G_s. If the R_i has been activated and GTP is bound to G_i, then adenylate cyclase is effectively inhibited (Gilman, 1984) (Fig. 2).

It is our working hypothesis that ethanol may increase cellular cAMP levels by direct action on adenylate cyclase. Ethanol could affect adenylate cyclase and thus increase cAMP concentrations via several possible mechanisms. For example, (1) Ethanol may inhibit the GTP'ase mechanism of the G_s subunit. It has been previously reported that some substances, such as choleragen, will inhibit this GTP'ase activity. If this occurs then the catalytic subunit will continually produce cAMP from ATP since GTP is constantly bound to the G_s (Moss and Vaughn, 1979). This has already been suggested by Luthin and Tabakoff (1984), who report that the G_s must be present and loaded with GTP in order for maximal ethanol activation. (2) Ethanol may modify the fluidity of the cellular membrane, thus dissociating the membrane-bound subunits. Increased cAMP may be the result if the G subunits are dissociated from the catalytic subunit (Shimizu and Daly, 1972; Whetton et al., 1983). (3) Another possibility is that ethanol could block the inhibitory arm of adenylate cyclase, therefore leaving only the stimulatory

Fig. 2. Diagrammatic sketch of adenylate cyclase and the reactions associated with it (Gilman, 1984). See text for explanation of abbreviations.


EXTRACELLULAR
PLASMA MEMBRANE
INTRACELLULAR
R_s
G_s
STIMULATORY MESSAGE
GTP
GDP + P_i
CATALYTIC SUBUNIT
ATP
cAMP + P-P
R_i
G_i
INHIBITORY MESSAGE
GTP
GDP + P_i

proteins operations. (4) Finally, ethanol may increase the hormonal sensitivity of the R_s as suggested by Kuriyama (1977).

This thesis observed the effect(s) of ethanol on growth and cAMP levels in cultured chick neural retina cells. Neural retina cells were selected for study because many studies indicate that ethanol affects the development of the central nervous system, especially the brain (Woodson and Ritchey, 1979; Samson and Diaz, 1981; Boyd et al., 1984). Utilizing cell culture, direct observations were made concerning the role of ethanol on cellular growth.

Ethanol has also been shown to increase cAMP in neuronal tissue (Kuriyama and Israel, 1973; Boyd, 1983). Thus, cAMP concentrations were observed in the cultured chick neural retina cells and then the cAMP data was correlated with the growth data.

In order to establish chick neural retina cells as a model for study, we observed three parameters. The first parameter was the ethanol metabolism of the neural retina cells in culture. This was a necessary step in order to determine if alchohol deydrogenase (ADH) was present in the culture system during the culture period. Secondly, any effects of ethanol on the growth of the cells was observed. In studying this area, changes in total protein and DNA of the cells was determined. Finally, the effects of ethanol on cAMP levels within the cell cultures was determined.

Therefore, if ethanol affects cAMP concentrations within the cells, does the cAMP in turn affect the growth?

PROCEDURES

Materials

Unincubated fertile eggs were obtained from Webber's Hatchery in Goldsboro, North Carolina. The Medium 199 (see Appendix I), horse serum, chick embryo extract and triple, distilled H_2O components of the chick neural retina media were purchased from Difco Laboratories, Detroit, Michigan. Deoxyribonuclease I (2000 units/mg), 1-methyl,3-isobutyl-xanthine, alcohol dehydrogenase, bisbenzimidazole, and calf thymus DNA standards were purchased from Sigma Chemical Company, St. Louis, Missouri. The protein assay kit was obtained from Bio-Rad Company, Richmond, California. The Rainen cAMP radioimmunoassay kit was purchased from DuPont Specialty Dianostics, North Billerica, Maine. Reagent grade 95% ethanol was purchased from the Chemistry Department of East Carolina University, Greenville, North Carolina. Other chemicals used in this study were obtained from Fisher Scientific Company, Raleigh, North Carolina.

Eggs Incubation

Unincubated fertile eggs were stored at 10°C, for no more than five days before the start of incubation. Eggs were incubated for seven days at 37.5°C in 70%-90% humidity in a forced air incubator (Sears Roebuck Co.) and turned once a day.

Cell Cultures

All cell culturing was performed as described by Dunson (1979) with minor modifications. Chick embryos were sacrificed at day seven of incubation, stage 30 as defined by Hamburger and Hamilton (1951). The eyes were dissected free and following an incision on the posterior of the eye, the vitreous body was removed. The neural retina was then dissected away from the pigmented retina. All dissections were done in a petri dish, while the embryos and eyes were suspended in calcium and magnesium-free (CMF) Hank's solution. The neural retina was collected from all the eyes (approximately 40 eyes) and held in a 35 x 10 mm petri dish containing Medium 199.

The tissue was transferred to a test tube with a large bore pipette. The cells were then dispersed, in the test tube, by enzymatic digestion for 10 minutes in approximately 10 ml of 0.1% trypsin (250 units/mg) dissolved in CMF Hank's solution while incubating at 37.5°C. At the end of the incubation, physical dispersion was accomplished by flushing the suspension 100 times with a flame-polished Pasteur pipette at room temperature (22°-24°C).

The cells were centrifuged at 900 rpm for 8 minutes to remove the dissociation medium. The cells were then washed as follows: (1) The cells were resuspended in CMF Hank's solution, centrifuged (900 rpm; 5 minutes) and the supernatant discarded. This step removed any of the remaining dissociation medium. (2) The pellet was

resuspended at room temperature in CMF Hank's solution containing 1 mg/50 ml deoxyribonuclease I (DNA'ase) for approximately 5 minutes. The DNA'ase removed any extraneous chromatin that resulted from broken cells during isolation and dispersion of the tissue. The suspension was immediately centrifuged (900 rpm; 5 minutes) and the supernatant discarded. (3) The pellet was again resuspended in CMF Hank's solution, centrifuged (900 rpm; 5 minutes) and the supernatant discarded.

After the third wash, the cellular pellet was resuspended in 5 ml nutrient media (see below). Cell number was determined using a hemacytometer, and cell viability was determined by exclusion of 0.4% trypan blue in CMF Hank's solution. The appropriate volume of the cell suspension was added to 5.5 ml of the nutrient media in 25 ml Erlemeyer flasks to give a final concentration of 10^6 cells/ml.

The innoculated flasks were gassed with 95% O_2 - 5% CO_2, and stoppered. The flasks were placed in a New Brunswick gyratory growth chamber and incubated at 37.5°C while agitating at 70 rpm for four days.

Preparation of Solutions and Nutrient Media

Solutions and the nutrient media were prepared as follows: (1) Chick Ringer's solution (Howard, 1983) consisted of NaCl (8.0 g/L), KCl (0.37 g/L) and $CaCl_2$ (0.18 g/L), pH 7.2. (2) CMF Hank's solution (Paul, 1972) contained NaCl (8 g/L), KCl (0.4 g/L), $K_2HPO_4 \cdot 2H_2O$ (0.06

g/L), and KH_2PO_4 (0.06 g/L). Both solutions were autoclaved at 120°C for 25 minutes. Glucose (1 g/L) and $NaHCO_3$ (0.35 g/L) were added to the CMF Hank's solution immediately before use. (3) The nutrient media (Lee et al., 1974) consisted of 60 parts (v/v) Medium 199 (Morgan et al., 1950), 20 parts horse serum (v/v) and 20 parts (v/v) chick embryo extract. Lyophilized horse serum and chick embryo extract were reconstituted to 30 ml/vial and 10 mg/vial, respectively, with triple, distilled H_2O. The nutrient media was supplemented with L-glutamine (1.46 g/ml) plus streptomycin (100 ug/ml) and penicillin (100 units/ml).

Test Agents and Dosing Procedures

The test agents used in this study were 95% reagent grade ethanol and 1-methyl,3-isobutylxanthine (MIX), a phosphodiesterase inhibitor (Beavo et al., 1980).

Ethanol dosages (50 and 200 mg/dl) were administered on day one of the culture period immediately preceding incubation. Using Medium 199 as a solvent for the ethanol, 0.28 ml of ethanol was diluted to 10 ml in a volumetric flask, resulting in a 2150 mg/dl stock solution. Using a micropipette, 0.5 ml of the stock solution was transferred to 5.0 ml of the culture media containing the cells. This resulted in a final volume of 200 mg/dl in the incubation media. In order to obtain the 50 mg/dl dosages, the stock ethanol solution (2150 mg/dl) was diluted 3:1 with Medium 199 and administered to the culture as described above. The

stock ethanol solutions were made up on the day of culture and held no more than 20 minutes prior to dosing.

Three MIX dosages (11.5, 115 and 1150 ug/ml) were used to determine the maximum dose of MIX that would not significantly affect the total protein content of the cell culture. A stock solution was prepared by dissolving 126.5 mg of the MIX into 10 ml of sterile distilled H_2O resulting in a final concentration of 12650 ug/ml. To facilitate the dissolving of the MIX, a small volume of 1 N NaOH was added with a Pasteur pipette until the MIX was dissolved. From this stock, 0.5 ml was added to the 5.0 ml culture system. The other MIX dosages were obtained by serially diluting the stock in ten fold increments and administered as described above.

The final volume in the culture system was always maintained at 5.5 ml. Vehicles contained 5 ml nutrient media and dosed with 0.5 ml sterile, distilled H_2O. Control groups received 5.5 ml nutrient media only. The gassing procedure asjusted the pH in all the groups to approximately 7.0.

Cell Harvest

The cells were harvested at the end of the four day culture period. The flasks were removed from the incubator and the cells were scraped from the walls of the flasks with a rubber policeman. Using a Pasteur pipette, the media containing the suspended cells was transferred from the 25

ml Erlemeyer flasks to corresponding test tubes. These suspensions were centrifuged (900 rpm; 10 minutes) and the media discarded. The cellular pellets were washed three times in CMF Hank's solution and centrifuged (600 rpm; 5 minutes each) to remove the media.

Ethanol Assay

To confirm that ethanol was not metabolized during the incubation period, ethanol assays were performed as described by Mendenhall and co-workers (1980). This assay utilizes alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD^+) in the following reaction:

$$CH_3CH_2OH \text{ (ethanol)} + NAD^+ \xrightarrow{ADH} CH_3CHO \text{ (acetaldeyde)} + NADH + H^+$$

The increase in absorbance at 340 nm, which occurs when NAD^+ is reduced to NADH, was used to quantitate the amount of ethanol in the sample.

A stock NAD^+-ADH reagent was prepared by diluting 30,000 units of ADH (75 mg protein) with 7.5 ml cold distilled H_2O. This stock was then pipetted (0.3 ml) into polypropylene vials and frozen until time of use. On the day of the assay, a working NAD-ADH solution was prepared by diluting each vial used with 24.7 ml glycine buffer (0.5 M glycine; 0.1 M semicarbazide; pH 9.0) and adding 10 mg NAD^+.

Ethanol standards were prepared from a stock solution that contained 3.0 ml 95% ethanol diluted to 100 ml with

distilled H_2O. This solution was stored for no more than five days. On the day of the assay, a working solution was prepared by diluting 12.9 ml of the stock ethanol solution with distilled H_2O to 100 ml in a volumetric flask. The final ethanol concentration was 300 mg/dl in the working solution.

The standard curve was prepared by pipetting 0.5 ml of the working ethanol solution into a test tube containing 0.5 ml distilled H_2O and vortexed. This was the 150 mg/dl standard. The 75 mg/dl standard was prepared by diluting 0.5 ml of the 150 mg/dl ethanol standard with 0.5 ml distilled H_2O. The 37.5 mg/dl ethanol standard was prepared by transferring 0.5 ml of the 75 mg/dl standard into a test tube containing 0.5 ml distilled H_2O. The standard curve was prepared fresh for each assay.

Samples were prepared by removing 0.05 ml aliquots of the nutrient media from the culture flasks to be sampled each day of the incubation period and placed in microcentrifuge tubes. Two tenths ml of 6.24% TCA was added to the samples and they were vortexed to precipitate any protein that was present in the medium. The samples were centrifuged in an Epindorff high speed centrifuge for three minutes and held at 4°C for no more than four hours assayed.

Three ml of the NAD-ADH working solution was transferred to appropriately labelled test tubes containing 0.1 ml of the standards or TCA supernatant from the samples. The tubes were vortexed, and incubated at ambient

temperature for 10 minutes. The contents were transferred to cuvettes and the absorbance was measured at 340 nm on a Coleman 124 spectrophotometer. All samples were ran in duplicate.

The absorbance of the known standards were plotted against their concentration on standard linear graph paper. The absorbance values for the unknowns were averaged and the concentration read from the graph.

Protein Assay

Total protein content of the cells was determined as a parameter of growth. Protein was assayed using a kit purchased from Bio-Rad that is based on the methods of Bradford (1976). The Bio-Rad protein assay is based on the observation that an acidic solution of Coomassie Brillant Blue G-250 dye shifts from an absorbance maximum of 465 nm to 595 nm when bound to protein.

Protein samples were obtained by disrupting the cellular pellet obtained at cell harvest with 1 ml of 1 N NaOH, then incubating in a 30°C water bath for 30 minutes. After the incubation, the samples were allowed to equilibrate to room temperature. At this time, 4.2 ml of ice-cold 16.3% TCA was added to the samples and they were placed in an ice bath (4°C) for 10 minutes. The samples were centrifuged (Sorvall Model RC-3; 2100 rpm; 15 minutes; 4°C), supernatant discarded and the resulting protein pellet was held at 4°C until time of the assay.

At the time of assay, the protein dye reagent, supplied in the assay kit, was diluted with H_2O and filtered through a No. 1 Whatman Filter. The protein pellets obtained as described above were resolubilized in 3 ml of 0.2 N NaOH. Five ml of the diluted dye reagent was added to test tubes containing either 0.1 ml of the protein standard (20-140 ug/ml) or unknown protein samples. After a 5-10 minute incubation at room temperature, protein standards and samples were then transferred to cuvettes and the absorbance was read on a Bausch and Lomb Spectronic 88 spectrophotometer at 595 nm.

Optical density (595 nm) for the known protein standards of bovine serum albumin were plotted on standard linear graph paper of optical density (595 nm) versus ug of protein. Unknown protein concentrations were read from the standard graph. Protein values for the neural retina cells in culture are reported as total milligrams (mg) per flask.

Cyclic AMP Assay

Cyclic AMP was measured using a radioimmunoassay (RIA) kit purchased from Rainen. This is a double antibody kit based on the methods of Steiner and co-workers (1972). The sensitivity of the RIA was increased by acetylation of the samples as described by Harper and Brooker (1975).

Standards were prepared by diluting the 5,000 pm/ml stock cAMP standard supplied in the kit fifty-fold, resulting in a 100 pm/ml cAMP solution. A 40 pmol/ml cAMP

solution was prepared by adding 1 ml of the 100 pm/ml cAMP solution to 1.5 ml of 0.05 M sodium acetate buffer (pH 6.2). From the 40 pmol/ml cAMP solution, 0.2 ml was pipetted into a clean test tube and acetylated by the addition of 0.01 ml of the acetylation reagent that contained two parts triethanolamine plus one part acetic anhydride. After a three minute incubation at room temperature, 1.8 ml of a sodium acetate buffer (0.05 M; pH 6.2) were added, resulting in a 4.0 pmol/ml cAMP standard. Cyclic AMP standards ranging from 0.25 to 4.0 pmol/ml cAMP were then prepared by serial dilutions using the sodium acetate buffer containing 0.5% acetylating reagent. One tenth ml of the standards were transferred to clean tubes and assayed.

For the cAMP samples, cell harvest was performed as described earlier but the samples were held in an ice bath throughout the assay. To the pellet of the freshly harvested cells, 0.5 ml of ice-cold 6% TCA was added. The samples were disrupted with a Brinkman Polytron apparatus by two 10 second pulses at maximum speed. The samples were centrifuged (IEC Centra 7-R; 2100 rpm; 15 min; 4°C) and 0.4 ml of the TCA supernatant was retained for the cAMP assay; the remaining supernatant was discarded. The resulting pellets were stored at 4°C until assayed later for protein and/or DNA.

The 0.4 ml TCA supernatants were extracted by the addition of 0.4 ml of a 1:4 solution of tri-n-octylamine and triflourotrichloroethane, the final pH of the two phases

before separation was 6.8. From the aqueous phase, two 0.02 ml samples were transferred to assay tubes. These were acetylated by adding 0.005 ml of the acetylation reagent and were allowed to incubate at room temperature for at least three minutes. Then 0.08 ml of the (0.05 M) sodium acetate buffer (pH 6.2) was added to maintain a 0.1 ml volume of sample that was assayed.

A working tracer solution was prepared by diluting one volume of succinyl cAMP tyrosine methyl [^{125}I] to one volume of cAMP carrier serum. All samples and standards received 0.1 ml of the working tracer solution. Each assay tube then received 0. 1 ml of the antibody. Assay controls included duplicate tubes that received only working tracer solution (Total Count tubes) and two duplicate Blank tubes that received only sodium acetate buffer with 0.5% acetylating reagent, working tracer solution, and antibody. All test tubes (except Total Count tubes) were vortexed and then incubated for 16-18 hours at 4°C.

After the incubation period, 0.5 ml of ice-cold sodium acetate buffer was added to each test tube (except Total Count tubes) to precipitate the bound antibody. The test tubes were vortexed and centrifuged at 2000xg for 20 minutes at 4°C and the supernatants discarded. All the test tubes were counted twice for a one minute counting period each on an Intertechnique GC 4000 gamma counter.

The counts were averaged for each assay tube and the average net counts for the standards and samples were

expressed as a percentage of the "O" standard (B_o) and calculated as follows:

$$\%B/B_o = \text{(Average net counts of standard or sample/Average net counts of Zero standard)} \times 100$$

The $\%B/B_o$ for the standards were plotted on the y-axis of semilog graph paper with the corresponding cAMP concentrations plotted on the log x-axis. The unknown values for the samples were read directly from the graph. Cyclic AMP values for the cultured neural retina cells are expressed as picomoles (pmol) of cAMP per milligram (mg) of protein. All standards and samples were assayed in duplicate.

DNA Assay

Quantitative DNA determinations were made using a flourescent dye-binding technique as described by Labarca and Paigen (1980). This method is based on the observation that bisbenzimidazole (H 33258) increases flourescence when bound to DNA.

Standards of calf thymus DNA were solubilized in 0.2 N NaOH and serially diluted to 25 ug/ml. One tenth ml of the standards were transferred to the assay tubes. Samples were obtained from the protein/DNA pellet that was recovered in the cAMP extraction (see above). The pellet was resolubilized in 3 ml of 0.2 N NaOH and 0.1 ml was removed

to be assayed. Standards and samples were assayed in duplicate.

H 33258 was dissolved in phosphate-saline (0.05 M $NaPO_4$, 2 M NaCl) buffer, pH 7.4, to a final concentration of 1 ug/ml. Five ml of this solution was added to each assay tube. The flourescence was read immediately on a Perkin-Elmer Flourescence Spectrophotometer, Model 512.

The standards were plotted on standard linear graph paper with the flourescence units against the corresponding DNA concentrations. The unknown values for the samples were read directly from the graph. The DNA values are expressed as total micrograms (ug) per flask.

Statistical Analysis

The effects of ethanol and MIX on the growth and cAMP levels of the neural retina cells was evaluated by comparing the vehicle and control groups with the MIX, ethanol, or MIX plus ethanol dosed groups. Analysis of variance (ANOVA) was used to determine if significant differences existed between more than two independent groups of data. The t-distribution was used to determine any significant differences between the means of two independent samples. Standard error of the mean (SEM) was used as an index of variability.

For all statistics, $p < 0.05$ was accepted as significant.

RESULTS

Repeated ethanol assays revealed that over the 4 day incubation period, a 200 mg/dl ethanol dose, administered on day one remained present in the neural retina cell cultures (Fig. 3). Ethanol concentrations in samples taken from the cell cultures ranged from 200.0 to 189.5 mg/dl.

Protein values for the chick neural retina cells dosed with 11.5 ug/ml MIX were not significantly different compared with the control or vehicle dosed group (Fig. 4). The 115 or 1150 ug/ml MIX dose did significantly decrease the protein content relative to the controls and vehicles (Fig. 4). The largest MIX dose (1150 ug/ml) reduced the total protein content 28% below the vehicle values and 31% below the control (no treatment) group.

In the absence of MIX, the protein content of the neural retina cell cultures was not significantly affected by ethanol concentrations up to 800 mg/dl (see Table 8 in appendix for experiment containing protein data for greater than 200 mg/dl ethanol). Individually, 200 mg/dl ethanol or 11.5 ug/ml MIX did not significantly affect the protein content of the chick neural retina cells. However when combined, the same dosages of ethanol and MIX reduced the total protein content 23% below the vehicles ($p < 0.001$; Fig. 5). Furthermore, even though a 50 mg/dl dose of ethanol did not appear to significantly affect the protein content of the cells, the combination of low dose ethanol (50 mg/dl)

Fig. 3. Ethanol levels over a 4 day period in chick neural retina cell cultures dosed initially with 200 mg/dl ethanol.

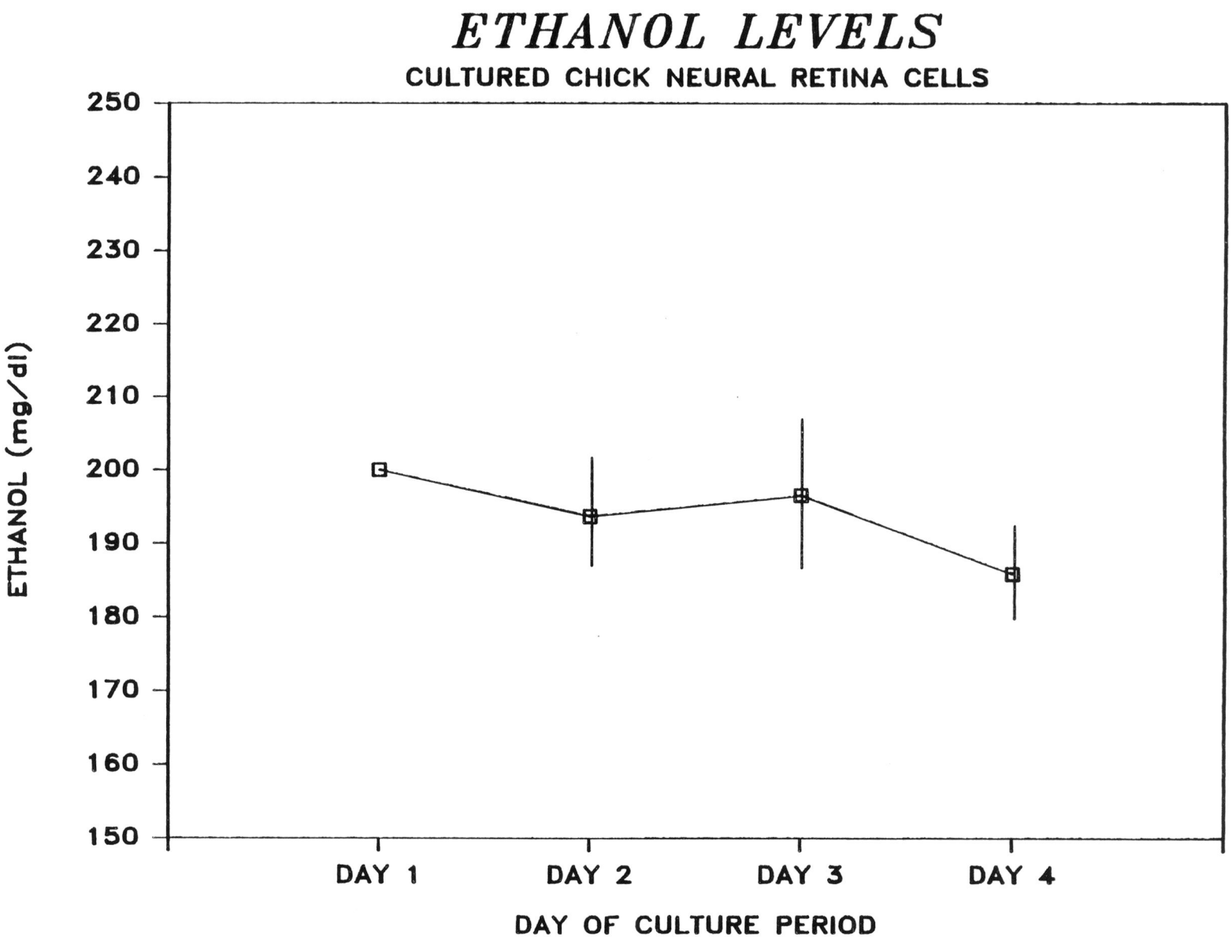

ETHANOL LEVELS
CULTURED CHICK NEURAL RETINA CELLS
250
240
230
220
210
200
190
180
170
160
150
ETHANOL (mg/dl)
DAY 1
DAY 2
DAY 3
DAY 4
DAY OF CULTURE PERIOD

Fig. 4. The effects of 1-methyl,3-isobutylxanthine (MIX) on the protein content of chick neural retina cells. Astericks indicate significant differences between the means of the experiment group and the control/vehicle groups. Bars represent plus/minus the SEM.

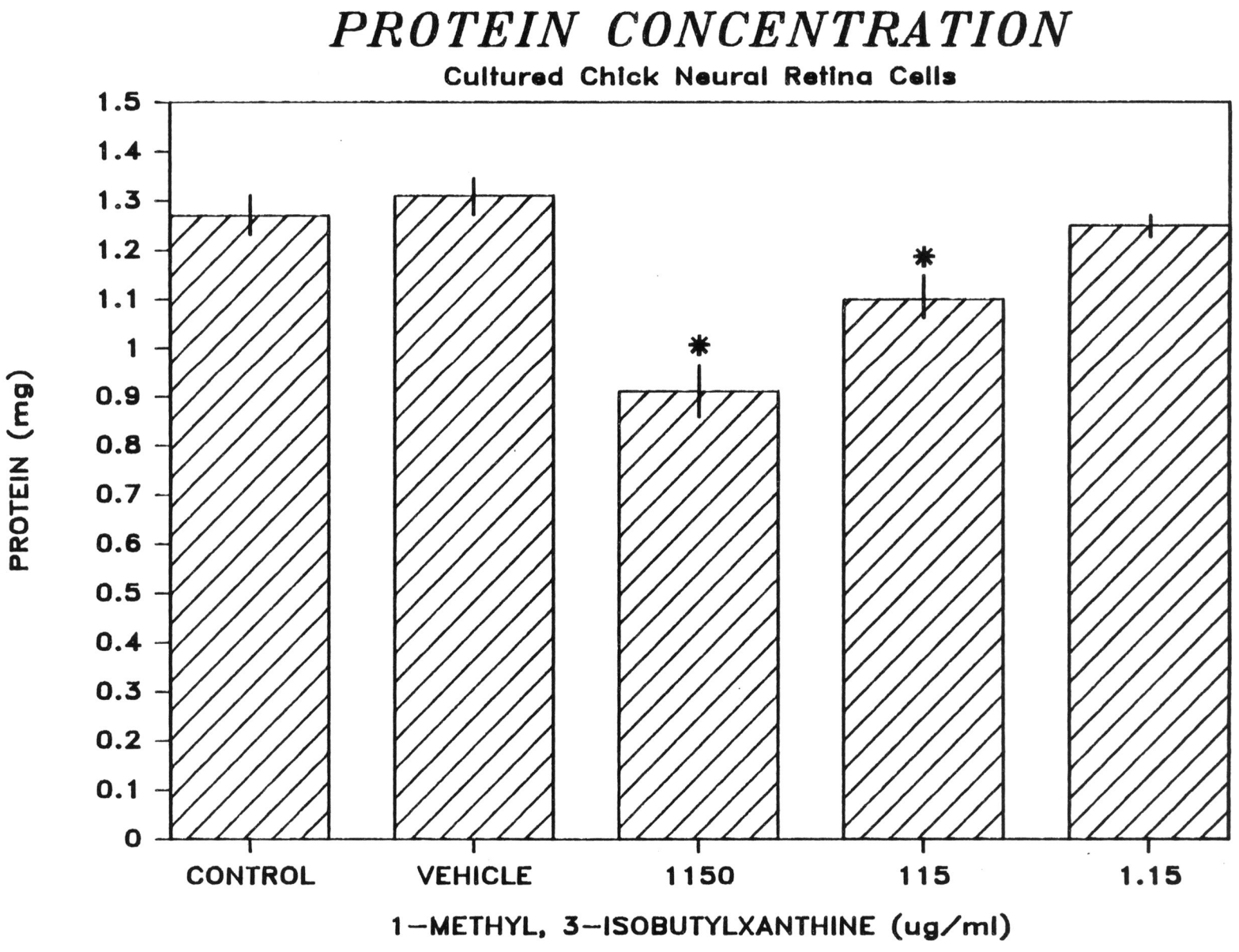
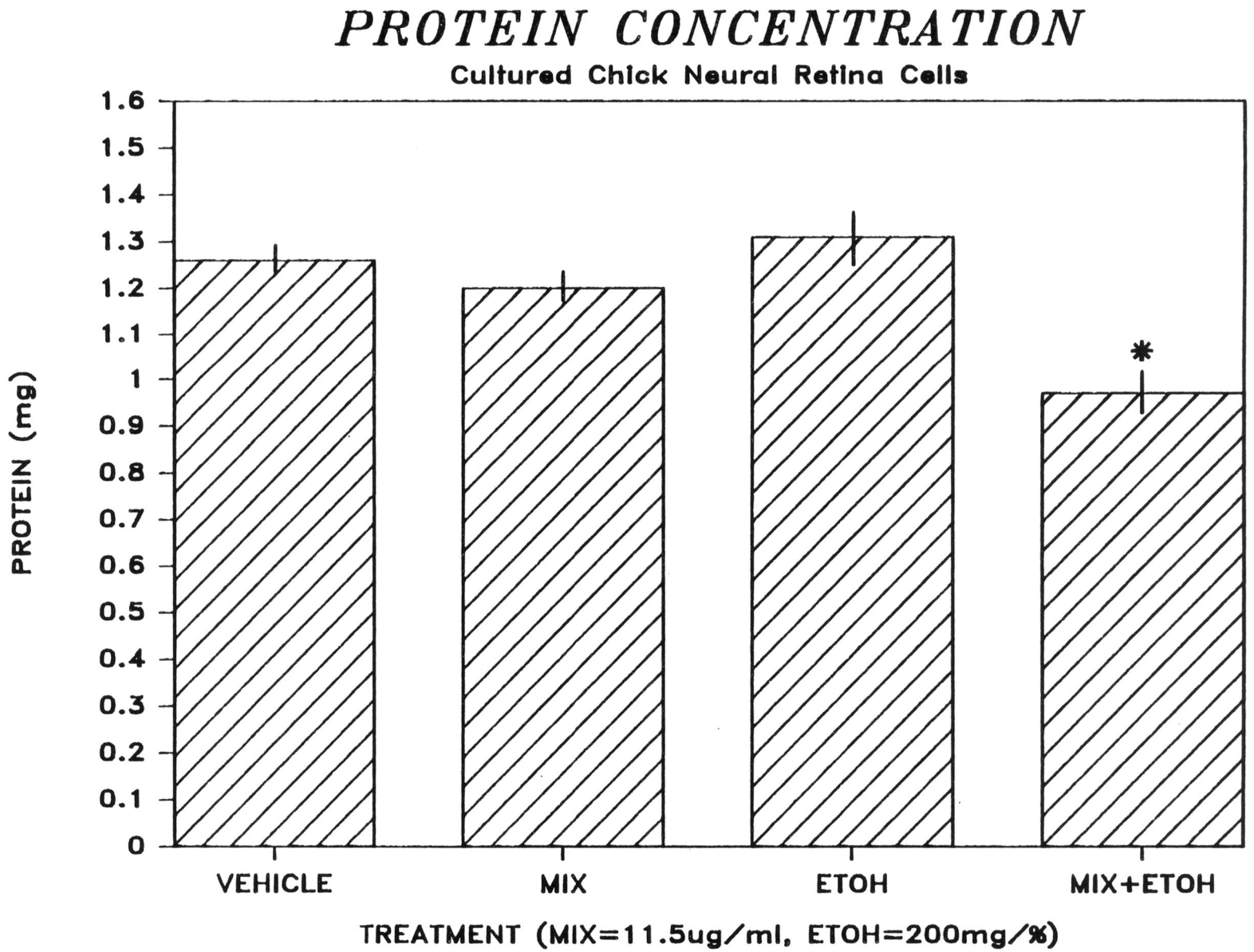

PROTEIN CONCENTRATION
Cultured Chick Neural Retina Cells
PROTEIN (mg)
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
CONTROL
VEHICLE
1150
115
1.15
1-METHYL, 3-ISOBUTYLXANTHINE (ug/ml)

Fig. 5. The influence of 200 mg/dl ethanol (ETOH), 11.5 ug/ml MIX, or ethanol (200 mg/dl) plus MIX (11.5 ug/ml) on the protein content of cultured chick neural retina cells. Astericks indicate significant differences between the means of the experimental group and the vehicle groups. Bars represent plus/minus the SEM.


PROTEIN CONCENTRATION
Cultured Chick Neural Retina Cells
PROTEIN (mg)
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
VEHICLE
MIX
ETOH
MIX+ETOH
TREATMENT (MIX=11.5ug/ml, ETOH=200mg/%)

and MIX (11.5 ug/ml) did significantly reduce the cellular protein (Fig. 6). Although not significantly different, a single experiment indicated that 50 mg/dl ethanol and 11.5 ug/ml MIX reduced DNA content 20% below the vehicle group, paralleling the results of the protein experiments. Lack of statistical significance may be attributed to the low number of samples and large variation within the groups (Fig. 7).

Cyclic AMP radioimmunoassays revealed that high levels of MIX (115 ug/ml) administered on day one increased the cAMP levels of the neuronal cells in primary tissue culture compared with low dose MIX (11.5 ug/ml) or the vehicles (Fig. 8). However, 11.5 ug/ml MIX did not significantly alter cAMP levels relative to the vehicle group (Fig. 8).

When exposed to 50 mg/dl ethanol, the cAMP concentrations in the chick neural retina cells increased significantly over the 11.5 ug/ml MIX or vehicle group. However, there was no significant difference between the 50 mg/dl ethanol or the 50 mg/dl ethanol plus 11.5 ug/ml MIX groups (Fig. 9).

Fig. 6. The effects of 50 mg/dl ethanol (ETOH), 11.5 ug/ml MIX, or 50 mg/dl ethanol plus 11.5 ug/ml MIX on the protein content of chick neural retina cells in culture. Astericks signify statistical differences between the means for the experimental and vehicle groups. Bars represent plus/minus the SEM.

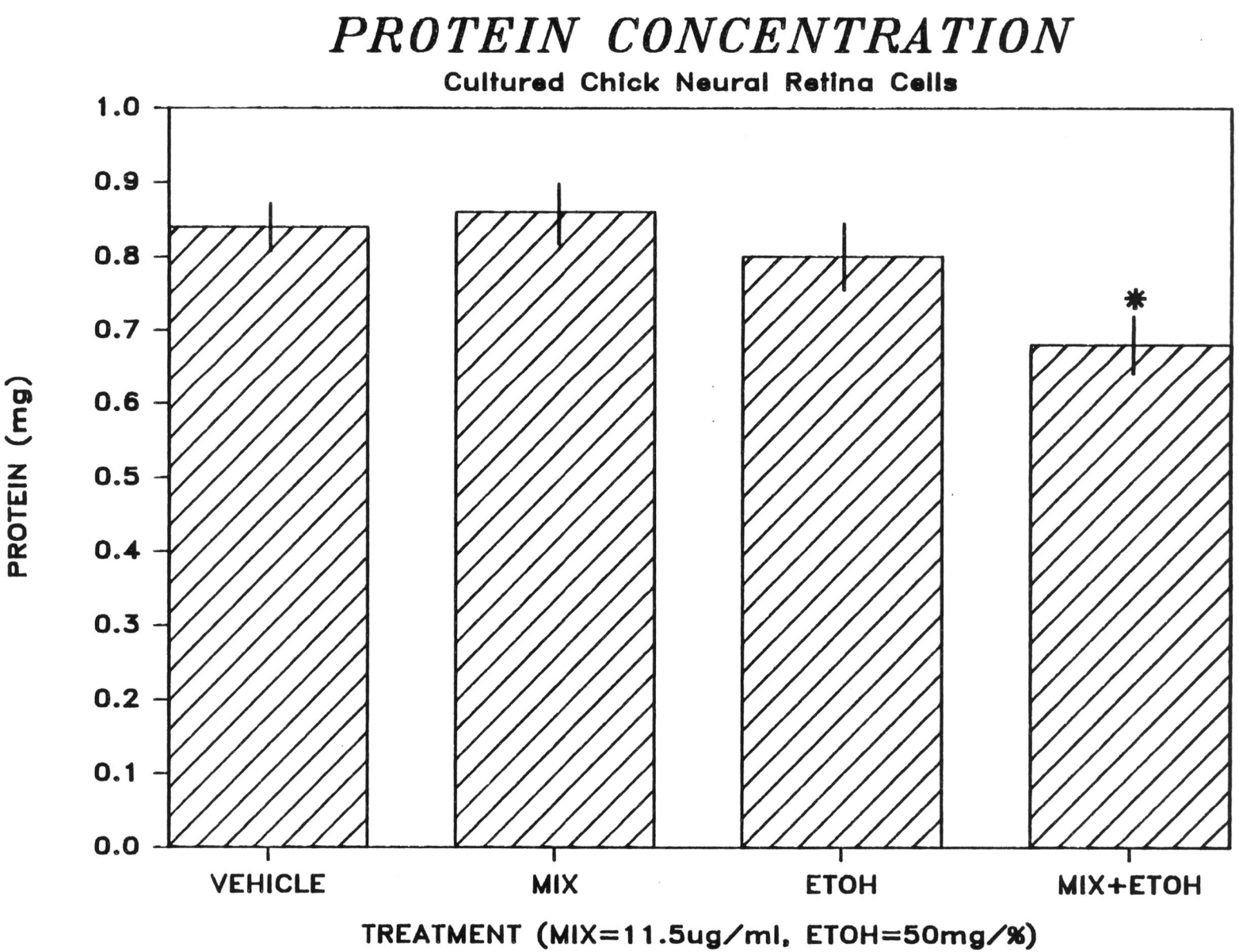

PROTEIN CONCENTRATION
Cultured Chick Neural Retina Cells
PROTEIN (mg)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
VEHICLE
MIX
ETOH
MIX+ETOH
TREATMENT (MIX=11.5ug/ml, ETOH=50mg/%)

Fig. 7. DNA content of the neural retina cells in response to 50 mg/dl ethanol (ETOH), 11.5 ug/ml MIX, or 50 mg/dl ethanol plus 11.5 ug/ml MIX. No significant differences existed between the samples. Bars represent plus/minus the SEM.

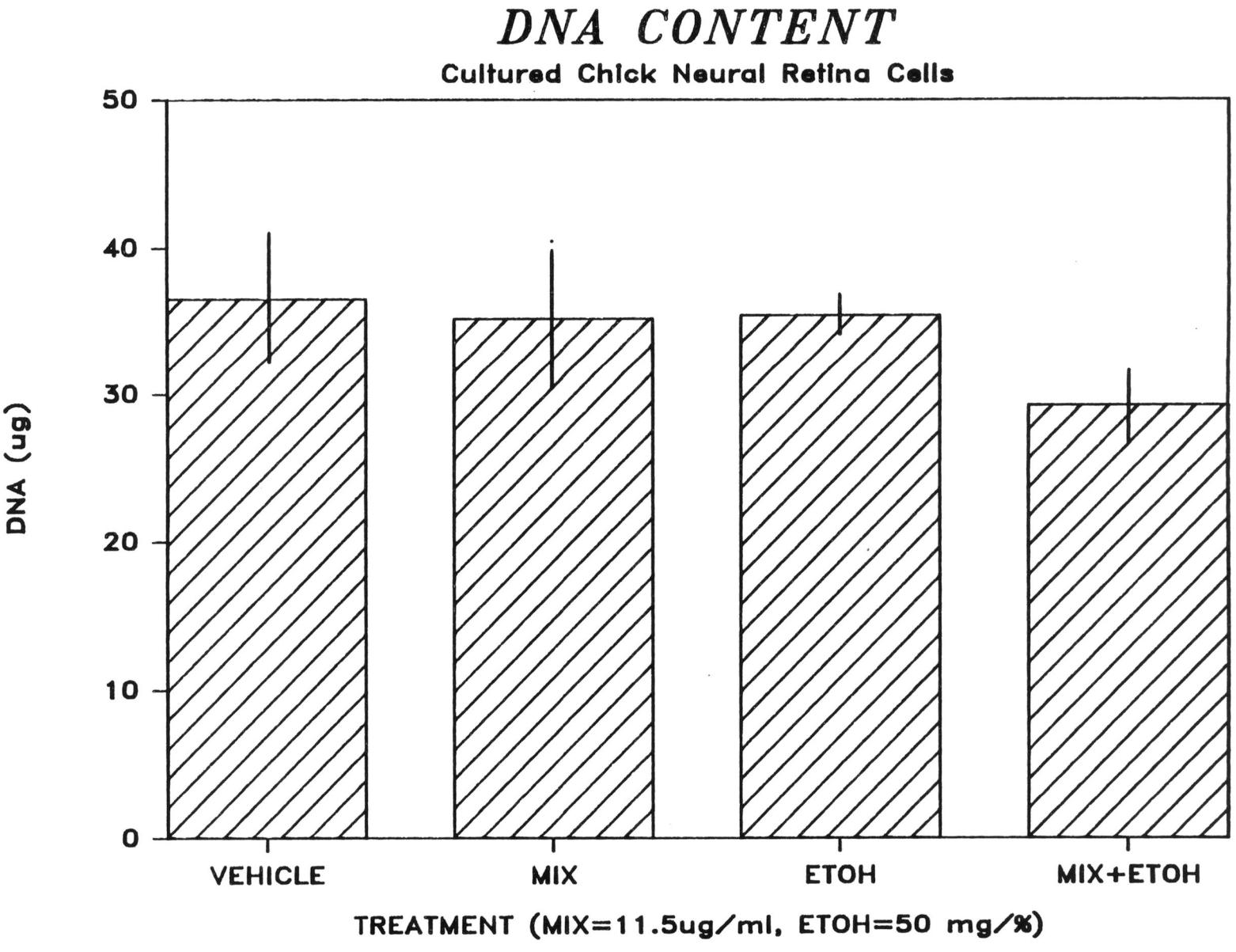

DNA CONTENT
Cultured Chick Neural Retina Cells
DNA (ug)
50
40
30
20
10
0
VEHICLE
MIX
ETOH
MIX+ETOH
TREATMENT (MIX=11.5ug/ml, ETOH=50 mg/%)

Fig. 8. The effects of 115 ug/ml or 11.5 ug/ml MIX on cAMP levels in the cultured neural retina cells. Significant differences between the means of the experimental groups and vehicle group are indicated by astericks. Bars represent plus/minus the SEM.

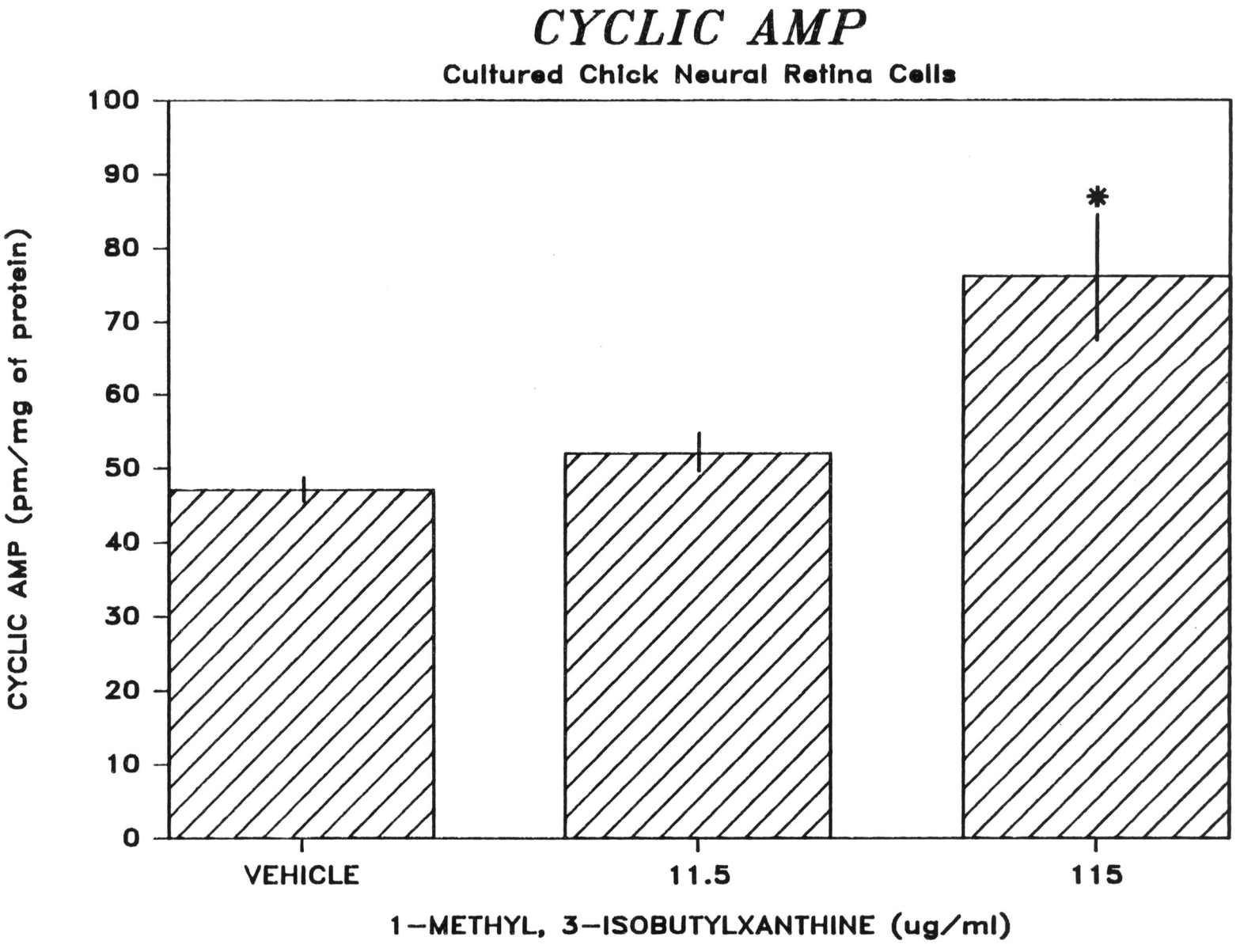
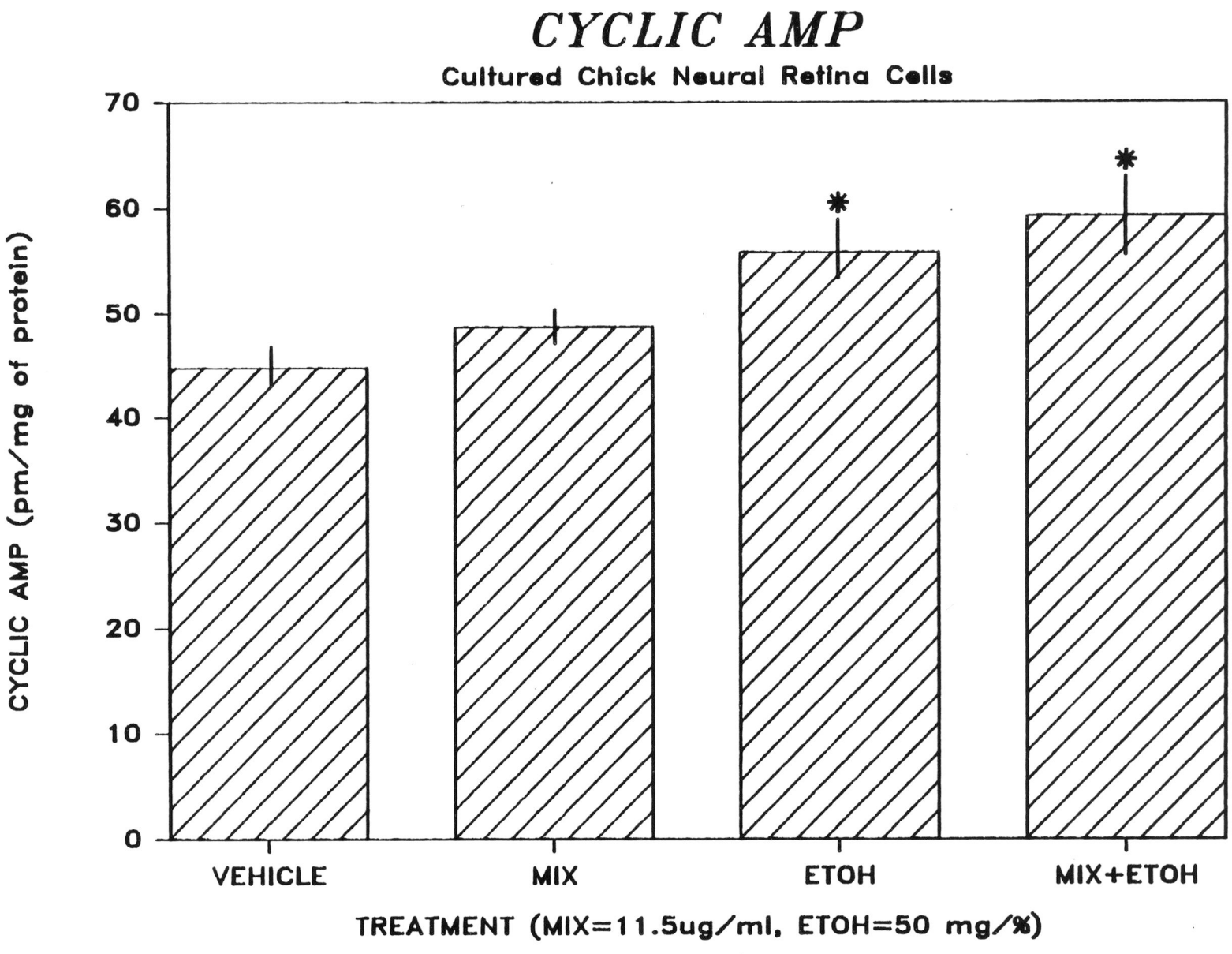

CYCLIC AMP
Cultured Chick Neural Retina Cells
CYCLIC AMP (pm/mg of protein)
100
90
80
70
60
50
40
30
20
10
0
*
VEHICLE
11.5
115
1-METHYL, 3-ISOBUTYLXANTHINE (ug/ml)

Fig. 9. Cyclic AMP concentrations in response to 50 mg/dl ethanol (ETOH), 11.5 ug/ml MIX, or 50 mg/dl ethanol plus 11.5 ug/ml MIX. Astericks indicate significant differences in the means between the experimental and vehicle groups. Bars represent plus/minus the SEM.


CYCLIC AMP
Cultured Chick Neural Retina Cells
CYCLIC AMP (pm/mg of protein)
70
60
50
40
30
20
10
0
*
*
VEHICLE
MIX
ETOH
MIX+ETOH
TREATMENT (MIX=11.5ug/ml, ETOH=50 mg/%)

DISCUSSION

It has been hypothesized that ethanol-induced growth suppression observed in FAS offspring is associated with an in cellular cAMP levels (Pennington, 1985). By affecting cAMP concentrations, ethanol is hypothesized to activate intracellular protein kinases that either directly or indirectly regulate the enzymes responsible for protein and/or DNA synthesis (see literature review). This thesis is an attempt to determine the effects of ethanol on cellular growth and associated changes in cellular cAMP concentrations in chick neural retina cells grown in culture.

The effects of ethanol on the chick neural retina cells were determined by observing three parameters. First, ethanol metabolism was assayed in the neural retina cell cultures to determine if ethanol remained in the systems during the culture period. It was demonstrated that ethanol was not metabolized in the culture system, therefore the results obtained were related directly to ethanol's effects and not an ethanol metabolite (e.g., acetaldehye). Secondly, using a dye-binding method of protein analysis, growth was determined by assaying the total protein content of the cells. Cellular growth were also confirmed by assaying for total DNA content. The data showed that ethanol alone (50 or 200 mg/dl) did not significantly reduce the total protein content of the cells (Figs. 6 and 7).

Furthermore, for a single 50 mg/dl dose of ethanol, DNA content was not significantly affected (Fig. 8). However, when a low level of a phosphodiesterase inhibitor (MIX 11.5 ug/ml) was present in conjunction with the ethanol, growth was significantly reduced (Figs. 6, 7, and 8). Finally, intracellular cAMP concentrations were assayed in response to varying pharmacological treatments. These experiments revealed that ethanol alone (50 mg/dl) with or without MIX (11.5 ug/ml) significantly increased cAMP in the cells.

By assaying ethanol levels remaining after four days of culture, it was concluded that ethanol remained present in the culture system (Fig. 3), indicating that there was no detectable ADH activity in the cultured cells. Therefore, the observations were due to the direct effects of ethanol and not acetaldehyde or other ethanol metabolite(s). The small fluctuations in ethanol levels seen in Figure 3 are attributable to the volatile nature of ethanol and other experimental errors. Wilson (1982) demonstrated that ADH was not detectable in the embryonic chick until the ninth day of development. In the same study, the majority of the early (prior to day 10) ADH activity was in the liver and did not appear in eye tissue prior to day 11.

Having established that ethanol remained present in the neural retina tissue cultures, it was then necessary to determine the effect(s) of ethanol on the growth of the cells. Total protein estimates indicated that ethanol levels of 50 and 200 mg/dl did not significantly affect cell

growth (Figs. 5 and 6). In another study chronic ethanol exposure (80 and 400 mg/dl) failed to inhibit protein synthesis in glial-enriched cell cultures (Davies and Vernadakis, 1984). Finally, protein synthesis was not significantly affected for hepatocytes growth in tissue culture when and obtained from ethanol-intoxicated rats (chronic blood alcohol levels between 200-220 mg/dl).

As an alternate measure for cell growth, DNA levels did not appear to be affected by a 50 mg/dl dose of ethanol (Fig. 8). Freund and Forbes (1976) reported that ethanol at a physiologic concentration (130 mg/dl) actually stimulated incorporation of DNA precursers into primary tissue cultures of rat splenic cells. In cultured glial cells, 81 mg/dl and 400 mg/dl ethanol after four days of exposure, did not significantly affect DNA synthesis (Davies and Vernadakis, 1984). Brain DNA content of 15 day old offspring obtained from rats that were chronically exposed to ethanol, was not significantly different from control groups (Salinas and Fernandez, 1983).

The working hypothesis is that ethanol affects growth via a cAMP mechanism. Therefore, if ethanol increased cAMP concentrations (Fig. 9) but did not inhibit growth, it is possible that high levels of phosphodiesterase could prevent a cAMP-mediated response in the cells. This has been previously established in cultured cells where cAMP analogs induced phosphodiesterase activity in the cells (Schwartz and Passonneau, 1974). Thus, if phosphodiesterase activity

was induced in the neural retina cells in response to increased cAMP levels, it might be necessary to inhibit this activity in order to elicit a cAMP-mediated response. To test this hypothesis MIX, a potent phosphodiesterase inhibitor, was added in low concentrations (11.5 ug/ml) with and without ethanol to inhibit phosphodiestenase in the cells. It was observed that this 11.5 ug/ml concentration of MIX, when added independent of the ethanol, did not significantly increase cAMP (Fig. 8) or affect total protein content (Fig. 4). However, when 11.5 ug/ml MIX was added to cultures containing 50 or 200 mg/dl ethanol, significant reductions in total protein (Figs. 5 and 6) and DNA content were seen.

Associated with this reduction in growth, 50 mg/dl ethanol, not supplemented with MIX, increased cAMP 25% over the vehicle values. Atkinson and co-workers (1977) observed in human lymphocytes that 80 to 4,000 mg/dl ethanol significantly increased cAMP levels over the control groups.

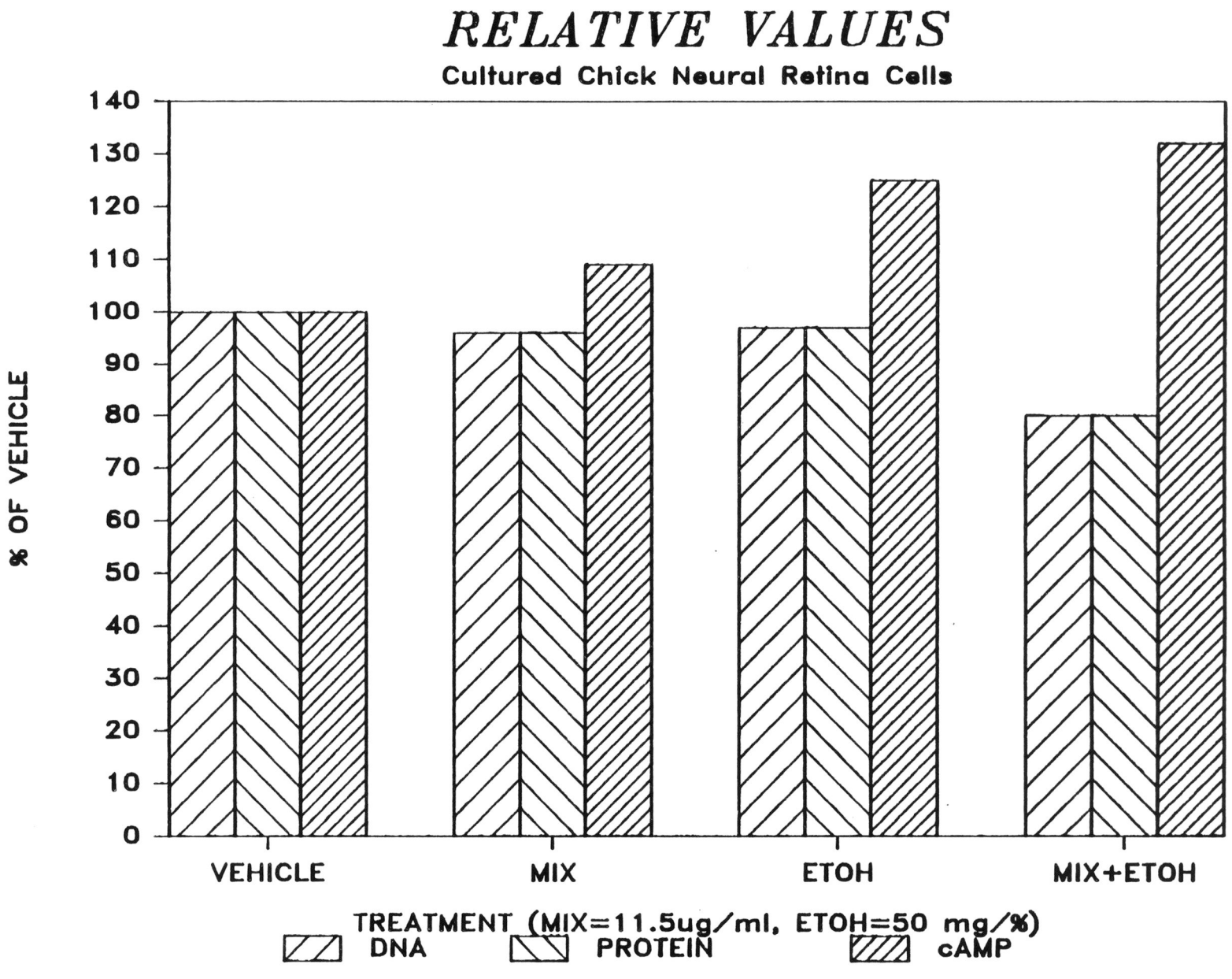
Investigations of chronic ethanol-induced increases in cAMP content comparable to the findings of these present experiments, have been reported in several animal models. These models include: rat striatum (Lucchi et al., 1983); whole embryonic chick brain (Boyd, 1983); and rat cerebrum (Kuriyama and Israel, 1973; Shen et al., 1977).

High levels of cAMP as a result of both ethanol (50 mg/dl) and MIX (11.5 ug/ml) significantly reduced the total protein and DNA content (i.e., growth) of the neural

retina cells (Fig. 10). It has been previously confirmed that high levels of cAMP and cAMP analogs (.5 and 5 mM) significantly reduce protein and DNA synthesis in cultured chick neural retina cells (Kalmus et al., 1982). In a similar study, mouse adrenal tumor cells exposed to either cAMP or disbutyryl cAMP showed reduced thymidine incorporation (Masui and Garren, 1971). In an article reviewing the role of cyclic nucleotides in growth control, Pasten and co-workers (1975) remarked that a rapid decline in cAMP levels was required before quiescent cells have the ability to synthesize DNA.

In conclusion, although significant increases in cAMP occurred as a result of 50 mg/dl ethanol exposure, it appeared that slightly higher levels of cAMP were necessary to elicit a growth inhibition in the neural retina tissue cultures. This was confirmed when the ethanol-dosed (50 mg/dl) cultures were supplemented with MIX (11.5 ug/ml). The protein and DNA content in these cultures was reduced (Fig. 4) although the cAMP levels in this group were only slightly higher than the cultures containing only 50 mg/dl ethanol (Fig. 3). As shown in Figure 10, as the cAMP concentration went up, the corresponding DNA or protein value went down. Therefore, it was concluded that cAMP levels must be maintained at a minimal level in order to elicit a growth response in the chick neural retina cells grown in culture.

Fig. 10. Protein, DNA and cAMP data expressed as a percentage of their respective vehicles. Note that as the cAMP levels increased, the protein and DNA values were down.


RELATIVE VALUES
Cultured Chick Neural Retina Cells
% OF VEHICLE
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
VEHICLE
MIX
ETOH
MIX+ETOH
TREATMENT (MIX=11.5ug/ml, ETOH=50 mg/%)
DNA
PROTEIN
cAMP

SUMMARY AND CONCLUSIONS

The overall objective of this thesis was to establish chick neural retina cells in primary culture for use in ethanol research. The three main parameters observed were ADH activity, growth, and cAMP concentrations in response to ethanol.

It was demonstrated that ethanol was not metabolized in the culture system. Therefore, any effect(s) observed were in response to ethanol not any ethanol metabolites.

Ethanol (either 50 mg/dl or 200 mg/dl) alone does not reduce the growth of cultured chick neural cells. However, ethanol in association with a phosphodiesterase inhibitor (11.5 mg/dl MIX) reduced the total protein of the cells. Similar results were obtained from the DNA data using 50 mg/dl ethanol and/or 11.5 mg/dl MIX.

Cyclic AMP levels responded to a 50 mg/dl dose of ethanol and increased significantly above the vehicle and 11.5 mg/dl MIX groups. Accordingly, ethanol (50 mg/dl) plus 11.5 mg/dl MIX also significantly increased cAMP concentrations over the vehicle and 11.5 mg/dl MIX groups. The cAMP values in relation to the protein data appears to indicate that phosphodiesterase inhibition is required in the system to maintain the cAMP levels.

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APPENDIX I

Composition of Medium 199 (Morgan *et al.*, 1950).

Amino Acid Components	grams/liter
DL-alanine	0.050
L-arginine . HCl	0.070
DL-aspartic acid	0.060
L-cysteine free base	0.0001
L-cysteine . aHCl	0.026
DL-glutamic acid	0.150
L-glutamine	0.100
glycine	0.050
L-histidine . HCl . H_2O	0.025
L-hydroxyproline	0.010
DL-isoleucine	0.040
DL-leucine	0.120
L-lysine free base	0.070
DL-methionine	0.030
DL-phenylalanine	0.050
L-proline	0.040
DL-serine	0.050
DL-threonine	0.060
DL-tryptophan	0.020
L-tyrosine . 2Na	0.05766
DL-valine	0.050

Metals	grams/liter
calcium chloride . $2H_2O$	0.0265
ferric nitrate . $9H_2O$	0.00072
magnesium sulfate	0.100
potassium chloride	0.400
sodium acetate	0.050
sodium chloride	6.800
sodium phosphate monobasic	0.125

Vitamins and Other Components	grams/liter
adenine sulfate	0.010
adenosine triphosphate . 2Na	0.001
adenylic acid	0.0002
alpha tocopherol phosphate . 2Na	0.00001
ascorbic acid	0.00005
biotin	0.00001
calciferol	0.0001
cholesterol	0.0002
choline chloride	0.0005
deoxyridbose	0.0005
folic acid	0.0001
glutathione	0.00005
guanine . HCl	0.0003
hypoxanthine	0.0003
menadione (sodium bisulfate)	0.000016
myo-inositol	0.00005
niacinamide	0.000025
nicotinic acid	0.000025
PABA	0.00005
D-pantothenic acid Ca	0.00001
polyoxyethylene sorbitan monooleate	0.020
pyridoxal HCl	0.000025
pyridoxine HCl	0.000025
riboFlavin	0.00001
ribose	0.00005
thiamine HCl	0.00001
thymine	0.0003
uracil	0.0003
vitamin A acetate	0.00014
xanthine . Na	0.000344

Additional Components	
glucose	1.000
phenol red (indicator)	0.0213

APPENDIX II

Tables of ethanol, protein, DNA and cAMP values.

Table 1. Ethanol levels taken from 200 mg/dl dosed samples of cultured chick neural retain cells over a 4 day incubation period.

DAY OF CULTURE	ETHANOL[a] x (±SEM)	STD. DEV.	n
1	200[b]	-	12
2	193.7 (±11.8)	41.0	12
3	196.6 (±14.7)	51.0	12
4	185.9 (±7.4)	23.3	10

[a] milligrams per deciliter

[b] Cultures were dosed on day 1 of the culture period, therefore assumed to be 200 mg/dl.

Table 2. The effects of 1-methyl,3-isobutylxanthine (MIX) on the protein content of the chick neural retina cells in culture.

TREATMENT	PROTEIN[a] x (±SEM)	STD. DEV.	n
CONTROL	1.27 (±0.041)	0.191	22
VEHICLE	1.31 (±0.032)	0.137	18
MIX (1150 ug/ml)	0.91 (±0.043)[b]	0.179	17
MIX (115 ug/ml)	1.10 (±0.044)[c]	0.183	17
MIX (11.5 ug/ml)	1.25 (±0.018)	0.081	20

[a] milligrams of protein

[b] $P < 0.001$

[c] $P < 0.01$

Table 3. The effects of ethanol (200 mg/dl) and MIX (11.5 ug/ml) on the protein content of the chick neural retina cells in culture.

TREATMENT	PROTEIN[a] x (±SEM)	STD. DEV.	n
VEHICLE	1.26 (±0.049)	0.194	16
MIX (11.5 ug/ml)	1.20 (±0.041)	0.158	15
ETHANOL (200 mg/dl)	1.31 (±0.057)	0.190	11
MIX + ETHANOL	0.97 (±0.028)[b]	0.121	19

[a] milligrams of protein

[b] $P < 0.001$

Table 4. The effects of ethanol (50 mg/dl) and/or MIX (11.5 ug/ml) on the protein content of chick neural retina cells.

TREATMENT	PROTEIN[a] x (±SEM)	STD. DEV.	n
VEHICLE	0.84 (±0.027)	0.114	18
MIX	0.86 (±0.033)	0.140	18
ETHANOL (50 mg/dl)	0.80 (±0.043)	0.160	14
MIX + ETHANOL	0.68 (±0.031)[b]	0.127	17

[a] milligrams of protein

[b] $P < 0.001$

Table 5. The effects of 50 mg/dl ethanol and/or 11.5 ug/ml MIX on the DNA content of the neural retina cells.

TREATMENT	DNA[a] x (±SEM)	STD. DEV.	n
VEHICLE	36.53 (±3.47)	6.93	4
MIX (11.5 ug/ml)	35.18 (±4.08)	8.16	4
ETHANOL (50 mg/dl)	35.40 (±1.49)	2.98	4
MIX + ETHANOL	29.20 (±2.80)	6.86	6

[a] micrograms of DNA

Table 6. Cyclic AMP in cultured chick neural retina cells in response to 11.5 and 115 ug/ml MIX.

TREATMENT	cAMP[a] x (±SEM)	STD. DEV.	n
VEHICLE	47.08 (±2.48)	5.56	5
MIX (11.5 ug/ml)	52.00 (±3.33)	7.45	5
MIX (115 ug/ml)	76.18 (±8.21)[b]	18.36	5

[a] picomoles cAMP per milligram of protein

[b] $P < 0.01$

Table 7. The effects of 50 mg/dl ethanol and/or 11.5 ug/ml MIX on cAMP levels of cultured chick neural retina cells.

TREATMENT	cAMP[a] x (±SEM)	STD. DEV.	n
VEHICLE	44.77 (±2.14)	7.44	12
MIX (11.5 ug/ml)	48.68 (±2.11)	7.31	12
ETHANOL (50 mg/dl)	55.81 (±3.16)[b]	11.40	13
MIX + ETHANOL	59.28 (±3.69)[b]	11.68	10

[a] picomoles cAMP per milligram of protein

[b] $P < 0.01$

Table 8. Protein data for a single experiment where ethanol values were ▷ 200 mg/dl in the chick neural retina cells.

ETHANOL	PROTEIN[a] x (±SEM)	STD. DEV.	n
VEHICLE	1.48 (±0.075)	0.167	5
300 mg/dl	1.61 (±0.059)	0.133	5
400 mg/dl	1.53 (±0.158)	0.315	4
600 mg/dl	1.47 (±0.150)	0.212	2
800 mg/dl	1.58 (±0.015)	0.021	2

[a] milligrams of protein

APPENDIX III

RIA standard curve

Cyclic AMP[a]	CPM[b]	%B/Bo[c]
0.0	9467	100
0.10	7885	83.3
0.25	6411	67.7
0.50	4999	52.8
1.0	3425	36.2
2.0	2498	26.4
4.0	1758	18.6

[a] picomoles cAMP per milliliter

[b] average counts per minute (n = 6 assays)

[c] percentage antibody bound divided by "o" bound