John William Boyd. THE FETAL ALCOHOL SYNDROME: INFLUENCE OF ETHANOL ON CHICK EMBRYO BRAIN cAMP AND PROTEIN KINASE. (Under the direction of Dr. Gerhard W. Kalmus and Dr. Sam N. Pennington.) Department of Biology, July, 1983.

Maternal ethanol consumption during pregnancy is known to be detrimental to embryonic (and/or fetal) growth. A broad range of developmental abnormalities including growth deficiency, central nervous system dysfunction, atypical facial appearances and various other malformations (cardiovascular, renogenital, and skeletal) are characteristic of ethanol's influence on development. This group of malformations is termed the Fetal Alcohol Syndrome (FAS). Although the morphological characteristics of FAS are well defined, the underlying molecular mechanisms responsible for FAS are not understood. The two objectives of this thesis were (1) to develop the chick embryo as a suitable model for study of FAS and (2) to study ethanol's influence on a molecular mechanism involved with normal growth and differentiation. The first objective was accomplished by dosing the chick embryo via the egg airspace and measuring the influence of ethanol on chick embryo growth (weight) at 8, 10, and 12 days of development. Ethanol injected into the egg airspace at the start of incubation and in concentrations of 33 and 65 mg ethanol/100 g whole egg resulted in a dose dependent growth suppression as compared to appropriate controls. Gas chromatographic determinations of ethanol in yolk, albumen and embryo blood indicated that ethanol diffused evenly throughout the egg, was cleared in a linear rate, and was detectable at 12 days of embryonic development. Because ethanol was known to alter fetal hormone levels it was our working hypothesis that the molecular mechanism of ethanol

induced growth suppression might be mediated via the influence of ethanol on a hormone-related intracellular mechanism. This intracellular mechanism involves the nucleotide cyclic adenosine 3',5'-monophosphate (cAMP) and the enzyme protein kinase. The influences of ethanol on cAMP levels, ability of protein kinase to bind cAMP (regulatory subunit of protein kinase) and protein kinase catalytic activity were studied. All biochemical studies were restricted to embryo brain as the tissue source because microcephaly and mental retardation are key characteristics of FAS. Brains were removed from embryos receiving the same ethanol dosages which had previously been shown to induce growth suppression. Ethanol was found to elevate brain cAMP levels in 8 and 10 day chick embryos. Ethanol increased the catalytic efficiency of protein kinase (in the presence of exogenous cAMP) in 8 day embryo brain but did not significantly alter the ability of 8 day chick embryo brain homogenate to bind cAMP. Previous investigators have correlated increased cAMP levels with growth inhibition, but few studies have examined the relationship between ethanol and protein kinase or the influences of ethanol on protein phosphorylation and overall growth. The results of this work support the hypothesis that ethanol does influence a hormone-related intracellular mechanism involved with growth.

THE FETAL ALCOHOL SYNDROME: INFLUENCE OF ETHANOL ON CHICK EMBRYO BRAIN cAMP AND PROTEIN KINASE

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THE FETAL ALCOHOL SYNDROME:

INFLUENCE OF ETHANOL ON CHICK EMBRYO

BRAIN cAMP AND PROTEIN KINASE

by

John William Boyd

APPROVED BY:

SUPERVISOR OF THESIS

Gerhard W. Kalmus, Ph.D.

THESIS COMMITTEE

Sam N. Pennington, Ph.D.

Everett C. Simpson, Ph.D.

W. James Smith, Ph.D.

James L. Smith, Ph.D.

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

Charles E. Bland, Ph.D.

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette, Ph.D.

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DEDICATION

This thesis is dedicated to my grandmother, Mrs. Lillian Payne, and to my aunt, Mrs. Ruby Stokes. I only hope that I have brought as much happiness into their lives as they have into mine. I will love them both forever.

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INTRODUCTION

Maternal ethanol consumption is known to be harmful to the developing embryo and/or fetus. Since the early 1970's maternal abstinence during pregnancy has been recommended as a way to prevent the embryonic and/or fetal teratogenic influences of ethanol (Jones et al., 1973). However, the concept that ethanol could be detrimental towards fetal and embryonic development is not a recent concern surrounding pregnancy. Centuries ago, the Greeks and Romans prohibited drinking of ethanol on their wedding nights to avoid any detrimental influences during conception (Randall, 1977). But it was not until the turn of this century that scientific correlations between ethanol consumption and abnormal fetal development were recorded. This occurred in 1899 when Sullivan reported higher rates of stillbirths and developmental abnormalities among children of alcoholic women as compared to children of nondrinking women.

Curiously, widespread concern within the scientific community about the harm caused by ethanol consumption during pregnancy has only been evident in the last fifteen years (Lemoine et al., 1968; Ulleland, 1972; Jones et al., 1973; Streisguth et al., 1981). Awareness of ethanol's influences during gestation occurred when a similar pattern of abnormal features was recognized among children of chronic alcoholic mothers (Jones and Smith, 1973). These features included growth deficiencies, central nervous system (CNS) dysfunction, atypical facial appearances, and various other malformations (cardiovascular, renogenital, and skeletal). This group of malformations is now termed the fetal alcohol syndrome (FAS), and characterizes the extreme influence of ethanol during gestation (Jones et al., 1973).

FAS may be prevented through maternal ethanol abstinence during pregnancy, but prevention is hindered by the drug dependence effect produced by ethanol (Shaw, 1980). Even if a woman were to abstain from ethanol once her pregnancy was realized, ethanol consumption could influence embryonic development during the developmental time between conception and realization of pregnancy. A rough estimation is that one birth in every 750 (unselected sample of predominantly white, middle class, married women) is diagnosed as suffering from FAS; however, the number of infants that sustain developmental damage from maternal ethanol consumption without manifesting the full syndrome is unknown (Hanson et al., 1978). This frequency for the occurrence of FAS may increase if the current rise in ethanol consumption by women in their reproductive years continues, posing many problems for their potential children (Shaw, 1980).

With the knowledge that maternal ethanol consumption is detrimental toward fetal development, the placental link between mother and fetus has been investigated for its role in FAS. The placenta is much more than an intimate association between mother and fetus. The placenta is a multiple organ system responsible for immunological protection of the fetus, production of hormones involved with the regulation of pregnancy, and transfer of substances essential to the development of the fetus (Dancis and Schneider, 1975). The placental barrier protects the fetus from many substances within the maternal blood supply, but unfortunately the placenta does not act as a barrier to ethanol. In 1899, Nicloux discovered the placenta to be no obstacle for ethanol transfer and autoradiographic methods have since confirmed the equilibrium of ethanol between mother

and fetus through placental transfer (Ho et al., 1972; Akesson, 1974).

When the embryo or fetus is exposed to ethanol via placental transfer FAS may occur, if FAS occurs, the most prevalent characteristic is ethanol induced growth deficiency (Abel and Greizerstein, 1979). This growth deficiency in neonates suffering from FAS is indicated by low birth weight, reduction in length and small head circumference (Ferrier et al., 1973). Although the morphological influences of ethanol are becomming well documented, the molecular mechanisms responsible for FAS remain elusive (Brown et al., 1979).

Much of the experimental work concerning the growth deficiency of FAS has utilized various animal models to understand ethanol's molecular involvement during prenatal differentiation and development (Randall and Riley, 1981). Several animal models had pointed to the dangers of ethanol consumption during pregnancy before recognition of FAS (Combemal, 1888; Stockard, 1910). Combemal exposed gravid female dogs to ethanol resulting in stillbirths and poorly developed litters. Stockard noticed abnormal CNS development when he bathed fish and chick embryos in ethanol. Recently, a variety of animal models has been used to study FAS. These include the chimpanzee (Pieper et al., 1972), the rhesus monkey (Ellis and Pick, 1969), the beagle dog (Ellis and Pick, 1970), rabbits (Schwetz et al., 1978), mice (Kronick, 1976), rats (Pennington et al., 1981), and the chick embryo (Shoemaker et al., 1980).

Animal models used to study FAS have one common disadvantage; few animals are willing to drink ethanol. Dosage of the animal with ethanol to study FAS then becomes a technical problem. Therefore, a variety of

dosage methods have been developed; ethanol has been introduced intravenously (Sandor and Amels, 1971), via gastric intubation (Cannon, 1974), and through inhalation of ethanol vapors (Roach, 1973). However, some animals will voluntarily drink ethanol. Certain strains of inbred rats and mice are widely used to study FAS since they simulate human consumption by willingly drinking ethanol (Friedman and Lester, 1975). Thus, dosage methods and animal models vary depending on the ethanol influence being studied.

This thesis is concerned with the development and use of the chick embryo as a model for the study of FAS. The influence of ethanol on the development of the chick embryo has been studied since the turn of this century. In 1894, Feré exposed hen eggs to ethanol vapors before incubation, the exposure resulted in a broad range of embryonic abnormalities. A typical pattern of CNS disturbances was reported by Stockard (1910) when chick embryos were bathed in an ethanol solution. Sandor and Elias (1968) also studied the effects of ethanol on early stages of chick embryo development and found ethanol to generate early maldevelopment with increased weight loss among ethanol treated animals. The chick embryo was recently employed as an animal model to study FAS when Koda et al. (1980) examined toxic effects of ethanol on the chicken embryo, when Linakis and Cunningham (1980) used the chick embryo to study prenatal ethanol influence on learning, and also in 1980 when Shoemaker et al. utilized the chick embryo to study the influence of ethanol on cerebellar development.

The advantages and experimental ease innate with the chick embryo make it an attractive and worthy model for study of FAS. A major advantage

of the chick embryo is the absence of maternal and placental factors common to mammals. Elimination of maternal and placental influences during gestation removes such variables as maternal nutrition, maternal behavior, maternal metabolism of ethanol and placental involvement with ethanol metabolites. Although maternal and placental metabolism of ethanol are not considered variables with the chick embryo model, the possible influence of embryo ethanol metabolism must be considered. Pennington et al.(1983) have studied ethanol metabolism in the developing chick embryo and their results indicate an inability of the chick embryo to metabolize ethanol prior to day 9 of development. Therefore, prior to day 9 of embryonic development, the influence of ethanol on the developing chick embryo may be attributed to ethanol alone and not ethanol metabolites.

The experimental ease surrounding the use of the chick embryo model for study of FAS is largely due to the egg. By supplying its own internal food source and waste removal, the chick egg eliminates much of the care required by mammals. Embryonic exposure to ethanol is also facilitated by the egg; ethanol can be injected directly into the airspace with dosage levels comparable to human blood ethanol levels (Pennington et al., 1983).

The first objective of this thesis was to realize an ethanol dose that would elicit chick embryo anomalies which characterize certain features of FAS. As mentioned earlier, the most prevalent characteristic of FAS is growth deficiency (Abel and Greizerstein, 1979). Therefore, an ethanol dose that would result in chick embryo growth deficiency was sought. Obtaining an effective ethanol dose involved: (1) determination of appropriate ethanol concentrations, (2) selection of an ethanol vehicle, (3)

choice of an ethanol injection time during development, and (4) evaluation of embryo ethanol levels resulting from injection.

The second objective of this thesis was to study the influence of ethanol on a molecular mechanism thought to be involved with cellular growth and differentiation. It is the working hypothesis of this thesis that ethanol, which induces growth suppression, should also elicit changes in the molecular events associated with growth and development.

Maternal ethanol consumption has recently been reported to influence the endocrine system and alter hormone metabolism in the fetus. Fetal thyroid function (Rose et al., 1981), and prostaglandin metabolism (Pennington et al., 1981) have been reported to be influenced by maternal ethanol consumption. These studies suggest that ethanol-induced alterations on fetal endocrine function may be related to ethanol's influence on fetal growth. Extracellular hormones are products of endocrine tissue and minute changes in hormone levels can drastically alter cellular responses (Tepperman, 1979). If ethanol could alter hormone levels of a developing embryo, the hormonal imbalance might influence embryonic development. The influence of ethanol on a hormone-related intracellular molecular mechanism involved with growth and development was studied utilizing the chick embryo.

The growth and development of embryonic tissues is thought to be dependent upon the sequential action of a number of hormones, some of which utilize the same intracellular messenger to effect the hormone-receptor signal (Zalin, 1975). The intracellular messenger utilized by many hormones is cyclic adenosine 3',5'-monophosphate (cAMP) (Malbon, 1978; Exton, 1972; Wicks, 1974; Wicks et al., 1974). Sutherland et al. (1968)

first discovered cAMP while studying hormonal regulation of glyconeogenesis in the liver. Cyclic AMP is chemically a simple nucleotide that plays a key regulatory role in many biological systems (Robison et al., 1971). The regulatory mechanism of cAMP occurs through cAMP activation of cAMP dependent protein kinase (cAMP-dPK) enzymes, which phosphorylate proteins (Kuo and Greengard, 1969; Krebs, 1972; Walsh and Ashby, 1973; Ogreid and Doskeland, 1980). Protein phosphorylation mediated by cAMP has a variety of important regulatory functions involving cell anabolism, cell division, and neurochemistry. For example, hormone triggered, cAMP mediated phosphorylation is known to regulate fatty acid synthesis (Yeaman and Cohen, 1975; Denton et al., 1975) and is thought to play an important role in polypeptide chain initiation and protein synthesis (Ochoa, 1979).

The phosphorylation of histone proteins is thought to influence cell division and is known to cause structural changes in DNA (Zeilig and Langan, 1980; Langan, 1973). Phosphorylation can modulate histone-DNA interactions and is thought to regulate the rate of cell division (Martinage, 1980). Phosphorylation of histones has also been correlated with mitosis and suggested to be fundamental to cell proliferation (growth) (Block and Atkinson, 1979).

The proteins phosphorylated by cAMP-dPKs in neural and brain tissue may be involved with neural transmission and thought processes. Phosphoproteins are known to regulate proteins at synaptic junctions (Reddington and Mehl, 1979) and to play an important role in memory (Rottenberg, 1979).

Based on the hypothesis that ethanol should elicit changes in the molecular events associated with growth and development, the influences of ethanol on the overall pathway between cellular recognition of hormone

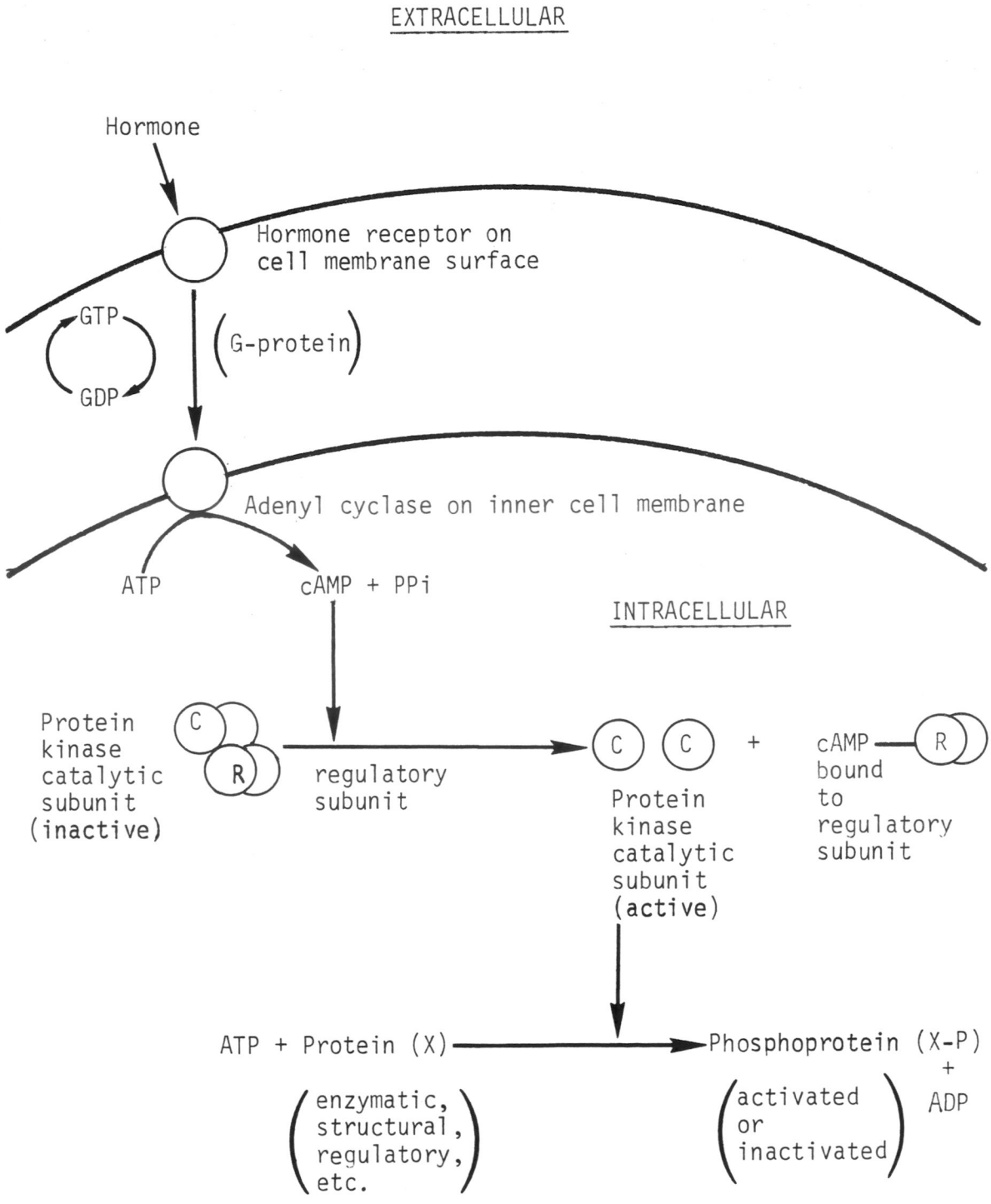
and resulting changes in protein phosphorylation were studied using the chick embryo.

The hormonal control of protein phosphorylation mediated by cAMP involves a biochemical cascade which has specific steps linking external hormonal stimulation of the cell with intracellular regulation via phosphorylation. A synopsis of this cascade type mechanism follows (figure 1).: (1) Hormone interacts with a receptor site of a specific target cell, binding to the cell surface receptor (Harrison and Lunt, 1980). (2) The hormone bound extracellular surface receptor activates adenylate cyclase, an enzyme on the inner cell surface (Lehninger, 1975). Adenylate cyclase is activated indirectly by a guanine nucleotide regulatory protein (G protein) that mediates between the hormone receptor complex and adenylate cyclase (Stryer, 1981). The G protein is activated by guanosine triphosphate (GTP) when a hormone receptor complex exists and deactivated by guanosine diphosphate (GDP) when the complex does not exist (Ross and Gilman, 1980). These events enable a target specific hormone to activate the catalytic subunit of adenylate cyclase within the target cell. (3) Active adenylate cyclase converts adenosine triphosphate (ATP) into cyclic 3',5'-adenosine monophosphate (cAMP) through ester formation with release of inorganic pyrophosphate (Butcher, 1968). (4) Intracellular regulation by cAMP involves protein phosphorylation (Rosen and Krebs, 1981). As described, protein phosphorylation is accomplished by a class of enzymes activated by cAMP, the cAMP-dPKs (Builder et al., 1981).

The cAMP-dPKs are a broad family of enzymes which phosphorylate a variety of substrate proteins (Langan, 1973). The first cAMP-dPK was discovered in rabbit skeletal muscle by virtue of its ability to

FIGURE 1

A representation of how extracellular hormones may regulate intracellular events via protein phosphorylation.


EXTRACELLULAR
Hormone
Hormone receptor on cell membrane surface
GTP
GDP
G-protein
Adenyl cyclase on inner cell membrane
ATP
cAMP + PPi
INTRACELLULAR
Protein kinase catalytic subunit (inactive)
C
R
regulatory subunit
C
C
+
cAMP
R
bound to regulatory subunit
Protein kinase catalytic subunit (active)
ATP + Protein (X)
enzymatic, structural, regulatory, etc.
Phosphoprotein (X-P)
+
ADP
activated or inactivated

phosphorylate and activate the enzyme phosphorylase kinase (Walsh et al., 1968). Shortly after Walsh et al. recognized the first cAMP-dPK in rabbit skeletal muscle, Kuo and Greengard (1969) reported a widespread occurrence of cAMP-dPKs in various tissues of different animals. Early experiments with cAMP-dPKs were carried out with partially purified preparations causing the substrate specificities of cAMP-dPKs to be misleading (Walsh et al., 1968). Homogenous preparations of cAMP-dPKs are now available and some cAMP-dPKs are known to be very substrate specific, they can even distinguish between similar proteins that catalyze the same reaction (Hjelmquist et al.,1974; Edlund et al., 1975). However, some cAMP-dPKs still exhibit a very broad substrate specificity (Weller, 1979). (5) Protein kinases that require cAMP for activation are composed of two subunits; a regulatory subunit and a catalytic subunit (Granot and Kaiser, 1980). When inactive, protein kinase is considered a holoenzyme with regulatory and catalytic subunits intact (Corbin et al., 1975). The inactive regulatory and catalytic subunits of cAMP-dPKs are protein dimers resulting in a tetrameric form of the inactive holoenzyme (Beavo et al., 1974; Rubin et al., 1974).

Activation of, and sequential protein phosphorylation by the cAMP-dPKs is initiated by intracellular cAMP binding to the regulatory subunits of protein kinase (Walsh and Ashby, 1973). Once activated by cAMP, the tetrameric holoenzyme dissociates to form one regulatory cAMP bound dimer and two free catalytic (phosphotransferase) subunits (Ogreid and Doskeland, 1980). The free catalytic subunits of cAMP-dPKs are responsible for phosphorylation of substrate proteins usually at serine residues (Rudolph and Krueger, 1979). The source of the incorporated phosphate is the terminal

(gamma) phosphate of adenosine triphosphate (ATP) (Corbin and Reimann, 1974).

This thesis research attempted to show that if ethanol influenced cAMP content in the developing chick embryo brain then it would follow that the cAMP mediated aspects of the previously outlined growth related cascade mechanism should also be influenced (i.e., cAMP-dPK should also be altered as a result of ethanol induced growth suppression). Previous *in vitro* studies have related changes in cAMP levels with growth inhibition (Kram *et al.*, 1973, Kalmus *et al.*, 1982) and *in vivo* studies have shown that ethanol can alter tissue levels of cAMP (Weitbrecht and Cramer, 1980). These studies suggest a link between ethanol induced growth suppression and cAMP levels in various tissues. Although the relationship between growth inhibition and cAMP is becomming clear in the literature, there are few, if any, studies which correlate growth inhibition with changes in protein kinase (regulatory or catalytic subunit) levels.

The research described in this thesis studied the influence of ethanol on levels of cAMP, cAMP binding protein (protein kinase regulatory subunit) and protein kinase catalytic activity in the developing chick embryo. All biochemical studies were restricted to a single embryonic tissue, brain. Brain tissue was chosen as the tissue of study since microcephaly and mental retardation are key characteristics of FAS (Jones and Smith, 1975). By restricting biochemical assays to a single tissue the meaning of any ethanol induced alterations could be better interpreted as compared to whole animal or multiple organ system interpretation of the results. However, studies measuring ethanol's influence

on growth utilized whole embryos and these embryos were the source of brain tissue.

It is the intention of this thesis to correlate changes in overall embryonic growth with alterations in both brain cAMP content and brain protein kinase (regulatory and catalytic subunit) levels. By studying the influence of ethanol on these intracellular components of molecular growth and differentiation a better understanding of how ethanol influences embryonic development may be achieved.

METHODS AND MATERIALS

Injection of Eggs

Unincubated fertile Black Sex Linked hen eggs were obtained from Newman's Hatchery in La Grange, North Carolina. Eggs were stored at 10°C upon receipt until time of use (within five days).

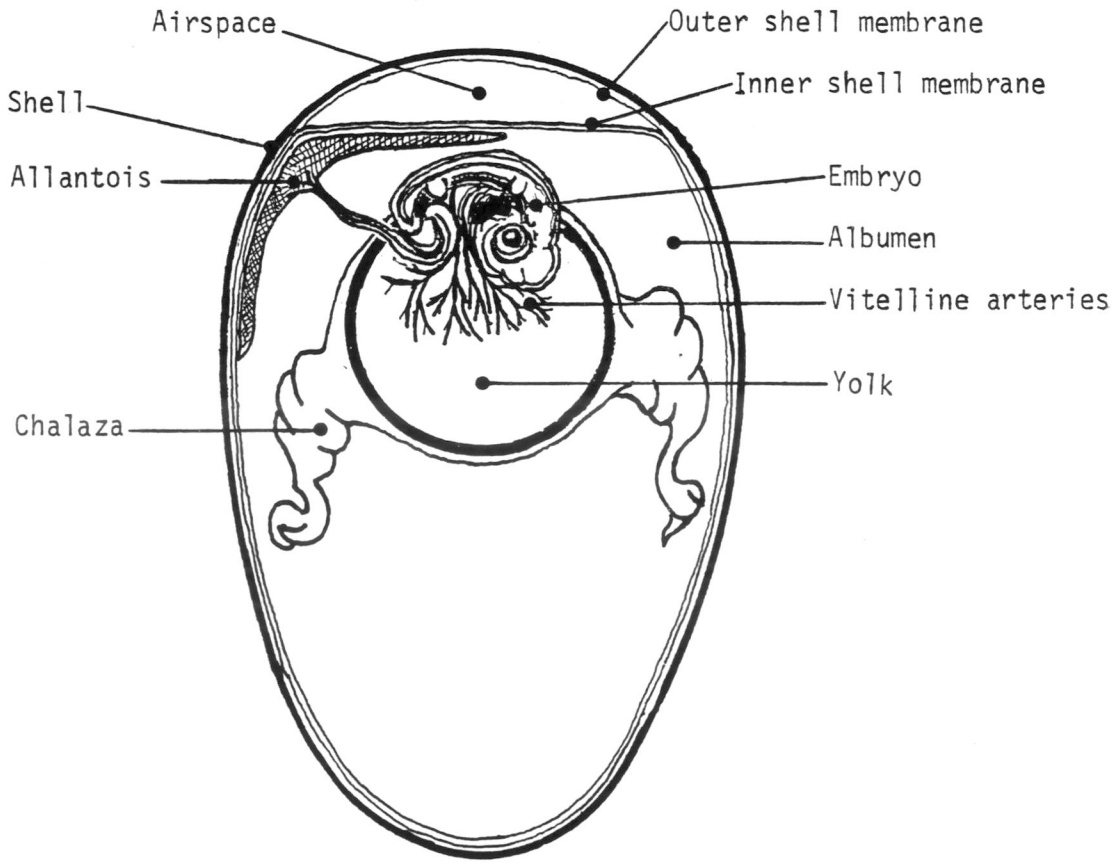
Eggs were selected at random, sequentially numbered, and weighed within a tenth of a gram before incubation. Egg weight was known to influence embryo weight (Romanoff, 1967) thus variation in egg weights was recorded. Variation in egg weights was also considered when expressing the dose levels administered to each embryo. Experiments consisted of two groups of ethanol treated animals (a high dose and a low dose) and one group of control animals, all randomly selected. Ethanol treated animals received ethanol in a solution of chick Ringer's, while control treated animals received only chick Ringer's.

Dosage of the animals was accomplished via injection of the ethanol solution or vehicle solution into the egg airspace (Figure 2). Each experiment utilized a single injection per egg prior to incubation except for the experiments designed to maintain a constant level of ethanol within the egg during incubation. In these experiments, multiple injections were delivered throughout development.

Eggs were injected by puncturing the egg shell with a sharp-pointed probe, making a small hole at the apex of the egg blunt end (directly above the airspace). The pointed tip of the probe was held in a 70% solution of ethanol in chick Ringer's between usages to prevent cross contamination between eggs. A constant volume of 200 microliters (μl) of ethanol or vehicle solution was injected into the airspace of each

FIGURE 2

Representation of an incubated egg containing an eight day developed chick embryo.


Airspace
Outer shell membrane
Inner shell membrane
Shell
Allantois
Embryo
Albumen
Vitelline arteries
Yolk
Chalaza

egg using a 250 µl gastight syringe with a 26 gauge, 3/8 inch disposable needle. The needle of the syringe was allowed to only enter 1/2 centimeters (cm) into the egg. Immediately after injection, the injection site was sealed with hot paraffin applied with a cotton swab. The paraffin was then allowed to cool and harden. A minimum amount of paraffin was used to seal the injection site so as not to disturb the egg airspace gas exchange between embryo and atmosphere. After being sealed, each egg was placed on a plastic holding tray to keep the airspace in an upright position. To prevent contamination, the needles used for injection were sterilized prior to each experiment by autoclaving in petri dishes; this allowed recycling of needles. The same syringe was used during each experiment and the syringe was autoclaved between experiments. Fresh solutions of ethanol and vehicle for injection were prepared for each experiment in autoclaved, 25 ml Erlenmeyer (E)-flasks fitted with septum stoppers. The chick Ringer's solution was autoclaved after preparation (Appendix A). Autoclaved chick Ringer's (20 ml) was decanted into the control E-flask and sealed with a sterilized rubber septum stopper. Into one of the remaining sterile E-flasks was placed 17.5 ml of chick Ringer's, the other E-flask received 15.0 ml of the sterile Ringer's. These two E-flasks were both brought to a 20.0 ml volume with 95% ethanol (analytical reagent grade) resulting in 12.5 and 25% solutions of 95% ethanol in chick Ringer's by volume. The two E-flasks containing ethanol solutions were then sealed.

A standard means of expressing the dose per animal weight was sought. The average egg weighed 60 grams with an overall standard error of 3.0 grams (Appendix B). This weight varied depending on the time of year;

eggs were smaller in the spring of the year and larger in the fall. Variation in egg weights between dosage groups was the same since selection of eggs for all dosage groups was a random process. Based on a density of 0.781 gm/ml, the 200 µl injection volumes of 12.5 and 25% ethanol solution contained 19.5 mg and 39.1 g of ethanol respectively. The dose of ethanol was expressed as mg ethanol per 100 g whole egg. Therefore, 19.5 mg ethanol/60 ± 3 g egg was 32.5 ± 1.7 mg ethanol/100 g egg and 39.1 mg ethanol/60 ± 3 g egg was 65 ± 3.0 mg ethanol/100 g egg. For ease of expression, the two ethanol doses were referred to as 33 and 65 mg ethanol/100 g egg. The eggs dosed only with chick Ringer's were referred to as vehicles (control animals).

Incubation of the three injected groups within each experiment began at the same time. All eggs were incubated in the plastic holding trays previously described. Eggs were incubated at 37.5°C with 60-70% humidity by a forced air incubator and turned once a day until the developmental stage to be studied was attained.

Stages of development were determined by comparing external features of the dosed chick embryos with a complete developmental series of uninjected embryos day 1 through 20 or 21 (hatching). The complete developmental series was staged according to Hamburger and Hamilton (1951).

Ethanol Determinations

At various stages of embryonic development, embryo and egg ethanol content were determined by gas chromatography. Embryo ethanol levels were measured within embryo blood, internal egg levels were measured within yolk and albumen.

All samples for ethanol determinations were taken from eggs immediately after being removed from incubation. Embryo blood samples were obtained from the omphalomesenteric artery. To sample embryo blood, the following method was used: 1) With the egg held blunt end up, the egg shell surrounding the egg airspace was removed. 2) The shell membrane was removed, exposing the living embryo. 3) While the chick embryo heart was beating a pointed scalpel severed the omphalomesenteric artery. 4) A plastic conical container fashioned from a needle cap was held beneath the bleeding artery to obtain the embryo blood. 5) A heparin coated calibrated capillary tube was used to withdraw 50 µl aliquots from the concial container. The 50 µl blood aliquot was delivered into a 400 µl Beem capsule, sealed and kept cold (4°C) before preparation for gas chromatography. Yolk and albumen samples (0.5 ml) were removed from eggs containing viable embryos using 1.0 ml tuberculin syringes (no needle). Yolk and albumen samples were sealed and kept cold (4°C) in 5 ml screw top vials before preparation for gas chromatography.

Egg yolk, egg albumen and embryo blood ethanol content was measured by gas chromatography. Chromatography was performed on a Hewlett Packard 5730A gas chromatograph coupled to a Varian CDS 111 integrator. Standard curves were generated for varying ethanol levels, using propanol as an internal standard. A standard curve was prepared for each determination of ethanol content. Preparation of each standard curve was accomplished as follows: 1) The propanol stock solution was made by pipeting 15.0 ml of reagent grade 1-propanol (propanol) into a 500 ml volumetric flask and bringing to volume with distilled water. From this stock propanol

solution a working propanol solution was prepared in a volumetric flask. 2) An ethanol stock solution was made by diluting 3.16 ml of 95% reagent grade ethanol to 100 ml with distilled water in a volumetric flask. The working solutions of ethanol 0.250 ml, 0.500 ml, 1.00 ml, 2.00 ml, and 3.00 ml aliquots of stock ethanol were diluted to 100 ml with distilled water in separate volumetric flasks. These dilutions of stock ethanol resulted in standard ethanol concentrations of 1.2, 2.4, 6.0, 12.0, 23.7, 47.4, and 71.1 mg ethanol/100 g of distilled water in each volumetric flask of working ethanol solution. 3) Each ethanol standard was made by combining 1 part of an ethanol working solution (0.5 ml of one of the various standard ethanol concentrations) to 3 parts of the propanol working solution (1.5 ml of working propanol solution). This resulted in various ethanol concentrations and equal propanol concentrations for each known standard. The standards (1.2 - 71.1 mg ethanol/100 g distilled water) were kept cold (4°C) and sealed while awaiting analysis.

The experimental samples of blood, yolk, and albumen were also combined with propanol. Like the standard samples 1 volume of blood, yolk, or albumen was mixed with 3 volumes of propanol. This corresponded to 50 µl blood mixed with 150 µl propanol, 0.5 ml yolk mixed with 1.5 ml propanol, and 0.5 ml albumen mixed with 1.5 ml propanol.

Embryo Weights

The growth of the chick embryo is directly related to chick embryo weight (Romanoff, 1960; Romanoff, 1967). Thus, whole embryo wet weights of control and treated animals were compared to determine if embryonic growth was influenced by treatment with ethanol. Three stages of embryonic development (8, 10, and 12 days) were studied. Within each

developmental stage three dosage groups were used (vehicle control, 33 mg ethanol/100 g egg, and 65 mg ethanol/100 g egg). A dose-response relationship was expected if ethanol exhibited an influence on embryo growth within a developmental stage.

Each chick embryo was removed from the egg and the weight recorded using the following procedure: 1) The egg was held blunt end up. 2) The egg shell surrounding the egg airspace was removed. 3) The shell membrane covering the embryo was removed. 4) Using forceps the embryo was held gently by the neck and slowly removed from the egg. 5) All extra embryonic membranes were removed from the embryo and all blood supplies were separated by removal of the umbilicus. 6) The embryo was weighed to the nearest one-hundreth of a gram on a tared and calibrated balance. 7) After weighing and recording the embryo wet weight, the egg which contained the embryo was examined for egg number and the egg number was recorded. 8) The embryo was then used experimentally or discarded.

To assure no experimental bias between the recording of embryo weights and the assessment of treatment, the injected eggs were randomly shuffled before embryos were removed to eliminate pooling of animals with the same treatment. Further, the egg number which corresponded to treatment was not read until after the respective embryo was weighed.

Embryo Brain Biochemical Measurements

Brain Removal. The following method was used to obtain all embryo brains used for biochemical measurements. Embryos to undergo brain removal were removed from the egg and weighed as previously described. The embryo weight and treatment was then recorded. As the embryos were sacrificed each embryo was placed into an aluminum cup with several perforations

in the floor of the cup. These aluminum containers were previously numbered and each embryo was assigned a container number. The entire process of embryo removal, weighing, recording, and placing into a container took no longer than 10 seconds per embryo.

Once the embryo was within the container, the edge of the aluminum container was held with hemostats and the container with embryo was submerged into liquid nitrogen. The embryo was kept submerged for at least 10 seconds to insure complete and thorough freezing. Frozen embryos were stored at -80°C in the same labeled aluminum container until brain dissection was performed.

Whole embryo brain was dissected away from the embryo. Dissection involved the use of a sharp scalpel to shave frozen tissue away from embryo brain, brain tissue could be easily differentiated from other cephalic embryo tissue. Dissection was accomplished within a refrigerated room (5°C) with the frozen embryo on dry ice.

After removal, each brain was weighed on an analytical balance to the nearest milligram. Weighing of brains was done in a refrigerated room (5°C) and brains were continuously kept on dry ice to preserve frozen brain, except when on the balance pan. After each brain was weighed, it was wrapped in aluminum foil, labeled, and stored at -80°C until an aspect of brain biochemistry was studied.

Radioimmunoassay of cAMP. The amount of cAMP within whole embryo brain was measured by radioimmunoassay (RIA). Brain cAMP levels were determined using a double antibody RIA kit purchased from New England Nuclear (Boston, Massachusetts). The New England Nuclear RIA kit was

adapted from the procedures of Steiner et al. (1972).

A standard curve was prepared with each new experimental determination of unknown cAMP levels. Eight levels of cAMP were prepared as standard values and each point on the curve was the average of duplicate values. Concentrations of cAMP ranged from 25.0 picomoles (pmol) to 0.10 pmol. These concentrations were made by serial dilution of a 5000 pmol of cAMP/ml solution in 0.05 Molar sodium acetate buffer, pH 6.2. Disposable borosilicate glass test tubes (5 ml) received 100 µl of the various cAMP standard concentrations. After the cAMP standards were added, a 100 µl solution of radioactive cAMP (Succinyl cAMP tyrosine methyl ester-[^{125}I] or simply [^{125}I]-cAMP) in 1.0% normal rabbit serum (NRS) was added to each standard tube. Then, a 100 µl aliquot of antiserum complex (pre-reacted first and second antibody in 0.1 M sodium phosphate buffer, pH 6.2) was added to each standard tube. As the test tubes with known cAMP concentrations were prepared three other duplicate sets of test tubes were prepared. These were the blank tubes (received only 200 µl of sodium acetate buffer) to measure possible background radiation, the total counts tubes (received only 100 µl of the cAMP-I^{125} in 1.0% NRS) to measure maximum possible activity and efficiency of antibody binding to form antigen-antibody complexes, and the zero standard tubes (received only [^{125}I]-cAMP with NRS, antiserum, and buffer).

All tubes except the total counts tubes were mixed, using a vortex mixer (vortexed). All tubes were then incubated 16-18 hours at 2-8°C. After incubation, one ml of sodium acetate buffer (0.05 M, pH 6.2) was added to all tubes except the total counts tubes (these were set aside until counting). Tubes were then vortexed and centrifuged for 15 min at

2000 x g. Supernatant was decanted into a radioactive waste container and all test tubes were counted in a Beckman Gamma-4000 counter with a counting time of one minute per tube; an instrument blank was included.

Each set of counts from duplicate test tubes was averaged. Average counts from the blank tubes and instrument background counts were substrated, resulting in net counts per tube. The "0" standard net counts represented maximum counts bound to antibody. The "0" standard resulted in 40-50% of [^{125}I-cAMP] actually binding to antibody (maximum actually bound) however, this was set to 100% binding as a reference for maximum possible antigen bound. Based on 100% binding of "0" standard all net counts were expressed as normalized percent bound or $\%B/B_0$:

$$\%B/B_0 = \frac{\text{average net counts}}{\text{average net counts of "0" standard x 100}}$$

Semilog paper was used to plot $\%B/B_0$ versus cAMP known standard concentrations. Unknown values of cAMP were measured by determining $\%B/B_0$ and then interpolating the cAMP concentration from the standard curve.

Values of cAMP were determined in chick embryo brain for two developmental stages, i.e., eight and ten days of development. Within each developmental stage, embryos were dosed as described above to measure the influence of ethanol dose on embryo brain cAMP levels. Embryos were sacrificed, weighed, brains removed and embryos stored as described earlier.

Each frozen brain was homogenized with a ground glass homogenizer (1 g/20 ml) in 7% perchloric acid ($HClO_4$). Homogenization was performed in a refrigerated room (5°C) and after homogenization all solutions were kept on ice. An aliquot of each homogenate was neutralized for immuno-

assay within two hours. Each sample homogenate was neutralized by adding 3 M potassium carbonate (K_2CO_3) in 0.5 M triethanolamine (TEA). The addition of K_2CO_3 in TEA resulted in a pH of 6.2 for each $HClO_4$ brain sample (as measured by close range calibrated pH paper). The neutralized brain samples were kept on ice until sampled (within one hour). Duplicate 100 µl aliquots were taken from each neutralized brain homogenate. The 100 µl brain samples containing an unknown amount of cAMP were treated exactly as standard 100 µl samples of known cAMP used to generate a standard curve (discussed above). The counts from each experimental tube were expressed as $\%B/B_0$ and cAMP concentration in the samples was determined by interpolation from the standard curve. Brain cAMP levels were expressed as picomoles (pmol) of cAMP per gram of whole brain.

Assay of Protein Kinase Enzyme Activity. The effect of ethanol on protein kinase activity in the chick embryo brain was determined. Embryos were dosed and brain was removed and stored as discussed earlier. One developmental stage was studied (8 days) with three embryo treatment groups (vehicle, 33 mg ethanol/100 g egg, 65 mg ethanol/100 g egg) within the developmental stage.

The influence of ethanol on both binding (regulatory) subunits and phosphorylating (catalytic) subunits of protein kinase was studied. Measurement of the ability of the regulatory subunit of protein kinase to bind cAMP and measurement of the catalytic subunit to phosphorylate protein required two distinct assays, each with its own set of conditions. Both assays were performed on each embryo brain.

Since binding and phosphorylation assays required different sets of conditions and both assays were performed using a single brain, the initial brain homogenate was the sample source for both assays. Individual frozen embryo brains were homogenized with a glass-teflon homogenizer (1 g/3 ml) in 40 mM phosphate buffer, pH 6.5, containing 0.3 mM ethyleneglycol-bis (β-aminoethylether) N_1N'-tetraacetic acid (EGTA), 30 mM sodium fluoride (NaF) and 1.0 mM theophylline using a glass-teflon homogenizer. A portion of this initial brain homogenate was frozen at -80°C for the binding assay and the remaining portion was retained on ice to be used for the phosphorylation assay.

Measurements of the phosphorylating ability of protein kinase were accomplished by modifying the methods of Miyamoto et al. (1969) and Gilman (1970). The phosphorylation assay involving protein kinase was based on the transfer of the gamma phosphate of ATP into substrate protein by the catalytic subunit of protein kinase. The gamma phosphate of ATP was labeled with ^{32}P and the incorporation of phosphate from ATP into substrate protein resulted in ^{32}P labeled protein. By measuring the incorporation of ^{32}P into protein from [γ-^{32}P]ATP the catalytic activity of protein kinase was assessed.

The phosphate brain homogenates were used in the phosphorylation assay within 2 hrs after homogenization. The initial homogenates were diluted with 40 mM phosphate buffer, pH 6.5 containing 0.3 mM EGTA, 30 mM NaF, 1.0 mM theophylline, and 10 mM $MgCl_2$. The final dilution was 1 g/40 ml buffer based on the initial brain weight.

Each homogenate was assayed in triplicate and an assay blank was run for each brain. Therefore, each brain experimental determination of

protein phosphorylation consisted of three experimental tubes to measure enzyme activity and three blank tubes as a reference.

Borosilicate test tubes received 100 µl of 40 mM phosphate buffer (pH 6.5) which contained 0.3 mM EGTA, 30 mM NaF, 1.0 mM theophylline, 10 mM $MgCl_2$, 25 µM cAMP, 100 µM ATP, 5 mg/ml histone type IIA, and 1 microCurie (µCi) of [γ-^{32}P] ATP with a specific activity of 10.0 Curies/millimole. Blank tubes received 2.0 ml of 5% trichloroacetic acid (TCA) after the addition of [γ-^{32}P] ATP.

After adding the brain homogenate (200 µl) the reaction mixture (experimental and blank) was incubated for 10 minutes at 37°C. The experimental reaction was terminated by adding 2.0 ml of 5% TCA to only the experimental tubes. All tubes were then centrifuged for 10 min at 500 x g. The supernatant was decanted into a radioactive waste container. The resulting pellet was washed and resuspended with 2.0 ml of 5% TCA. After the resuspension, each sample was again pelleted and washed three times in 5% TCA. The final pellet in each tube was resuspended in 1.0 ml of 1.0 normal (N) potassium hydroxide (KOH) and transferred to a 5 ml mini vial for counting of radioactive ^{32}P. Before counting, the KOH suspension of protein was further diluted in the mini vials by adding 4.0 ml of distilled water.

Cerenkov counting was employed to measure ^{32}P incorporation (Rapkin, 1973). Triplicate blank and experimental counts were averaged. Each blank value was subtracted from the respective experimental value resulting in net counts per sample of embryo brain.

The net counts for each 10 min incubation were expressed as net counts per min (CPM). The phosphorylating ability of protein kinase was

expressed in two ways; femtamoles (fmol) [γ-^{32}P] transferred/min/mg brain protein or pmol [γ-^{32}P] transferred/min/gram embryo brain. When expressing moles of [γ-^{32}P] transferred from [γ-^{32}P]ATP the following formula was used:

$$\frac{\text{net CPM}}{\text{reaction time x specific activity of ATP x counting efficiency}} = \frac{\text{moles } [\gamma\text{-}^{32}\text{P}] \text{ transferred}}{\text{min}}$$

Cerenekov counting efficiency was determined by comparing CPM from 10 µl aliquots of ^{32}P solubilized in 5 ml distilled water (Cerenekov counting) or 5 ml liquid scintillation cocktail (8.25 g 2,5-diphenyloxazole (PPO), 25.0 mg 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP) in 1000 ml of toluene). Liquid scintillation counting of ^{32}P solubilized in cocktail was assumed to be 98% efficient (Brown, 1971). Cerenekov counting efficiency equaled Cerenekov CPM divided by cocktail CPM multiplied by 100.

Embryo brain protein was determined by the method of Lowry et al. (1951) by sampling directly from the (1 g/40 ml) brain homogenate.

After the enzymatic phosphorylation of embryo brain protein was measured, the ability of the regulatory subunit of protein kinase to bind cAMP was studied. Protein kinase affinity for cAMP was measured by modifying the methods of Gill and Walton (1979) and Gilman (1970). The binding of cAMP to protein kinase was assayed using radioactively labeled cAMP ([^{3}H]-cAMP) and measuring the binding of [^{3}H]-cAMP by brain protein under appropriate conditions.

The initial phosphate homogenate, stored frozen at -80°C, was the source of chick embryo brain protein kinase used in the protein kinase

binding assay. The homogenates were kept no longer than four days at -80°C before being used. The initial brain homogenates (1 g/3 ml in buffer) were thawed, kept on ice, and vortexed immediately before use. An aliquot of each brain homogenate was diluted with 150 mM sodium acetate buffer, pH 4.0 to yield a 1 g/6 ml homogenate (based on original brain weight). The final pH of this 1 g/6 ml homogenate was 4.0 (as measured by close range calibrated pH paper). This homogenate was used as the protein source for the binding assay. All binding assays were performed in borosilicate disposible test tubes and each brain was assayed in triplicate.

Each assay tube received 25 µl of 150 mM sodium acetate buffer, pH 4.0, containing 20 mM $MgCl_2$ and 3 x 10^{-7} M [^{3}H]-cAMP (specific activity = 35 Ci/mmole). To each tube containing buffer and [^{3}H]-cAMP, an 80 µl aliquot of the appropriate homogenate was added. Addition of homogenate initiated the binding reaction. The reaction mixture (buffer, [^{3}H]-cAMP, and homogenate) was incubated for one hour at 0°C. The reaction was stopped by adding 2.0 ml of 50 mM sodium acetate buffer to each tube. After termination, the reaction mixture was decanted onto a GN-6 Gelman Metricel membrane filter (0.45 µM pore size, 25 mm in diameter). The membrane filter was held in place by a Hoeffer apparatus. The Hoeffer apparatus allowed ample reservoir for buffer and permitted aspiration of the reaction mixture through the membrane filter. Each filter was subjected to a vacuum of not more than 15 inches of mercury. The suction produced by the Hoeffer apparatus drew the buffer and unbound [^{3}H]-cAMP through the membrane filters. Brain protein (and protein bound [^{3}H]-cAMP) remained bound to the cellulose fibers of the filters. After

decanting the initial reaction mixtures onto the appropriate membrane filters and applying vacuum, the assay tubes were each washed twice with 2.0 ml aliquots of 50 mM sodium acetate buffer and decanted onto the previously suctioned membrane filter. After filtering, each membrane was placed in a 5.0 ml mini vial and allowed to air dry.

When dry, 5.0 ml of liquid scintillation cocktail (same as described previously) was added to each membrane filter in the mini vials. Vials were then sealed and the bound [^{3}H]-cAMP trapped on the filters was counted.

The binding of [^{3}H]-cAMP to embryo brain protein was expressed in two ways; pmoles cAMP bound/mg protein and pmoles cAMP bound/g brain. The conversion of counts to moles cAMP was accomplished using the same method mentioned earlier for determining moles protein phosphorylated from [γ-^{32}P] counts.

The efficiency of counting ^{3}H in liquid scintillation cocktail was determined by comparing the CPM with the disentigrations per minute (DPM) of a ^{3}H sample with known DPM. Efficiency of counting ^{3}H in cocktail equaled CPM divided by DPM multiplied by 100. Liquid scintillation counting of ^{3}H and ^{32}P employed a Beckman LS-233 counter using preset windows for each radiolabeled nucleotide.

Embryo brain protein was determined by the method of Lowry et al. (1951) by sampling directly from the 1 g/6 ml brain homogenate.

Purified cAMP-dPK from rabbit skeletal muscle was used to verify binding and phosphorylation activity in response to purified enzyme. This was accomplished following the discussed procedures for both assays except purified protein kinase was used instead of brain tissue.

Chemicals and Reagents

The following chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri: rabbit muscle protein kinase, equine muscle ATP, calf thymus histone type IIA, theophylline, and sodium fluoride. Radiolabeled nucleotides [γ-^{32}P]-ATP, [^{3}H]-cAMP, and [^{125}I]-cAMP (as part of the previously mentioned RIA kit) were purchased from New England Nuclear, Boston, Massachusetts. Ethanol was purchased from the Chemistry Department of East Carolina University, Greenville, North Carolina. All other chemicals and reagents used in this study were purchased from Fisher Scientific Company, Raleigh, North Carolina.

Statistical Analysis

The influence of ethanol on chick embryo weight was determined by comparing weight differences between ethanol and vehicle treated animals. Three developmental periods were studied. Each developmental period was statistically analyzed separately to determine if ethanol did influence embryo weight at that stage of development. Numerous individual experiments were performed with exactly the same procedures within each developmental period. This allowed a very large number of animals to be studied at each stage of development. The statistical analysis system (SAS) at Triangle University Computing Center (TUCC) in Research Triangle Park, North Carolina was used to determine statistical differences concerning embryo weight and dosage. A two way analysis of variance determined if a difference in embryo weights existed between different developmental stages or animals receiving different treatments (ethanol or vehicle). For each developmental stage, differences in treatment means were analyzed by one way analysis of variance in conjunction with Duncan's Multiple

Range Test.

Standard curves for ethanol and brain protein determinations were expressed as straight lines obtained from least-square analysis. Unknown ethanol concentrations were measured by determining the ratio of ethanol to propanol within the sample and interpolating ethanol concentration from the standard curve. Protein standard curves consisted of known protein (bovine serum albumen) content plotted along the abscissa and optical density (spectrophotometrically obtained) plotted along the ordinate. Unknown protein content was determined by measuring the sample optical density and interpolating protein content from the standard curve.

One way analysis of variance was utilized to determine possible brain biochemical differences between chick embryos dosed with ethanol or vehicle solutions. If there was a significant biochemical difference between ethanol and vehicle dosed animals the Newman's-Keuls Multiple Range Test was employed.

In all analyses, statistical significance was assumed if $P < 0.05$.

RESULTS AND DISCUSSION

Because growth deficiency is the most prevalent characteristic of FAS (Abel and Greizerstein, 1979), the first objective of this thesis was to determine an ethanol dose that would elicit growth deficiency in the chick embryo model. The influence of ethanol on chick embryo growth had previously been studied by several researchers with different methodologies, leading to differing results. For example, Sandor and Elias (1968) injected ethanol in the airspace of eggs to study ethanol's influence on development. They injected a 500 µl volume of 60% ethanol in saline into 60 g eggs (400 mg ethanol/100 g egg) and noted that ethanol produced embryo growth retardation and a variety of physical malformations during embryonic development. Sandor and Elias also reported a 50% mortality rate after 8 days of incubation with ethanol injected eggs. In contrast, Shoemaker et al. (1980) and Koda et al. (1980) did not find ethanol induced growth retardation when embryo growth was measured at hatching. Shoemaker et al. dosed the embryo via the airspace on day 4 of embryonic development and then studied the dosed embryos within 24 hr after hatching. Their dose was a single 160 µl injection of 25% ethanol in water (volume to volume) per egg airspace (53 mg ethanol/100 g egg). Shoemaker et al. determined that egg airspace injection volumes above 320 µl produced a high mortality rate during embryonic development, which may explain the high incidence of mortality reported by Sandor and Elias who used a 500 µl injection volume. Koda et al. also dosed the embryo via the airspace but varied injection time (day 0 through day 16) and ethanol concentrations (4-600 mg ethanol/100 g egg) while

maintaining an injection volume similar to Shoemaker et al. Using these conditions, Koda et al. found ethanol to be most toxic when administered between days 1 to 4 of incubation.

By comparing the results of Sandor and Elias, Koda et al. and Shoemaker et al. inference can be made that ethanol induced chick embryo growth deficiencies may be elicited if ethanol is injected into the egg airspace before incubation and embryos are studied before hatching. Further, a high incidence of mortality can be avoided if the injection volume does not exceed 320 µl.

This thesis studied the influence of ethanol on embryo growth by dosing chick embryos via the egg airspace immediately before incubation and measuring embryo growth before hatching. A single injection volume was used (200 µl) with three dosages 0 mg ethanol/100 g egg (vehicle), 33 mg ethanol/100 g egg, and 65 mg ethanol/100 g egg (as previously described).

At each developmental stage studied (8, 10, and 12 days) ethanol exposure influenced the growth (weight) of the developing embryo (Figures 3, 4, 5, Table I) and the ethanol injections did not increase embryo mortality (Table II) as compared to vehicle controls. Embryos treated with 65 mg ethanol/100 g egg weighed significantly less than controls at days 8 and 10 (Figures 3 and 4, Table I). However, at 12 days of development both the 33 and 65 mg ethanol/100 g egg treated embryos weighed significantly less than the vehicle controls (Figure 5, Table I). At longer developmental periods the influence of ethanol on embryo growth was dose dependent (Figures 3, 4, 5, and 6, Table I). Pennington et al. (1983) have investigated the cell division time of

FIGURE 3

Ethanol influence on chick embryo growth at 8 days of development. Numbers within bars represent number of embryos. Significant difference between weights of ethanol and vehicle dosed embryos represented by an asterisk.

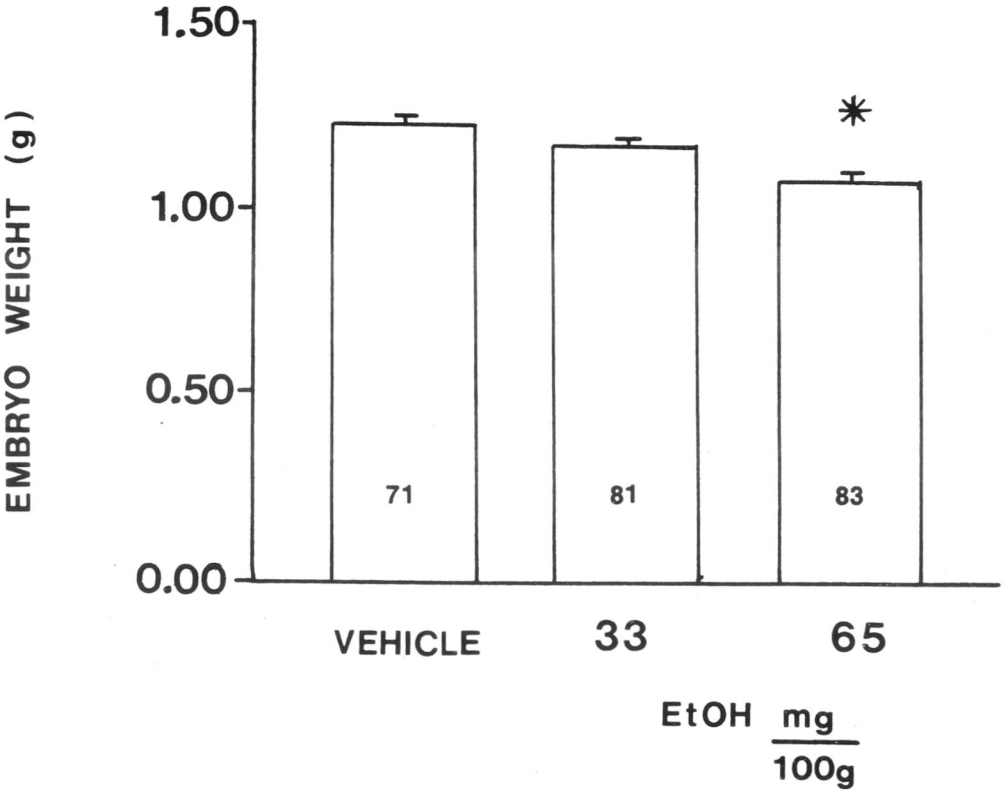

EMBRYO WEIGHT (g)
1.50
1.00
0.50
0.00
71
81
83
*
VEHICLE
33
65
EtOH mg/100g

FIGURE 4

Ethanol influence on chick embryo growth at 10 days of development. Numbers within bars represent number of embryos. Significant difference between weights of ethanol and vehicle dosed embryos represented by an asterisk.

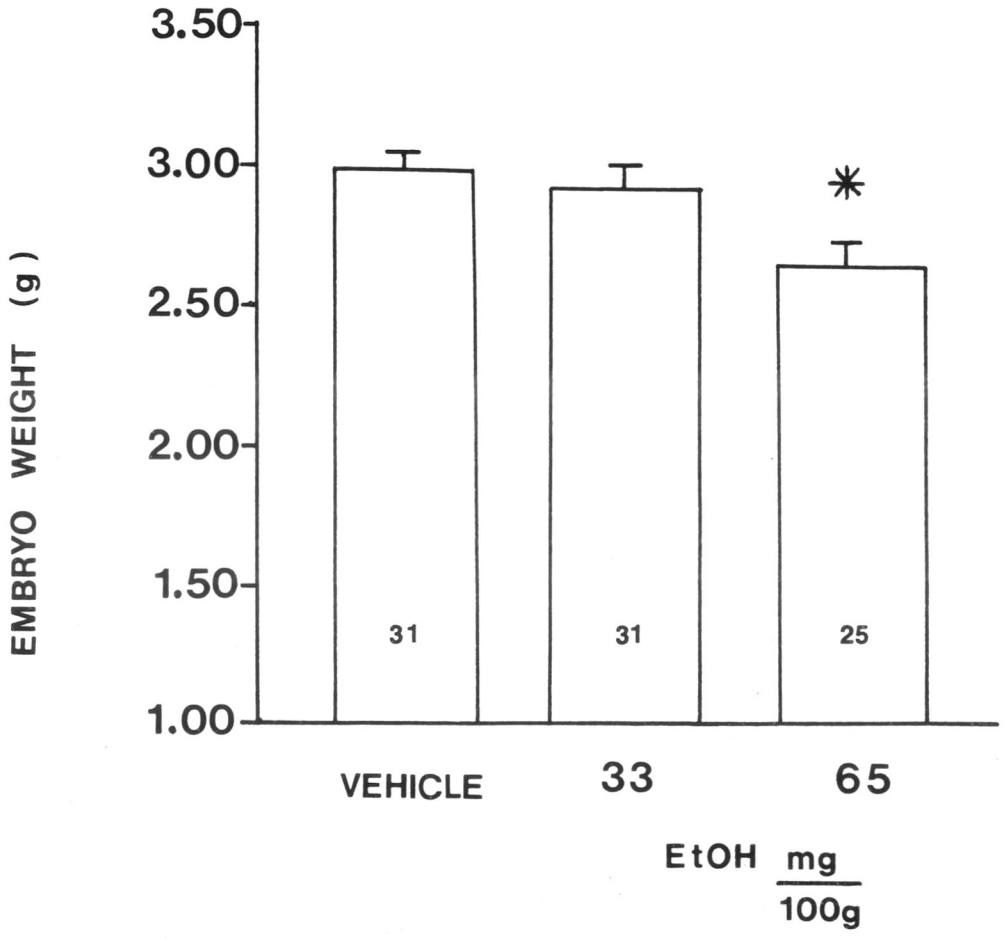

3.50
3.00
2.50
2.00
1.50
1.00
EMBRYO WEIGHT (g)
31
31
25
*
VEHICLE
33
65
EtOH mg/100g

FIGURE 5

Ethanol influence on chick embryo growth at 12 days of development. Numbers within bars represent number of embryos. Significant differences between weights of ethanol and vehicle dosed embryos represented by an asterisk.

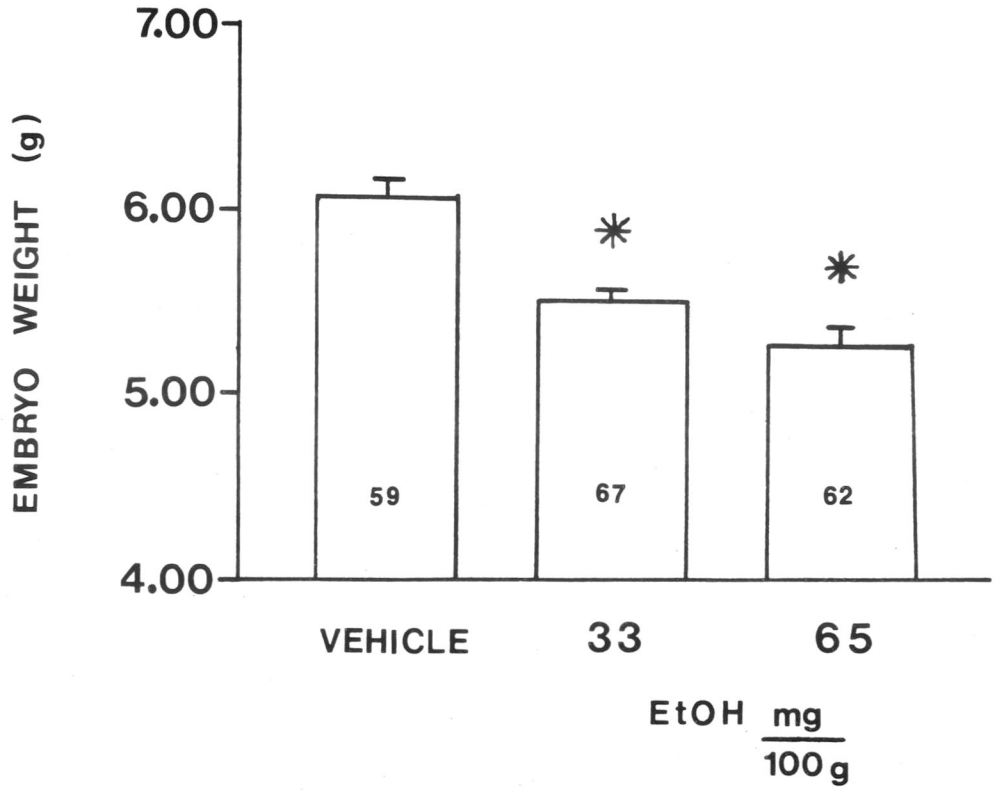

7.00
6.00
5.00
4.00
EMBRYO WEIGHT (g)
59
67
62
*
*
VEHICLE
33
65
EtOH mg/100g

FIGURE 6

Ethanol influence on chick embryo growth at 8, 10, and 12 days of development. Each point represents a mean value. This graph is an expression of the data in Table III.

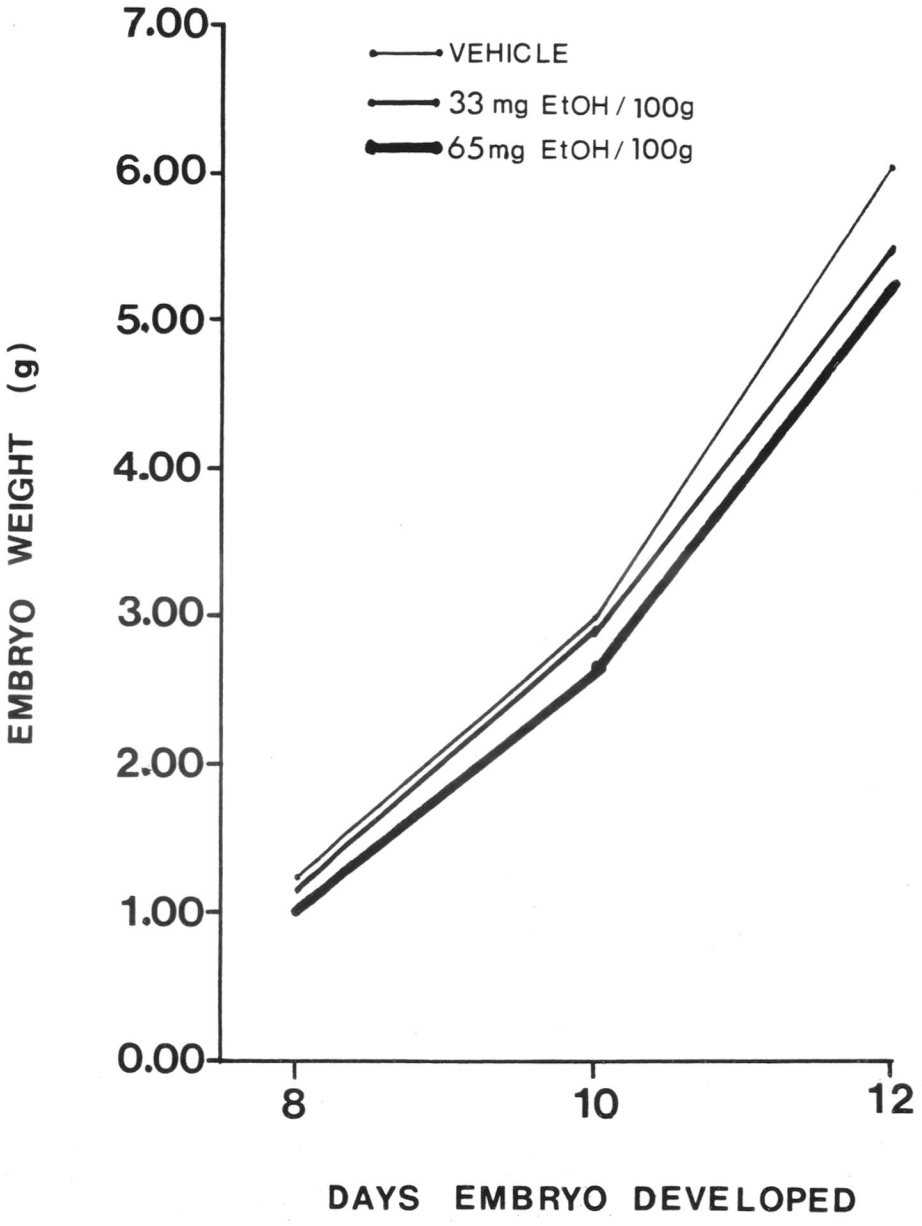

7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
EMBRYO WEIGHT (g)
VEHICLE
33 mg EtOH / 100g
65mg EtOH / 100g
8
10
12
DAYS EMBRYO DEVELOPED

Table I. Chick embryo wet weights (grams)[1]

Treatment	8 Day	10 Day	12 Day
Vehicle	1.23±0.02 (N=71)	2.99±0.07 (N=31)	6.06±0.12 (N=59)
33 mg etOH/100 g egg	1.17±0.02 (N=81)	2.92±0.09 (N=31)	5.50±0.11* (N=67)
65 mg etOH/100 g egg	1.09±0.02* (N=83)	2.68±0.09* (N=25)	5.27±0.14* (N=62)

[1] Each value represents mean ± S.E.M., three developmental periods vs. three dosages.

* Statistically different from vehicle control values ($P < 0.05$).

Table II. Comparison of viability between control and ethanol treated embryos.[1]

Treatment	Number of Embryos Examined	Number of Viable Embryos[2]	Percentage Viable
Vehicle	60	53	88%
33 mg ethanol/100 g egg	62	50	81%
65 mg ethanol/100 g egg	68	57	84%

[1] Eggs injected only once before incubation, all embryos were studied after 12 days of incubation.

[2] Viability was defined as an embryo with a heartbeat.

chick embryos developed 12 days and dosed in the same way as described in this study. They reported that ethanol decreased the number of cell divisions in ethanol treated embryos without altering cell size. Therefore, the ethanol treated embryos are probably smaller than controls due to fewer cells.

After appropriate ethanol doses were found to elicit embryo growth deficiency, the second objective of this thesis was addressed. This objective was to use the chick embryo as a model to study the mechanism by which ethanol could influence embryonic growth, i.e., the molecular mechanisms involved with retardation of cell growth and division. In order to quantitate these studies, the relationship between ethanol dosage and egg ethanol levels needed to be determined. Therefore, while the dosage administered into the egg airspace was known, the ethanol dosage reaching the embryo was uncertain.

Ethanol content in yolk, albumen, and embryo blood, resulting from ethanol injected into the airspace at the start of incubation, was compared throughout embryonic development. Egg albumen and egg yolk ethanol levels resulting from dosages of 33 mg ethanol/100 g egg into the airspace were found to be the same (Figure 9, Table III). Therefore, the diffusion of ethanol through the inner shell membrane into the albumen appeared to result in an equilibrium of ethanol content between egg albumen and egg yolk. Egg yolk and embryo blood ethanol levels were also found to be the same (Table IV). Inference can thus be made that the internal levels of ethanol within the egg are the same within albumen, yolk, and embryo blood. This inference is strongly supported by studied proving that physiological membranes

are not a barrier to ethanol (Akesson, 1974; Ho et al., 1972).

A single injection of ethanol at the start of incubation was cleared at a linear rate from the egg yolk and albumen (Figures 7, 8, 9, and 10; Tables III and IV). Repeated injections of ethanol prior to day 12 of embryonic development maintained a constant level of ethanol within the egg (Figure 11, Table V). Nonetheless, neither single nor multiple injections of ethanol could sustain detectable levels of ethanol within the egg past 12 days of development (Figures 7-11, Tables III-V). Eggs reinjected until 12 days of embryonic development contained 21-27 mg ethanol/100 g egg during the first 12 days of development but despite an injection of 65 mg ethanol/100 g egg on day 12, the egg contained no measurable amount of ethanol on day 13 (Table V). This rapid clearance of ethanol from the egg after day 12 was thought to involve ethanol metabolism by the embryo. Metabolism of ethanol by the developing embryo was considered in a separate thesis (Wilson, 1982). Wilson studied the chick embryo's ability to metabolize ethanol by measuring the levels of ADH within the chick embryo. This study indicated an inability of the embryo to metabolize ethanol via ADH before day 9 of embryonic development, and reported a logarithmic rise in embryo ADH activity after day 9 of embryonic development. The rapid clearance of ethanol between days 12 and 13 was thus attributed to metabolism of ethanol via ADH.

Once the relationship between ethanol dosage and egg (embryo) ethanol levels was determined, the influence of ethanol on the previously described hormone mediated growth control mechanism was studied. This hormone mediated growth regulatory mechanism was of interest because

FIGURE 7

Egg yolk ethanol clearance during embryonic development, resulting from a single preincubation injection of ethanol, arrow. After initial, subsequent points represent gas chromatographic determinations, each representing the mean determined in duplicate ± S.E.M.

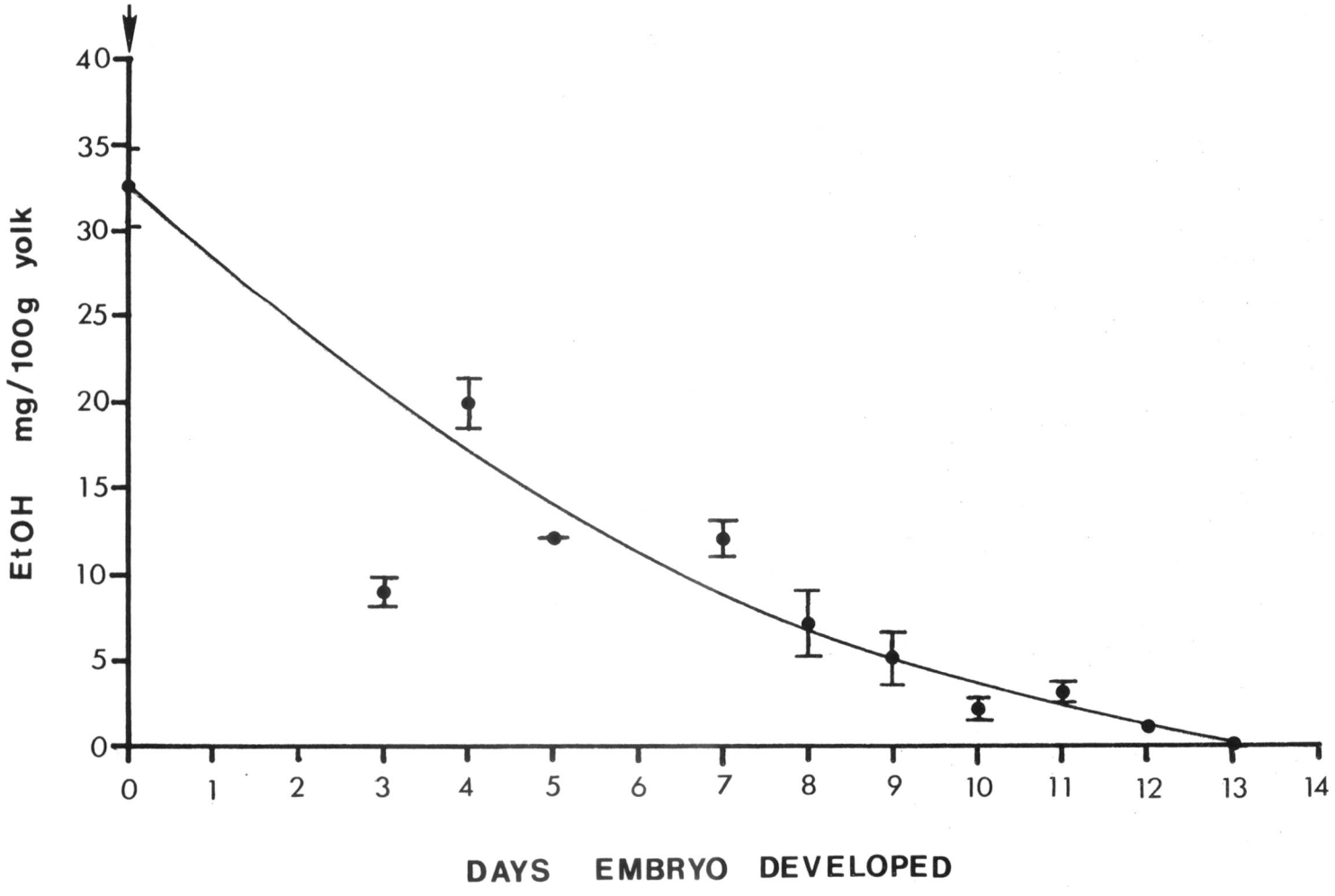

EtOH mg/100g yolk
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS EMBRYO DEVELOPED

FIGURE 8

Egg albumen ethanol clearance during embryonic development, resulting from a single preincubation injection of ethanol, arrow. After initial, subsequent points represent gas chromatographic determinations, each representing the mean determined in duplicate ± S.E.M.

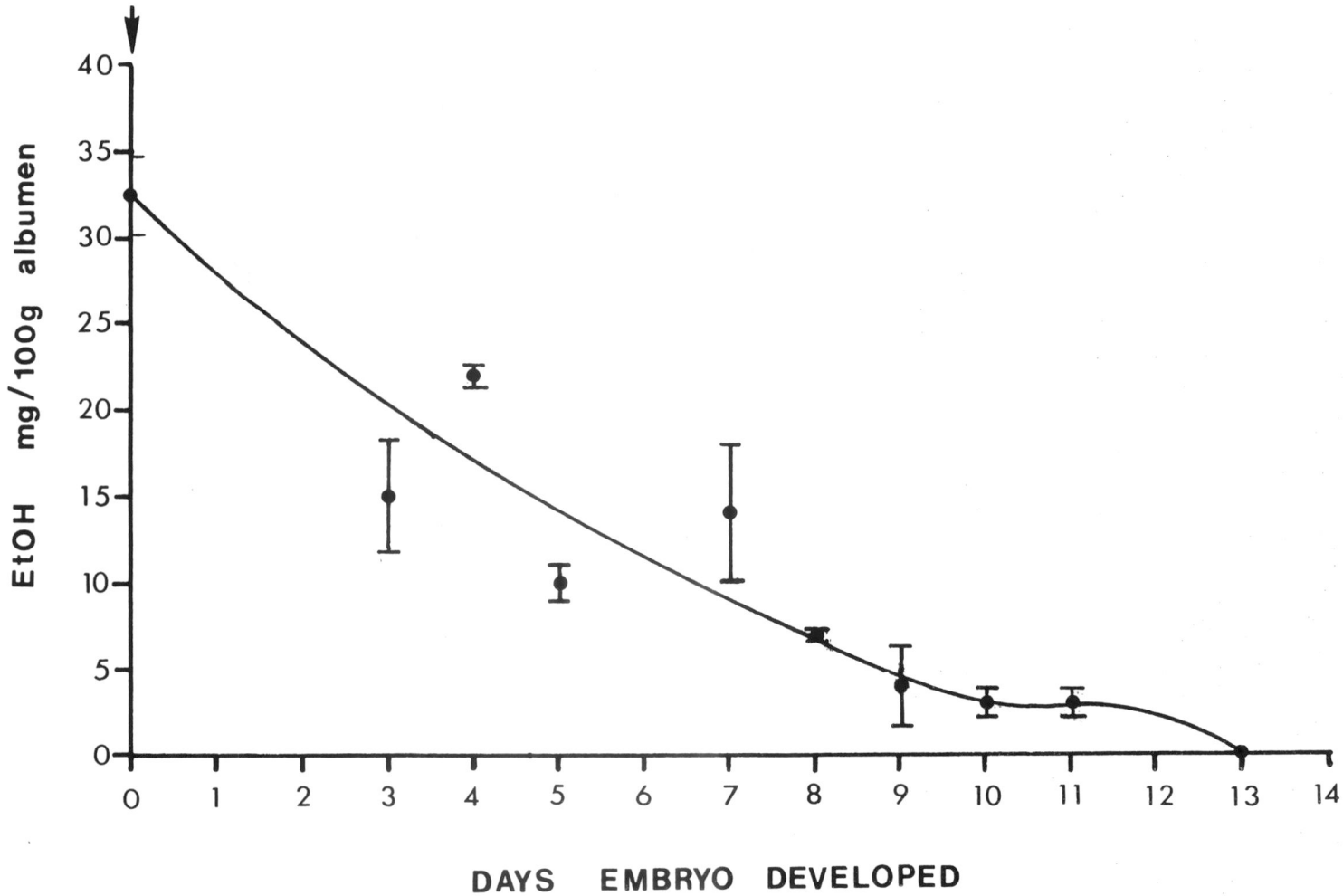

EtOH mg/100g albumen
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS EMBRYO DEVELOPED

FIGURE 9

Egg yolk and albumen ethanol clearance during embryonic development, resulting from a single preincubation injection of ethanol, arrow. After initial, subsequent points represent gas chromatographic determinations, each representing the mean determined in duplicate ± S.E.M.

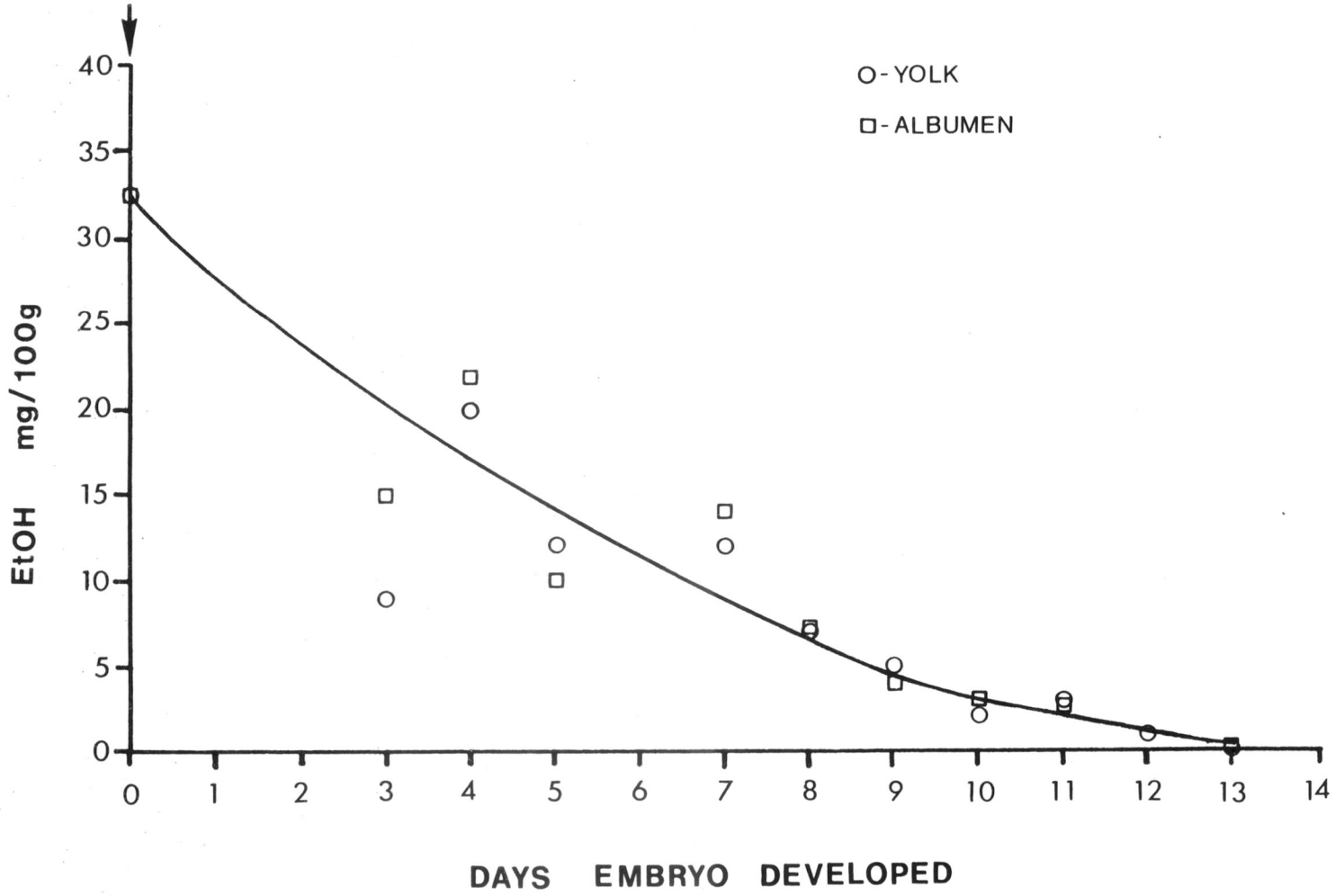

O - YOLK
□ - ALBUMEN
EtOH mg/100g
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS EMBRYO DEVELOPED

Table III. Ethanol clearance during embryonic development, resulting from a single preincubation injection of 33 mg ethanol/100 g egg.

Days Embryo Developed Upon Ethanol Determination	Ethanol Determined in Yolk[1] (mg/100 g egg)	Ethanol Determined in Albumen[1] (mg/100 g egg)
3	9±0.7	15±3.3
4	20±1.5	22±0.4
5	12±0.0	10±1.0
7	12±1.0	14±4.0
8	7±2.0	7±0.3
9	5±1.6	4±2.1
10	2±0.6	3±0.7
11	3±0.5	3±0.9
12	1±0.0	--
13	0	0

[1] Each value represents the mean ± S.E.M., each value also represents an N ≥ 3 with a duplicate of each N.

FIGURE 10

Egg yolk ethanol clearance during embryonic development, resulting from a single preincubation injection of ethanol, arrow. After initial, subsequent points represent gas chromatographic determinations, each representing the mean determined in duplicate ± S.E.M.

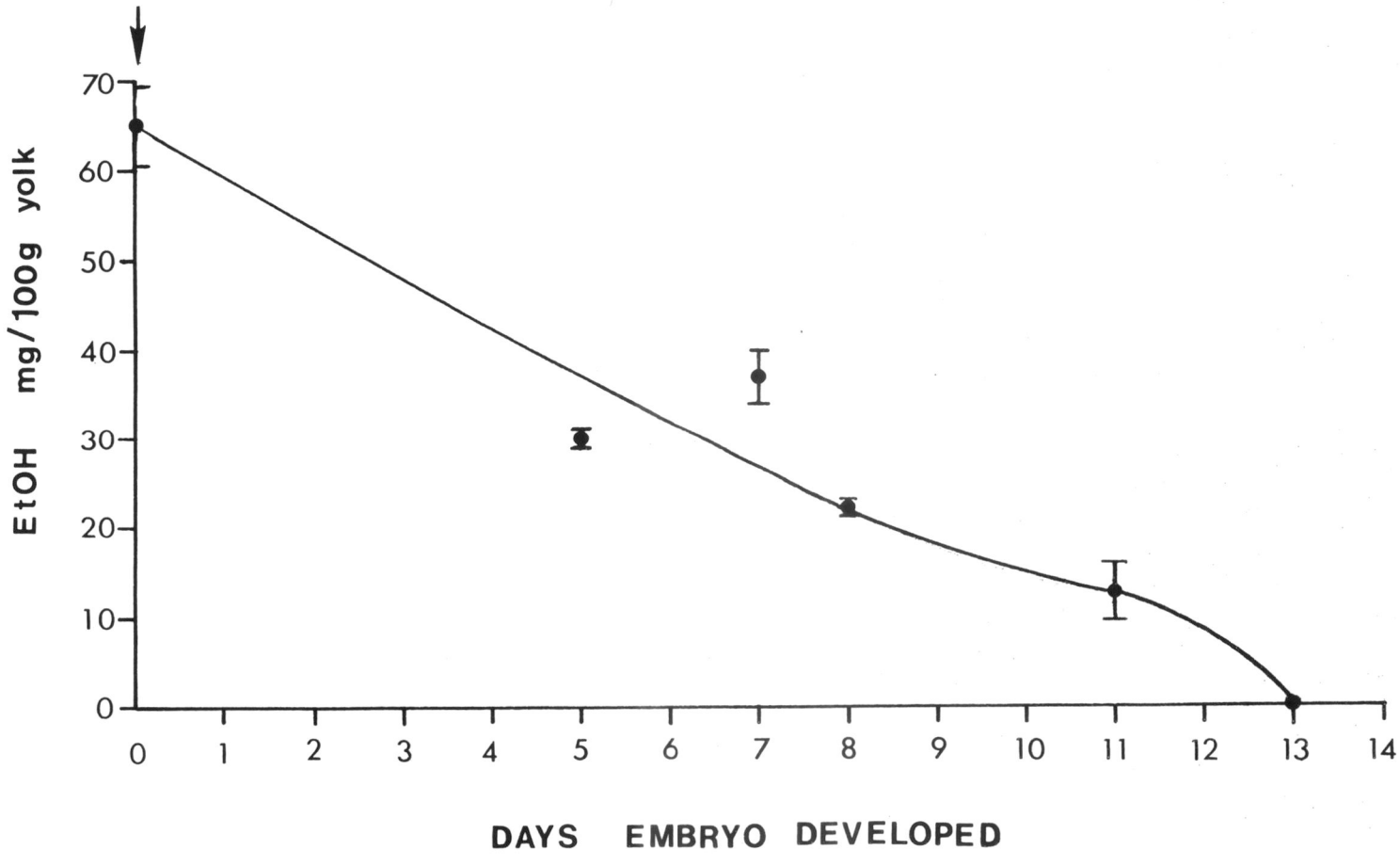

EtOH mg/100g yolk
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS EMBRYO DEVELOPED

Table IV. Ethanol clearance during embryonic development resulting from a single preincubation injection of 65 mg ethanol/100 g egg.

Day of Development on which Ethanol was Determined	Ethanol Determined in Yolk (mg/100 g egg)[1]	Ethanol Determined in Embryo Blood (mg/100 ml)[1]
5	30±0.5	--
7	37±3.3	--
8	22±0.6	21±2.0
11	13±2.7	12±4.0
13	0	0

[1] Each value represents the mean ± S.E.M., each value also represents an N ≥ 3 with a duplicate of each N.

FIGURE 11

Sustained ethanol level during embryonic development (prior to day 13), resulting from a preincubation injection of ethanol (33 mg ethanol/100 g egg, first arrow) and subsequent injections of ethanol (65 mg ethanol/100 g egg, remaining arrows) during embryonic development. After initial, subsequent points represent gas chromatographic determinations, each representing the mean determined in duplicate ± S.E.M.

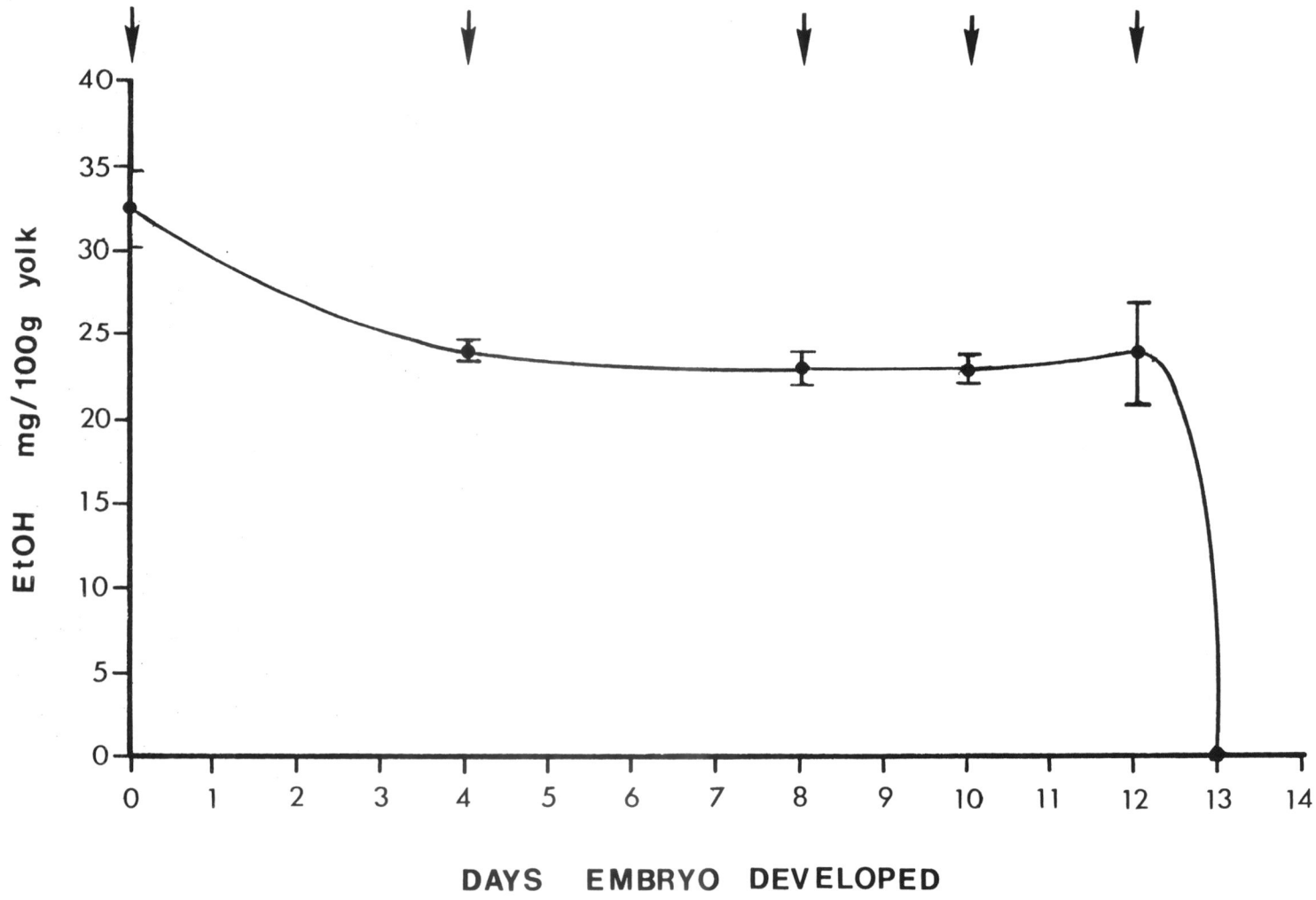

EtOH mg/100g yolk
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS EMBRYO DEVELOPED

Table V. Sustained ethanol level during embryonic development

Day(s) of Injection(s)	Amount of Ethanol Injected (mg/100 g egg)[1]	Day on Which Ethanol Determined	Ethanol in egg yolk (mg/100 g)[2]
0	33	4	24±0.5
0, 4	33, 65	8	23±1.0
0, 4, 8	33, 65(2)	10	23±0.9
0, 4, 8, 10	33, 65(3)	12	24±3.0
0, 4, 8, 10, 12	33, 65(4)	13	0

[1] Each egg injected with 33 mg etOH/100 g egg initially, reinjected with 65 mg etOH/100 g egg during development.

[2] Each value represents the mean for 3 or more animals determined in duplicate plus or minus the standard error of the mean.

ethanol is thought to alter fetal hormone metabolism (Pennington et al., 1982; Rose et al., 1981). Thus, alterations in fetal hormone levels could influence cellular growth at the molecular level.

The hormone-mediated mechanism studied involves a biochemical cascade by which extracellular hormones elicit intracellular changes (see Introduction). The influence of ethanol on three aspects of the hormone mediated cascade was studied; (1) brain cAMP levels, (2) brain cAMP binding protein, and (3) brain protein kinase catalytic activity. Ethanol's influence on cAMP levels was studied because a number of hormones can alter cAMP tissue levels and because cAMP is an important mediator between hormone influence and tissue growth (Robison, 1971). The only known mediators of cAMP's influence on growth are the cAMP-dependent protein kinases (Ogried and Doskeland, 1980). Therefore, the influence of ethanol on the brain's ability to bind cAMP and thus activate cAMP dependent protein kinases (cAMP-dPKs) was also studied. Ethanol's influence on protein kinase activity was studied by measuring the changes in tissue protein phosphorylation. Protein phosphorylation, catalyzed by the catalytic subunits of protein kinase, was studied since it controls and/or regulates many cellular events involved with cell growth (Weller, 1979).

All biochemical studies related to ethanol induced growth suppression used whole embryo brain as the tissue source. Brain tissue was selected for several reasons: microcephaly and mental retardation are characteristics of FAS (Jones and Smith, 1975), abnormalities in brain morphogenesis have also been correlated with FAS (Sterling et al., 1978),

and ethanol exposure is detrimental towards learning ability in both the rat (Abel, 1978) and chick embryo (Linakis and Cunningham, 1980). Separate brain regions of embryo brain were not studied since dissecting these regions from small (less than 1 g), frozen brain tissue was technically prohibitive.

Ethanol dosed whole embryos were smaller than controls (Figures 3, 4, 5, Table I), and brains from ethanol dosed embryos were suspected to be smaller than those of control embryos. However, no statistical correlation between ethanol induced embryo weight loss and smaller brain size in ethanol treated embryos could be made (Table VI). Although the brains of ethanol dosed animals appeared to be smaller than those of controls, the dissection of embryo brain from frozen involved an unmeasurable amount of human error.

The influence of ethanol on embryo brain cAMP levels was studied at 8 and 10 days of embryonic development. At both 8 and 10 days of development the embryos dosed with 65 mg ethanol/100 g egg had significantly higher levels of brain cAMP as compared to brain cAMP levels of vehicle dosed embryos (Figures 12, Table VII). Brain cAMP levels of 8 day embryos displayed a dose dependent response to egg ethanol levels (Figure 12, Table VII). The brain cAMP levels of 10 day embryos were elevated by ethanol, i.e., both ethanol doses (33 and 65 mg ethanol/100 g) produced significantly higher levels of embryo brain cAMP relative to control animals but the response was not dose dependent (Figure 12, Table VII). Levels of brain cAMP were not measured prior to day 8 since embryo brain tissue before day 8 lacked sufficient development to be certain that other cephalic tissues were not being dissected along

Table VI. Weight of whole chick embryo brains used in all experiments[1]

Treatment	8 day (cAMP RIA)	8 day (phosphorylation and binding assays)	10 day (cAMP RIA)
Vehicle	0.388±0.035 (N=6)	0.391±0.016 (N=10)	0.687±0.024 (N=6)
33 mg etOH/100 g egg	0.343±0.024 (N=6)	0.382±0.032 (N=10)	0.693±0.026 (N=6)
65 mg etOH/100 g egg	0.338±0.020 (N=7)	0.332±0.029 (N=10)	0.605±0.046 (N=7)

[1] Weights (gm) expressed as means ± S.E.M.

FIGURE 12

Influence of ethanol on chick embryo brain cAMP levels at 8 and 10 days of embryonic development. Numbers within bars represent number of brains assayed. Significant differences between ethanol and vehicle treatments represented by an asterisk.

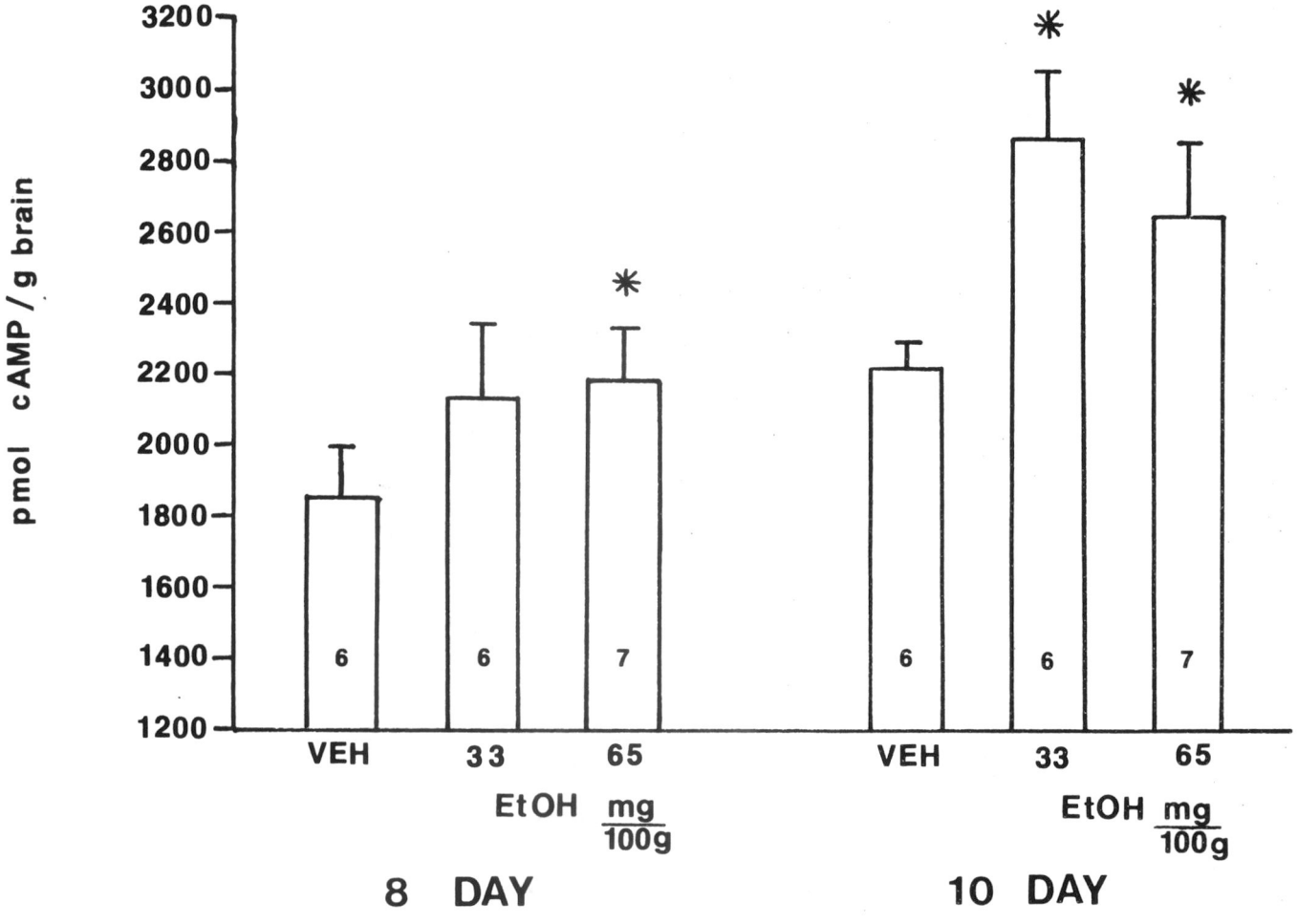

pmol cAMP / g brain
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
6
6
7
6
6
7
*
*
*
VEH
33
65
EtOH mg/100g
VEH
33
65
EtOH mg/100g
8 DAY
10 DAY

Table VII. RIA of chick embryo brain cAMP.

Treatment	8 Day			10 Day		
	CPM[1]	%B/B_o[2]	pmol cAMP/g brain[3]	CPM[1]	%B/B_o[2]	pmol cAMP/g brain[3]
Vehicle	769	9.2	1847±146 (N=6)	939	11.1	2220±85 (N=6)
33 mg etOH/100 g egg	889	10.7	2133±2.8 (N=6)	1198	14.4	2879*±180 (N=6)
65 mg etOH/100 g egg	910	10.9	2177*±152 (N=7)	1101	13.3	2651*±203 (N=7)

[1] Average CPM - blank and background.

[2] Percent [^{125}I]-cAMP bound divided by percent [^{125}I]-cAMP not bound.

[3] Each value represents mean ± S.E.M.

* Statistically different from vehicle control values ($P < 0.05$).

with embryo brain. Although ADH metabolism of ethanol at day 10 might be considered as an influence on brain cAMP levels (Wilson, 1982), measurable amounts of ethanol are still present 10 days after initial ethanol injection (Table III).

The results indicate that ethanol administered in vivo significantly increases levels of brain cAMP in 8 and 10 day chick embryos. Few studies have previously measured ethanol's influence on embryo brain cAMP levels and in vivo studies of ethanol's influence on adult brain cAMP levels are not in agreement. Thus, ethanol has been reported to increase adult brain cAMP levels in mice and rat (Israel et al., 1972; Kuriyama and Israel, 1973), to lower adult brain cAMP levels in rat and human cerebrospinal fluid (Volicier and Gold, 1975; Orenberg, 1976) and not to change adult brain cAMP levels in rat (Redos et al., 1976).

While the literature is uncertain regarding the in vivo influence of ethanol on adult cAMP levels, a definite relationship exists between changes in cAMP levels and rates of cell division (growth). Several studies done in vitro have reported that increases in cAMP levels inhibit cellular growth in numerous cell lines including mouse embryo (Kram et al., 1973), human fibroblast (Froehlich and Rachmeler, 1974), rat anterior pituitary gland (Hertelendy, 1974), chick retinal epithelium (Newsome et al., 1974) and chick embryo neural cells (Kalmus et al., 1982). The results of this thesis support a correlation between growth inhibition and increases in tissue levels of cAMP.

The influence of ethanol on embryo brain protein kinase activation and protein kinase catalytic activity was studied at 8 days of embryonic development. Brain protein kinase assays used crude whole brain homogenates as a source of cAMP-dPK enzymes. Therefore, the activation of cAMP-dPK, not the absolute catalytic activity of cAMP-dPK was determined. The assay of cAMP-dPK thus involved two assays, one to measure activation ([^{3}H]-cAMP binding) and another to measure catalytic activity ([^{32}P] incorporated) of chick embryo brain protein kinase.

Expressed as either nanomoles (nmol) cAMP bound per mg protein or as nmol cAMP bound per g brain, no significant difference was found between vehicle or ethanol dosed embryo brain homogenates in their ability to bind [^{3}H]-cAMP (Figure 13, Table VIII). When interpreting the binding assay results it is important to mention the possible influence of competition between exogenous radioactive and endogenous cAMP for binding sites on homogenate protein. Since assay levels of radioactive cAMP were kept constant, any fluctuation in binding of cAMP to embryo brain homogenate may represent alterations in brain cAMP levels or alterations in brain homogenate ability to bind cAMP (or both). Although the brain homogenate samples for the binding assays contained adenyl cyclase and phosphodiesterase inhibitors, the actual level of physiological cAMP in the embryo brain homogenate at the time of assay is uncertain. If endogenous cAMP levels were higher in ethanol treated animals during the binding assay, the [^{3}H]-cAMP binding to brain homogenate protein binding sites might have been lower in ethanol treated animals but no difference in the binding of [^{3}H]-cAMP between ethanol and control animals was found (Figure 13,

FIGURE 13

Influence of ethanol on [^{3}H]-cAMP binding to whole chick embryo brain homogenate at 8 days of embryonic development. Numbers within bars represent number of brains assayed. No significant difference between ethanol and vehicle treatments.

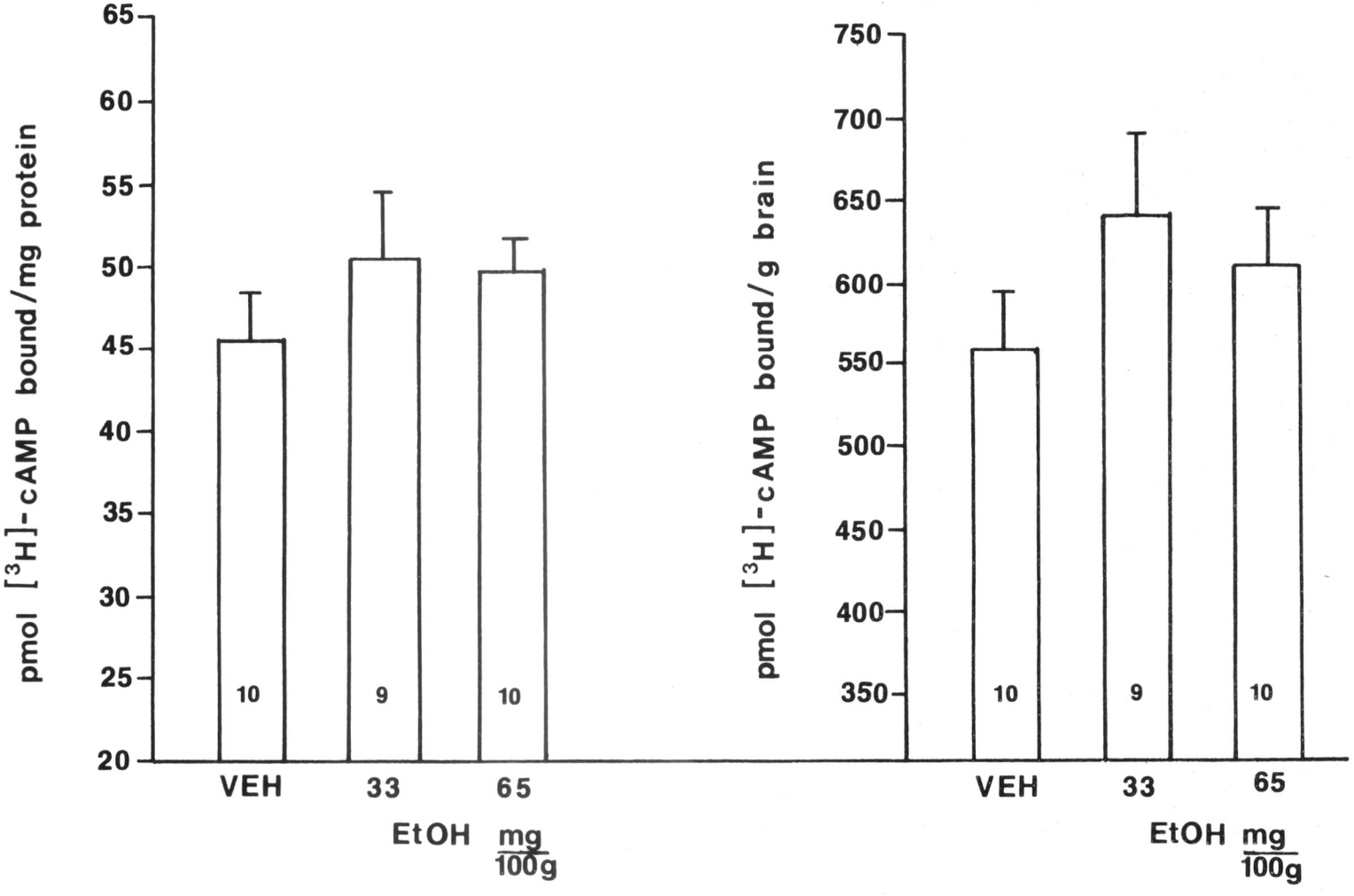

pmol [3H]-cAMP bound/mg protein
65
60
55
50
45
40
35
30
25
20
10
9
10
VEH
33
65
EtOH mg/100g
pmol [3H]-cAMP bound/g brain
750
700
650
600
550
500
450
400
350
10
9
10
VEH
33
65
EtOH mg/100g

Table VIII. Assay of [^{3}H]-cAMP binding by 8 day chick embryo brain homogenates.

Treatment	DPM	pmol cAMP	mg protein	pmol cAMP bound[1] / mg protein	pmol cAMP bound[1] / g brain
Vehicle (N=10)	55,541	7251	0.160	45.3±3.2	558±38
33 mg etOH/100 g egg (N=9)	64,555	8428	0.163	51.7±4.6	648±44
65 mg etOH/100 g egg (N=10)	60,536	7903	0.161	49.1±2.6	406±37

[1] Each value represents mean ± S.E.M., no significant difference exist between different treatments.

Table VIII). The binding of [^{3}H]-cAMP to brain homogenates from ethanol and control animals could have also been altered by ethanol's influence on the binding (regulatory) subunit of cAMP-dPK within the brain homogenate, but again, no difference between ethanol and control brain homogenate ability to bind cAMP was observed.

Phosphorylation of embryo brain homogenate protein, as a measure of cAMP-dPK catalytic activity, was significantly different in ethanol treated embryo brain as compared to controls (Figure 14, Table IX). Both of the ethanol doses (33 mg and 65 mg ethanol/100 g) elicited a significant increase in brain homogenate ^{32}P incorporation as compared to controls (vehicle dosed) (Figure 14, Table IX). Incorporation of ^{32}P expressed per g brain exhibited a slight dose dependent response to ethanol treatment (Figure 14). However, when expressed per mg protein the incorporation of ^{32}P did not exhibit a dose dependent response (Figure 14). The different responses to dose described above were attributed to the fluctuations in protein content among different treatment groups (Table IX).

Phosphorylation assays were performed with an excess of nonradioactive cAMP (2.5 μmole per tube) as compared to the possible physiological level of cAMP (10 pmol per tube based on 2000 cAMP/g brain). Higher than physiological levels of cAMP were required in the phosphorylation assay to produce measurable levels of protein kinase activity. Therefore, any changes in the physiological levels of cAMP under assay conditions would not have influenced kinase catalytic activity, measured as phosphorylation, since additional (over 1000 times physiological levels) cAMP was added during the phosphorylation assay. Thus increases

FIGURE 14

Influence of ethanol on ^{32}P incorporation into whole chick embryo brain homogenate at 8 days of embryonic development. Numbers within bars represent number of brains assayed. Significant differences between ethanol and vehicle treatments represented by an asterisk.

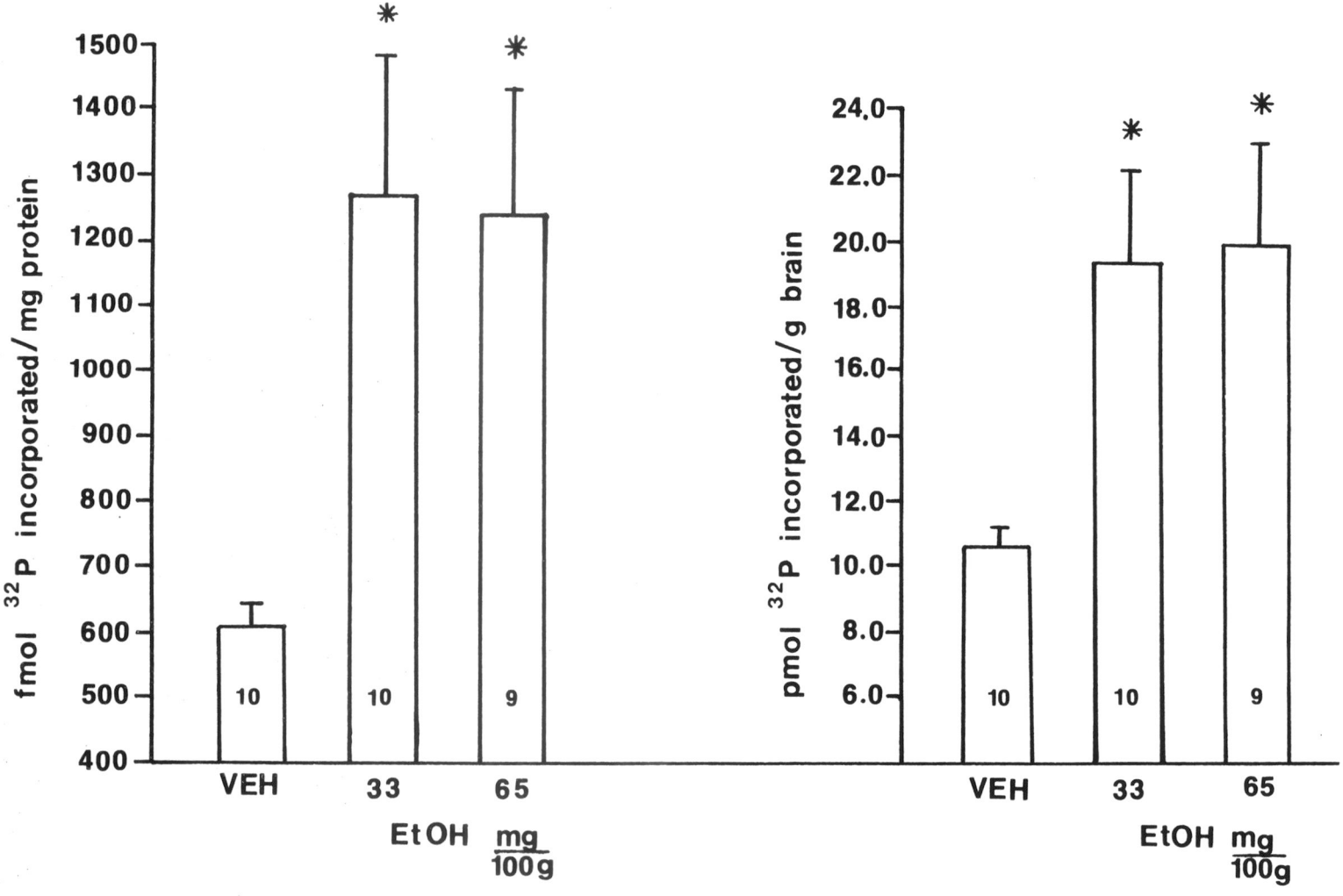

fmol ^{32}P incorporated/mg protein
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
10
10
9
VEH
33
65
EtOH mg/100g
pmol ^{32}P incorporated/g brain
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
10
10
9
VEH
33
65
EtOH mg/100g

Table IX. [^{32}P] incorporated by 8 day chick embryo brain homogenates.

Treatment	DPM	fmol	mg protein	fmol incorporated/mg protein[1]	pmol incorporated/g brain[1]
Vehicle (N=10)	41,185	53.0	0.088	606±33	10.6±0.6
33 mg etOH/100 g egg (N=10)	75,268	96.8	0.080	1266*±215	19.4*±2.7
65 mg etOH/100 g egg (N=9)	77,295	99.4	0.082	1239*±190	19.9*±3.1

[1] Each value represents mean ± S.E.M.

* Statistically different from vehicle control values ($P < 0.05$)

in protein phosphorylation in ethanol treated embryo brain homogenates, measured by this assay, cannot be directly attributed to increases in brain cAMP (Figure 14, Table IX). Results from this study do indicate that ethanol administration influences the catalytic efficiency of protein kinase within chick embryo brain homogenate in the presence of excess cAMP.

In the assay of protein kinase catalytic activity via ^{32}P protein phosphorylation, the primary substrates for phosphorylation were purified histone (added to the reaction mixture, as described in "Methods") and chick embryo brain homogenate proteins. The relative amount of ^{32}P incorporated into chick brain protein as compared to ^{32}P incorporated into histone protein was not determined. Therefore, the combined protein phosphorylation of both embryo brain and purified histone protein was an indirect measure of protein kinase activity in chick embryo brain toward endogenous proteins.

Few, if any, studies have previously studied the influence of ethanol dosed *in vivo* on embryo brain protein kinase, but Kuriyama *et al.* (1976), have reported insignificant increases in adult mice brain protein kinase activity after dosing the animals with ethanol as compared to controls. Further studies concerning ethanol's influence on embryo brain protein kinase are needed to establish a firm link between embryo ethanol exposure and alterations in embryo brain protein kinase activity.

Although it is uncertain how increases in embryo brain phosphorylation may affect the developing embryo, this report links increases in brain protein phosphorylation with decreases in embryo growth both

resulting from ethanol exposure during embryonic development. Perhaps reduced brain growth (microcephaly) reported as a common FAS occurrence (Jones and Smith, 1975b) is related to increases in embryo brain cAMP and protein phosphorylation reported in this study. This is possible since phosphorylation (mediated by cAMP-dPK) is thought to regulate many aspects of cellular growth and division (Martinage et al., 1980; Block and Atkinson, 1979; Walton and Gill, 1974; Kish and Kleinsmith, 1973). A better understanding of why ethanol administered in vivo is able to alter embryo cAMP levels and influence protein phosphorylation within the brain may be an important step towards better understanding the molecular mechanisms involved with FAS.

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APPENDIX A

Chick Ringer's Solution

NaCl	7.19 g
KCl	0.37 g
$CaCl_2$	0.18 g

Bring to a total volume of 1000 ml with distilled water, then adjust pH to 7.2 with HCl/NaOH.

APPENDIX B

Egg Weights

Total number of eggs containing embryos used in this study equals 606. Average ($\overline{X}$) weight of eggs equaled 60 grams per egg with standard error of the mean (S.E.M.) equal to ± 3.0 grams.

i.e., Total N = 606, $\overline{X}$ = 60 g, S.E.M. = ± 3.0 g

APPENDIX C

RIA Standard Curve

CPM[1]	$\frac{\text{pmol cAMP}^2}{100\ \mu\text{l sample}}$	$\%B/B_o$[3]
3521	0.00[4]	100.0
3190	0.10	90.5
3112	0.25	88.4
2754	0.50	78.2
2458	1.00	69.8
2006	2.50	56.9
1674	5.00	47.5
1287	10.00	36.6
659	25.00	18.7

[1] Average CPM - blank and background.

[2] Mean duplicate determinations.

[3] Percent [^{125}I]-cAMP bound divided by percent [^{125}I]-cAMP not bound.

[4] No cAMP added, "0" standard.

APPENDIX D

Presentations and Publications

PUBLICATION

Pennington, S. N., J. W. Boyd, G. W. Kalmus and R. W. Wilson. The molecular mechanism of Fetal Alcohol Syndrome (FAS) 1. Ethanol-induced growth suppression. Neuro Behavioral Toxicology and Teratology 5: 259-262 (1983).

PRESENTATIONS

S. N. Pennington, J. W. Boyd, and G. W. Kalmus. The influence of ethanol on the development of the avian embryo.

The fetal alcohol syndrome (FAS) is a term widely used to express the many abnormalities resulting from fetal ethanol exposure. We have developed an avian model, the chick embryo, to study FAS. Solutions of 25% and 12.5% ethanol were injected into the airspace. A 200 µl constant volume was maintained. Wet weights of whole embryos were compared between vehicle and ethanol treated animals at 4, 6, 8, 10, and 12 days of development. Statistical weight difference between vehicle and 25% ethanol treated animals was observed in all 12 day embryos; only half of the 10 and 8 day embryos; and no difference in 6 and 4 day embryos. Weight loss among all except 4 day embryos was dose dependent; ethanol treated embryos were smaller (weighed less). A 29% increase in abnormalities was seen in the 25% ethanol treated animals as compared to vehicle controls. Common abnormalities among ethanol treated animals included protruding mesencephalon, external viscera, and abnormal facial features.

Presented at the annual meeting of the North Carolina Academy of Science held at UNC-Wilmington, Wilmington, North Carolina on March 26-27, 1982 (J. Elisha Mitchell Sci. Soc., in press).

S. N. Pennington, J. W. Boyd, and G. W. Kalmus. The developing chick embryo as a model for the fetal alcohol syndrome: Gas chromatographic determinations of egg ethanol levels.

The term "fetal alcohol syndrome" (FAS) defines a multitude of developmental abnormalities resulting from exposure of the unborn to ethanol. Various animal models have been used to better understand the effects ethanol has on both embryo and fetus. Mammalian models are widely used to study FAS. However, the selection and use of mammals is often hampered by dosage problems since most mammals (except man) refuse to drink ethanol. The chicken embryo as a model for FAS offers distinct advantages compared to mammalian models: easily administered ethanol dosages, no maternal factors to consider, short period for complete embryonic development, and a low cost factor. Gas chromatography was employed to measure yolk and albumen ethanol levels. Dosage of the eggs was via injection into the airspace. Concentrations of 0.03 and 0.06 g ethanol per 100 g whole egg were injected. The injection vehicle was chick Ringers with a constant volume of 200 µl per egg. Ethanol levels of yolk and albumen within the same egg were equal. Sustained levels of ethanol could not be maintained beyond 10 days of development. Enzymatic metabolism of ethanol is thought responsible for rapid ethanol clearance beyond 10 days of development.

Presented at the annual meeting of the New Jersey Academy of Science at William Patterson College, Wayne, New Jersey on April 3, 1982 (Bull. New Jersey Acad. Sci. 27:33, 1982).

S. N. Pennington, G. W. Kalmus, and J. W. Boyd. The fetal alcohol syndrome and brain cAMP: The influence of varied ethanol concentrations on cAMP levels in the chick embryo brain.

Embryonic development is known to be retarded when the embryo is exposed to ethanol. Ethanol exposure during gestation causes significant central nervous system dysfunction. Cyclic 3',5'-adenosine monophosphate (cAMP) is known to regulate many enzyme systems. Cyclic AMP is thought to play an important role in cell differentiation, growth, regulation of brain protein synthesis and many other metabolic events. Experimental chick embryos were dosed on initial incubation via the airspace with various ethanol concentrations. Controls were dosed with vehicle. After 10 days of development, cAMP whole brain levels were quantified with radioimmuno techniques. Results indicate a positive dose response correlation between ethanol concentrations and cAMP levels. Ethanol induced elevations in cAMP levels may play an important role in the control of brain cell differentiation. Research funded in part by Sigma Xi.

Presented at the 43rd annual meeting of the Association of Southeastern Biologist held at Eastern Kentucky University, Richmond, Kentucky on April 14-17, 1982 (ASB Bull. 29:77, 1982).

S. N. Pennington, J. W. Boyd, G. W. Kalmus and R. W. Wilson. The molecular mechanism of FAS. 1. Ethanol induced growth suppression.

Of the birth defects associated with alcohol consumption during pregnancy, *in vitro* growth retardation which results in neonates that are small for gestational age is the most common observation in both humans and animal models. A variety of alcohol-induced alterations in maternal, placental and/or fetal physiology have been proposed as the basis for this retarded fetal growth. The molecular mechanism of this retardation, however, is obscure; and it remains to be determined whether the growth suppression is the result of the action of ethanol or its metabolites on embryonic, maternal or placental tissue. Using the embryonic chick as a model which circumvents changes in maternal and placental function, we have measured ethanol-induced growth suppression as a function of embryonic age and ethanol dosage. The data suggest that in the absence of measurable alcohol dehydrogenase activity, ethanol *per se* suppresses the rate of cell division in embryonic tissue including brain. The result is fewer cells/organ for a given time of gestation. The suppression of cell division is proportional to the ethanol dose and appears to be related to ethanol-induced changes in the metabolism of the prostaglandin hormones and resulting changes in the cyclic-AMP levels of the developing embryos.

Presented at the annual meeting of the American Society of Biological Chemists at New Orleans, Louisiana on April 15-23, 1982 (Fed. Proc. 41:924, 1982).