EFFECT OF ETHANOL ON THYMIDINE INCORPORATION IN CHICK NEURAL TISSUE

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by

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ABSTRACT

Richard D. Barrow, II. THE EFFECT OF ETHANOL ON THYMIDINE INCORPORATION IN CHICK NEURAL TISSUE. (Under the direction of Dr. Gerhard W. Kalmus) Department of Biology, April 1984.

Maternal ethanol consumption during pregnancy has been demonstrated to result in deformity of the developing fetus. This condition has been labeled Fetal Alcohol Syndrome (FAS) and is characterized by facial deformity, neural defects, small birth size, and other malformations.

Ethanol has been shown to be highly teratogenic in most of the animal models (rat, mouse, sheep and chick embryo) that have been studied. The current research was an attempt to establish the effect of ethanol on the processes of DNA replication by studying thymidine incorporation into chick neural tissue. Firstly, it was determined if ethanol effected the rate of thymidine incorporation into DNA and if so, was it dose related? Secondaly, if ethanol did effect thymidine incorporation into DNA, which step of incorporation was affected?

Results demonstrate that ethanol adversely influences chick embryo development. Ethanol exposure in-ovo resulted in a reduction in viability, embryo weight and brain weight. A reduction in the amount of thymidine incorporated into DNA has also been demonstrated. Further study is required to determine at which step in the DNA synthesis does ethanol exhibit its influence.

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INTRODUCTION

Clinical Manifestation of Fetal Alcohol Syndrome

The most obvious clinical observations of Fetal Alcohol Syndrome (FAS) in human infants are changes in both pre and postnatal growth patterns, specifically in the head, face and central nervous system (Jones and Smith, 1973; Smith, 1979). Children affected by FAS often are small in stature, particularly in regards to length relative to weight (Newman and Correy, 1980). Specific physical manifestations commonly noted in FAS children are: flattening of the bridge of the nose, the absence of the philtrum and thin vermillion line of the lip, the presence of epicanthic folds, microphthalmia, joint anomalies, micrognathia, microcephaly, and maxillary hypoplasia (Newman and Correy, 1980). Other characteristics of FAS include poor finger articulation, delay in demonstrating hand dominance, and a weak grasp (Beagle, 1981). These children often are jittery, irritable or lethargic and suffer from tremors, spontaneous seizures, a weak suckling reflex, and increased muscle tone and respiration rate (Newman and Correy, 1980). The IQ range for normal children is 90-110 while the range for children suffering from FAS is 15-105 with the mean being 65. The majority of children suffering from FAS do not undergo a major change in IQ during the developmental period, however, exceptions to this

pattern have been reported (Newman and Correy, 1980; Beagle, 1981). More females than males tend to express FAS but this could be due to a higher mortality rate in the affectecd males (Newman and Correy, 1980).

There appears to be a dose response to ethanol consumption which increases proportionally with the increase in average daily ethanol consumption. Evidence indicates that ethanol effects the transfer of nutrients across the placenta and this once was thought to be the reason for the smallness of offspring from mothers that consumed ethanol during pregnancy (Newman and Correy, 1980). Embryonic malnutrition will produce impaired body growth but not necessarily impaired head growth. This impairment is normally corrected postnatally during a "catch-up phase" presuming that proper nutrition is provided. Children suffering from FAS do not undergo this typical "catch-up phase" and tend to be small in stature for the rest of their lives (Newman and Correy, 1980; Beagle, 1981).

Historical Perspective

The term Fetal Alcohol Syndrome (FAS) was not coined until 1973 when it was used to describe a pattern of abnormalities in children of alcoholic women (Jones and Smith, 1973; Jones *et al.*, 1973). Early work in the field of FAS was complicated by problems such as the lack of nutritional control, lack of reproducible data, genetic variation in the experimental

animals, and little control of the amount of ethanol consumed prenatally (Randall and Riley, 1981). Prior to these studies, some observations had been noted that associated maternal ethanol consumption with fetal abnormalities. In early Greek mythology, the first evidence that alcohol could produce a teratogenic effect was that Vulcan, the blacksmith to the Gods, was believed to have been deformed due to parental intoxication at the time of his conception. Later, Carthiginian law forbade the drinking of alcohol on the wedding night by a bridal couple to prevent the conception of defective children (Newman and Correy, 1980). Evidence in 1834 before a Select Committee of the British House of Commons suggested that infants born to alcoholic mothers had a "starved , shrivelled, and imperfect look". In 1899, a researcher named Sullivan discovered that mortality and morbidity in infants was higher in children of alcoholic mothers than in children of non-alcoholic relatives (Newman and Correy, 1980). Research into the area of FAS underwent periods of inactivity until the 1970's when it became a very popular area of concentration. Researchers have studied FAS using a variety of models to study the effect of ethanol on fetal development.

Fetal Alcohol Syndrome Studies

Ethanol has been shown to produce lower body weights, shorter body lengths, lower internal organ weights (heart,

kidney and liver), and lower viability in rats, mice and sheep (Tze and Lee, 1975; Henderson and Schenker, 1977; Henderson et al., 1979; Lochery et al., 1980; Potter et al., 1980; Sulik et al., 1981). Some researchers randomly culled pups from litters so that there would be the same number of animals in each group, thus assuring valid statistical analysis. The offspring that were stillborn or that were culled for statistical purposes were found to have a much higher incidence and severity of teratology (Lochry et al., 1980; Potter et al., 1980; Sulik et al., 1981). The lower body weights of offspring from ethanol dosed mothers was not compensated during the post-natal period. This lack of weight gain is typical of infants born with FAS (Lochry et al., 1980). Tze and Lee (1975) found that maternal ethanol exposure produced offspring that suffered from cracked, dry and loose skin, microcephally, reddened areas on the head and body, and a shrivilled appearance. It has been demonstrated that the abnormalities that are produced in embryos by ethanol exposure depends upon the developmental stage at the time of exposure of the fetus to ethanol. The severity of the deformity is directly related to the level of maternal ethanol exposure (Lochry et al., 1980).

Total brain cell numbers, RNA, and DNA are reduced in embryos exposed to ethanol. This decrease has been shown to be directly related to ethanol exposure and not the result of a decreased energy intake (Woodson and Ritchey, 1979).

Acetaldehyde, an ethanol metabolite, has been demonstrated to further reduce ^{3}H-Thymidine incorporation into DNA of fetal rat tissues, especially in tissues of the central nervous system (Dreosti et al., 1981). This suggest that the metabolic products of ethanol may be more teratogenic than ethanol itself (Dreosti et al., 1981). Whichever is responsible for this disruption, proper DNA synthesis is essential for proper fetal development.

Ethanol Metabolism

Fetal Alcohol Syndrome is caused by maternal consumption of ethanol. Since ethanol is rapidly metabolized, metabolic by-products must be considered in the study of FAS. Ethanol is metabolized to a final product of acetyl-CoA (Lehninger, 1975) by a series of reactions which are shown in Figure 1. Initially ethanol is converted to acetaldehyde by the enzyme alcohol dehydrogenase with the reduction of NAD^{+}. Acetaldehyde is further metabolized to acetate by the enzyme aldehyde dehydrogenase which also uses NAD^{+} as a coenzme. Acetate is finally converted to acetyl Co-A by the enzyme Acetyl-CoA synthetase which produces acetyl adenylate as an intermediate product. This final reaction requires that ATP and Mg^{++} be present for the reaction to occur.

FIGURE 1: CHEMICAL REACTIONS BY WHICH ETHANOL IS METABOLIZED

$$EtOH + NAD^{+} \xrightarrow{(1)} Acetaldehyde + NADH + H^{+}$$

$$Acetaldehyde + NAD^{+} \xrightarrow{(2)} Acetate + NADH + H^{+}$$

$$Acetate + 2ATP + Mg^{++} \xrightarrow{(3)} Acetyl\ Adenylate \xrightarrow{(3)} Acetyl\ CoA$$

(1) Alcohol Dehydrogenase
(2) Aldehyde Dehydrogenase
(3) Acetyl CoA Synthetase

Thymidine Metabolism

In vivo labeled thymidine can be used to label DNA that is undergoing synthesis. Normally, deoxythymidine 5′-triphosphate (dTTP), which is required for DNA synthesis, is produced by a series of reactions which use deoxyuridine 5′-triphosphate (dUTP) as the precursor (White *et al.*, 1970). In the presence of water, dUTP and the enzyme deoxyuridine 5′-triphosphate diphosphohydrolase, will produce deoxyuridine 5′-phosphate (dUMP) and pyrophosphate (PPi). N5,N10-methylenetetrahydrofolate plus dUMP and the enzyme thymidylate synthetase and the cofactor Mg^{++} yield deoxythymidine 5′-phosphate (dTMP) and dihydrofolate. Another source of dTMP is the conversion of thymine via the Salvage pathway and this is the pathway that permits the use of labeled thymidine to label the DNA. This pathway involves the conversion of thymine and deoxyribose 1′-phosphate to deoxythymidine (dT) and organic phosphorus (Pi) by the enzyme thymidine phosphorylase. Deoxythymidine is then reacted with ATP by thymidine kinase which produces dTMP. Regardless which mechanism is used to produce dTMP, it is then converted to deoxythymidine 5′-diphosphate (dTDP) and then to dTTP by either specific deoxyribonucleotide kinase or nonspecific nucleoside diphosphate kinase. Deoxythymidine 5′-triphosphate is then incorporated into DNA by DNA polmerase (Figure 2).

These reactions are controled by the fact that large amounts

FIGURE 2: BIOSYNTHESIS OF DEOXYTHYMIDINE 5′-TRIPHOSPHATE

Normally:

Deoxyuridine 5′-triphosphate (dUTP) + H2O ---(1)---> Deoxyuridine 5′-monophosphate (dUMP) + PPi

dUMP + N5,N10-methylenetetrahydrofolate ---(2)---> Deoxthymidine 5′-monophosphate (dTMP) + Dihydrofolate

Salvage Pathway:

Thymine + Deoxyribose 1′-phosphate ---(3)---> Thymidine (dT)+ Pi

Thymidine + ATP ---(4)---> Thymidine 5′-monophosphate (dTMP) + ADP

dTMP ---(5 or 6)---> Deoxythymidine 5′-diphosphate (dTDP)
dTDP ---(5 or 6)---> Deoxythymidine 5′-triphosphate (dTTP)

(1) Deoxyuridine triphosphate diphosphohydrolase
(2) Thymidylate synthetase + Mg^{++}
(3) Thymidine phosphorylase
(4) Thymidine kinase
(5) Specific deoxyribonucleotide kinase
(6) Nonspecific nucleoside diphosphate kinase

of ATP are necessary for the production of dTTP thus when the ATP level is depressed, the rate of synthesis slows. The presence of ADP and GDP inhibit the activity of ribose 5-phosphate pyrophosphokinase which produces 5-phosphoribosyl-1-pyrophosphate (PRPP). This is a key intermediate since it is required as the starting point for all nucleotides.

Current Research

The present study is an effort to establish the relationship of ethanol and its effect on the processes of DNA replication. In an effort to limit as many variables as possible, the chick model was chosen for this study. By using the chick model prior to day 8 of incubation, the effect of ethanol, rather than acetaldehyde, on the embryo can be elucidated since the chick embryo does not produce alcohol dehydrogenase until after day 8 (Wilson, 1983). The chick embryo is an ideal model because there is no interaction between the developing embryo and the mother. Ethanol is not supplied through the mother but directly administered to the egg. Since all nutrients essential for development are contained in the egg, ethanol would appear to have little or no effect on the nutrient uptake in the chick embryo. In contrast, ethanol causes an alteration in the placental uptake mechanism of mammals for amino acids which may affect maternal-fetal nutritional transfer (Wiener, 1980).

Scientific literature presents conflicting data on the effect of ethanol on chick development but there is consistancy in that most researchers reported that ethanol caused a decrease in viability, body weight, and organ weight (Koda et al., 1980; Sandor and Ellas, 1970). Sandor and Ellas (1970) reported ethanol to be highly teratogenic to the chick embryo while Koda and coworkers (1980) reported that it is not grossly teratogenic at a dose compatable with life.

The current research was an effort to answer the following questions: 1. Does ethanol affect the rate of thymidine incorporation into the DNA of embryonic chick neural tissue?; 2. If there is a response to ethanol exposure, is it a dose related response?; and, 3. If there is a response to ethanol exposure, at which stage during the process is incorporation of thymidine into DNA affected?

MATERIALS AND METHODS

Eggs

Fertile chicken eggs of the Black Sex Linked strain were purchased from Newman's Hatchery, La Grange, North Carolina. Eggs were stored at 10-12°C for no longer than six days prior to use.

Treatment

At the time of incubation, the eggs were divided into groups and were injected with 200 microliters (µl) of either physiological saline (chick Ringer's, see Appendix 1), 0.3 g or 0.6 g ethanol (EtOH) per kilogram (Kg) of egg weight. The 0.6 g EtOH solution was prepared from 95% EtOH and chick Ringer's in sufficient quantity to prepare 100 dosages. To determine the proper concentration, the average egg weight was multiplied by the concentration to be prepared and then divided by the specific gravity of EtOH.

$$\frac{\bar{X} \text{ egg weight} \times \text{EtOH concentration}}{\text{Specific Gravity of EtOH}} \times 100 = \text{ml of EtOH per 100 dosages}$$

0.6 g EtOH/Kg egg weight = EtOH concentration
0.816 = Specific gravity of EtOH at 25°C

The final multiplication by 100 is to allow for 100 doses. This final volume of EtOH is then diluted with chick Ringer's to provide a total volume of 20 ml. The 0.3 g EtOH solution was prepared by a 1:1 dilution of the 0.6 g EtOH stock solution with chick Ringer's. An additional group received handling only (no injection) and this group was labeled Sedentary Control Group. All glassware, chick Ringer's, syringes, and needles were sterilized prior to use. The injections were administered with a 250 µl syringe using a #26Gx3/8" needle. The dose was injected through a small opening (Figure 3) made with a

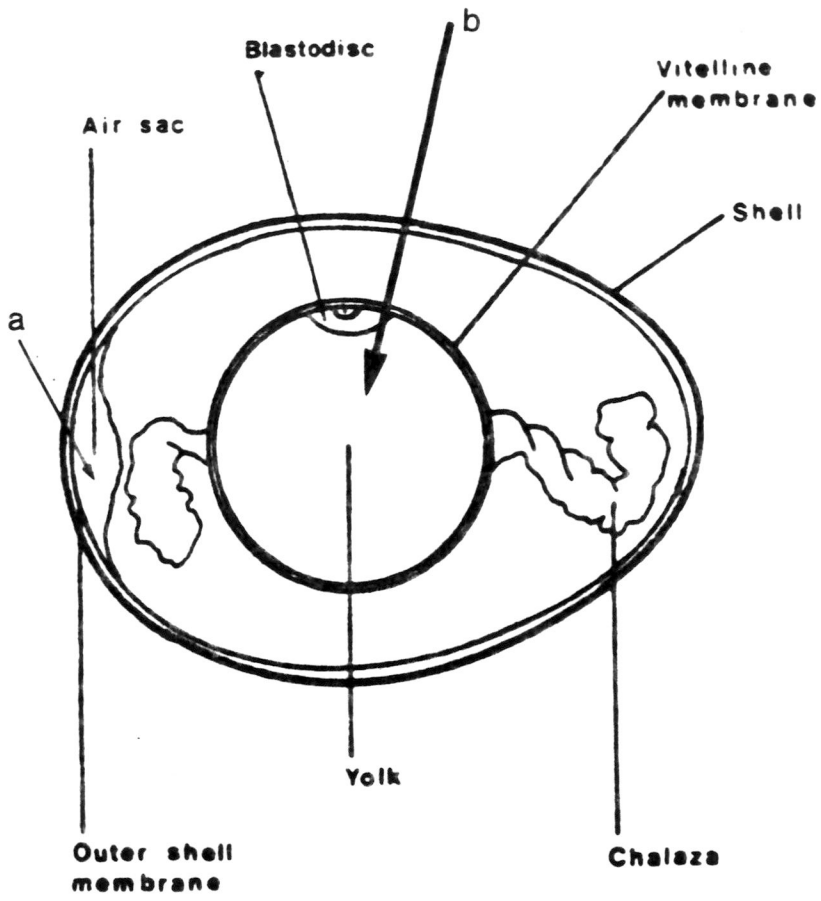


FIGURE 3: SITES OF ETHANOL AND LABELED THYMIDINE INJECTION

a Ethanol injection
b Thymidine injection

dissecting needle into the egg air space. This opening was sealed with hot paraffin after injection. The eggs were incubated at 37.5°C, 50-60% relative humidity, in a forced air egg incubator and turned daily.

On day seven the eggs were injected with 1 μCi of ^{3}H-thymidine in chick Ringer's at a concentration of 5 μCi/ml. The injection volume of 200 μl was injected into the yolk using a #21Gx1" needle. To determine proper needle placement, the syringe was aspirated prior to injection to determine if the needle was in the yolk (Figure 3). After injection, the eggs were sealed as before and then reincubated for six, twelve, or twenty-four hours. At appropriate incubation times, the embryos were isolated, sacrificed in liquid nitrogen and stored at -80°C in a Revco freezer until the time of analysis.

Growth Determination

Brains were dissected from frozen embryos by decapitation and enucleation at room temperature (23°C), weighed, and then homogenized in a teflon-glass homogenizer with cold 0.1 M phosphate buffer (pH 7.4) in a 10:1 ratio (V/W). A two ml alliquot was removed from the homogenate and was precipitated by adding an equal volume of 10% trichloroacetic acid (TCA). The tubes were centrifuged at 3000xG in a Beckman tabletop centrifuge for twenty minutes and the pellet washed twice with two ml of 5% TCA followed by centrifugation after each wash.

The final pellet was resuspended in 0.1 M phosphate buffer. This TCA precipitation removed all unincorporated ^{3}H-Thymidine (Freemen and Gottleib, 1980). A 0.5 ml portion of the TCA-insoluble fraction was oxidized in a Packard Tri-Carb Sample Oxidizer using Mono-phase 40 (Fisher) as the scintillation cocktail and glass counting vials (Kimble). The products were then counted in a Beckman 100C liquid scintilation counter for ten minutes. To ensure that the labeled compound was being incorporated into the DNA, 1000 Kuntz units of DNase I, type III, was added to a 0.5 ml portion from selected samples. This was incubated for twenty-four hours at 25°C with constant agitation. After incubation, these samples were precipitated in the same manner as described above.

An additional portion of the TCA-insoluble fraction was assayed for DNA concentration using a fluorometric technique (Labarca and Paigen, 1980) which is based upon the enhancement of fluorescence seen when bisbenzimidazole binds to DNA. The fluoresence was measured in a Turner Fluorometer. This procedure was performed in duplicate using 0.5 ml of the sample and 4.5 ml of a phosphate saline buffer composed of 0.5 M $NaPO_4$ and 2 M NaCl at pH 7.4. Five μl of the bisbenzimidazole dye (Hoechst No. 33258) was added to the tubes and then measured in a fluorometer with the excitation and emission set at 356 nm and 458 nm, respectively, and the sensitivity set at 10. A zero to 50 microgram (μg) standard curve was obtained using calf thymus

DNA dissolved in the phosphate-saline buffer. The results were recorded in counts of thymidine incorporated per ug of DNA.

Double Isotope Labeling Technique

The point of inhibition of thymidine incorporation into DNA was determined using a Double Isotope Technique (Chae et al., 1968; Chae et al., 1970). Control eggs and experimental eggs were injected with ^{3}H-thymidine and ^{14}C-thymidine, respectively. Brains were dissected as before and one ml of the sample was precipitated with one ml of 10% TCA. After centrifugation the TCA-soluble fraction was saved and the TCA removed by extracting two times with ether. The ether was removed by a constant stream of nitrogen for twenty minutes in a fume hood. The TCA soluble fraction from a control brain was combined with the TCA soluble fraction from an ethanol dosed brain and the mixture was lyophilized in a Vertis model 10 MR-TR lyophilizer. The lyophilized residue was resuspended in 0.5 ml of distilled water. Samples from this solution were then placed in single spots of 3 mm diameter on a starting line on PEI-cellulose plates (Brinkman). Along with this sample, standards of thymidine (dT), thymidine 5'-monophosphate (dTMP), thymidine 5'-diphosphate (dTDP), and thymidine 5'-triphosphate (dTTP) were loaded onto the plate. The plates were developed vertically in a closed tank in a mixture of 2 N formic acid and 0.6 M LiCl in a 1:1 ratio. Incubation time was approximately two hours.

Migration of the thymidine nucleotide standards was determined by observation under ultraviolet light. The regions on the plates that corresponded to the nucleotide standards were cut from the plate and placed into scintillation vials, and the nucleotides were eluted with 2 ml of 0.6 N formic acid for two hours. Eighteen ml of Scintiverse E was added to each vial. The vials were counted for ten minutes in a Beckman 3303 liquid scintillation counter with ^{3}H and ^{14}C being counted simultaneously on separate channels. The ratio of ^{3}H:^{14}C for DNA and each metabolic intermediate was determined.

Chemicals and Reagents

Nonlabeled-thymidine, dTMP, dTDP, dTTP and DNase I, Type III, were purchased from Sigma Chemical Company, St. Louis Mo. Labeled thymidine was purchased from New England Nuclear, Boston, Ma. Polgram Cel 300 PEI-cellulose plates were purchased from Brinkman Company, Westbury, N.Y. To ensure the purity of the radioactive compounds, thin layer chromatography was performed on PEI-cellulose plates with the labeled compounds being loaded in single spots of 3 mm diameter. Loaded next to the spots was nonlabeled thymidine. The plates were developed as in the Double Isotope Labeling Technique and examined under ultraviolet light (Randerath and Randerath, 1964). Ethanol was purchased from the Department of Chemistry at East Carolina University, Greenville, N.C. All other chemicals and reagents

were purchased from Fisher Chemical Company, Raleigh N.C.

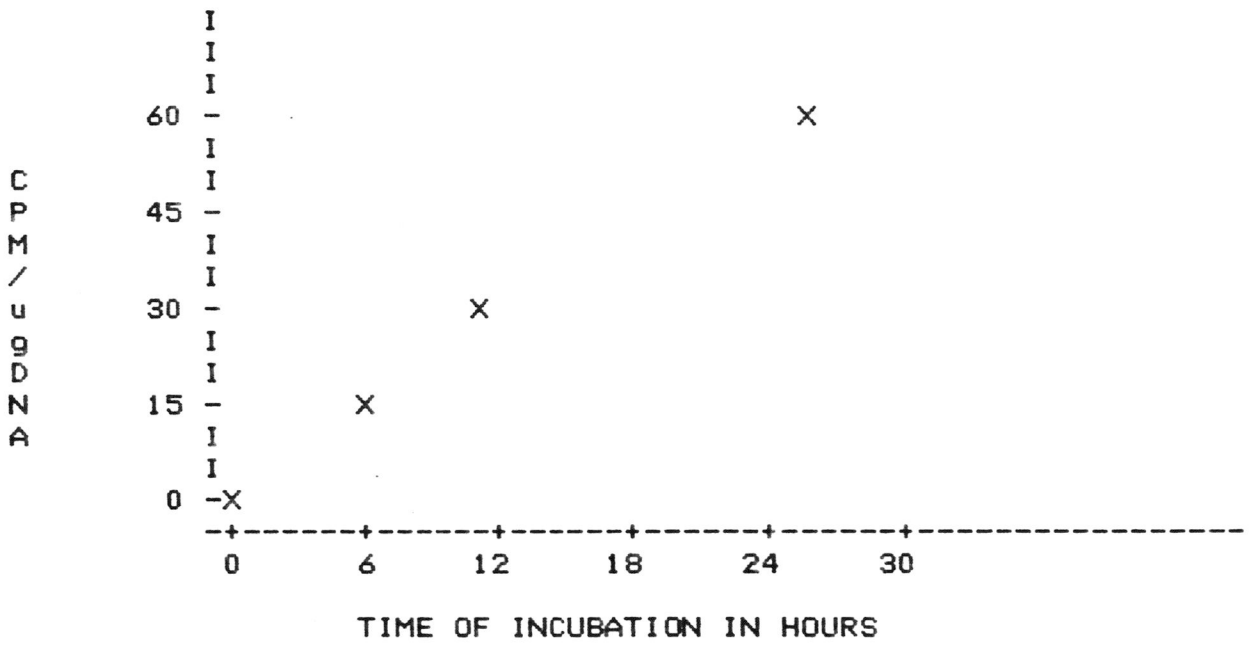
Statistical Analysis

Statistical analysis of data was performed on a Hewlett Packard 97 Calculator. The standard curve for the DNA assay was performed using the Hewlett Packard Curve Fitting Program. Analysis of variance was determined using the Hewlett Packard ANOVA program followed by a standard F-test and a Multiple Range test (Newman-Keuls Test). Both the Curve Fitting and ANOVA programs were part of a statistical package purchased from Hewlett Packard and were stored on magnetic cards. All statistics were calculated at the $p<0.05$ level.

RESULTS

A post thymidine injection incubation of twelve hours was chosen for several reasons. Graph 1 indicates that at twelve hours the rate of incorporation was constant whereas at twenty-four hours the rate had started to decline. This indicates that the ^{3}H-thymidine supply had started to be depleated or that the tridium had started to undergo nuclear

GRAPH 1: COUNTS OF ^{3}H-THYMIDINE INCORPORATED PER MICROGRAM OF DNA VERSUS INCUBATION TIME. (SEDENTARY CONTROL EGGS ONLY)



exchange. A twelve hour incubation was chosen over six hours because it allowed for a higher specific activity in the TCA-insoluble fraction.

A typical dose response was seen in viability, average embryo and brain weights (Table 1). A drop in viability from 82.8% in the sedentary control group to 73.9% in the vehicle control group is directly attributable to death due to contamination since 2 out of 23 (8.3%) eggs in the vehicle control group were contaminated with mold at the time of sacrifice. Low EtOH dosage caused a decrease in viability to 75.0% while the high EtOH dosage caused a further decrease in viability to 65.5% This inverse relationship of EtOH dosage to viability is consistant with reported expectations for ethanol treatment (Henderson and Schenker, 1977;, Henderson et al., 1979;, Dreosti et al., 1981). The percentage of infertile eggs was included in Table 1 to explain the depressed percentage of viability in the control eggs.

An average embro weight of 1.18 grams (g) and an average brain weight of 0.45 g for the vehicle control group was not statistically different from an average embryo weight of 1.21 g and an average brain weight of 0.45 g for the vehicle control group. The low EtOH dosage resulted in embryo and brain weights of 1.00 g and 0.36 g, respectively, while the high EtOH dosage resulted in weights of 0.83 g and 0.31 g, respectively, for embryo and brain weights. Both low and high EtOH dosage

TABLE 1: PERCENT VIABILITY, AVERAGE EMBRYO WEIGHT AND AVERAGE WEIGHT VERSUS TREATMENT.

Treatment	Viability	Infertility	$\bar{X}$ Embryo Weight e	$\bar{X}$ Brain Weight e
Sedentary	82.8% (24/29)	10.3% (3/29)	1.18+- 0.09	0.42+- 0.06
Vehicle	73.9% (17/23) d	4.3% (1/23)	1.21+- 0.14	0.45+- 0.04
0.3 g EtOH	75.0% (18/24) a,b	0.0% (0/24)	1.00+- 0.16 a,b	0.36+- 0.06 a,b
0.6 g EtOH	65.5% (19/29) a,b,c	3.4% (1/29)	0.83+- 0.10 a,b,c	0.31+- 0.04 a,b,c

a statistically significant from sedentary control group
b statistically significant from vehicle control group
c statistically significant from 0.3 g EtOH group
d two eggs were contaminated with mold
e weight in grams

resulted in weights which were statistically lower than either control group and the high EtOH dosage resulted in weights which were statistically lower than the low EtOH dosage weights.This weight depression is consistant with symptoms of FAS (Sandor and Ellas, 1970; Henderson et al., 1979; Woodson and Ritchey, 1979).

Ethanol administration resulted in an inhibition of thymidine incorporation into the DNA of embryonic chick neural tissue. Specific activities for the DNA from each group is presented as counts per minute of thymidine incorporated per microgram of DNA (cpm/ugDNA) per twelve hours of incubation. The specific activity of DNA (Table 2) from the low EtOH dosed embryos was 24.2 cpm/ugDNA and the high EtOH dosed embryo was 15.9 cpm/ugDNA. Both of these specific activities were statistically lower than the specific activities of 33.9 and 33.1 cpm/ugDNA for the sedentary control group and vehicle control group, respectively. The high EtOH dosage value was statistically lower than the low EtOH value while there was no difference between the two control groups.

Data (in text on p.23) from the samples which had DNase I, type III, added indicated that the ^{3}H-thymidine was, in fact, being incorporated into the DNA. Only control samples were used for this determination since they had the highest specific activity, viability, and there were more samples in this group. The average counts per minutes (cpm) for the 0.5 ml DNase treated sample was 33.4 while the background cpm for the vial

TABLE 2: SPECIFIC ACTIVITY OF ^{3}H-THYMIDINE INCORPORATED INTO DNA BY TREATMENT

Treatment	Counts/.5 ml Homogenate	DNA*/.5 ml Homogenate	Specific Activity
Sedentary	390.4+-172.6	11.3+-3.1	33.9+-8.3
Vehicle	320.6+-106.2	9.8+-3.7	33.2+-4.3
0.3 g EtOH	292.2+-33.5	11.7+-3.1	24.2+-4.3 a,b
0.6 g EtOH	74.6+-47.2	5.4+-3.9	15.9+-10.4 a,b,c

a statistically significant from sedentary control
b statistically significant from vehicle control
c statistically significant from 0.3 g EtOH group

* DNA in micrograms

with only monophase was 30.3. The average µg of DNA per 0.5 ml of DNase treated sample was 1.5 µg. This data gives a specific activity of 2.07 cpm/µgDNA which indicates that virtually all incorporated ^{3}H-thymidine had been removed from the TCA-insoluble fraction. Since DNase caused the specific activity to decrease as such, it can be assumed that the specific activity of the TCA-insoluble fraction is in fact due to labeling of the DNA with ^{3}H-thymidine.

The results from the double isotope technique are presented in Appendix 2. This data is not incorporated in this portion of the thesis since insufficient data was collected.

DISCUSSION

Results demonstrated that ethanol effects the viability of the chick embryo, the average embryo weight, and the average brain weight. It has been shown that ethanol produces a dose response in that it causes a decrease in viability and that the higher the concentration, the higher the mortality. Increasing the ethanol concentration injected into the egg results in a decrease in average embryo and brain weights.

Ethanol has also been demonstrated to reduce the amount of ^{3}H-thymidine incorporated into the chick neural tissue. This indicates that ethanol inhibits the synthesis of DNA by blocking one of the enzymes that catalize the reaction associated with incorporation of thymidine into DNA.

Validity of these results is supported by Sandor and Ellas (1970) who reported that a high ethanol dosage, greater than 0.6 g EtOH/Kg egg weight, resulted in an average embryo weight of 0.88 g while the control egg average embryo weight was 1.11 g. The values reported in the present research are in close agreement with these values. Novikoff and Potter (1947) reported that an eight day embryo had an average of 240 µg of DNA in the untreated embryo. The present study has shown that in the control embryo there was an average of 94.9 µg of DNA in the brain region alone. Assuming that the amount of DNA is constant (not necessarily so) in all tissues, this gives a total body concentration of 266 µg. This DNA concentration corroborates the ones reported in this research.

Dreosti and coworkers (1981) studied the effect of ethanol on the incorporation of ^{3}H-thymidine in regenerating rat liver. They reported that control animals had an average specific activity of 28.3+-4.2 cpm/µg DNA in the regenerating tissues while 0.2 g and 0.5 g of EtOH/Kg of body weight resulted in specific activities of 20.8+-3.6 and 15.5+-1.8cpm/µg DNA, respectively. Although the present research was in the chick model using neural tissue instead of rat liver, rapid DNA synthesis was observed in both models. The values from the regenerating rat livers are very close to those reported in the present research on the chick embryo.

Preliminary data from the Double Isotope Labeling Technique

is presented in Appendix 2. The data indicates that there is no difference between the ratios for the various nucleotide intermediates, thus indicating that the ethanol may be having an inhibitory effect at the DNA polymerase level. However, the data is not supported by a sufficient number of observations to perform any standard statistical analysis.

These results can be substantiated best by trying to demonstrate that the inhibition is due to some reason other than the ethanol treatment. Since the results from the present research has demonstrated that ethanol inhibits incorporation of thymidine into the neural tissue of the chick embryo, the next logical direction to proceed with this research should be, to determine if the inhibition of thymidine incorporation is due to inhibition of an enzyme used to incorporate thymidine into DNA or is it due to some other reason. The first parameter to examine is, does ethanol affect the permeability of the inner shell membrane and the vitelline membrane to thymidine? Should ethanol affect the permeability, then the results of this research would be void. To determine if the transport is affected, control and ethanol dosed, fertile chick eggs must be injected with labeled thymidine and then allowed to stand for varying periods of time. After standing, the albumin and yolk must be assayed for radioactivity and thymidine content. To support this research, there must be no difference in the radioactivity and thymidine concentration in the albumin and

yolk from control and ethanol dosed eggs. Another method to rule out the possibility of ethanol affecting the membranes permeability to thymidine would be to use a cell free system to examine the effect of ethanol on thymidine incorporation. Culturing chick neural cells in suspension would eliminate the vitelline and inner shell membranes which may inhibit thymidine passage in the presence of ethanol. The second parameter to investigate should be, is the incorporation of thymidine into neural tissue proportional with protein content and DNA content? Under constant conditions, the rate of incorporation should be proportional to the protein content and especially the DNA content. A third aspect to examine is, to determine if incorporation of thymidine into the neural tissue can be inhibited with known inhibitors of DNA synthesis. This could be approached by injection into the egg, prior to injection with labeled thymidine, an enzyme inhibitor that affects one of the enzymes that convert thymidine to dTTP or DNA polymerase which incorporate dTTP into DNA. If the inhibitor prevents the incorporation of labeled thymidine into the neural tissue, this would indicate that the radioactivity found in the TCA-insoluble fraction is due to an enzyme mediated process and not due to non-specific uptake of the labeled thymidine. The results of the current research will be supported if it can be demonstrated that thymidine incorporation can be prevented by these inhibitors.

APPENDICES

Appendix 1: Composition of Chick Ringers.

NaCl---------- 9.0 g/L

KCl----------0.42 g/L

CaCl2----------0.25 g/L

Adjust pH to 7.4 with HCl or NaOH.

Appendix 2: Results of Double Isotope Labeling Experiment

RATIO OF ^{3}H TO ^{14}C FOR THE THYMIDINE NUCLEOTIDE INTERMEDIATES

Intermediate	Ratio
Thymidine	1.44
Thymidine 5′-monophosphate	1.55
Thymidine 5′-diphosphate	1.25
Thymidine 5′-triphosphate	1.29

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