A STUDY OF THE PATHOGENESIS OF FETAL DAMAGE CAUSED BY ETHANOL IN THE EXPERIMENTAL MOUSE

by

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SUMMARY

In an attempt to determine mechanisms of certain aspects of ethanol-induced fetal damage, I have established a mouse model of the fetal alcohol syndrome based on the work of Chernoff (1977), using inbred C3H mice. Ethanol or its metabolite, acetaldehyde, was administered to female mice prior to and throughout gestation. Ethanol in doses of 6%, 10% and 20% ethanol derived calories and acetaldehyde 3.9mg and 11.8mg were administered daily in a nutritionally balanced liquid diet.

An acute dose study was also undertaken, in which pregnant C3H mice were given "binge" doses of 1mℓ of a 7.35% solution of ethanol, twice daily through an orogastric tube, on days one and eight or four and twelve of gestation.

The mice were sacrificed on day eighteen of gestation and the fetuses weighed and examined macroscopically. Some were sectioned using Wilson's method of free-hand razor blade sectioning (Barrow and Taylor, 1969), and the skeletons of the others were examined using a modified Dawson's method of skeletal preparation (Richmond and Bennett, 1938).

A separate in vitro model based on the work of New (1967) was established, in which embryos of eight or nine days' gestation were explanted with visceral yolk sac intact from normal C3H mice. They were cultured for twenty-eight hours in rat serum containing various concentrations of ethanol or acetaldehyde (ethanol 1500, 3000 and 6000mg/ℓ and acetaldehyde 7.4, 19.7 and 39.4mg/ℓ). During the last four hours of the culture period the embryos were labelled with one microcurie of tritiated thymidine (specific activity 5curies/mmol). At the end of the culture period the embryos were assessed morphologically, and then prepared for liquid-scintillation counting to determine DNA synthesis by measuring tritiated thymidine uptake. Small numbers of embryos from each group were used for autoradiographic studies in an attempt to quantitate the uptake of label in the various parts of the embryo.

I found that ethanol given in chronic dosage in vivo was embryotoxic in all three doses studied. There was no evidence of maternal toxicity other than hyperactivity at the highest dose used and maternal jaundice in a small number of the 10% EDC and 20% EDC mice. Acetaldehyde given in chronic dosage in vivo produced no toxic effects on mothers or fetuses, other than a reduction in placental weights.

Acute "binge" ethanol dosage of mothers on days one and eight or four and twelve of gestation did not appear to have any adverse effects on mothers or fetuses, apart from changes in placental weights. These findings should be viewed with caution, as the in vitro studies did not produce a corresponding result. In the latter study there was a marked time-related response, particularly for acetaldehyde.

Ethanol given in vitro produced little evidence of toxicity except at dose levels which in the corresponding in vivo situation were extremely toxic to the mothers.

Acetaldehyde, given in vitro in minute fractions of the harmless doses given to mothers in vivo, proved to be highly toxic to 8-day embryos and relatively non-toxic to 9-day embryos. This difference in sensitivity indicates that there must be some protective factor intervening between eight and ten days gestation - possibly the developing placenta may have a role here.

From these findings I would suggest that acetaldehyde is a true teratogen, and the abnormalities produced in the chronic ethanol in vivo study were probably largely due to the action of acetaldehyde.

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The main purposes of teratology are to find out why some babies enter the world suffering from physical, mental or metabolic handicaps from which the majority of their contemporaries are free, and with the knowledge thus acquired, to remove or, at least, alleviate the effects of these conditions.

(Woollam, 1966:7).

INTRODUCTION

I set out to investigate various aspects of drug-induced fetal damage with two particular objectives in mind:

(a) to gain further understanding of mechanisms of drug-related teratogenesis, with particular reference to effects on the developing nervous system.

(b) to develop techniques for evaluation and prediction of teratogenic potential of new medicines.

These represent critical problems in pharmacology and therapeutics.

The history of teratology and contemporary knowledge concerning known teratogens and their mechanisms of action are reviewed. The human fetal alcohol syndrome, and animal models of it which have been described by other researchers are also discussed.

I include detailed descriptions of the in vivo and in vitro models which I have used, the mode of administration of ethanol and its metabolite, acetaldehyde, and the determination of dose-response effects of acute and chronic administration of ethanol. Assessment of fetal damage by macroscopic, histopathological and radiolabelling techniques is also included.

Comparisons are drawn between the in vitro and in vivo models, and ways in which the findings in this series of experiments might reflect possible mechanisms of ethanol induced damage to human fetuses are discussed.

CHAPTER 1

TERATOLOGY AND THE EFFECTS OF ALCOHOL ON THE FETUS

1.1. A BRIEF HISTORY OF TERATOLOGY

Throughout the ages man has displayed a peculiar fascination for the 'abnormal' in his fellow man, and, depending on the religious beliefs and cultural norms of the time, people having congenital deformities have been either reviled or revered.

The first permanent records of congenital malformations were sculptures or images. The oldest known to us, depicting a double headed creature, was found at the Neolithic site of Catal Hüyük in Asia Minor. It dates from approximately 6 500B.C. (Mellaart, 1963:29).

The earliest known written records of congenital malformations were Babylonian, found on clay tablets in the library of Ashurbanipal, who lived in the 7th century B.C. It is thought that the original texts may be as much as 4 000 years old and were kept for the purpose of divination (Ballantyne, 1904).

In many instances the etiology of congenital malformations is yet to be explained, but myths and theories as to their cause have abounded throughout history. Belief in the Theory of Supernatural Causes has spanned time dating from pre-history to the present day (Barrow, 1971). The Theory of Maternal Impressions has been accepted for thousands of years, and today some behavioural scientists believe that pre-natal maternal stress can affect fetal development (Joffe, 1965). The Hybrid Theory which persisted through the dark ages had little scientific foundation and caused great suffering to both the parents and child in many cases.

More recent, and having a stronger scientific base, have been the Mechanical, Fetal Disease, Developmental Arrest and Genetic

Theories (Barrow, 1971). All these theories can explain the etiology of some congenital malformations but they provide only partial answers to the problem.

A number of specific factors are now recognised in the causation of congenital anomalies, namely:-

chromosomes, infections, irradiation, nutrition, hormones and drugs (Robson and Sullivan, 1968).

Experimental investigation of congenital malformations is not new. Saint-Hilaire (1820) was an early pioneer in this field. He applied physical insults to chick embryos, his son Isidore documented this work and also described a classification of monsters. It was Isidore Saint-Hilaire who first coined the term 'teratology'. The latter half of the 19th century saw experimental embryology placed on a firm foundation, and experimental work and publications on this subject proliferated (Barrow, 1971). Leaders in experimental teratology were Lehnert (1909) who treated pregnant rabbits with strontium, and Stockard (1921:115) who showed that environmental agents could produce various types of monsters among treated fish embryos. Because these generations of scientists prepared the ground so thoroughly, experimental teratology has made significant advances in the past 25 years.

1.2. PRINCIPLES OF TERATOLOGY

The outcome of any teratogenic study is dependent upon a number of factors:-

1.2.1. the genotype of the embryo
1.2.2. the stage of development at which the insult is applied
1.2.3. the mode and duration of action of the drug
1.2.4. the mechanisms of action of the agent on the developing organism
1.2.5. the nature of the agent concerned
1.2.6. the dosage of the agent

1.2.1. The genotype of the embryo

The differences in reaction to the same potentially harmful

agents by individuals, or strains, or species has been observed many times and it is thought to depend on genetically determined variations in biochemical or morphological makeup (Wilson, 1977a.).

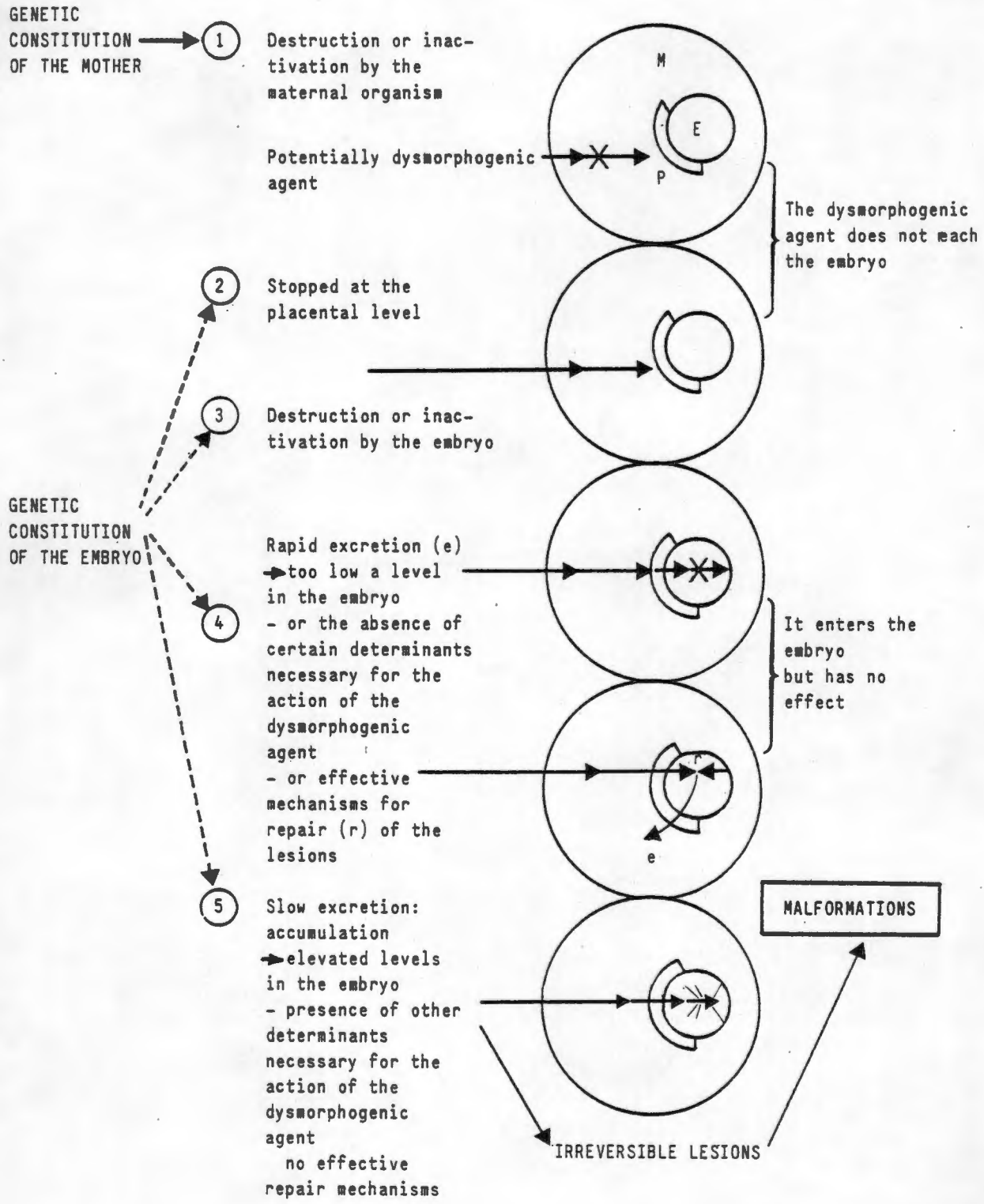

Fig 1.1 Mechanisms which might explain the difference in susceptibility to potential dysmorphogenic agents between individuals, ethnic groups and animal species.
(Tuchmann-Duplessis, 1975:39).

1.2.2. The stage of development at which the insult is applied (phase specificity)

When investigating a potential teratogen it is usual to divide pregnancy into three stages: pre-implantation, embryogenic and fetogenic. During the pre-implantation stage prior to gastrulation there is little likelihood of any teratogen producing a malformation as cell differentiation has not yet occurred, and the agent will either kill enough cells to abort the embryo or it will have no effect. The embryogenic stage is the period of maximum sensitivity to teratogens, resulting in gross malformations. This is the period of organogenesis and depending on the time of exposure specific organs will be particularly susceptible to teratogenic damage. Fetogenesis follows the development of the embryo and during this stage growth and histological differentiation occur. Teratogenic effects at this juncture are likely to be manifested as disturbances of function. These may be no less devastating in terms of future growth and development of the child than the major structural abnormalities which occur during the embryogenic period. Therefore it is extremely important in any study to know accurately the timing of these stages in the animal model used (Robson and Sullivan, 1968).

The period of maximum sensitivity to teratogens occurs during early organogenesis. It is possible to cause damage to a specific organ by administering a teratogen during the early stages of development of that organ, after cell differentiation has taken place. It has been shown in experimental teratology that one cannot produce predictable localised defects until after tissue and organ differentiation has begun (Wilson, 1977:51).

Thalidomide embryopathy is a good example of sensitivity to insult over a limited period of time. Mothers who took thalidomide between thirty five and fifty days after menstruation had a probable risk, greater than 50%, of producing an infant with thalidomide embryopathy. This apparently comprises a wide spectrum of abnormalities, but the morphological type of the malformation is essentially a function of the time of intake, and no other factors have been shown to be involved. (Lenz, 1966).

1.2.3. The mode and duration of action of the drug

Short acting drugs given over a brief period of time would be expected to produce very specific defects at a given stage of development. Teratogenic agents often have a specific action on some particular aspect of cellular metabolism (Robson and Sullivan, 1968). They may cause an increase in embryonic mortality in the absence of any harmful effect on the mother. thalidomide is a good example of such an agent.

1.2.4. The mechanisms of action of the agent on the developing organism

Teratogenic agents act in specific ways on developing cells and tissues to initiate abnormal embryogenesis.

Wilson (1977:55) has described nine different mechanisms by which an agent can initiate teratogenic damage:

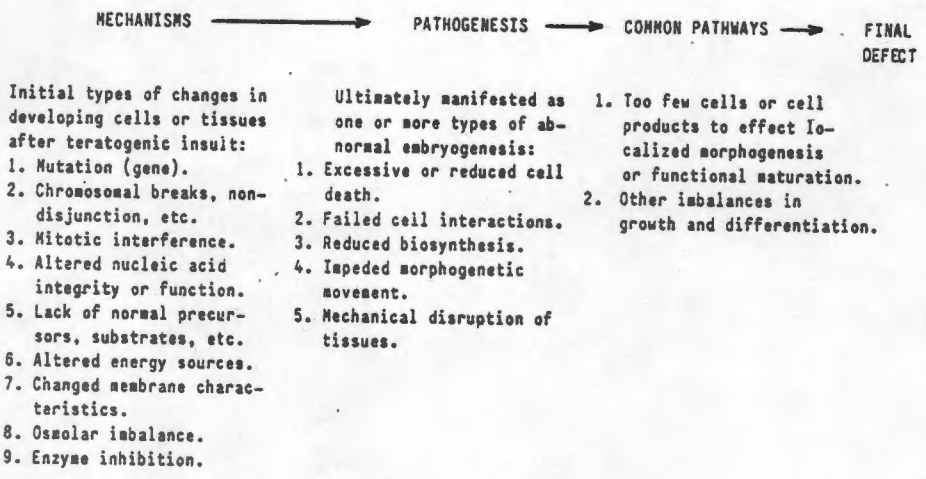

Fig. 1.2.Diagram of the successive stages in the pathogenesis of a developmental defect, beginning with the initial types of changes in developing cells or tissues (the mechanism) and continuing to the final defect. One or more mechanisms is initiated by the teratogenic cause from the environment. This leads to changes in the developmental system which become manifested as one or more types of abnormal embryogenesis. This in turn leads into pathways that seem often to be characterized by too few cells or cell products to effect morphogenesis or functional maturation, but the suggestion that this is a single or common pathway for all developmental defects is conjecture. (Wilson, 1977:55)

1.2.5. Nature of the agent concerned

The access of adverse agents to developing tissues depends on this. There are two routes of access:

1. By direct traverse of the maternal tissue without modification

eg. ionizing radiation and ultrasound.

2. Via the maternal circulation, eg. drugs or their metabolites. The placenta does not in most cases serve as a barrier to protect the embryo from foreign chemicals, as virtually all unbound chemicals in maternal plasma can cross the placenta (Wilson, 1977:59).

> Regardless of how it may be mediated the ultimate action of all teratogens seems to be to produce either cell death or an alteration in the rate of cell growth (mitosis).
> (Wilson, 1959:126).

1.2.6. The dosage of the agent

Teratogenic damage usually increases as the dosage increases, with a range from no effect at a low dose to lethal effect at a high dose.

Neubert and Barrach (1977) have described three aspects which are important when the dose response relationship of a teratogenic action is assessed:-
phase specificity
drug specificity
dose specificity
Phase specificity has already been discussed in section 1.2.2.

Drug specificity

It is possible to distinguish two types of teratogens
-"general teratogens" which act on a basic metabolic process
-"specific teratogens" which induce definite types of malformation but not a broad spectrum of all possible abnormalities.

Dose specificity

A special problem arises when we attempt to evaluate dose-response relationships in teratology. In a defined developmental system the application of a given dose of a drug may produce a specific abnormality but an increase in the dose often results in a more severe defect which prevents the original abnormality from being expressed. This phenomenon may be called "dose specificity" because the particular defect caused is not characteristic of the drug type but is critically dependent on the dose applied.

1.3. KNOWN TERATOGENIC AGENTS

Drugs established as embryotoxic in man are surprisingly few in number, namely:

- thalidomide
- androgenic hormones
- folic acid antagonists.

Agents suspected of being embryotoxic in man:

- anti-convulsants
- neurotropic-anorectic drugs
- oral anticoagulants
- alkylating agents
- oral hypoglycaemics.

Drugs possibly embryotoxic in man:

- female sex hormones
- tranquillisers
- salicylates
- antibiotics
- anti-tuberculous drugs
- quinine and other antimalarials
- anaesthetics
- insulin
- lithium carbonate

(Wilson, 1977b.).

Considering the evidence which has been gathered in the past two years I would suggest that maternal alcoholism could well be included in the first group.

1.4. A HISTORY OF ALCOHOL EFFECTS ON OFFSPRING

Frequent warnings about the dangers of alcohol to the unborn child have been sounded over many centuries. For example in about 1100 B.C. an angel appeared to Samson's mother and said to her

> Behold, you are barren and have no children; but you shall conceive and bear a son. Therefore beware, and drink no wine or strong drink, and eat nothing unclean.....
>
> (Judges 13:3-4 The Holy Bible. Revised Standard Version.)

The ancient Greeks knew of the risks involved and Aristotle wrote

> foolish drunken and harebrained women, most part bring forth children like unto themselves, moroses et languidos. (Burton, 1621).

Carthage and Sparta had laws prohibiting the use of alcohol by newly married couples (Burton, 1621).

More recently the Gin Epidemic (1720 - 1750) in England drew attention to the harmful effects of alcohol on the fetus,

> half the train of chronical diseases, with which we see children afflicted, are only the secondary sighs and groanings, the evidential Marks, and Reproaches, of parentive ill-spent Life..... The Consequences may, nay without doubt, will be brought on Infants, by the Debauchery of the Mother..... so that from the whole, the Regulation of the Mother, during her Pregnancy, is an affair of the highest Moment and Consideration. (Sedgewick 1725).

Many other writers echoed these sentiments. Parliament was petitioned by the College of Physicians in 1726, requesting control of the distilling trade, calling Gin

> a cause of weak, feeble and distempered children (George, 1925).

Stephens (1857) also pointed out that alcohol produced mentally defective offspring.

These warnings were not confined to England. In the U.S.A. Samuel Howe (1848) studied institutionalized mental defectives. He studied the family histories of three hundred idiots and found that one hundred and forty five had intemperate parents.

Interest and research continued into the early part of this century. Ballantyne (1898), author of the Manual of Ante Natal Pathology and Hygiene, divided pregnancy into three stages - the germinal, embryonic and fetal, and postulated that during the second stage - which included most of the first trimester - structural abnormalities were produced by alcohol. During the fetal stage, which encompassed the second and third trimesters,

alcohol would act by causing disease or abortion (Ballantyne, 1907). Later Ballantyne (1917) published the first English language bibliography on relationships between alcohol intake and its effects on offspring.

Unfortunately we have short memories and in spite of all the attention given to the problem in the nineteenth century, the English-speaking world chose to forget, ignore or ridicule all that had gone before. Little interest was shown in the subject, although work continued in other countries, until Jones et al. (1973: 1267) described the Fetal Alcohol Syndrome. They wrote

> eight unrelated children of three different ethnic groups, all born to mothers who were chronic alcoholics, have a similar pattern of craniofacial, limb and cardiovascular defects associated with pre-natal onset growth deficiency and developmental delay.
>
> This seems to be the first reported association between maternal alcoholism and aberrant morphogenesis in the offspring.

In fact Lemoine et al (1968) had described 127 offspring of alcoholic parents and his observations were strikingly similar to those described by Jones. Unfortunately this report was not widely read as it was not included in Index Medicus.

1.5. FEATURES OF THE FETAL ALCOHOL SYNDROME (Smith, 1979)

1.5.1. Commonly occurring features:-

Facial characteristics:

Eyes: short palpebral fissures

Nose: short, upturned, hypoplastic philtrum

Maxilla: hypoplastic

Mouth: thinned upper vermilion border, retrognathia in infancy, micrognathia or relative prognathia in adolescence.

Central nervous system dysfunction:

Intellectual: mild to moderate mental retardation

Neurologic: microcephaly, poor co-ordination, hypotonia

Behavioural: irritability in infancy, hyperactivity in childhood.

Growth deficiency:

Pre-natal: less than two standard deviations for length and weight

Post-natal: less than two standard deviations for length and weight

Disproportionately diminished adipose tissue.

1.5.2. Frequently associated features:-

Eyes: ptosis, strabismus, epicanthal folds

Mouth: prominent lateral palatine ridges

Heart: murmurs especially in early childhood, usually due to atrial septal defects.

Renogenital: labial hypoplasia

Cutaneous: haemangiomas

Skeletal: aberrant palmar creases, pectus excavatum.

1.5.3. Other features occasionally associated with the fetal alcohol syndrome:-

Eyes: myopia, clinical microphthalmia, blepharophimosis

Ears: poorly formed concha, posterior rotation

Mouth: cleft lip or cleft palate, small teeth with faulty enamel

Heart: ventricular septal defect (VSD), great vessel anomalies, Tetralogy of Fallot

Renogenital: hypospadias, renal defect

Cutaneous: hirsutism in infancy

Skeletal: limited joint movements, nail hypoplasia especially fifth polydactyly, radioulnar synostosis, pectus carinatum, bifid xiphoid, Klippel Feil anomaly, scoliosis

Muscular: hernias of the diaphragm, umbilicus or groin, diastasis recte.

1.5.4. Additional Abnormalities reported in the literature:-

1. Liver abnormalities (Habbick et al, 1979).
2. Tetraectrodactyly, club feet, short metatarsals and metacarpals (Herrmann et al, 1980).
3. Neural tube defect (Goldstein and Arulantham, 1978).

1.6. INCIDENCE OF THE FETAL ALCOHOL SYNDROME (F.A.S.)

Approximately one-third to half the offspring of chronic alcoholic mothers exhibit the F.A.S. to some degree (Hanson et al, 1976; Corrigan, 1976; Jones et al, 1974).

In France it is estimated that different degrees of the F.A.S. appear in one per three hundred deliveries (Dehaene et al, 1977; Samaille-Vilette and Samaille, 1977). The U.S.A. sees an incidence of the F.A.S. of one or two live births per one thousand (Abel, 1980).

> Maternal abuse of ethanol appears to be the most frequent known teratogenic cause of mental deficiency in the Western world.
>
> (Clarren and Smith, 1978:1066).

1.7. ALTERNATIVE OR CONTRIBUTORY CAUSES OF THE F.A.S.

Many questions have been raised in this regard but few answered. Is the damage due directly to alcohol or are its metabolic products, eg. acetaldehyde, also implicated? This remains to be clarified. Do other factors complicate the picture? eg. maternal malnutrition, smoking, drug abuse, etc.? While these may compound the teratogenic effect of alcohol none of them alone has reportedly produced the pattern of malformation occuring in the F.A.S. (Ouellette and Rosett, 1976).

Is the type of alcoholic beverage abused important?
It is possible that agents other than ethanol, which occur in alcoholic beverages, may also be damaging to the fetus eg. - aldehydes and aromatic moieties, or low levels of heavy metals may cross the placenta and be responsible for some of the defects which occur in the F.A.S. (Palmisano et al, 1969).

The effects of beer, wine, whiskey and ethanol on pregnant rats and their offspring were studied by Abel et al. (1981) but their findings suggested that the congeners present in these beverages did not potentiate the effects of ethanol on embryonic/fetal development in rats.

Cellular and cell free synthesis of RNA are inhibited by alcohols and aldehydes and this decrease in RNA synthesis may contribute to the development of F.A.S. malformations (Obe and Ristow, 1979:252).

Hypoglycaemia occurs after alcohol intake and it may be that this is a compounding factor or it may have a teratogenic action of its own (Erb and Andresen, 1978:648). It is well known that infants of diabetic women have a higher incidence of congenital abnormalities than the general population, but these children do not usually resemble the infants of alcoholic mothers.

Another theory which has been advanced is that chronic alcoholism causes depletion of dihomogamma-linoleic acid (DGLA)and prostaglandin E1 (PGE1), and that it is a deficiency of PGE1 which results in many of the alcohol-induced abnormalities. If this is the case then prevention should be relatively easy even if the mother does not reduce her alcohol intake (Horrobin, 1980).

Maternal alcohol intake. Is there a safe dose?
Unfortunately we do not yet know whether there is a safe dose or safe time during pregnancy when alcohol will not have adverse effects on the fetus. The University of Washington, using prospective studies, has estimated that one to two ounces of absolute alcohol per day may affect the fetus. The Food and Drug Administration (FDA) bulletin recommends no more than two drinks per day (Randall, 1978) but no one has actually defined a safe level of alcohol intake. Studies suggest the risk of an infant being born with the F.A.S. increases proportionately with the average daily alcohol intake of the mother. If maternal ingestion is less than one ounce absolute alcohol per day the risk to the fetus appears to be low. In the range of one to two ounces absolute alcohol per day the risk may increase to 10% and with intake over two ounces of absolute alcohol per day 19% of drinking mothers may expect to have abnormal infants.

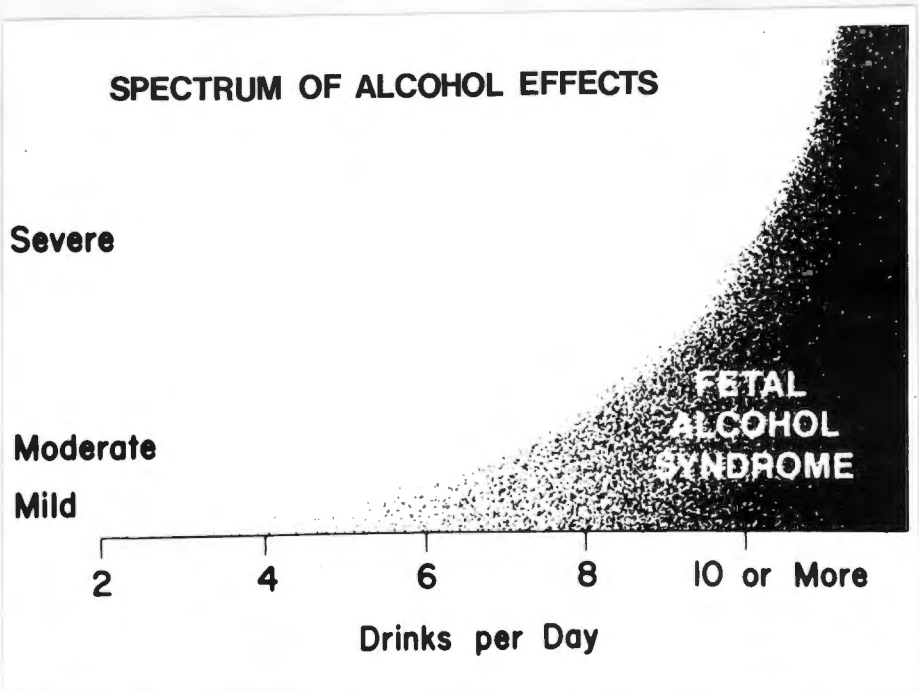

Fig. 1.3.
The spectrum of potential alcohol effects on the fetus range from very mild to severe in degree and extent. The fetal alcohol syndrome represents the most severe end of the spectrum and commonly occurs in women who are chronically alcoholic. By Ms. Jan Norbisrath, Department of Obstetrics and Gynecology, University of Washington Medical School (Smith, 1980:75).

The evidence suggests that quantity and timing of alcohol intake in the earliest stages of pregnancy may have important implications for fetal growth and development (Hanson et al, 1978). The tragedy is that severe damage may be done to the fetus at a time when the woman is not aware that she is pregnant. Thus it is of paramount importance that those contemplating pregnancy be aware of the risks in even moderate alcohol intake.

1.8. ANIMAL EXPERIMENTS

Many animal studies have been carried out in attempts to answer some of the questions listed in section 1.7. Hodge (1903) was the first English-speaking scientist to investigate the effects of alcohol on animal offspring using cocker spaniels. He found that the progeny of the alcoholic mothers showed less vigor and

vitality than the control animals. Rodent experiments followed soon after with studies by Nice (1912) and Stockard (1913). Using a guinea pig model Stockard studied treated animals for over four generations. He administered the alcohol by inhalation and found that the offspring of the treated animals had deformities, growth retardation, low birth weight, high infant mortality and low fertility.

Further studies on mice, rabbits and rats followed with similar results (Cole and Davis, 1914; MacDowell and Vicari, 1917). These early rodent studies focussed on the effect on offspring of paternal alcoholism.

Recently the teratogenic potential of alcohol has been studied in chickens (Sandor and Elias, 1968), guinea pigs (Papara-Nicholson and Telford, 1957), rabbits (Schwetz et al, 1978), rats (Sherwin et al, 1979; Sandor and Amels, 1971; Tze and Lee, 1975), mice (Chernoff, 1978; Kronick, 1976; Schwetz et al, 1978; Randall and Taylor, 1979), and beagle dogs (Ellis and Pick, 1976). Sandor injected an ethanol/distilled water (3:2) mixture into the air space of hens' eggs at zero and twenty-three hours incubation, and observed marked changes in early development with loss of the normal growth and differentiation rhythm and general retardation of development. He also observed a wide range of early structural malformations and a mortality rate of 50% in the first group and 95% in the second. Histological examination revealed severe necrotic changes within the central nervous system associated with morphogenetic disturbances and evidence of repair. There was also defective differentiation of the paraxial mesoderm primordia.

Papara-Nicholson and Telford (1957:438) treated pregnant guinea pigs with ethanol in a dose of less than three millilitres per kilogram three to four times weekly and found that the offspring of the treated mothers had low birth weights, poor movement and sucking and reduced haemoglobin levels. Male fetuses were most seriously affected. On histological examination of the brains he found consistent cellular lesions and retardation of myelination

at birth. Edema and dilatation of blood vessels with haemorrhagic areas were also frequently found. The number of immature newborns, stillbirths and neonatal deaths was markedly increased in the treated animals.

Schwetz (1978) and his co-workers treated mice, rats and rabbits with 15% ethanol in their drinking water during the period of major organogenesis. They achieved blood alcohol levels of 200mg% in non pregnant mice and 25 to 50 mg% in non pregnant rats and rabbits. Although maternal toxicity was evident in decreased liquid intake and reduced body weight they did not observe teratogenic effects in any of the three species studied. Randall and Taylor (1979) treated C57BL/6J mice with a liquid diet in which 17%, 25% or 30% of the calories were derived from ethanol, from day five to day ten of gestation. The fetuses were examined at term and the findings were a dose related increase in fetal resorptions and congenital abnormalities in the treated groups.

1.9. MOTIVATION FOR USING AN ANIMAL MODEL

The problems in predicting teratogenicity of an agent in man on the basis of animal data alone are well known and need not be discussed here. Most known human teratogens have been diagnosed as such only after human exposure to the agents.

In this study I was fortunate in dealing with a known human teratogen. The purposes of my experiments were to improve our understanding of how this teratogenic effect is produced, to determine whether there is any safe level of dosage or safe period during gestation, and whether ethanol itself is the teratogen. The results indicate a reasonable degree of success in attaining these goals, and it would have been extremely difficult if not impossible to obtain this information from human studies. However, I am very aware that extrapolation of these findings to man is difficult.

> The action of a dysmorphogenic agent on the conceptus depends mainly on three conditions: the developmental stage of the embryo, the genetic susceptibility of the embryo and the physiological or pathological status of the mother.
> (Tuchmann-Duplessis, 1975:40).

To a large extent it is possible in animal studies to control all three of these factors. Thus, while animal experiments in the past have not been markedly useful in averting human tragedies, they do have a significant role in elucidating mechanisms of teratogenicity in sensitive species. It follows logically that when one understands how a particular agent works it is easier to plan a course of action which will prevent its producing adverse effects in man. The difficulties of extrapolation from animal studies to man will remain but as our understanding of mechanisms of teratogenicity grows their importance will diminish.

CHAPTER 2

EXPERIMENTAL METHODS : IN VIVO MODELS

2.1. CHOICE OF A SUITABLE ANIMAL MODEL FOR THIS STUDY

The mouse was selected because of its high fertility and known sensitivity to teratogens in general (Schardein, 1976:22). Another consideration was the established fact that mice of various strains are sensitive to the teratogenic effects of ethanol, and that the abnormalities produced resemble those seen in the F.A.S. in humans (Randall and Taylor, 1979; Chernoff, 1977; Kronick, 1976). All strains of mice studied so far have demonstrated a characteristic dose response effect ranging from no effect at low levels of ethanol consumption to lethal effects at high levels of intake, although the zero effect or safe range of dosage has differed between strains (Chernoff, 1979).

The disadvantage in using mice is a high spontaneous abnormality rate which in C3H mice is 3.3% (Schardein, 1976:23; Heinecke, 1972). In this respect the rat would have been a more suitable model as it also has a high fertility rate, is genetically stable, and has a spontaneous malformation rate of less than one percent. Unfortunately the response of the rat to the teratogenic effect of ethanol has been rather variable with some conflicting reports (Sandor and Amels, 1971; Tze and Lee, 1975; Abel, 1978:5). (I selected C3H mice and based my chronic ethanol dose study on the model established by Chernoff (1977) in which he used C3H and CBA mice).

2.2. EXPERIMENTAL CONDITIONS

The animals were housed in one large room which was artificially lit, the air temperature thermostatically controlled at a mean of 24°C, and maximum and minimum temperatures were checked daily and recorded. The room was lit for fifteen hours each day starting at 7.00 a.m. with reduced intensity for the final four to five hours.

The female animals were housed individually in cages lined with white paper towel and the males in communal cages with vermiculite bedding. All animals initially were fed Epol Rat cubes whose constituents are listed below, and were allowed water ad libitum.

Table 2.1.
Constituents of Epol Rat Cubes

Total digestible nutrients	75.95%
Protein	20.8%
Fat	5.17%
Crude fibre	3.84%
Vitamin A	11 000 iu/kg
Vitamin D_3	2 750 iu/kg
Vitamin E	60 iu/kg
Vitamin K	5.7 parts/million
Pyridoxine	6 parts/million
Thiamine	3.9 parts/million
Riboflavine	5 parts/million
Pantothenic Acid	12 parts/million
Niacin	50 parts/million
Vitamin B_{12}	0.11 parts/million
Folic Acid	9.5 parts/million
Choline	500 parts/million
Calcium	0.726%
Phosphorus	0.6%
Sodium Chloride	0.9%
Cobalt	1 part/million
Copper	10 parts/million
Iron	100 parts/million
Zinc	50 parts/million
Iodine	0.2 parts/million
Manganese	75 parts/million
Metabolisable energy	12.48 Megajoules/kg

Once the females had settled down, usually after two to three days, they were started on a liquid diet plus rat cubes. When it was observed that they were feeding normally on the liquid diet the rat cubes were withdrawn. The liquid diet was based on that described by Chernoff (1977) who used chocolate

flavoured Metrecal, a product not available to me. The diet which I formulated using locally available products is described in detail in section 2.3. and table 2.2.

The cages were cleaned and fresh water given three times a week. All food and water containers were sterilised in Biocide D (300 parts/million chlorine in solution) and rinsed thoroughly after sterilisation.

For the duration of the experiment the female mice were weighed once or twice weekly using a Dial-O-Gram scale. Young adult mice aged about eight weeks were used for all the experiments, and male stock was changed every four to six months.

2.3. THE LIQUID DIET

I prepared this after consultation with a chemist and a dietician and a careful study of the local products. The following mixture was decided upon, as there was no single product which fulfilled all the nutritional requirements of the mouse (the first two items were kindly donated by Bristol Mead Johnson).

The mixture contained:-
2 grams of Sobee
1 gram of Nutrament T
12mg disodium phosphate
5mg potassium chloride
and ethanol or isocaloric amounts of sugar added to make up a total daily intake of 19.8 calories/mouse. Each feed was mixed with water to a volume of 10 millilitres. It was estimated that each animal would require 8 millilitres of this mixture per day to meet its normal requirements. 10 millilitres was given to the animals in the ethanol experimental and control groups to allow for loss due to spillage.

The feeds were prepared forty at a time, mixed with the appropriate amount of water and dispensed into 20-millilitre plastic syringes fitted with rubber bungs and stainless steel ball tipped feeding tubes (see figure 2.1.).

Fig. 2.1. Feeding tube.

Note - calibrated syringe
- availability of water
- paper towel bedding

(Photo: Mrs. E. Fuller)

TABLE 2.2. NORMAL DAILY DIETARY REQUIREMENTS FOR THE LABORATORY MOUSE

(Mice normally consume ± 4gms solid feed/day)

Normal Daily Requirement/4gm Diet	Range	Constituents of Diet found in 2g. Sobee 1g. Nutrament T. 12mg. Disodium Phosphate 5mg. Potassium Chloride	Deficiency or Excess Teratogenic
Protein	0.28 - 1g.	0.66g	
Fat	0.08 - 0.48g	0.365g	
Carbohydrate	1.8 - 2.2g	1.72g	
Crude fibre	0.16 - 0.2g	0.02g	
Ash	0.2 - 0.24g	0.09g	
Vitamin A	1.2 - 60iu	.34 - 76iu	Deficiency or excess teratogenic in the rat
Vitamin E	0.08 - 0.6mg	0.2mg	Deficiency teratogenic in the rat
Vitamin K	0 - 0.04mg	0	
Vitamin B_1	0.012 - 0.08mg	0.0154mg	Deficiency teratogenic in the rat
Vitamin B_2	0.016 - 0.04mg	0.016mg	Deficiency teratogenic in the rat and mouse
Vitamin B_3	0.024 - 0.2mg	0.109mg	Deficiency teratogenic in the rat
Vitamin B_6	0.008 - 0.06mg	0.017mg	
Nicotinic Acid	0 - 0.2mg	0.245mg	Excess teratogenic in the rat
Biotin	0 - 0.8µg	0.746µg	Deficiency teratogenic in the rat
Folic Acid	0 - 0.04mg	0.004mg	Deficiency teratogenic in the mouse and rat
Vitamin B_{12}	0.004 - 0.12µg	0.055µg	Deficiency teratogenic in the rat
Choline	4 - 6mg	1.27mg	Deficiency or excess not teratogenic in the mouse
Vitamin D	0 - 4iu	6.58mg	Deficiency teratogenic in the rat
Copper	0.02mg	0.02mg	
Fluorine	0.000028-0.002mg	0	
Iodine	0.00008-0.002mg	0.0012mg	
Iron	0.1 - 0.4mg	0.331mg	
Molybdenum	0.00008-0.00016mg	0	
Magnesium	2mg	2.816mg	
Phosphorus	20mg	21.47mg	
Potassium	20mg	15mg	
Selenium	0.0016mg	0mg	
Sodium	20mg	15.84 mg	
Sulphur	1.2mg	0mg	
Zinc	0.048 - 0.08mg	0.126mg	
Calcium	24mg	23.34mg	
Chloride	2mg	5.83mg	

TABLE 2.2. (Continued)

Normal Daily Requirement/4gm Diet		Constituents of Diet found in 2g. Sobee 1g. Nutrament T. 12mg. Disodium Phosphate 5mg. Potassium Chloride	Deficiency or Excess Teratogenic
Manganese	0.2mg	0.018mg	
Boron	0.00016mg	-	
Essential fatty acids	40mg	-	
Sugar	-	1.5gms	
Calories	13 - 22	19.8	

Table 2.2. shows the normal daily requirements for the laboratory mouse compared with the constituents of the liquid diet. The normal daily requirements were calculated from tables in Mitruka et al (1976:24), Lane-Petter and Pearson (1971), and Williams (1976:45). The teratogenic potential of deficiency or excess of certain constituents of the diet was obtained from Wilson (1959). Every effort was made to ensure that those components, whose deficiency or excess was known to be teratogenic, were present in the liquid diet in the correct concentration.

2.4. MATING PROGRAM

The animals were mated once weekly, males being placed singly in the female cages. Mating was restricted to a one hour period between 7.30 a.m. and 9.30 a.m. The onset of estrus in a normal female mouse is four to six hours after dark. In our animal room estrus would have started between 2.00 a.m. and 4.00 a.m. and ovulation occurred two to three hours later (Rugh, 1968:34). The choice of an early morning mating period thus ensured a maximum chance of pregnancy resulting. The liquid diets were removed from the cages during the mating period in order to prevent the males from being exposed to ethanol.

Towards the end of the study it became necessary to put the animals together more frequently (three or four times weekly under the same conditions), owing to pressure of time. These matings were observed and records kept.

The pregnant animals were sacrificed using an overdose of ether on day eighteen of gestation. Day one of pregnancy was counted as the day following mating.

2.5. PROCEDURE FOR STAINING FETAL SKELETONS BASED ON THE MODIFIED DAWSON METHOD (Richmond and Bennett, 1938)

The fetus was eviscerated through a small midline abdominal incision. For convenience it was eviscerated after fixation in ten percent buffered formalin although the preferred procedure is to eviscerate the fresh fetus as this permits free access of fixatives

and prevents liver pigments from staining the final mounting solution. Particular care was required in the evisceration of the thoracic contents to avoid damage to the ribs and spinal column. The brain was not removed. The specimen was then rinsed in tap water and placed in one percent potassium carbonate for four weeks or longer.

The soft parts were cleared by immersion in ten percent potassium hydroxide for about one month. Sometimes the tissues became too soft, and as the appendages were in danger of breaking off, it was necessary to harden the specimen in a solution of equal parts of glycerol, ninety five percent ethanol, and water, for twelve to twenty-four hours before returning the specimen to the clearing solution. Specimens left in the clearing solution for too long disintegrate and would even dissolve completely. It was therefore important to check their condition regularly.

The specimens were washed in running tap water overnight, after which they they were stained by immersion for thirty to sixty minutes, in a solution of Alizarin Red S, to which six to ten drops of one percent potassium hydroxide had been added. After staining, the specimens were again washed in running tap water for thirty minutes.

The soft parts were decolorized by immersion in an aqueous solution of twenty percent glycerol and one percent potassium hydroxide for two weeks or longer.

The specimens were dehydrated by leaving them for two to three days at a time in increasingly concentrated ethanol and glycerol solutions according to the following scheme:

Solution number	1	2	3	4	5	
95% ethanol	10	20	30	40	50	(mℓ)
Glycerol	20	20	30	40	50	(mℓ)
Water	70	60	40	20	0	(mℓ)

The specimens were then stored in screw cap glass vials and assessed using a dissecting microscope.

2.6. WILSON'S SECTIONING TECHNIQUE (Barrow and Taylor, 1969)

The fetuses were fixed in ten percent buffered formalin for a period of at least two weeks, and then sectioned using the free-hand razor blade technique described by Wilson (1965), although as Barrow and Taylor did, I modified the thoracic sections to allow fine dissection of the heart.

Sections of the head were obtained following Wilson's technique. The first cut was made transversly through the mouth and ears. The tongue was removed and the palate examined. Then with the palatal surface placed downwards three frontal sections were made: one anterior to the eyes, one through the eyes and one through the brain at the widest diameter of the head. Each section was examined under a dissecting microscope. From the head sections it was possible to diagnose or exclude cleft palate, anophthalmia, microphthalmia and internal hydrocephalus.

Next, transverse sections of the trunk were made with the fetus in a supine postion. The first section was cut through the mid portion of the neck after which the thorax was left intact for careful dissection of the heart. A transverse section was made through the diaphragm, transecting the base of the lungs. Transverse sections approximately three millimetres apart were continued caudally for examination of the liver, stomach, spleen, adrenals and kidneys. In the pelvic area the gastro intestinal viscera were removed revealing the ureters, bladder and sex organs (see figures 2.2., 2.3., 2.4., 2.5., and 2.6.).

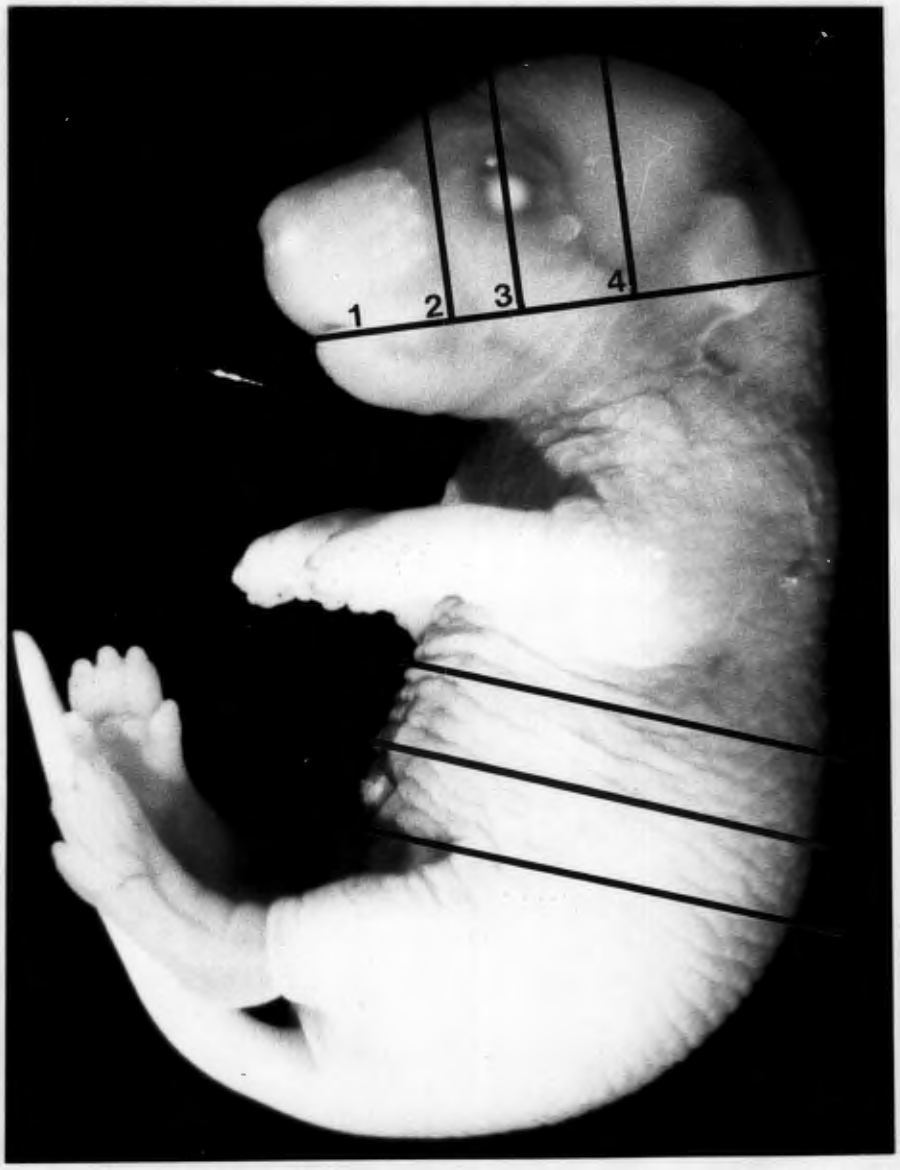

Fig. 2.2.

Preparation of Wilson's section: 18-day fetus

Sections are made as indicated by the lines on the photograph.

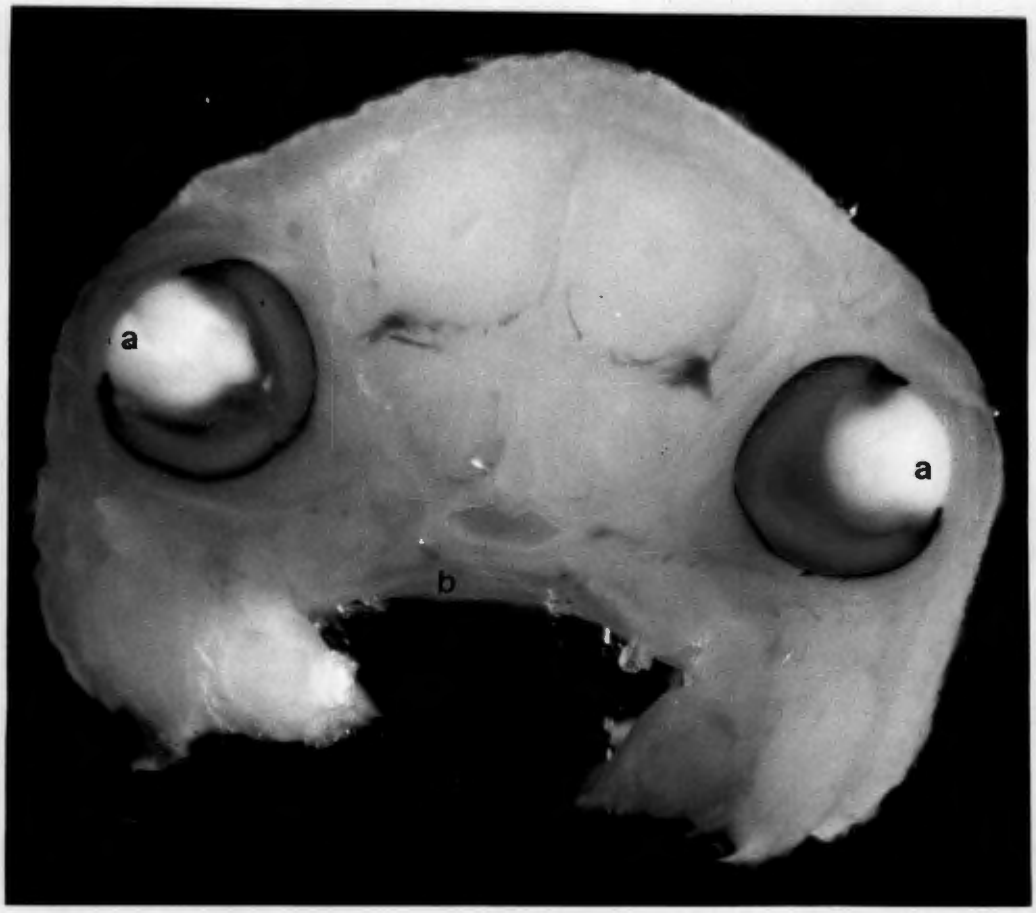

Fig. 2.3.

Wilson's section through the eyes: 18-day fetus.

Note - normal eye development (a)
- intact palate (b)

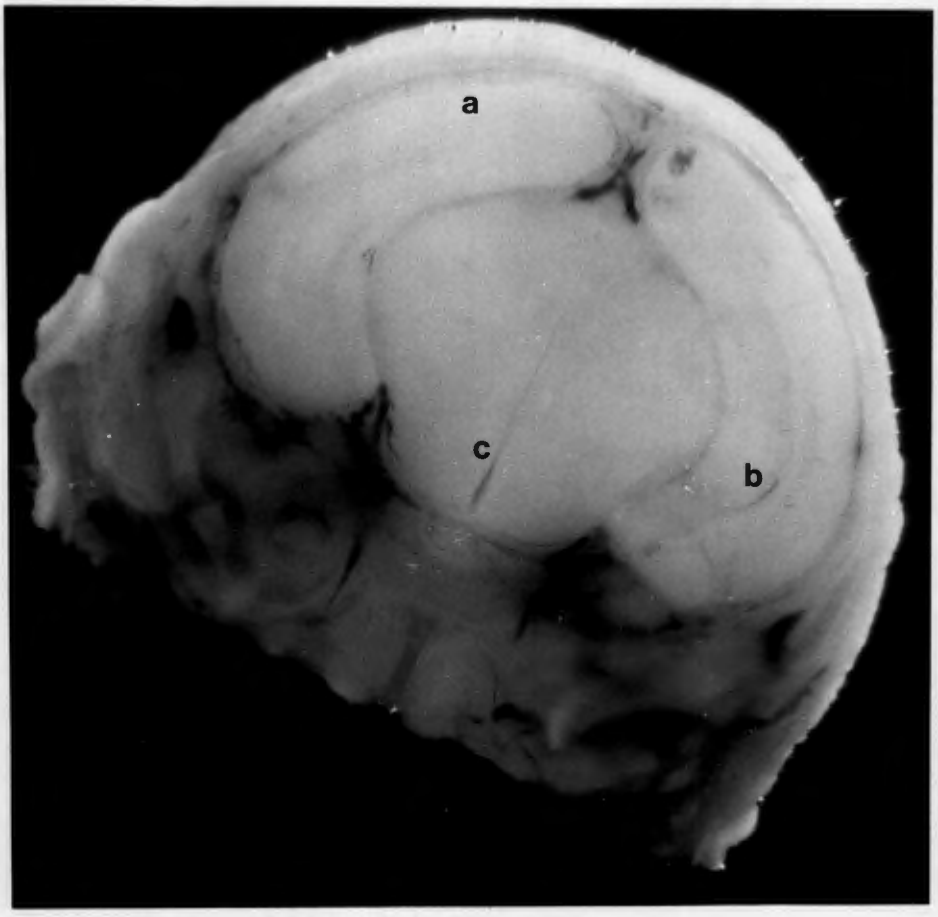

Fig. 2.4.

Wilson's section through the brain: 18-day fetus.

Note - normal cortex (a)

- normal lateral ventricles (b)
- normal third ventricle (c)

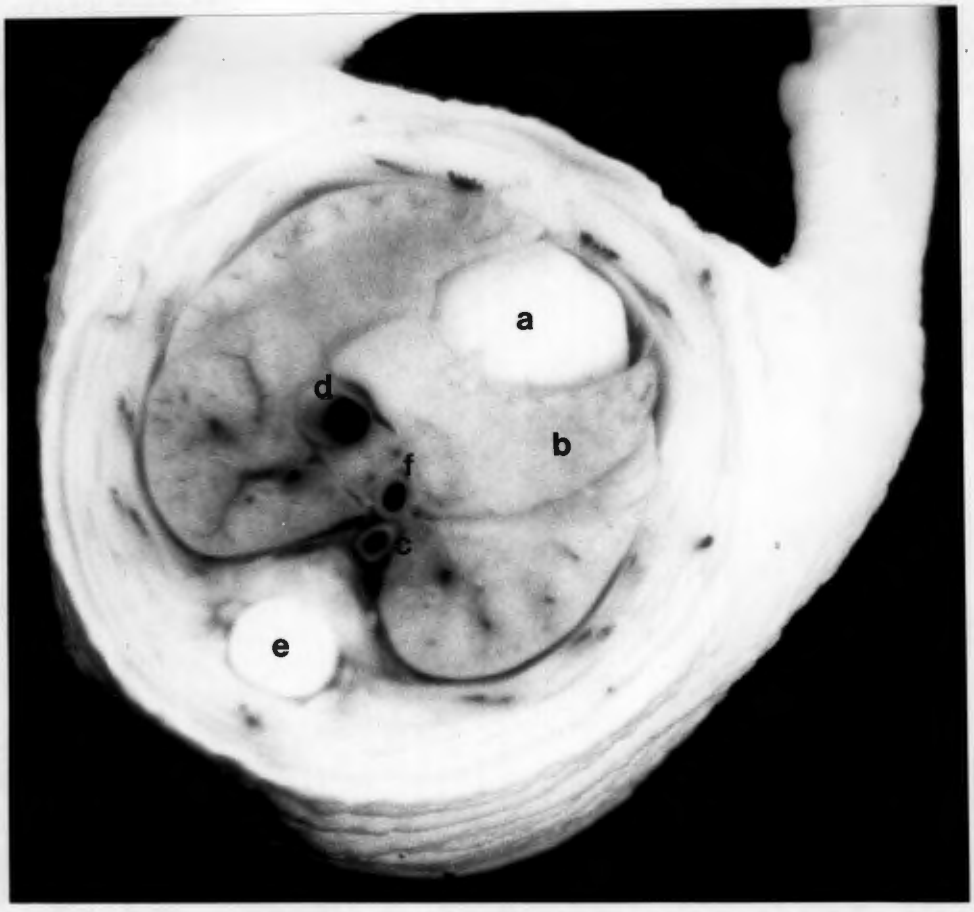

Fig. 2.5.

Wilson's section through the diaphragm: 18-day fetus.

Note - apex of heart (a)
- lungs (b)
- great vessels - aorta (c)
- inferior vena cava (d)
- spinal cord (e)
- esophagus (f)

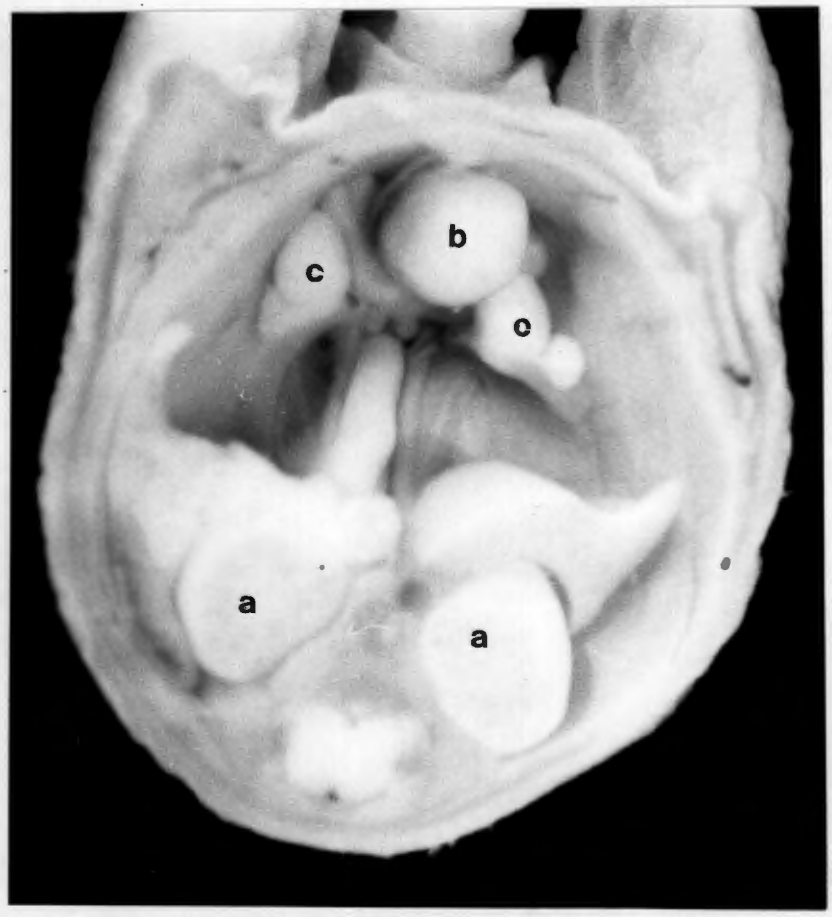

Fig. 2.6.

Wilson's section through the pelvis: 18-day fetus.

Note - normal kidneys (a)
- bladder (b)
- testes (c)

Heart dissection (Folb, 1981)

The thoracic portion of the trunk was opened ventrally with a mid-sagittal cut as described in Barrow and Taylor (1969) exposing the heart lungs and thymus gland.

The heart and lungs were dissected free as a unit from the thoracic cage. The two lobes of the thymus gland were separated along the midline allowing visualisation of the two main vessels - the aorta and the pulmonary artery, and their main branches.

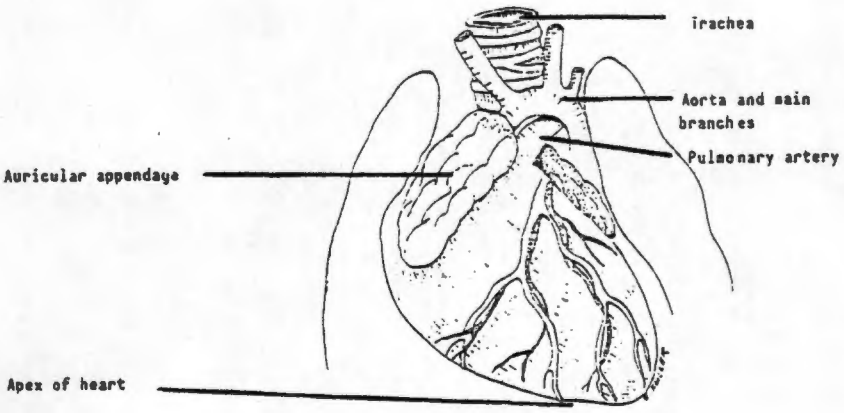

Figure 2.7.1. Exposure of the great vessels.

The lungs and diaphragm were then pushed aside from the base of the heart with fine forceps and the pericardium overlying the diaphragmatic surface of the heart exposed and incised. The pericardium was then stripped from the myocardium and the main coronary arteries inspected.

Using a fine pair of scissors the cardiac apex was excised at a point one to two millimetres from the outermost tip of the heart. In this manner the left and right ventricular cavities were exposed.

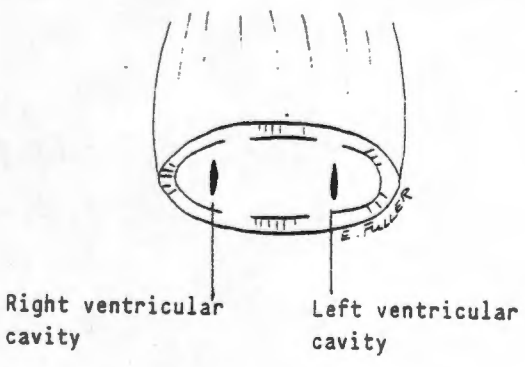

Figure 2.7.2. Transection of the heart.

Both cavities were then cut along their lengths as far as the aorta and the main pulmonary artery respectively.

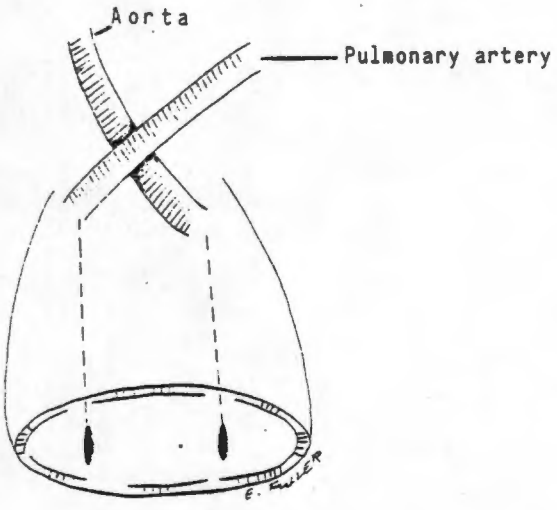

Figure 2.7.3. Longitudinal heart section to expose the ventricular cavities.

The interventricular septum, the two ventricular cavities and the aorta and pulmonary valves were then visualised directly. At this point the external appearance of the right and left atria and their appendages was examined and an incision made along the length of the two atria. Any blood clot present was removed, permitting inspection of the atrial walls and interatrial septum from within (wide variation of the normal appearances of the interatrial septum was noted).

Finally a close inspection was made of the spinal cord, the spinal column and the thoracic cage, the sternum and ribs, and the internal mammary arteries, intercostal vessels, and the paravertebral blood supply.

The entire dissection was performed under a dissecting microscope.

2.7. MEASUREMENT OF BLOOD ETHANOL LEVELS

Blood for ethanol estimation was collected at the time of sacrifice either by cardiac puncture or from the thoracic cavity after incision of the heart. During the pilot experiment blood was collected from the tail veins of non-pregnant mice.

The analyses were performed in the department analytical laboratory by laboratory staff.

Method: This was the standard method used in our analytical laboratory as described by Rottanburg, (1981).

2.7.1. INTRODUCTION

Gas Chromatography (GC) is limited to those substances or their derivatives which vaporize without decomposing. The most widely used detector in GC, responding with high sensitivity to all organic compounds, is the flame ionization detector which measures the ionization current generated when a compound emerges from the chromatographic column and burns in a hydrogen flame. The sensitivity of this detector enables 10^{-9} g of many components to be detected and does not respond to inorganic gases or water (Andrews, 1970).

GC allows the detection and identification of a variety of volatile substances, including the common alcohols. A suitable internal standard is selected whose elution properties resemble those of the substance to be determined and known concentration of the internal standard is added to the sample under investigation. The relative response of internal standard compared with that of alcohol to be measured allows an accurate result to be established.

GC has become a widely accepted analytical technique for the detection and quantification of various alcohols in blood, tissue and plasma. The advantages of GC in this context are its capacity for measuring volatiles, its speed and the micro-volume sample required (Freudiger and Vignau, 1966; Mather and Assimosis, 1965; Davis, 1966; Andrews, 1970).

2.7.2. INSTRUMENTATION AND OPERATING CONDITIONS

A Gow-Mac Series 750 Gas Chromatograph, Model 69-752 (Gow-Mac Instrument Co. New Jersey, USA) fitted with a flame ionization detector was used for the detection of ethanol in plasma.

A 1,2m, 2mm i.d. glass column packed with 0,2% carbowax 1500 on carbopack A 60/80 mesh was used under the following conditions: injection port temperature at 120°C, the detector at 140°C and the column oven at 115°C. Nitrogen was used as the carrier gas with a flow rate of 20mℓ/minute, hydrogen was set at a flow rate of 30mℓ/minute and oxygen at 300mℓ/minute.

A Hewlett Packard Automation System 3385A Integrator/recorder was used to record the chromatograms.

2.7.3. REAGENT AND SAMPLE PREPARATION

(i) Solvent test solution
1mℓ each of methanol, ethanol, acetone, isopropanol, and n-propanol were mixed and diluted to 500mℓ with distilled water. All reagents were guaranteed Reagent Grade (Merck, Darmstadt, Germany). To give final concentrations of 157mg%, 157mg%, 157mg%, 156mg%, 160mg% respectively.

(ii) Internal standard
1mℓ isopropanol and 1mℓ n-propanol were mixed and diluted to 1 000mℓ with distilled water to give concentrations of 78,1mg% and 80,2mg% respectively.

(iii) Ethanol control solution

1mℓ absolute ethanol was diluted to 500mℓ with distilled water. The resulting solution had a concentration of 158mg%.

(iv) Standard ethanol solutions

2,0mℓ absolute ethanol was diluted to 500mℓ with distilled water. The resulting solution had an ethanol concentration of 316mg%; this was labelled Solution A. Various subsidiary standards containing 158mg%, 79mg%, and 31,6mg% were made by dilutions of Solution A.

200µℓ of the standard solutions were mixed with 200µℓ of internal standard solution and the tubes were sealed with parafilm. 5 aliquots of each standard solution were prepared.

From the blood sample drawn from each patient 200µℓ serum was mixed with 200µℓ internal standard in a tube and sealed with parafilm.

2.7.4. ASSAY PROCEDURE

(i) 1µℓ of the test solution was injected directly onto the column and the retention times (R_T) were recorded. The solvents elute in the following order: methanol, ethanol, acetone, isopropanol, n-propanol.

(ii) The syringe was rinsed 20 times with distilled water.

(iii) 1µℓ of the prepared sample from each standard solution was injected directly on to the column and the R_T values were recorded. The syringe was washed 20 times between each injection.

(iv) The plasma samples were injected in the same way.
Following the determination of the standard solutions, 1µℓ of the prepared sample from each patient was assayed in the same way.

The ratios of ethanol to isopropanol were calculated for standard and serum samples. The ratios for the standards were used to prepare a standard curve which, in turn, was used in the determination of ethanol concentrations in the plasma samples.

Ethanol Concentration of Standard Solutions (mg%)	Number of Samples	Mean Ethanol/ Isopropanol Ratio	Standard Deviation	Coefficient of Variation (%)	Observed Concentration
0	4	0,00	0,00	0,00	0,00
31,6	4	0,344	0,010	2,9	30,5
79	4	0,937	0,005	0,53	79,5
158	4	1,910	0,011	0,57	160
316	4	3,780	0,010	0,26	315

Validation of ethanol determination by Gas Chromatography

Slope = 0,012
Y intercept = 0,020
r = 0,9998

2.8. PILOT STUDY

Chronic alcoholic model

The pilot study was performed using ICR (albino) and C3H inbred mice. The aim was to determine the maximum dose of ethanol which could be tolerated without undue toxic effects on the mother. The animals were housed and established on the liquid diet as described in sections 2.2. and 2.3.

Eight ICR and seven C3H mice were used. Weights at the start of the experiment ranged from 20 to 27 grams in the ICR group and 19 to 25 grams in the C3H group. All animals were fed the control diet for the first ten days of the experiment and thereafter the animals received increasing doses of ethanol to a maximum of thirty percent ethanol derived calories (EDC). The dose of ethanol started at two percent EDC and the ethanol content was increased by two percent every two or three days. See table 2.3. At weekends, initially, the mice were given the option of taking liquid feed or rat cubes, and as they showed a preference for the solid feed, it was decided to provide the entire dietary intake in liquid form. Records of daily feed intake were kept and the animals were weighed three times a week.

They remained healthy and gained weight satisfactorily until the eighteen percent EDC level of dosage. At this point they became hyperactive during the day. At the twenty percent EDC level some of the animals displayed loss of coordination and unsteady gait. At the twenty-four percent level they seemed more able to tolerate the ethanol and besides hyperactivity showed no evidence of toxicity.

Blood ethanol levels were measured in four mice (Blood was taken from their tails at 2.00 p.m. Diets were dispensed every morning between nine and ten o'clock).

Mouse		Blood ethanol level
ICR	1	0mg/ℓ
	2	992mg/ℓ
C3H	1	1950mg/ℓ
	2	264mg/ℓ

TABLE 2·3·

ICR group and C3H group. Mean weights at a given dose of ethanol.

Experiment day	Diet	Mean weight of ICR mice in grams	Mean weight of C3H mice in grams
0	Control + pellets	23.10± 2.58	22.20± 2.17
4	Control + pellets	23.29± 2.69	23.80± 2.59
6	Control	23.14± 2.73	23.80± 2.28
8	Control	24.29± 2.21	24.71± 2.43
11	Control	26.00± 2.65	24.71± 2.81
13	2% E D C.	24.71± 2.12	22.36± 1.60
15	4% E D.C	24.64± 2.15	24.43± 3.05
18	8% E.D C	27.64± 2.63	26.47± 3.16
21	10% E D C.	24.10± 2.41	23.14± 3.18
22	10% E D·C.	23.30± 2.63	22.64± 3.00
25	10% E·D·C.	25.20± 2.41	24.64± 2.55
27	12% E·D.C.·	25.40± 2.03	23.86± 2.61
29	14% E D C.	25.10± 1.84	23.00± 2.72
32	16% E D C.	23.80± 2.45	22.16± 2.53
34	18% E D C.	25.60± 1.63	23.36± 2.43
36	18% E D. C	24.90± 2.58	23.29± 2.64
39	20% E.D C.	24.90± 2.23	23.36± 3.36
41	22% E D.C.	25.50± 2.32	24.03± 2.76
43	22% E D. C.	26.40± 2.16	23.74± 3.28
46	24% E.D C.	27.30± 2.30	26.58± 1.30
49	24% E.D C.	27.30± 2.35	26.88± 2.74
53	26% E.D C.	27.40± 2.05	26.00± 3.17
61	30% E.D C.	26.90± 1.04	26.00± 3.17
67	30% E.D.C.	21.40± 1.78	19.14± 2.03
74	28% E.D C.	17.80± 1.35	18.20± 1.71
76	26% E.D.C.	18.60± 2.21	16.94± 1.72
79	20% E.D C.	20.90± 2.12	20.68± 1.60
82	20% E D C.	21.20± 3.38	21.40± 2.83
85	20% E D C	22.30± 2.65	20.30± 1.13
88	20% E D.C.	24.10± 3.09	22.45± 0.92
90	20% E D. C.	26.70± 1.87	24.25± 1.04
95	20% E.D.C	27.80± 1.57	24.45± 0.07
99	20% E.D.C.	29.30± 2.01	27.00± 0.57
102	20% E D.C.	31.07± 2.16	27.2 ± 0.42
106	20% E D.C.	28.3 ± 1.25	25.25± 1.06
110	20% E D C.	30.80± 1.97	25.25± 1.63
112	20% E D C	31.37± 1.37	25.80± 1.13
117	20% E.D.C.	31.20± 2.62	25.50± 0.71
119	20% E.D C	31.03± 2.24	25.70± 0.42
124	20% E D C.	31.63± 2.20	25.90± 2.12
126	20% E D C	31.53± 1.75	26.30± 0.99
131	20% E D C.	32.20± 1.85	27.25± 1.06
133	20% E D C	31.10± 1.83	25.45± 0.07
140	20% E.D C.	32.37± 2.79	26.5 ± 0.71
145	20% E.D C.	32.27± 2.30	25.2 ± 0.28
147	20% E D C	32.10± 3.00	25.85± 0.64
152	20% E D C.	31.17± 3.67	26.15± 0.21
155	20% E D C	31.17± 3.00	26.6 ± 0.57
158	20% E.D C	32.97± 2.24	26.00
161	20% E D C.	33.53± 2.15	26.75± 1.77
166	20% E D C	32.60± 3.00	25.30± 0.14

At the thirty percent EDC level all the animals showed definite signs of toxicity ranging from hyperactivity to loss of consciousness. There was also evidence of marked weight loss (see fig. 2.8.1. and 2.8.2.). Feed consumption was reduced, (see table 2.4.) and they became dehydrated. One ICR mouse had seizures and died after one week at this level. At this point it was decided to dilute the feed with an additional two millilitres of water but the animals remained ill and dehydrated, and some had to be withdrawn from the ethanol and given control diet to prevent further deaths. There was no evidence of any withdrawal syndrome in those whose ethanol intake was abruptly stopped. While on the diluted diet a second ICR mouse died, and one C3H mouse had a gastrointestinal bleed. By the end of two weeks on the thirty percent EDC diet a total of six mice (three C3H and three ICR) had been withdrawn from ethanol intermittently because of toxic effects. In spite of these measures two mice (one ICR and one C3H) died during this time and two more (one ICR and one C3H) a few days after being withdrawn from the diet.

Post mortems on two of the mice revealed bilateral pneumonic changes in the lungs. Because of these findings I changed the vermiculite bedding to paper towel as it appeared that the long periods of unconsciousness allowed inhalation of the vermiculite dust which, in their debilitated state, resulted in pneumonia.

Blood ethanol levels were checked on four mice receiving the thirty percent EDC diet.

Mouse	Blood ethanol concentration	Dietary intake in the preceding 5 hours	Level of consciousness
ICR 6	3920mg/ℓ	6mℓ	Semi conscious
ICR 8	5380mg/ℓ	6mℓ	Deeply unconscious
C3H 5	4480mg/ℓ	7mℓ	Semi conscious
C3H 6	5040mg/ℓ	7mℓ	Semi conscious

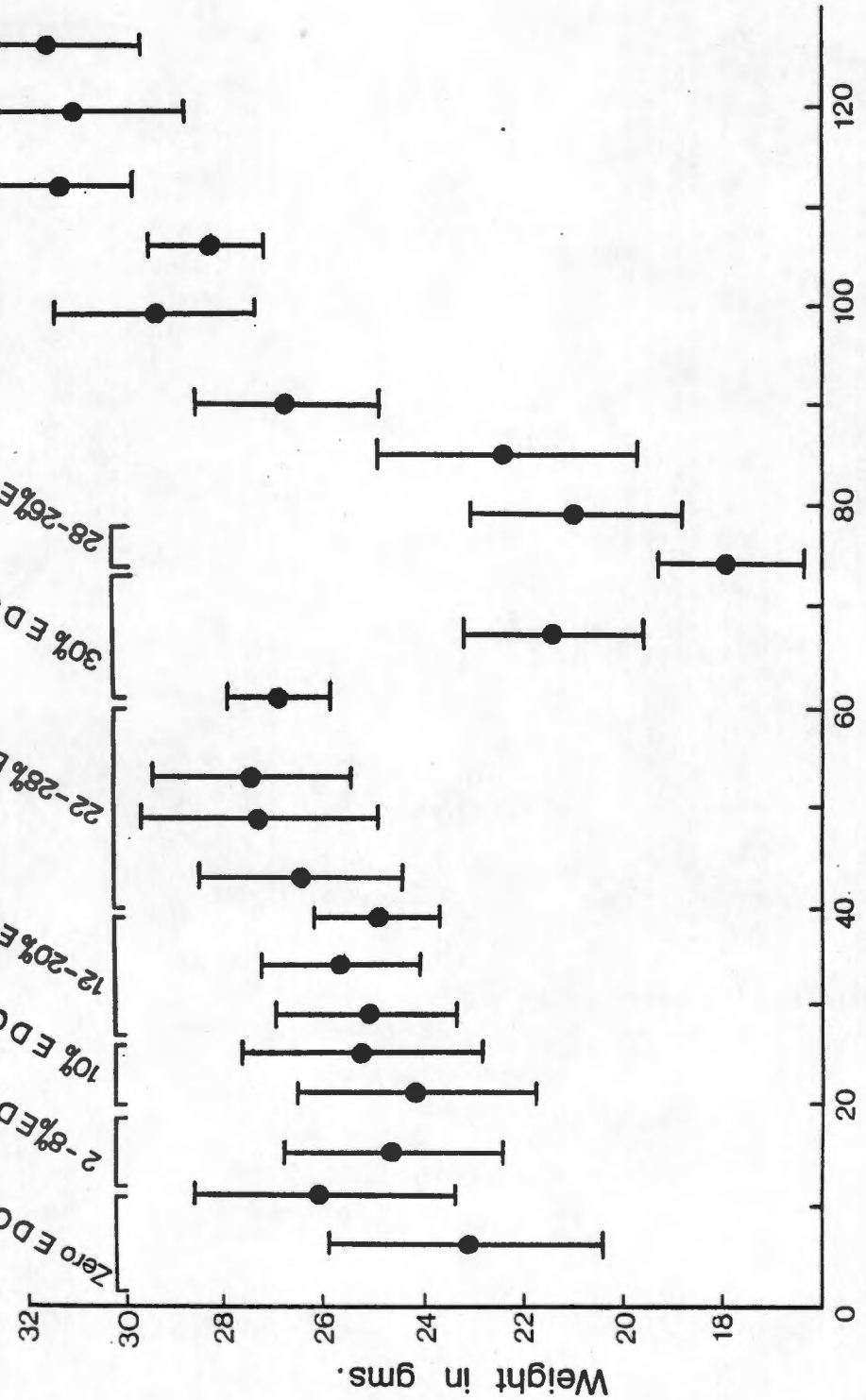

Fig. 2.8.1. Effects of varying amounts of ethanol on weight gain in ICR mice

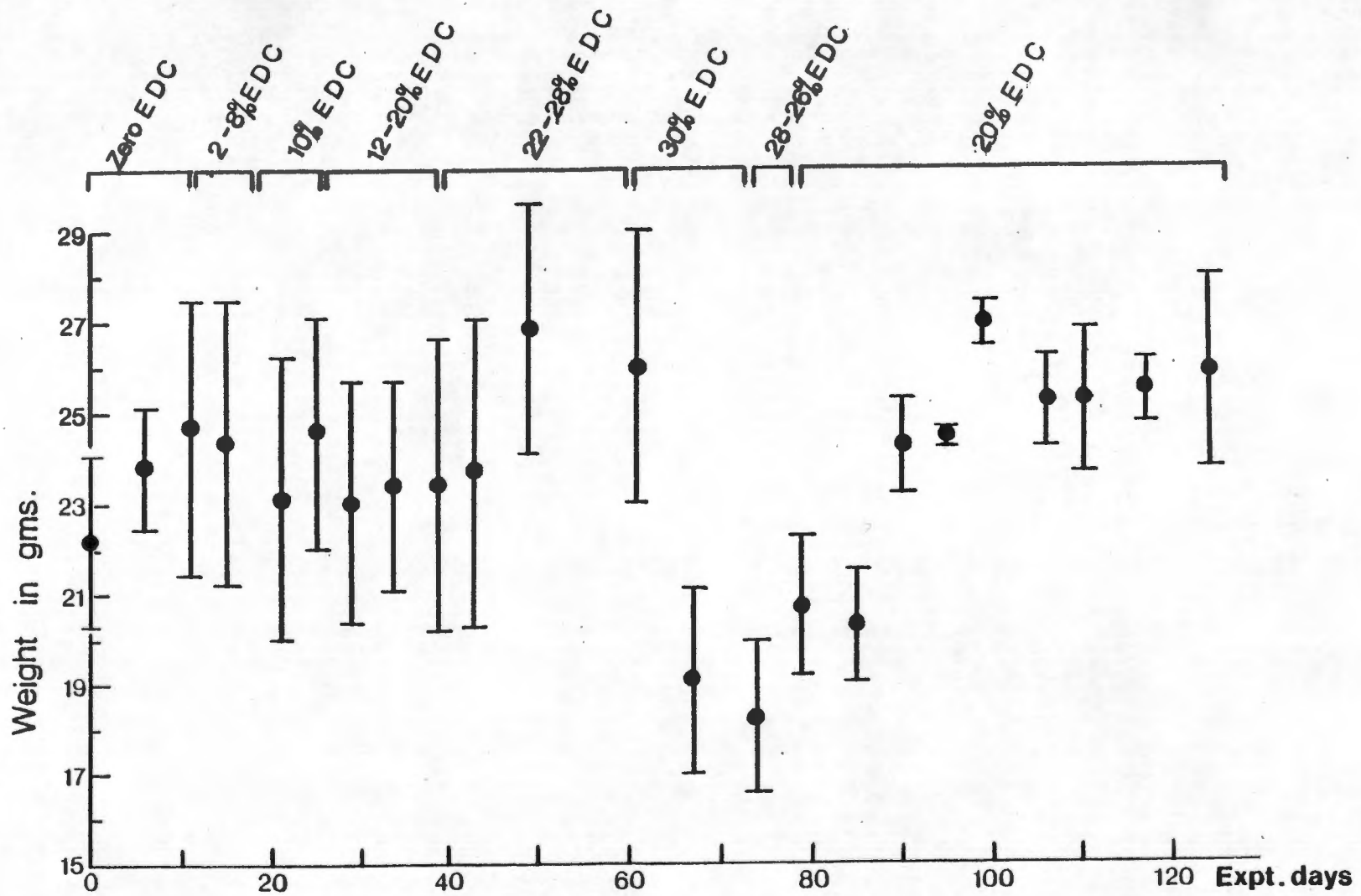

Fig. 2.8.2. Effects of varying amounts of ethanol on weight gain in C3H mice

Table 2.4. Mean dietary intake by ICR and C3H mice.

Mouse no.		Ethanol dose 0-20% EDC Mean intake in mℓ	Ethanol dose 20 - 30% EDC Mean intake in mℓ
1		8.85	7.07
2		9.05	7.55
3		8.83	7.69
4	ICR	9.00	7.40
5		9.03	7.50
6		8.63	7.07
7		8.69	7.10
8		9.22	7.81
9		7.89	6.93
10		8.11	7.00
11		8.60	6.85
12	C3H	8.71	7.46
13		8.63	6.96
14		7.97	-
15		8.52	-

There was a significant difference ($p = 0.05$, Mann-Whitney Test) between the mean dietary intake of the C3H and ICR mice combined at the 0-20% EDC level compared with the 20-30% EDC level.

At this stage the surviving animals were placed on the 20% EDC diet and gained weight and remained healthy. One pregnancy occurred in the ICR group within two months of return to the 20% EDC diet. There were four apparently normal fetuses in this litter and three resorptions.

After two months on the 20% EDC diet the remaining animals were sacrificed.

2.9. CONCLUSIONS

1. In order to guarantee intake of the ethanol it was necessary to feed only the liquid diet.
2. Ethanol in dosages above the 20% EDC level proved too toxic to the mothers to be of value in a study of ethanol induced teratogenicity.
   At the 20% EDC level the mice remained healthy and gained weight satisfactorily.

I was unable to reproduce the high levels of dosage of ethanol used by Chernoff (1977). He fed C3H and CBA mice a liquid diet containing 15%-35% EDC for at least thirty days before and throughout gestation.

CHAPTER 3

ETHANOL DOSAGE IN VIVO

3.1. CHRONIC ALCOHOL MODEL : METHOD

The experiment extended over a two year period and a total of one hundred and thirty-five test animals and sixty-seven control animals were used.

The C3H female mice were housed in individual cages and fed the liquid diet described in section 2.3. Three doses of ethanol were studied, namely six percent EDC (thirty-five mice), ten percent EDC (forty-seven mice) and twenty percent EDC (fifty--three mice). The mice in the ten percent EDC group became very obese making diagnosis of pregnancy difficult.

The six percent, ten percent and twenty percent EDC diets contained the same basic ingredients as the control diet described in section 2.3,the only variations being in the sugar and ethanol content.(see table 3.2.)

Table 3.1. Sugar and ethanol content of 6%, 10% and 20% EDC diets

Group	Sugar content in grams	Ethanol (100%) content in mℓ	Total Calories
Control	1.5	0	19.8
6% EDC	1.203	0.169	19.8
10% EDC	1.005	0.283	19.8
20% EDC	0.51	0.566	19.8

An attempt was made to increase the dose of ethanol to twenty-four percent in the twenty percent EDC group, however some of the mice showed signs of toxicity with evident drunkenness, loss of consciousness and weight loss. Five animals in this group died, two shortly after receiving this higher dose of ethanol. The maximum dose of ethanol in this experiment was set at

twenty percent EDC because this was the highest level at which the majority of the mice remained healthy and showed no toxic effects other than hyperactivity and jaundice in six of the mice studied. At the ten percent EDC level there was no evidence of toxicity, other than jaundice in four of the mice studied, and no recordable blood ethanol level in the animals tested. The six percent EDC dose was considered to be sufficiently low to constitute a 'safe' dose in mice. Thus it was hoped to cover the range from non teratogenic through mildly embryotoxic to teratogenic.

All animals received the liquid diet at the ethanol dose level being studied for at least one month prior to mating and the test groups were given the ethanol in graded increases of dosage until their particular level had been reached as described in the pilot experiment. The animals were allowed access to water ad libitum.

The pregnant mice were sacrificed on day eighteen of gestation using an overdose of ether anaesthesia. Day one of pregnancy was counted as the day following mating. Pregnancy was terminated rather than allowing normal delivery at term as mice are known to cannibalize any abnormal offspring.

Table 3.2. Chronic dose ethanol study. Total mice studied.
Maternal wastage

Group	Total mice	Premature births	Deaths	Wrong diagnosis of pregnancy. Sacrificed in error
Control	67	-	-	2
6% EDC	35	1 litter cannibalised	-	1
10% EDC	47	1 litter of 4 fetuses. 2 observed to be cyanosed.	1 sacrificed because of bizarre circling behaviour.	7
20% EDC	53	1 litter of 3 apparently normal fetuses.	1 due to pneumonia. 4 cause not known. 1 Sacrificed because of massive uterine tumour.	1

Blood was taken for ethanol determination from some of the animals. It was taken either by cardiac puncture or from the thoracic cavity after incision of the heart at the time of sacrifice. Blood was not taken from the living animals during pregnancy (as was done in the pilot experiment), as it was felt that this procedure was too stressful to the pregnant animals.

Uteri were removed intact and placed in normal saline and the fetuses, membranes and placentas removed. After this the uterus was examined for resorptions. The membranes were removed, fetal viability was assessed and they were examined for obvious external abnormalities. They were then mopped dry and each fetus and its placenta was weighed on a Sartorius chemical balance. After weighing, the fetuses were fixed in ten percent saline-buffered formalin. All fetuses obtained during the first year of experimentation were sectioned using Wilson's method (Barrow and Taylor, 1969) and examined under a dissecting microscope. During the second year of study all fetuses were treated in identical fashion to that described above but instead of sectioning they were prepared for skeletal examination using the modified Dawson's method (Richmond and Bennett, 1938) described in section 2.5.

3.2. RECORDS OF MATERNAL AND FETAL PARAMETERS STUDIED

Records were kept of the duration of each experiment, the ethanol level at the time of sacrifice, the pre-pregnant maternal weights, the daily dietary intake and the physical state of the mother (see tables 3.3., 3.4., 3.5. and 3.6. - pages 69-111).

3.3. RESULTS OF CHRONIC ETHANOL DOSAGE

3.3.1. Maternal findings (see table 3.7.)

There was no significant difference between test and control groups in regard to pre-pregnancy maternal weights indicating that although dietary intake was lower than the controls in the six percent and twenty percent EDC groups all test animals were adequately nourished. I can offer no explanation for the reduced food intake by the six percent EDC group, but noted that in the twenty percent EDC group the feeding tubes often were blocked.

TABLE 3.7

IN VIVO STUDY

EFFECTS OF CHRONIC ETHANOL ADMINISTRATION ON MATERNAL AND PLACENTAL WEIGHTS AND DIETARY INTAKE

Dose of Ethanol	Pre Pregnant Maternal Weight	Mean Dietary Intake	Placental Weight
6% E D C	No significant difference in weight (Test One way A.O.V.) Control N = 32 Test N = 24	Intake significantly reduced in test group (Test One way A.O.V.) Control N = 32 Test N = 24	Placental weight significantly reduced in test group (Test One way A.O.V.) Control N = 191 Test N = 132
10% E D C	No significant difference in weight (Test One way A.O.V.) Control N = 32 Test N = 20	No significant reduction in intake (Test One way O.A.V.) Control N = 32 Test N = 20	Placental weight significantly increased in test group (Test One way A.O.V.) Control N = 191 Test N = 68
20% E D C	No significant difference in weight (Test One way A.O.V.) Control N = 32 Test N = 26	Intake significantly reduced in test group (Test One way A.O.V.) Control N = 32 Test N = 26	Placental weight significantly increased in test group (Test One way A.O.V.) Control N = 191 Test N = 75

Statistical Test used : One way analysis of variance. Multiple comparison among means p = 0.05.

The tubes were shaken up each afternoon in an attempt to overcome this problem. A small number of mice in the 10% and 20% EDC groups were noted to be jaundiced at the time of sacrifice. It is possible that this may have been due to alcoholic liver damage but the cause was not determined (see tables 3.5. and 3.6.).

3.3.2. Placental findings

Placental weights were significantly different from the controls in all three groups. In the six percent EDC group placental weights were significantly reduced ($p = 0.001$) and in the ten percent and twenty percent EDC groups they were significantly increased ($p = 0.001$). I did not do any histology on the placentas and can only postulate that in the six percent EDC group the reduced placental weights may be consistent with general growth retardation which occurred in the ethanol treated fetuses (see table 3.8.). At the ten percent and twenty percent EDC levels it is possible that damage proceeded a stage further resulting in edema of the placental cells, with significantly increased weights while, in the twenty percent EDC group, still producing low birth weight fetuses.

3.3.3. Fetal findings (see table 3.8.)

Fetal weights, resorptions, total abnormal live births, alterations in sex ratio and total implants were assessed.

Total fetal abnormalities were identified by:

- macroscopic examination
- Wilson's sectioning
- heart dissection
- skeletal examination

The only macroscopic abnormalities which were observed were exomphalos, exencephaly and the 'monsters' or late resorptions.

The 'monsters' were grossly abnormal often with multiple defects and development halted at between twelve and fourteen days gestation (see fig. 3.3.). Open eyelids were common. Sometimes only one eye was identifiable and limb development was often retarded.

TABLE 3.8
IN VIVO STUDY

RESULTS OF CHRONIC ETHANOL DOSAGE
Fetuses assessed at eighteen days' gestation

Dose	Fetal Weight Live Births Only	Early Resorptions	Abnormal Live Births	Altered Sex Ratio (Only live fetuses assessed)	Total Implants
Ethanol 6% E.D.C.	Significant reduction in weight (Test One Way A.O.V.) Control N = 211 Test N = 145	No significant increase in resorptions Control N = 262 Test N = 194	No significant increase in abnormalities Control N = 149 Test N = 94	No significant alteration Control N = 130 Test N = 57	No significant reduction (Test One Way A.O.V.) Control N = 32 Test N = 24
Ethanol 10% E.D.C	Significant increase in weight (Test One Way A.O.V.) Control N = 211 Test N = 96	Very significant increase in resorptions $p<0.001$ Control N = 262 Test N = 154	No significant increase in abnormalities Control N = 149 Test N = 62	No significant alteration Control N = 130 Test N = 30	No significant reduction (Test One Way A.O.V.) Control N = 32 Test N = 21
Ethanol 20% E.D.C.	Significant reduction in weight (Test One Way A.O.V.) Control N = 211 Test N = 133	?Significant increase in resorptions $p<0.1 > 0.05$ Control N = 262 Test N = 183	Very significant increase in abnormalities $p<0.001$ Control N = 149 Test N = 69	No significant alteration Control N = 130 Test = 57	Significant reduction in implants in test group (Test One Way A.O.V.) Control N = 32 Test N = 26

Statistical Tests. Except where otherwise indicated the Chi^2 test was used (two tailed test).
One Way A.O.V. = One way analysis of variance. Comparison among means $p=0.05$
Deaths in utero were assessed using the Chi^2 test but there was no significant increase in deaths compared with controls in any of the groups studied.
Deaths in utero included monsters/late resorptions.

Incidence of macroscopic abnormalities:

Control group:

- one fetus with abnormal flexion of its hind limb
- three fetuses were born with exomphalos (see fig. 3.1.)
- nine monsters/late resorptions
- seven dead fetuses

Six percent EDC group:

- two fetuses with exomphalos
- one fetus with exencephaly
- five monsters/late resorptions
- twelve dead fetuses

Ten percent EDC group:

- three fetuses with exomphalos
- five monsters/late resorptions
- two dead fetuses

Twenty percent EDC group:

- three fetuses with exomphalos
- one fetus with exencephaly (see fig. 3.2.)
- six monsters/late resorptions (see fig. 3.3.)
- six dead fetuses

Wilson's sections (see table 3.9.)

Owing to poor fixation of the fetuses, accurate assessment of the brains and hearts in particular was very difficult. A number of fetuses examined in each group were classified according to Palmer's (1977:53) method as having common variants or minor visceral anomalies. These specimens showed relatively small structural changes which were not obviously detrimental to the fetus, for example, marginal dilatation of the brain ventricles, renal pelvic dilatation, slightly abnormal palatal architecture without any obvious cleft. These were the query abnormal sections shown in table 3.10. Histological sections of some of these specimens were prepared but this was fraught with problems because the original poor fixation made it very difficult to distinguish artifact from abnormality. Those query abnormal sections which were assessed histologically all proved to be normal (table 3.10. see page 53).

TABLE 3.9.
IN VIVO STUDY

EFFECTS OF CHRONIC ETHANOL DOSAGE ON FETAL DEVELOPMENT
Fetuses assessed at 18 days gestation

Group	Wilson Sections	Heart Sections		Skeletal Examination*	
	Normal	Normal	Abnormal	Normal	Abnormal
Control	Male 61 Female 69	101	1. High ventricular septal defect or cushion defect)	45	a.Absent or poorly developed supraoccipital bone. 1. b.Sternal bones missing or poorly developed. 0 c.11th and 12th Rib joined together on left side. 1. d.Cervical vertebral bodies absent. (In 1.upper ones only missing). 3. Combination of a. b. and d. above. 1. e.Occipital arch abnormal. 1.
6% E D C	Male 19 Female 38	39	1. (Large left ventricular cavity. Query cor unilocillare)	52	a.Sternal bones missing or poorly developed. 0 b.Absent or poorly developed supraoccipital bone. 1. c.Cervical vertebral bodies absent. 0 Combination of a. b. and c. above. 1.
10% E D C	Male 13 Female 17	13	0	45	a.Sternal bones missing or poorly developed. 0 b.Abnormal atlas. 0 c.Cervical vertebral bodies absent. (In 1.upper ones only missing). 2. Combination of a. and b. above. 1. Combination of a. and c. above. 1.
20% E D C	Male 36 Female 21	19	1. (Persistant truncus arteriosus)	29	a.Absent or poorly developed sternal bones. 0 b.Absent or poorly developed supraoccipital bone. 4. c.10th and 11th ribs fused. (See Fig. 3.4.) 0 d.Cervical vertebral bodies absent. 3. Combination of a. b. and d. above. 3. Combination of a. and d. above 2. Combination of b. and d. above. (See Fig. 3.5.2.) 1. Combination of b. and c. above. 1. Combination of a. and b. above. 2.

*Skeletal Examination - Normal = Only digit bones missing. All other bones present. Only live fetuses were included in this study.

Fig. 3.1.
18-Day fetus with exomphalos.
Mother treated with 20% E D C diet.

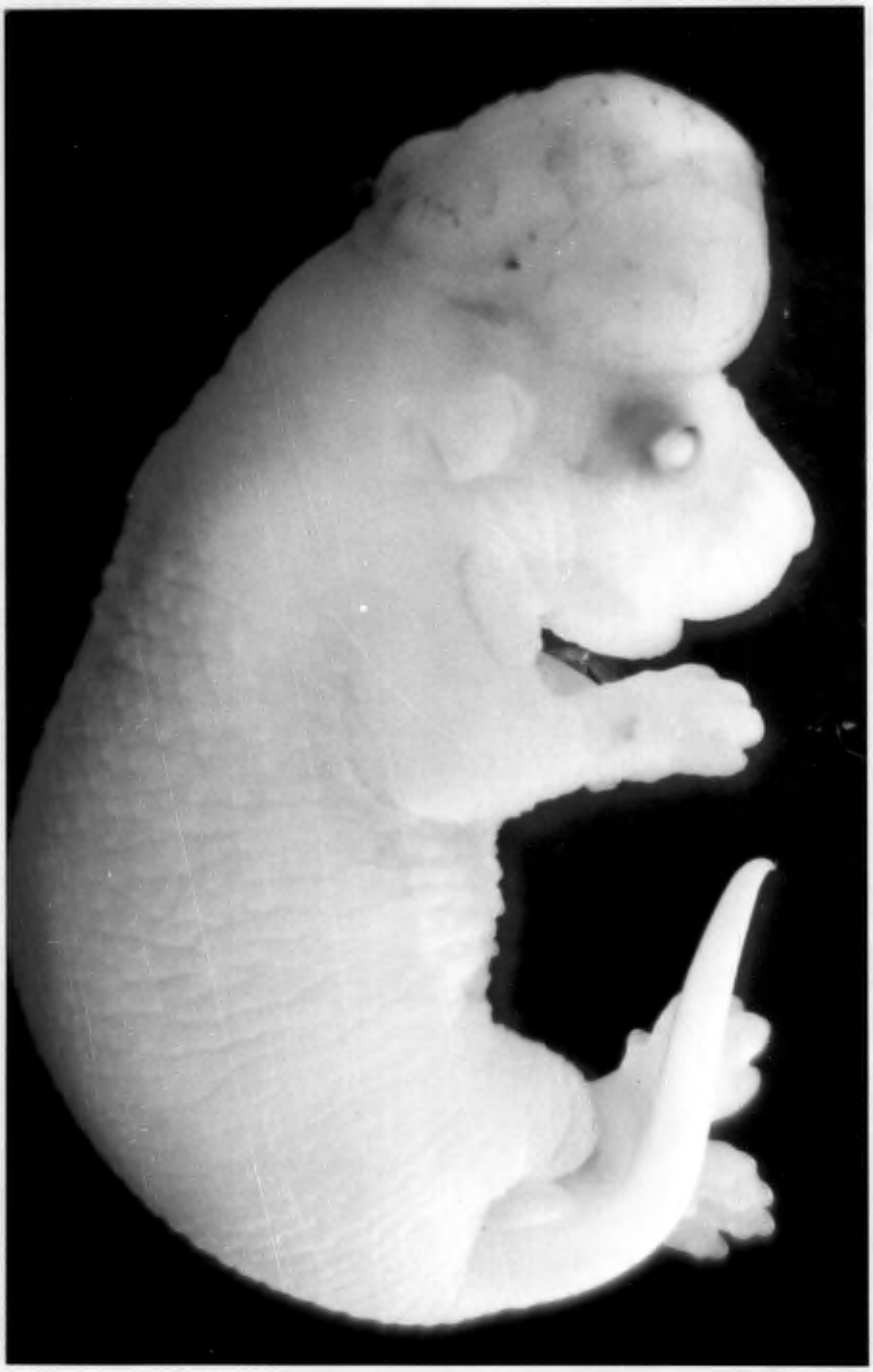

Fig. 3.2.

18–Day fetus with exencephaly.

Mother treated with 20% E D C. diet.

Note - open eyelids.

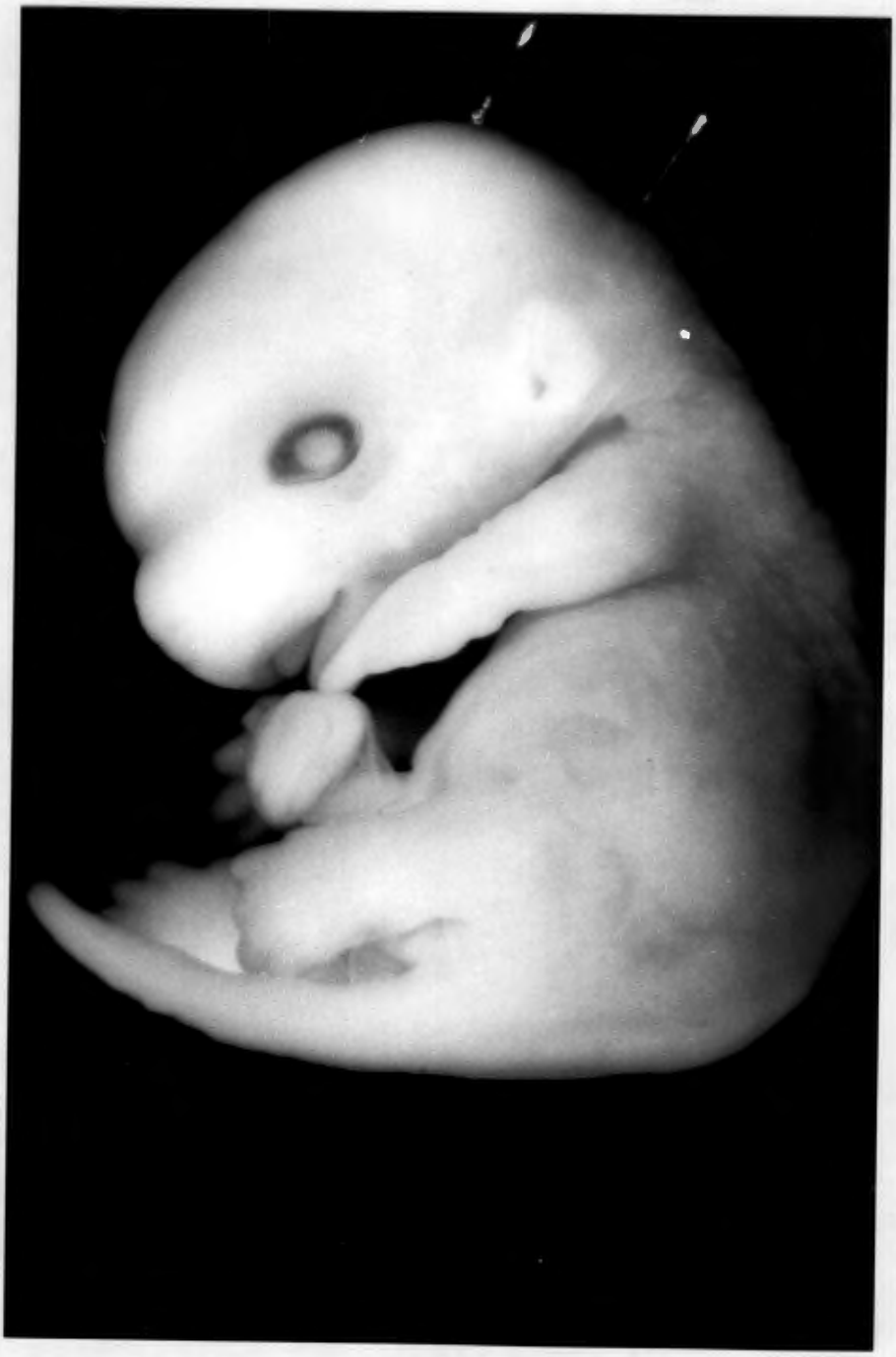

Fig. 3.3. 'Monster'

Mother treated with 20% E D C diet.

Note - open eyelids
- abnormal head shape
- protruding tongue
- retarded limb development

In my analysis of the Wilson's sections I included only those sections where it was possible to assess the entire fetus. If any part was damaged or distorted due to poor fixation the whole fetus was discarded for the purposes of this assessment.

The sex of the fetus was also defined and statistical tests done to determine whether there was any difference in the ratios of males to females between groups.

Table 3.10. Assessment of Wilson's sections in macroscopically normal live fetuses.

Statistical test - Fisher Exact Probability Test.

	Normal	Query abnormal
Controls	119	9
Six percent EDC	42	8
p = 0.0651		
Controls	119	9
Ten percent EDC	8	6
p = 0.000913		
Controls	119	9
Twenty percent EDC	28	3
p = 0.425		

As can be seen in table 3.10. there was no significant increase in abnormalities observed on Wilson's sections in the ethanol treated animals except in the 10% EDC group. As this group was very small the significance of this finding was doubtful, and, as the abnormalities observed were of a minor nature and could be classified as minor visceral anomalies, it was decided to include the query abnormal fetuses as normal in the analysis of total fetal abnormalities in table 3.8.

Heart dissection

It is known that different techniques of dissection may yield different incidences of abnormality. Palmer (1977:57) claims that micro-dissection techniques generally produce lower rates of ventricular septal defects (VSD) than the free hand sectioning methods of Wilson, and even with free hand sectioning the numbers of VSD's observed are smaller when fewer sections are cut. He recorded an incidence of eighty percent of fetuses with lethal cardiac abnormalities using a compound that was claimed to be non-teratogenic in another laboratory.

The heart dissection in our laboratory was performed using a micro-dissection technique, and the true incidence of congenital heart abnormalities may be considerably higher than we found. A total one hundred and two control, forty 6% EDC, thirteen 10% EDC and twenty 20% EDC hearts were assessed (see table 3.9.).

The only heart defects which we found in this group were a high ventricular septal defect in a control fetus, a large left ventricular cavity in one fetus in the 6% EDC group and a persistent truncus arteriosus in one fetus in the 20% EDC group.

Chernoff (1977), using a free hand sectioning technique, reported an incidence of seventy-eight percent in studies, with a maternal diet of twenty percent EDC, the main abnormalities being VSD's.

Skeletal assessment

In normal skeletal development described by Rugh (1965:220) the osseous skeleton develops from the chondrification centres which in turn arise from the membranous blastema. The mesenchymal stage is most vulnerable to environmental trauma, thus damage as early as seven and a half days' gestation may cause stunting through its effect on the mesenchyme which in turn affects chondrification, resulting in affected osteoblasts which arise from these centres. At fifteen days' gestation the basic ossification centres are sufficiently developed to be stained by Alizarin Red S. The centras chondrify at sixteen days' gestation and by seventeen days the sternum has formed, the cranial cavity is almost encased, the tympanic rings are identifiable and the

mandibular articulations at the zygomatic arches have developed. At eighteen days' gestation the appendage parts are stainable, the cranial bones have not yet fused and will continue to expand as the brain grows.

As the mice in this study were usually sacrificed during the first half of day eighteen of gestation it was decided to accept as normal those fetuses whose digit bones were not visible (or missing) because it was assumed that their invisibility was due to an inability to be stained by Alizarin Red S.

Table 3.11. Ossification centres in skeletal development (Rugh, 1968:222)

Bones	Ossification on gestation day
Supra occipital	16
Ribs	14
Sternebrae	16
Vertebrae:-	
cervical-arch	14
-body	18
thoracic-arch	15
-body	15
Metacarpals	16
Digits -proximal	18
-middle	18
-distal	17

Skeletal examination (see table 3.9.)

In the chronically treated ethanol group skeletal abnormalities which occurred could be classified as minor according to Palmer's (1977:53) method. The abnormalities seen in this study were probably not due to major structural changes but rather due to delayed ossification, and bones which I classified as absent or poorly developed, may simply have been invisible because of general retardation of development (see figures 3.4., 3.5.1. and 3.5.2.).

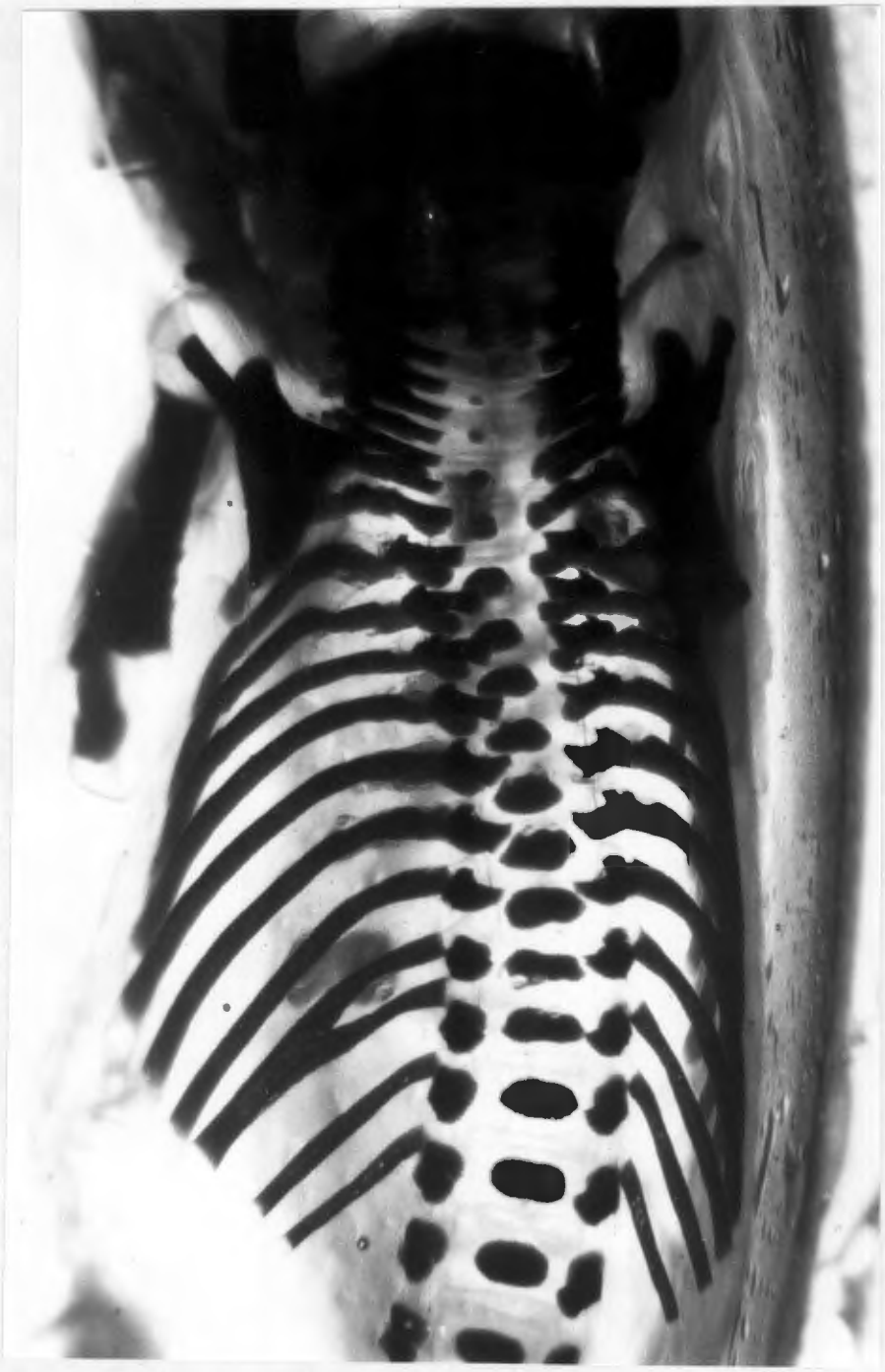

Fig. 3.4.

18-Day fetal skeleton with fused 10th and 11th ribs.
Mother treated with 20% E D C diet.

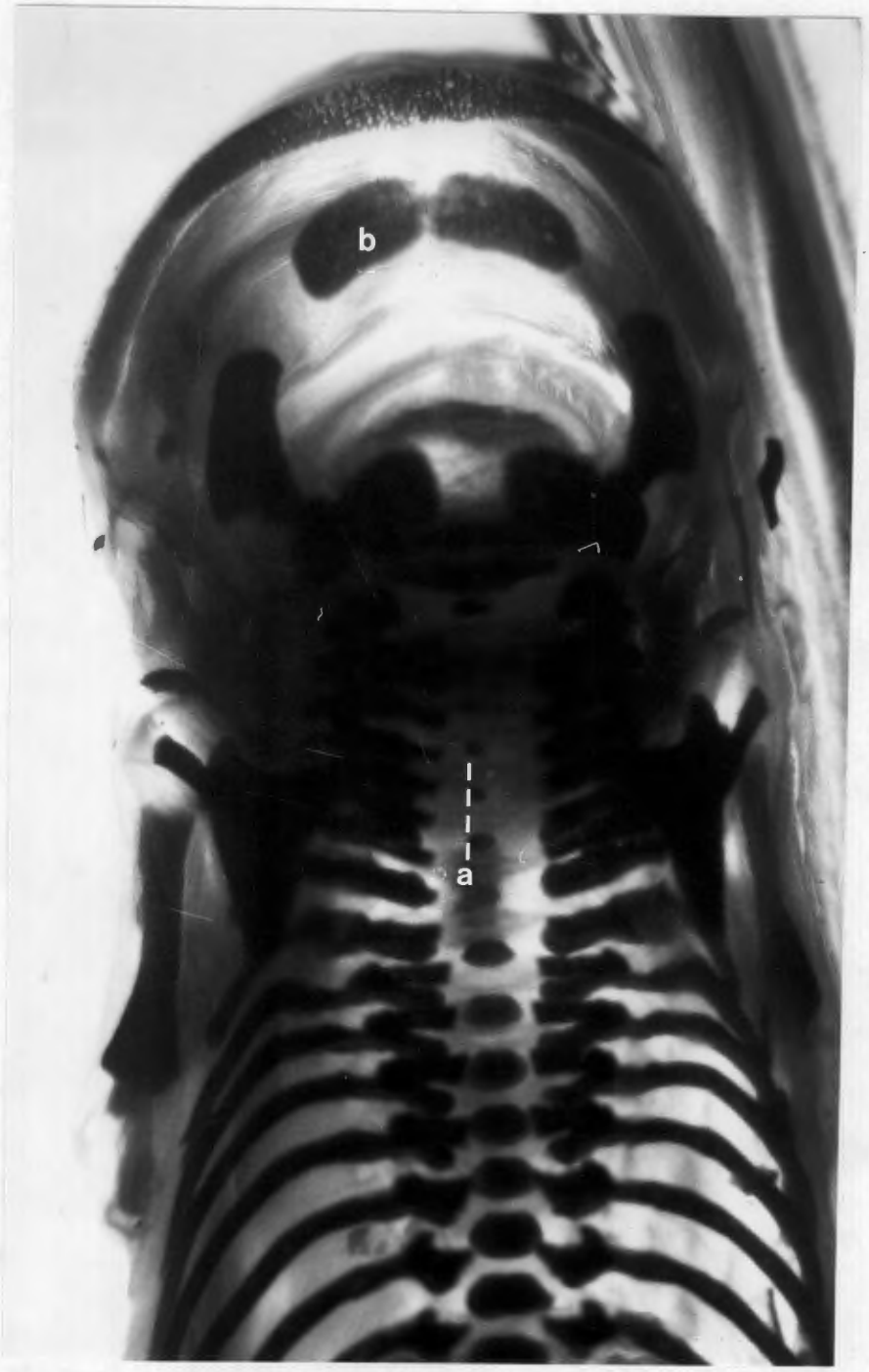

Fig. 3.5.1.

Normal 18-day fetal skeleton.

Note - cervical vertebral bodies (a)

- supra occipital bone (b)

Fig. 3.5.2.
Abnormal 18-day fetal skeleton.
Note - cervical vertebral bodies absent (a)
- supra occipital bone absent (b)
Mother treated with acetaldehyde 11.8mg.
(This abnormality also occurred in the ethanol treated animals).

Three 'grades' of abnormality were considered:

1. any bone invisible or absent;
2. any bone except digits absent;
3. any bone except digits and/or cervical vertebral bodies absent.

The Fisher Exact Probability Test was used in this assessment. In each grade there was a significant difference ($p = 0.05$) between the control group and the twenty percent EDC group and in the third grade p was 0.006. Abnormalities were increased in the test group.

In the skeletal study the numbers of test and control animals were approximately equal (see table 3.9.) and the above was considered to be a real effect caused by ethanol. The fetuses classified as abnormal in grade two were included in the assessment of total abnormalities shown in table 3.8.

The figures for total abnormal fetuses included only those which were macroscopically abnormal, or had abnormalities on Wilson's section and/or heart section, or had skeletal abnormalities. Fetuses which exhibited abnormalities in more than one area e.g. macroscopically abnormal and abnormal skeleton were included in one category only. Only live fetuses were assessed. In all the methods of assessment used I have only classified as abnormal those fetuses where abnormality was obvious and clearly recognisable. In doubtful cases they were placed in the normal category. Thus the incidence of fetal abnormality may be artificially low, and I have not overstated the risks incurred by ethanol intake during pregnancy.

Fetal weights.

Fetal weights were significantly different from the controls in all three groups studied. At the 6% and 20% EDC levels fetal weights were significantly reduced (see table 3.8.). This could be a reflection of reduced maternal dietary intake compared with the controls, but this seems unlikely in view of the similarity of maternal weights. Rather it is probable that this reduction in birth weight is evidence of ethanol toxicity to the fetus.

In the ten percent EDC group, the fetuses were significantly heavier than the controls, but considering the very significant ($p = 0.001$) increase in resorptions in this group, this effect is very probably due to the smaller litter size favouring fetal growth.

In the twenty percent EDC group there was a significant reduction in the total number of implants, making the lower fetal weights in this group all the more significant (see table 3.8.).

3.3.4. Discussion

These results indicate that ethanol given in chronic dosage produces adverse effects on the fetus even in the lowest dose studied, and that the severity of the effect increases with dosage, resulting at the twenty percent EDC level in reduction of fetal weights, increased incidence of fetal abnormalities and reduction in the number of implants. I believe that the doses of ethanol used in this experiment were adequate to illustrate its teratogenicity. Administration of a suitable dose of a teratogen generally results in the production of some normal, some malformed and some dead or resorbed offspring (Schardein 1976:12). My findings correspond with this.

Chernoff treated CBA and C3H mice with liquid diets containing 15%-35% EDC for at least thirty days before and throughout gestation. He examined the fetuses on day eighteen of gestation and found that prenatal death and maldevelopment increased with the level of alcohol intake. Deficient occiput ossification, neural anomalies and low fetal weights occurred with the low ethanol diets, and cardiac and eyelid dysmorphology with higher ethanol diets.

Although I found a similar range of ethanol induced effects on the fetuses I was not able to reproduce either the high levels of ethanol dosage or the high incidence of abnormalities found by Chernoff.

The findings in this study were very similar to those of Skosyreva (1973) who treated female rats from days eight to fourteen of pregnancy with ethanol forty percent in an oral dose

of five millilitres per kilogram. He assessed the fetuses on day twenty-one of gestation and found a significant increase in post-implantation deaths and resorptions, a reduction in fetal weights and general retardation of development, but no gross abnormal development of the internal organs or skeleton as assessed by Wilson's sections and skeletal examination using Dawson's method of skeletal preparation. He also found that placental weights of the treated animals were increased but not significantly so. He reasoned that these effects could be due to direct ethanol toxicity or due to indirect effects determined by maternal health and metabolism of ethanol and he proceeded to the use of an in vitro model to study the direct effects of ethanol exposure of the embryo.

3.4. ACUTE ETHANOL DOSE STUDY: METHOD

The purpose of this study was to determine whether 'binge' intake of ethanol during gestation would have any adverse effect on the fetus. Days one and eight in one group and four and twelve in another were chosen in an attempt to cover both the very early stages of pregnancy and the organogenesis period. It was thought that should any adverse effect occur on days one or four they would differ sufficiently from those produced on days eight and twelve to enable us to determine whether there was a critical period at which damage occurred (defects induced on day eight would also be different from those induced on day twelve enabling us to differentiate between them).

Young adult C3H female mice (± eight weeks old) were housed in communal cages with vermiculite bedding and fed Epol rat cubes. Twice a week they were mated with young adult C3H males. (Four or five females to two males). Those which were observed to mate were randomly allocated to two test and two control groups. The day following mating was designated as day one of pregnancy.

Test group one consisted of ten mice which were given one millilitre of a 7.35% solution of ethanol via an orogastric tube, twice on days one and eight of gestation (total ethanol dose was

0.157mℓ/day). The corresponding control group received one millilitre of water also via an orogastric tube at the same times and on the same days that the test group received ethanol. This dose of ethanol was less than the total daily dose given in the 6% EDC group but it was given in two bolus doses to mice who had not been exposed to ethanol before.

Test group two was treated in the same way as test group one, but the ethanol (or water in the case of the control group) was given on days four and twelve of pregnancy. This test group comprised eleven, and its control group nine mice. In all groups the treatments were given at 11.00 a.m. and 2.00 p.m.. In some cases the 2.00 p.m. dose of ethanol had to be omitted as the mice were too intoxicated to tolerate further treatment. In spite of the mice being of a highly inbred strain they showed very individual responses to identical doses of ethanol. This may have been due to variations in weight and feed intake in the different mice.

The pregnant animals were sacrificed on day eighteen of gestation and the fetuses assessed using the same methods as were used in section 3.3.3., the only difference being that the fetuses were randomly allocated numbers. The even numbered were used for skeletal assessment and the odd numbered for Wilson's sections.

3.5. RECORDS OF MATERNAL AND FETAL PARAMETERS STUDIED

No records were kept of maternal pre-pregnant weights as these mice were all receiving the normal diet of Epol rat cubes. All mice were healthy at the time of sacrifice.

Fetal and placental weights, number of resorptions and obvious external abnormalities were all recorded (see tables 3.12., 3.13., 3.14. and 3.15 - pages 112-128).

3.6. RESULTS OF ACUTE DOSE ETHANOL STUDY (see table 3.16.)

3.6.1. Maternal and placental findings

The only effect on the treated mice was a significant reduction in placental weights in the mice treated on days one and eight of pregnancy and a significant increase in placental weights in the mice treated on days four and twelve of pregnancy.

At eight and a half days gestation, placentation commences and it is possible that the ethanol may have had a toxic effect at this stage causing a reduction in the number of placental cells formed resulting in the reduced placental weight in the animals treated on days one and eight. I can offer no explanation for the apparent increase in placental weights in the animals treated on days four and twelve of gestation.

3.6.2. Fetal findings (see table 3.16.)

Fetal weights, resorptions, total abnormal live births, alterations in sex ratio and total implants were assessed.

Total fetal abnormalities were identified by:

- macroscopic examination
- Wilson's sectioning
- heart dissection
- skeletal examination

Incidence of macroscopic abnormalities

Control group day 1 and 8

- no macroscopic abnormalities
- four monsters/late resorptions

Binge ethanol group day 1 and 8

- two fetuses with exomphalos
- three monsters/late resorptions

Control group day 4 and 12

- one dead fetus but no macroscopic abnormalities observed
- No monsters/late resorptions

Binge ethanol group day 4 and 12

- three fetuses with exomphalos
- one fetus with abnormal flexion of its hind limb
- one monster/late resorption

It is interesting to note that although the abnormalities were not sufficiently frequent to give statistical significance, five out of six of the abnormalities were exomphalos.

In the mouse the foregut and hindgut are delineated between seven and a half days' and eight days' gestation and the hindgut is closed by the cloacal membrane between eight and a half and nine and a half days' gestation (Leone and Giavini, 1977:20). On day eleven of gestation the cloacal cavity is divided into urogenital and rectal portions, the post-anal gut regresses and an umbilical hernia is formed. On day twelve of gestation the umbilical hernia begins to be incorporated into the intestinal loop and by day fifteen the umbilical hernia is usually withdrawn (Rugh, 1968:262).

Bearing these developmental events in mind it is quite conceivable that ethanol played a part in the production of exomphalos in these mice.

The same criteria for assessment of Wilson's sections and skeletal abnormalities were applied as described in section 3.3.3.

Wilson's sections (see table 3.17.)

Only macroscopically normal fetuses were assessed and the fetuses were sexed and statistical tests performed as described in section 3.3.3.

TABLE 3.16

IN VIVO STUDY

RESULTS OF ACUTE 'BINGE' ETHANOL DOSAGE

Fetuses assessed at 18 days' gestation

Dose	Fetal Weights	Early resorptions	Altered Sex Ratio	Total Implants/Litter	Placental Weights
Ethanol 73.5µl Day 1 + 8	No significant reduction in weight. Two sample t-test (unpaired) Control N = 79 Test N = 63	No significant increase in resorptions. Control N = 90 Test N = 72	No significant alteration. Control N = 42 Test N = 25	No significant reduction Two sample t-test (unpaired) Control N = 12 Test N = 10	Placental weights were significantly reduced in the test. group. Two sample t-test (unpaired) Control N = 79 Test N = 65
Ethanol 73.5 µl Day 4 + 12	No significant reduction in weight. Two sample t-test (unpaired) Control N = 61 Test N = 64	No significant increase in resorptions. Control N = 70 Test N = 76	No significant alteration. Control N = 34 Test N = 20	No significant reduction Two sample t-test (unpaired) Control N = 9 Test N = 11	Placental weights were significantly increased in the test group. Two sample t-test (unpaired) Control N = 57 Test N = 55

Statistical tests. Except where otherwise indicated the Chi² test was used. (2 tailed test).

Live births only were studied unless otherwise indicated.

Deaths in utero and total abnormal live births were assessed using the Fisher Exact Probability Test and the Chi² test.

There was no significant increase in deaths or abnormalities in treated groups.

Deaths in utero included monsters/late resorptions.

TABLE 3.17
IN VIVO STUDY

EFFECTS OF ACUTE 'BINGE' ETHANOL DOSAGE ON FETAL DEVELOPMENT
Fetuses assessed at 18 days' gestation.

Group	Wilson Sections	Heart Sections		Skeletal Examination*	
	Normal	Normal	Abnormal	Normal	Abnormal
Binge Control Day 1 + 8	Male 21 Female 21	39	2. Large atrial septal defect. Dilated heart.	28	a.Absent or poorly developed supraoccipital bone. 1. b.Cervical vertebral bodies absent. 6. c.Sternal bones missing or poorly developed. 0 d.Combination of a. b. and c. above plus poorly developed interparietal bone. 1. Combination of a. b. c. and d. above plus missing upper thoracic vertebral bodies. 1.
Binge Ethanol Day 1 + 8	Male 11 Female 14	24	0	24	a.Absent or poorly developed supraoccipital bone. 0 b.Cervical vertebral bodies absent. 2. Combination of a. and b. above. 1.
Binge Control Day 4 + 12	Male 17 Female 17	22	1. Poorly developed atria.	19	a.Absent or poorly developed supraoccipital bone. 0 b. Cervical vertebral bodies absent. 8. Combination of a. and b. above. 1.
Binge Ethanol Day 4 + 12	Male 11 Female 8	18	0	20	Cervical vertebral bodies absent. 5.

*Skeletal Examination. Normal = only digit bones missing. All other bones present.
Only live fetuses were included in this study.

Table 3.18. Assessment of Wilson's sections in macroscopically normal live fetuses. (Statistical test: Fisher Exact Probability Test).

	Normal	Query abnormal
Control day 1 and 8	39	1
Ethanol day 1 and 8	23	1
p = 0.613		
Control day 4 and 12	30	3
Ethanol day 4 and 12	19	0
p = 0.247		

The query abnormal fetuses showed the same types of minor abnormalities as those described in section 3.3.3., i.e. slight renal pelvic dilatation and marginal dilatation of brain ventricles and were classified as normal in the analysis of total fetal abnormalities in table 3.16.

Heart dissection

Heart dissections were performed as described in section 2.6. The only abnormalities found in this study were one fetus with a large atrial septal defect and one fetus with a dilated heart, in the binge control group day one and eight, and poorly developed atria in one fetus in the binge control group day four and twelve. There were no abnormalities in the ethanol treated fetuses (see table 3.17.).

Skeletal examination

Skeletal examination was performed as described in section 3.3.3. Statistical evaluation of each grade of abnormality was carried out using the Fisher Exact Probability Test.

Grade 1. Any bone missing or invisible;
2. Any bone except digits missing;
3. Any bone except digits and/or cervical vertebral bodies missing.

There were no significant differences between test and control animals at any of the three grades of abnormality mentioned (see table 3.17.).

Fetal weights

There were no differences in fetal weights between any of the groups studied.

3.6.3. Discussion

Following acute doses of ethanol given to pregnant C3H mice on days one and eight or four and twelve of gestation I observed no significant increase in abnormalities, nor adverse effects on the fetus compared with the control group. The only findings in the treated mice which differed from the control animals were the effects on placental weights.

3.6.4. Conclusions

From this study it would appear that "binge" intake of ethanol on the days studied was not harmful to the fetuses. However it would be unwise to deduce that "binge" intake of ethanol at any time during pregnancy is harmless. It is possible that if I had used larger numbers of animals, or higher doses of ethanol, or administered the ethanol at different stages of gestation, I might have seen very different results.

Findings in mice, using acute ethanol dosage, appear to be variable. Schwetz et al. (1978) treated CF-1 mice with 15% ethanol in their drinking water, during organogenesis (days six to fifteen of gestation), which produced, in non pregnant animals, maximum blood alcohol levels of 200mg%.

They found no significant increase in the incidence of external or soft tissue abnormalities but noted a significant increase in the incidence of minor skeletal variants in the alcohol treated animals. They attributed this to retarded fetal growth rather than to the effects of ethanol.

Randall and Taylor (1979) found that the incidence of resorptions and congenital malformations increased in a dose-related manner in C57BL/6J mice fed a liquid diet containing 17,25% or 30% EDC from the fifth to tenth day of gestation. The anomalies included skeletal, neurological, urogenital and cardiovascular systems.

TABLE 3.3.

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	73	-	24.0		Healthy	1	0.9419	-	0	
						2	0.8685			
						3	0.9319			
						4	0.8503			
						5	0.8519			
						6	0.9160			
						7	0.9550			
2	48	-	24.0	9.67	Healthy	1	0.8463		0	0.1165
						2	0.8820			0.1418
						3	0.9576			0.1187
						4	0.9600			0.1479
						5	0.8765			0.1242
						6	0.7971			0.1243
						7	0.7996			0.1497
						8	0.8693			0.1339
						9	0.9666			0.1161

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
2						10	-	Monster or late resorption		-
3	48	-	25.6	9.75	Healthy	1	0.9617		0	0.1203
						2	1.0251			0.1417
						3	0.9428			0.1221
						4	1.0700			0.1399
						5	1.0359			0.1182
						6	0.9879			0.1321
						7	0.6395	Exomphalos	Twins	(-
						8	0.8058			(0.2050
						9	-	Monster or late resorption		-
4	61	-	25.7	9.74	Healthy	1	-	Monster or late resorption	0	0.1079
						2	0.9987			0.1130
						3	0.9788			0.1162
						4	0.9473			0.1090

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
4						5	0.7087			0.1000
						6	0.9341			0.1001
						7	0.9985			0.1115
						8	0.9600			0.1158
						9	1.0392			0.1078
5	61	-	21.5	8.64	Healthy	1	0.9575		0	0.1602
						2	1.0414			0.2199
						3	0.9864			0.1536
						4	0.5784		Twins	(0.2324
						5	0.9322			(-
						6	0.9307			0.1090
6	50	-	23.9	9.44	Healthy	1	1.0500		1	0.1329
						2	0.9244			0.1394
						3	1.0050			0.1598
						4	1.0000			0.1442
						5	0.8875	Dead		0.1258

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
6						6	0.9410			0.1448
						7	0.9257	Abnormal hind limb		0.1250
						8	0.9574			0.1251
7	66	-	27.8	9.5	Healthy	1	0.8183	Dead	2	0.1107
						2	1.0911			0.1540
						3	0.9591			0.1322
						4	1.1225			0.1223
						5	1.0988			0.1615
						6	1.1081			0.1500
8	73	-	24.0	9.2	Healthy	1	0.6632		0 Twins	(0.2200
						2	0.7549			(-
						3	0.9397			0.1197
						4	0.9399			0.1274
						5	0.8751			0.1063
						6	0.9600			0.1362
						7	0.8324			0.1159

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
8						8	0.9014			0.1428
						9	0.9133			0.1033
9	104	-	27.8	9.26	Healthy	1	0.9797		1 Twins	(0.3412
						2	0.6474			(-
						3	0.8857			
						4	0.9652			
						5	0.9693			
						6	0.9310			
10	82	-	26.8	9.22	Healthy	1	-	Monster or late resorption	1	-
						2	0.8892			0.1528
						3	0.9730			0.1600
						4	0.9800			0.1390
						5	0.9000			0.1570
						6	0.7949			0.1437
						7	1.0050			0.1257
						8	0.9383			0.1536

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
11	99	-	30.0	9.3	Healthy	1	1.0958		0	0.1331
						2	1.0677			0.1447
						3	1.0692			0.1455
						4	1.0548			0.1517
						5	1.0149			0.1451
						6	0.9861			0.1674
12	88	-	28.2	9.16	Healthy	1	-	Monster or late resorption	1	-
						2	1.0567			0.1614
						3	1.0579			0.1464
						4	1.0800			0.1300
13	100	-	27.0	7.1	Healthy	1	0.6647		0	0.1283
						2	0.9165			0.1447
						3	0.9933			0.1312
						4	0.9057			0.1473
						5	0.9236			0.1247
						6	0.8627			0.1722

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
13						7	0.9213			0.1411
14	92	-	30.7	9.52	Healthy	1	0.9109		2	0.1497
						2	0.9232			0.1444
						3	0.9263			0.1451
						4	0.9065			0.1214
						5	0.8947			0.1595
						6	0.8535			0.1332
						7	0.8554			0.1076
						8	0.9877			0.1192
15	93	-	25.0	9.2	Healthy	1	0.9312		2	0.1288
						2	0.8670			0.1195
						3	0.8950			0.1346
						4	0.8731			0.1327
						5	0.9506			0.1326
						6	0.5918			0.0899
						7	0.9377			0.1566

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
15						8	0.8211			0.1525
						9	0.9140			0.1265
16	85	-	24.1	9.81	Healthy	1	0.9400		1	0.1761
						2	0.8973			0.1313
						3	0.8304			0.1076
						4	0.8219			0.1155
						5	0.8300			0.1212
						6	0.7556			0.1280
						7	-	Monster or late resorption		-
						8	-	Monster or late resorption		-
17	91	-	28.2	9.01	Healthy	1	0.9700		2	0.0988
						2	0.7130			0.1361
						3	0.7876			0.1220
						4	0.9191			0.1496
						5	0.8195			0.1229

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
17						6	0.8186			0.1252
						7	0.9323			0.1409
						8	0.9046			0.1199
18	58	-	26.3	9.28	Healthy	1	-	Monster or late resorption	2	0.1194
						2	0.9898			0.1402
						3	0.9667			0.1332
						4	0.8877			0.1189
						5	0.9942			0.1300
						6	0.9100			0.1139
						7	0.8536	Exomphalos		0.1224
19	51	-	25.7	9.18	Healthy	1	0.9499		1 Twins	(0.2221
						2	1.0942			(-
						3	0.9470			0.1130
						4	0.8040			0.1500
						5	0.8100			0.1079
						6	0.9217			0.1070

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
19						7	0.9158			0.1318
						8	-	Monster or late resorption		-
20	53	-	23.0	9.42	Healthy	1	0.9279		0	0.1470
						2	0.9243			0.1359
						3	0.9010			0.1028
						4	0.9543			0.1082
						5	0.9451			0.1349
						6	0.9200			0.1168
						7	0.9639			0.1215
21	91	-	25.0	9.76	Healthy	1	0.8327		1	0.1018
						2	0.9258			0.1235
						3	0.8760			0.1115
						4	0.9415			0.1264
						5	0.8755			0.1382
						6	0.9316			0.1484
						7	0.7546			0.0891

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
21						8	0.9272			0.1480
22	84	-	29.0	9.43	Healthy	1	1.1400		2	0.1476
						2	1.2201			0.0900
						3	1.2229			0.1531
						4	1.2605			0.1318
						5	1.3038			0.1342
23	84	-	27.5	9.51	Healthy	1	1.1540		0	0.1174
						2	1.0380			0.1250
						3	1.1460			0.1680
						4	1.1806			0.1320
						5	1.1896			0.1209
						6	1.2128			0.1409
24	97	-	25.1	9.65	Healthy	1	0.8606		1	0.1700
						2	0.9124			0.1673
						3	0.9354			0.1619
						4	0.8684			0.1434

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
24						5	0.8835			0.1421
						6	0.8780			0.1554
						7	0.8533			0.1266
25	229	-	23.0	8.55	Healthy	1	0.9832		4	0.1547
						2	0.8957			0.0692
						3	0.9163			0.1661
						4	0.7591			0.1163
						5	0.9917			0.1529
26	38	-	24.5	9.26	Healthy	1	1.0687		3	0.1567
						2	0.8784			0.1463
						3	1.0872			0.1484
						4	1.0819			0.1364
						5	1.0154			0.1210
27	44	-	25.5	9.44	Healthy	1	0.4785	Dead	0	0.1119
						2	0.9395			0.1176
						3	0.9509			0.1126

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FŒTAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
27						4	0.9763			0.1072
						5	0.9800			0.1263
						6	0.9966			0.1027
						7	1.0406			0.0935
28	44	-	26.4	9.70	Healthy	1	0.6211	Dead	1	0.0900
						2	1.0275	Exomphalos		0.1769
						3	0.9445			(0.2487
						4	0.7342		Twins	(-
						5	1.1166			0.2029
						6	1.1381			0.1377
						7	1.0790			0.1729
29	59	-	25.0	8.93	Healthy	1	1.0526		2	0.1771
						2	1.1290			0.1452
						3	1.0743			0.1457
						4	1.1476			0.1534
						5	1.1450			0.1632

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FŒTAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
29						6	1.0961			0.1638
30	75	-	20.6	9.55	Healthy	1	0.4222	Dead	2	0.1068
						2	1.0451			0.1456
						3	1.0066			0.1412
						4	1.0438			0.1279
						5	1.0593			0.1218
						6	0.9940			0.1389
						7	1.0902			0.1517
31	79	-	25.2	9.68	Healthy	1	1.0200		2	0.1208
						2	0.9799			0.1348
						3	0.9703			0.1290
						4	1.0123			0.0970
						5	0.8900			0.1138
						6	1.0374			0.1185
						7	1.0815			0.1258

TABLE 3.3. (Cont.)

ETHANOL CONTROL GROUP

10mℓ of Liquid Diet Daily

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
32	83	-	27.5	9.76	Healthy	1	0.8480	Dead	1	0.1171
						2	0.7429	Dead		0.1095
						3	0.9832			0.1207
						4	0.9124			0.1540
						5	0.7648			0.1248
						6	0.8823			0.1316
						7	0.9430			0.1396

TABLE 3.4.

6% E.D.C GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	50	0	23.0	9.18	Healthy	1	0.7409		1	0.1538
						2	0.7112			0.1775
						3	0.8847			0.1646
						4	0.7991			0.1562
						5	0.7286			0.1356
						6	0.7558			0.1387
						7	0.7222			0.0987
						8	0.8739	Dead		0.1268
2	80	0	23.1	9.15	Healthy	1	0.7144			
						2	0.6825			
						3	0.8803			
						4	0.6022			
						5	0.8996			
						6	0.9291			
						7	0.9216			
						8	0.7605			

TABLE 3.4. (Cont.)

6% E D.C. GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
2						9	0.7210	Dead		
						10	-	Monster or late resorption		
3	79	0	24.4	9.2	Healthy	1	1.0187		1	0.1360
						2	0.8380			0.1132
						3	0.8307			0.1245
						4	0.7623			0.1240
						5	0.8992			0.1185
						6	0.9434			0.1130
						7	0.8575			0.1099
						8	0.9036			0.0995
						9	1.0011			0.1089
4	86	0	28.0	9.12	Healthy	1	0.6771		1	0.1049
						2	0.7452			0.1083
						3	0.9107			0.1721
						4	1.0060			0.1165

TABLE 3.4. (Cont.)

6% E.D.C. GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FŒTAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
4						5	0.8910			0.1482
						6	0.6856			0.1074
						7	0.8187			0.1417
5	86	0	27.9	9.34	Healthy	1	0.7276	Dead	3	0.1257
						2	0.9668			0.1032
						3	1.0866			0.1126
						4	0.9269			0.1303
						5	0.9078			0.1750
						6	0.9175			0.1225
						7	0.8517	Exomphalos		0.1172
6	86	0	29.0	9.51	Healthy	1	0.9289		4	0.1442
						2	0.7844			0.1441
						3	1.1038			0.1161
						4	0.9004			0.1635
7	86	0	24.0	9.55	Circling Behaviour	1	0.6231	Exencephaly	2	

TABLE 3.4. (Cont.)

6% E.D.C GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
7						2	0.6326			
						3	0.6745			
						4	0.6657			
						5	0.6510			
						6	0.6982			
						7	-	Monster or late resorption		
8	98	0	27.0	9.44	Healthy	1	-	Monster or late resorption	1	-
						2	0.8452			0.1446
						3	0.9652			0.1700
						4	1.0340			0.1465
						5	1.1160			0.1562
						6	0.9126			0.1761
						7	0.9349			0.1391
9	101	-	28.5	9.02	Healthy	1	0.9107		3	0.1299
						2	0.8560			0.1728

TABLE 3.4.(Cont.)

6% E D C. GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
9						3	0.7745			0.1443
						4	0.7657			0.1153
						5	0.7772			0.1276
						6	-	Marked Hydramnios Monster or late resorption		-
10	101	-	29.6	9.35	Healthy	1	0.7148	Dead	2	0.0983
						2	1.0645			0.1317
						3	1.0168			0.1324
						4	0.9378			0.1833
11	100	-	27.5	9.31	Healthy	1	0.4177	Dead	2	0.1122
						2	0.9619			0.1366
						3	0.9307			0.1513
						4	0.9040			0.1600
						5	0.8124			0.1277
12	108	-	29.1	9.28	Healthy	1	0.7847		3 Twins	(0.1964
						2	0.7524			(-

TABLE 3.4. (Cont.)

6% E.D C GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
12						3	0.9116			0.1101
						4	0.8183			0.1305
						5	0.8571			0.1294
						6	0.7952			0.1015
						7	0.9341			0.1407
13	119	-	29.5	8.86	Healthy	1	0.9289	Cystic masses present at both ends of uterine horns	2	0.1810
						2	1,0090			0.1353
14	51	0	26.0	8.78	Healthy	1	-	Monster or late resorption	0	-
						2	1.0619			0.1000
						3	1.0031			0.1400
						4	1.0493			0.1555
						5	1.0328			0.1415
						6	0.9631			0.1154
15	56	-	20.1	9.09	Healthy	1	1.0785		0	0.1613
						2	0.9027	Dead		0.1010

TABLE 3.4. (Cont.)

6% E D C GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
15						3	0.9122	Dead		0.1173
						4	0.9085			0.0960
						5	0.9886			0.0962
						6	1.1080			0.1216
						7	0.9916			0.1187
						8	1.0361			0.1209
16	51	-	23.5	8.43	Healthy	1	0.8741	Exomphalos	1	0.1314
						2	1.0351			0.1348
						3	0.9708			0.1211
						4	0.8800	Dead		0.1200
						5	0.9318			0.1353
						6	0.9579			0.1147
						7	0.9412			0.0954
17	51	-	20.0	8.67	Healthy	1	1.0866		0	0.1394
						2	0.6782	Dead		0.1087
						3	0.9531			0.1286

TABLE 3.4. (Cont.)

6% E D.C. GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
17						4	0.8642			0.0998
						5	1.0423			0.1195
						6	0.8583			0.1231
						7	0.9477			0.1413
						8	0.9633			0.1169
18	72	<1	24.6	8.85	Healthy	1	0.9680		1	0.1095
						2	1.0289			0.1016
						3	0.9381			0.1137
						4	0.9540			0.1007
						5	1.0201			0.1070
						6	0.9864			0.0858
19	91	0	24.4	8.40	Healthy	1	1.0397		0	0.1311
						2	0.8914			0.1342
						3	0.9986			0.1194
						4	0.9559			0.1174
						5	0.9580			0.1439

TABLE 3.4. (Cont.)

6% E.D.C GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
19						6	0.8478			0.1321
20	36	-	24.6	7.36	Healthy	1	0.9287	Dead	0	0.1473
						2	0.9700			0.1500
						3	0.9727			0.1250
						4	0.9298			0.1460
						5	0.9409			0.1297
						6	0.8028			0.1175
						7	0.7369			0.1200
						8	0.8784			0.1391
21	50	-	21.6	6.14	Healthy	1	0.9957		0	0.1242
						2	0.8961			0.0966
						3	1.0122			0.1194
						4	0.9354			0.1479
						5	0.9300			0.1250
						6	1.0321			0.1211
						7	0.9606			0.1306

TABLE 3.4. (Cont.)

6% E.D.C. GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FŒTAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
21						8	0.9618			0.1250
22	50	-	25.1	7.64	Healthy	1	0.9102		0	0.1412
						2	0.8467			0.1067
						3	0.8984			0.1431
						4	0.8908			0.1233
						5	0.8650			0.1133
						6	0.9664			0.1153
						7	0.8441			0.1231
						8	0.8560			0.1010
						9	0.8328			0.1167
23	50	-	24.0	7.26	Healthy	1	0.9552		2	0.1226
						2	0.9427			0.1493
						3	1.0431			0.1525
						4	0.9749			0.1404
						5	0.9102			0.1698
						6	1.0671			0.1328

TABLE 3.4. (Cont.)

6% E D C GROUP

Dose: Ethanol (100%) 0.169mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
23						7	0.9738			0.1537
24	56	–	26.6	8.91	Healthy	1	1.0577		2	0.1222
						2	1.0856	Dead		0.1100
						3	0.9738			0.1177
						4	1.0036			0.1106
						5	0.7949	Dead		0.1218
						6	1.0434			0.1184
						7	0.9472			0.1392

10% E D.C. GROUP

TABLE 3.5. Dose: Ethanol (100%) 0.283mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	71	-	25.7	9.68	Healthy	1	-	Monster or late resorption	4	
						2	0.9443			
						3	1.1475			
						4	1.0697			
						5	0.9597			
						6	1.0708			
2	71	-	30.6	9.04	Jaundiced	1	1.1707		2	
						2	1.0970			
						3	1.1300			
3	77	-	30.5	9.53	Healthy	1	-	Monster or late resorption	1	
						2	0.9718	Exomphalos		
						3	0.6780			
						4	0.8508			
						5	0.8867			
						6	0.9631			
						7	0.9746			

10% E D C GROUP

TABLE 3.5. (Continued) Dose: Ethanol (100%) 0.283 mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FŒTAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
4	78	0	32.0	9.81	Jaundiced	1	0.9234		7	
						2	1.0200			
5	133	0	24.8	9.36	Healthy	1	0.9825		7	
						2	0.9400			
6	91	0	25.6	9.68	Jaundiced	1	0.6927		2	
						2	1.0031			
						3	0.9649			
						4	0.8753			
						5	1.0000			
						6	1.1029	Exomphalos		
7	137	0	25.2	9.24	Jaundiced	1	0.9000		4	0.1457
8	137	0	31.5	9.32	Healthy	1	0.9621		2	0.1400
						2	0.9040			0.1217
						3	0.7719	Exomphalos		0.1293
						4	-	Monster or late resorption		-

10% E D C GROUP

TABLE 3.5. (Continued) Dose: Ethanol (100%) 0.283 ml/10ml Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/l	Pre Pregnant Weight in grams	Mean Dietary Intake in ml	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
9	108	0	30.3	9.58	Healthy	1	0.9432		6	0.1386
						2	0.8700			0.1387
						3	0.7933			0.1546
10	47	-	22.5	9.26	Healthy	1	1.1443		1	0.1400
						2	0.9854			0.1621
						3	1.0644			0.1273
						4	0.9210			0.1752
						5	1.1496			-
11	47	-	24.6	9.11	Healthy	1	0.8947	Dead	1	0.1328
						2	1.0619			0.1550
						3	1.0400			0.1664
						4	1.0086			0.1000
						5	1.0386			0.1615
						6	1.0448			0.1581
12	53	-	22.4	9.23	Healthy	1	1.0776		2	0.1223
						2	1.0755			0.1757

10% E D C GROUP

TABLE 3.5. (Continued) Dose: Ethanol (100%) 0.283mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
12						3	1.0753			0.1412
						4	1.0048			0.1443
						5	0.8827			0.1700
						6	0.8270			0.1511
13	68	< 10	22.5	8.68	Healthy	1	0.9013		1	0.1521
						2	1.0196			0.1494
						3	0.9833			0.1205
						4	0.9215			(0.2131
						5	0.9033		Twins	(-
						6	0.9062			0.1374
						7	0.9862			0.1492
14	68	9.8	26.1	9.32	Healthy	1	0.9953		1	0.1697
						2	1.0321			0.1361
						3	1.0051			0.1453
						4	0.9963			0.1421
						5	0.8643			0.1257

10% E.D.C. GROUP

TABLE 3.5. (Continued) Dose: Ethanol (100%) 0.283mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
14						6	1.0272			0.1692
15	87	-	28.4	9.37	Healthy	1	1.0046		2	0.1119
						2	0.9066			0.0934
						3	0.9670			0.1137
						4	1.0670			0.0955
16	95	-	23.5	8.65	Healthy	1	0.9009		3	0.0848
						2	0.9511			0.1089
						3	0.9344			0.1021
17	32	100	22.2	9.22	Healthy	1	1.0268		0	0.1300
						2	0.9123			0.1117
						3	1.0275			0.1231
						4	1.0054			0.1289
						5	0.9958			0.1446
						6	0.8654			0.1079
						7	0.9754			0.1552

10% E D.C. GROUP

TABLE 3.5. (Continued) Dose: Ethanol (100%) 0.283mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
18	62	-	24.0	9.25	Healthy	1	1.0185		4	0.1496
						2	1.1718			0.1560
						3	1.0259			0.1547
						4	1.0856			0.1443
						5	0.9699			0.1318
19	69	-	24.4	8.71	Healthy	1	-	Monster or late resorption	0	-
						2	0.6700	Dead		0.1433
						3	1.0096			0.1624
						4	1.0726			0.1935
						5	0.9317			0.1756
						6	0.9801			0.1696
						7	1.0281			0.1407
20	67	-	-	8.91	Healthy	1	1.1226		0	0.1427
						2	0.9494			0.1718
						3	1.0387			0.1349
						4	1.0685			0.1484

10% E D C GROUP

TABLE 3.5. (Continued) Dose: Ethanol (100%) 0.283 mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
20						5	1.0303			0.1250
						6	0.9813			0.1722
						7	-	Monster or late resorption		0.1312
21	75	-	24.9	9.32	Healthy	1	1.0327		1	0.1479
						2	0.8709			0.1419
						3	0.9566			0.1452
						4	0.9859			0.1485
						5	0.8340			0.1314
						6	0.9126			0.1216

20% E D C GROUP

TABLE 3.6. Dose: Ethanol (100%) 0.566mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	52	610	21.2	8.5	Healthy	1	1.0500		1	
						2	1.0000			
						3	0.9247			
						4	0.9219			
						5	0.6145			
						6	1.0300			
2	53	740	23.0	9.3	Jaundiced	1	0.8560		0	
						2	1.0421			
						3	0.9742			
						4	0.9086			
						5	1.0509			
						6	0.9155			
						7	0.9317			
3	66	710	21.9	9.2	Healthy	1	0.6428	Dead	0	
						2	0.8568			
						3	0.8269			

20% E D C GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.566mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
3						4	0.8173			
						5	0.7580			
						6	0.8787			
4	66	730	22.1	9.0	Jaundiced	1	0.8099		1	
						2	0.9406			
						3	0.9246			
						4	0.7438			
						5	0.8671			
						6	0.9149			
5	67	1650	22.9	9.3	Jaundiced	1	0.9701		1	
						2	0.9517			
						3	1.0215			
						4	0.9793			
						5	0.7996			
						6	0.7725			
						7	0.9033			

20% E.D C GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.566 mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
6	80	-	23.0	8.8	Healthy	1	0.6960		2	
						2	0.7087			
						3	0.7197			
						4	0.7086			
						5	-	Monster or late resorption		
7	152	1105	23.1	8.4	Healthy	1	0.8992		2	0.1618
						2	0.9689			0.1603
						3	0.9600			0.1655
						4	0.5130	Hydramnios Blood stained amniotic fluid		0.1169
						5	0.8721			0.1324
						6	0.8021			0.1202
8	152	1555	23.0	8.7		1	0.9739		2	0.1237
						2	0.8702			0.1098
						3	0.9057			0.1243
						4	0.9526			0.1265
						5	0.9070			0.1025

20% E D C GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.568 mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
9	152	0	25.2	8.82	Looked ill Feeding poorly	1	0.8583	Membranes appeared bile stained	4	0.1501
						2	0.7376			0.1445
						3	0.7772			0.1534
						4	0.6859			0.1600
10	69	0	22.0	9.1	Mildly jaundiced	1	0.8893		0	
						2	0.9838			
						3	0.9485			
						4	0.8560			
						5	1.0258			
						6	0.9857	Exomphalos		
11	69	810	26.6	9.35	Healthy	1	0.9635		1	
						2	0.8845			
						3	0.7335			
						4	0.7956			
						5	0.9642			
						6	0.8861			

20% E D C. GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.566 mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
12	69	890	25.0	9.15	Healthy	1	0.9819		2	
						2	0.9724			
						3	0.8879			
						4	0.8678			
						5	-	Monster or late resorption		
						6	-	Monster or late resorption		
13	69	1 140	23.2	9.59	Jaundiced	1	1.1495		2	
						2	-	Monster or late resorption		
14	83	-	26.0	8.99	Jaundiced	1	0.5396	Exomphalos	4	
						2	0.7500			
						3	0.6636			
15	164	-	25.0	8.63		1	0.8918		2	0.1190
						2	0.9254			0.1366
						3	0.9632			0.1295
						4	0.6200			0.1000

20% E D C GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.566mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
15						5	0.7279	Exomphalos		0.1554
16	173	986	24.1	7.52		1	0.7159		0	0.1267
						2	0.6600			0.1175
						3	0.5675			0.1284
						4	0.7129			0.1450
						5	0.6550			0.1420
17	36	810	23.6	8.22	Healthy	1	0.6044	Dead	0	0.1036
						2	0.7279			0.1324
						3	0.7325			0.0910
						4	0.6226			0.0959
						5	0.7251	Hydramnios Exencephaly		0.1091
						6	0.7239			0.1276
						7	0.8133			0.1283
						8	0.8111			0.1144
18	121	-	24.4	7.65		1	0.9312		2	0.1599
						2	0.8935			0.1141

20% E D C GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.566 mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
18						3	0.8032			0.1371
						4	0.9084			0.1368
						5	0.8849			0.1403
19	234	-	25.1	8.27		1	0.8980		2	0.1399
						2	0.8249			0.1800
						3	0.9134			0.1756
						4	0.8713			0.1464
						5	0.9670			0.1722
						6	0.9199			0.1401
20	53	-	20.7	8.45	Healthy	1	0.8515	Dead	1	0.1459
						2	1.0896			0.1500
						3	1.0016			0.1522
						4	0.9266			0.1032
						5	1.0821			0.1335
						6	1.0894			0.1937
21	53		21.5	8.96	Healthy	1	0.7744	Dead	0	0.1473

20% E.D.C GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.566mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
21						2	0.9687			0.1823
						3	0.9672			0.1408
						4	0.9560			(0.2703
						5	0.9563		Twins	(-
						6	0.9705			0.1344
						7	0.8424			0.1255
						8	0.9205			0.1391
22	56	-	24.0	8.87	Healthy	1	0.6188	Dead	1	0.0911
						2	0.6564	Dead		0.1000
						3	0.9707			0.1234
						4	0.8675			0.1667
						5	0.8988			0.1680
						6	1.0070			0.1381
						7	0.7100			0.1147
						8	-	Monster or late resorption		-

20% E D C GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.556mℓ/10mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
23	56	-	20.2	7.96	Healthy	1	1.0090		1	0.1390
						2	0.9648			0.1519
						3	0.7952			0.1400
						4	1.0929			0.1400
						5	1.0078			0.1591
24	57	-	25.5	8.57	Healthy	1	0.9014		2	0.1542
						2	0.7653			0.1494
						3	0.9494			0.1241
						4	0.9978			0.1622
						5	0.9719			0.1414
25	70	-	24.0	8.39	Healthy	1	0.9706		1	0.1337
						2	0.8837			0.1072
						3	0.9829			0.1308
						4	0.9104			0.1215
						5	0.8719			0.1322
						6	-	Monster or late resorption		0.0835

20% E.D.C GROUP

TABLE 3.6. (Continued) Dose: Ethanol (100%) 0.566mℓ/10mℓ Liquid Feed/House/Day

MATERNAL PARAMETERS						FETAL PARAMETERS				
Animal Number	Duration of Expmt in days	Ethanol Level in mg/ℓ	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus No.	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
26	69	-	26.0	8.72	Healthy	1	0.7812		4	0.1362
						2	0.9291			0.1655
						3	0.7586			0.1019

Table 3.12.

BINGE CONTROL GROUP

Water 2 mℓ given on days 1 + 8 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
1	Healthy	1	0.9773		0	0.1162
		2	0.9900			0.1085
		3	1.0033			0.0982
		4	1.0173			0.1262
		5	1.0082			0.0915
		6	1.0304			0.0705
		7	1.0838			0.1313
2	Healthy	1	0.6920		0	0.0800
		2	0.9671			0.1631
		3	1.0152			0.1146
		4	0.9725			0.1037
		5	1.0134			0.1173
		6	0.9635			0.1166
		7	1.0123			0.1406
3	Healthy	1	0.9929		0	0.1511
		2	1.1108			0.1292
		3	1.0600			0.1520
		4	1.0302			0.1689

TABLE 3.12 (Continued)

BINGE CONTROL GROUP

Water 2 mℓ given on days 1 + 8 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
3		5	0.9977			0.1532
4	Healthy	1	-	Monster or late resorption	1	-
		2	1.0459			0.1059
		3	1.0970			0.1385
		4	1.1100			0.1391
5	Healthy	1	0.9553		2	0.1429
		2	1.0599			0.1407
		3	1.0497			0.1442
		4	0.9035			0.1269
		5	0.9992			0.1354
		6	1.0657			0.1406
6	Healthy	1	0.9388		2	0.1111
		2	1.1029			0.1617
		3	1.0297			0.1300
		4	0.9679			0.1147
		5	1.0641			0.1430
		6	1.0048			0.1410

TABLE 3.12 (Continued)

BINGE CONTROL GROUP

Water 2 mℓ given on days 1 + 8 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
7	Healthy	1	1.0449		0	0.1466
		2	0.9300			0.1434
		3	0.9700			0.1402
		4	0.9000			0.1219
		5	1.0580			0.1312
		6	0.8110			0.0984
		7	1.0188			0.1557
		8	0.9269			0.1707
		9	0.9652			0.1131
8	Healthy	1	-	Monster or late resorption	0	-
		2	-	Monster or late resorption		-
		3	1.0391			0.1216
		4	1.1168			0.1243
		5	1.0069			0.1170
		6	1.1033			0.1253
		7	1.0216			0.1330
		8	1.1036			0.1179

IABLE 3.12 (Continued)

BINGE CONTROL GROUP

Water 2mℓ given on days 1 + 8 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
9	Healthy	1	-	Monster or late resorption	1	-
		2	0.8356			0.1630
		3	0.7759			0.1625
		4	0.7400			0.1582
		5	0.8556			0.1309
		6	0.8900			0.1649
		7	0.9252			0.1400
		8	0.9805			0.1632
10	Healthy	1	0.8011		0	0.1639
		2	0.8643			0.1639
		3	0.9005			0.1208
		4	0.9287			0.1684
		5	0.9484			0.1291
		6	0.9283			0.1283
		7	0.8751			0.1388
		8	0.8681			0.1352
		9	0.9061			0.1388

TABLE 3.12 (Continued)

BINGE CONTROL GROUP

Water 2 mℓ given on days 1 + 8 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
11	Healthy	1	0.9590		1	0.1237
		2	1.0348			0.1451
		3	0.9973			0.1207
		4	0.9649			0.1180
		5	0.9502			0.0934
		6	1.1518			0.1320
		7	1.0786			0.1370
12	Healthy	1	1.1381		0	0.1483
		2	0.9947			0.1384
		3	1.0180			0.1478
		4	1.0366			0.1429
		5	0.8751			0.1331
		6	0.9728			0.1296
		7	1.0031			0.1363

TABLE 3.13. BINGE ETHANOL GROUP

Ethanol 2mℓ of a 7.35% solution given on days 1 + 8 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	Healthy	1	0.9782		0	0.1241
		2	0.9416			0.0841
		3	1.0775			0.1203
		4	0.8591			0.0989
		5	0.9713			0.0904
		6	1.0492			0.1080
		7	1.0030			0.1095
2	Healthy	1	0.7431		0	0.1056
		2	0.8608			0.1154
		3	0.7842			0.1103
		4	0.8152			0.1148
		5	0.8139			0.1071
		6	0.8004			0.0860
3	Healthy	1	-	Monster or late resorption	0	0.1164
		2	-	Monster or late resorption		0.0848
		3	1.0121			0.1169
		4	0.8368			0.0794

TABLE 3.13. (Continued) BINGE ETHANOL GROUP

Ethanol 2mℓ of a 7.35% solution given on days 1 + 8 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
3		5	0.7345			0.0898
		6	1.0499			0.1369
		7	0.9340			0.1125
		8	0.9907			0.1345
4	Healthy	1	0.9300	Exomphalos	0	0.1350
		2	1.1087			0.1245
		3	0.8472			0.1222
		4	1.0485			0.1000
		5	1.0416			0.1246
		6	1.0873			0.1081
5	Healthy	1	1.1086		1	0.1218
		2	1.0871			0.1667
		3	0.9400			0.1555
		4	1.1154			0.1532
		5	1.0499			0.1752
6	Healthy	1	0.8942		1	0.1854
		2	0.9825			0.1365

TABLE 3.13. (Continued) BINGE ETHANOL GROUP

Ethanol 2mℓ of a 7.35% solution given on days 1 + 8 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
6		3	0.8806			0.1240
		4	0.9055			0.1567
		5	0.5823			0.1307
		6	1.0043	Exomphalos		0.1338
		7	0.8994			0.1414
7	Healthy	1	1.1152		1	0.1157
		2	0.9699			0.1301
		3	1.1078			0.1783
		4	1.0087			0.1309
		5	1.0230			0.1376
8	Healthy	1	0.9040		1	0.1259
		2	1.0710			0.1255
		3	1.0658			0.1221
		4	1.0009			0.1042
		5	0.9097			0.1188
		6	0.9560			0.0950
		7	1.0541			0.1135

TABLE 3.13. (Continued) BINGE ETHANOL GROUP

Ethanol 2mℓ of a 7.35% solution given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
9	Healthy	1	1.0615		1	0.1977
		2	0.9828			0.1448
		3	1.0957			0.1352
		4	0.9064			0.1280
		5	0.9815			0.1388
		6	0.9219			0.1542
		7	0.9603			0.1300
		8	0.9903			0.1146
10	Healthy	1	0.9651		0	0.1022
		2	0.9856			0.1077
		3	0.9446			0.1201
		4	1.1005			0.1312
		5	0.9283			0.1086
		6	1.0378			0.1491
		7	1.0303			0.1436
		8	1.0810			0.1231

IABLE 3.14

BINGE CONTROL GROUP

Water 2 mℓ given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
1	Healthy	1	0.7648		0	(0.2731
		2	0.7862		Twins	(-
		3	0.8360			(0.1652
		4	0.8588		Twins	(-
		5	0.8842			0.1220
		6	0.8182			0.1539
		7	0.7744			0.1217
2	Healthy	1	0.9445		1	0.1402
		2	0.9546			0.1695
		3	0.9519			0.1370
		4	0.9991			0.1275
		5	0.9887			0.1359
		6	1.0808			0.1402
3	Healthy	1	1.0522		2	0.1285
		2	0.8214			0.1140
		3	0.9365			0.1024
		4	0.9878			0.1258
		5	0.9260			0.1219

TABLE 3.14 (Continued)

BINGE CONTROL GROUP

Water 2 mℓ given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
4	Healthy	1	0.9475		3	0.0990
		2	0.8715			0.1122
		3	0.9293			0.1074
		4	0.9039			0.1642
		5	1.0280			0.1319
		6	0.8854			0.1422
5	Healthy	1	1.0053		1	0.1125
		2	1.0662			0.1200
		3	1.0158			0.1572
		4	0.9912			0.1123
		5	0.9559			0.1140
		6	1.0440			0.1551
		7	1.0990			0.1482
6	Healthy	1	1.0473		1	0.1287
		2	1.0042			0.1482
		3	1.1245			0.1488
		4	1.0200			0.1137
		5	1.1585			0.1553

IABLE 3.14 (Continued)

BINGE CONTROL GROUP

Water 2 mℓ given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
7	Healthy	1	0.5608	Dead	0	0.1065
		2	1.0392			0.1209
		3	0.9792			0.1215
		4	1.0178			0.1489
		5	0.9800			0.1437
		6	0.9444			0.1195
		7	0.8966			0.1146
		8	0.9231			0.1514
		9	1.1085			0.1399
		10	0.9961			0.1154
8	Healthy	1	1.1159		0	0.1241
		2	1.1187			0.1373
		3	1.0218			0.1258
		4	1.0640			0.1090
		5	1.0520			0.1080
		6	1.0585			0.1360
		7	1.0436			0.1245

TABLE 3.14 (Continued)

BINGE CONTROL GROUP

Water 2 mℓ given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious external Abnormality or Death	Resorptions	Placental Weight in grams
9	Healthy	1	0.9604		0	0.1300
		2	0.9476			0.1222
		3	1.1126			0.1436
		4	0.9417			0.1187
		5	0.9580			0.1138
		6	0.9023			0.1177
		7	1.0011			0.1427
		8	0.8990			0.1277
		9	1.0108			0.1738

TABLE 3.15. BINGE ETHANOL GROUP

Ethanol 2mℓ of a 7.35% solution given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	Healthy	1	1.0306		1	0.1479
		2	0.9770			0.1097
		3	1.0824			0.1300
		4	1.0040			0.1500
		5	1.1399			0.1200
2	Healthy	1	-	Monster or late resorption	2	(0.1993
		2	0.7731		Twins	(-
		3	0.8304			0.1500
		4	0.7300			0.1569
		5	0.7800			0.1480
		6	0.9236			0.1457
		7	0.8916			0.1528
3	Healthy	1	0.8833		1	(0.2476
		2	0.7647		Twins	(-
		3	0.8856			0.1626
		4	1.0299			0.1215

TABLE 3.15.(Continued) BINGE ETHANOL GROUP

Ethanol 2mℓ of a 7.35% solution given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
3		5	0.9503			0.1394
		6	1.0050			0.1427
		7	0.9063			0.1664
4	Healthy	1	1.0902		1	0.1443
		2	1.1197			0.1426
		3	1.1288			0.1639
		4	1.0590			0.1063
		5	1.1326			0.1204
5	Healthy	1	0.9029	Abn. hind limb	0	(0.3236
		2	0.9431		Twins	(-
		3	0.9852	Exomphalos		0.1652
		4	1.0803			0.2054
6	Healthy	1	0.8900	Exomphalos	0	(0.2850
		2	1.0656	Exomphalos	Twins	(-
		3	0.9776			0.1337
		4	1.0759			0.1172
		5	1.1664			0.1116

TABLE 3.15.(Continued) BINGE ETHANOL GROUP

Ethanol 2mℓ of a 7.35% solution given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
6		6	1.0563			0.1391
7		1	1.1054		1	0.1545
		2	1.1800			0.1400
		3	1.1480			0.1148
		4	0.9848			0.1461
		5	1.1025			0.1548
		6	0.9952			0.1500
		7	1.0288			0.1104
8	Healthy	1	0.8953		1	0.1674
		2	0.9274			0.1805
		3	0.9263			0.1449
		4	0.8324			0.1130
		5	0.9209			0.1060
		6	0.9371			0.1329
		7	0.9800			0.1702
9	Healthy	1	1.0097		1	0.1801
		2	1.0200			0.1348

TABLE 3.15.(Continued) BINGE ETHANOL GROUP

Ethanol 2mℓ of a 7.35% solution given on days 4 + 12 of gestation

Animal Number	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
9		3	1.0989			0.1647
		4	1.0015			0.1504
		5	0.9902			0.1649
		6	1.0133			0.1271
		7	1.1260			0.1348
10	Healthy	1	0.9545		3	(0.2891
		2	0.8261		Twins	(-
		3	1.0499			0.1503
		4	0.9308			0.1600
		5	1.0341			0.1267
11	Healthy	1	1.0930		0	0.1545
		2	1.0429			0.1430
		3	1.0655			0.1324
		4	0.9902			0.1332
		5	1.1230			0.1770

CHAPTER 4

ACETALDEHYDE DOSAGE IN VIVO AND COMPARISON OF CONTROL GROUPS

4.1. CHRONIC ACETALDEHYDE DOSAGE : METHOD

The purpose of this study was to determine whether acetaldehyde given daily to pregnant mice produced any adverse effects on the offspring. Young adult C3H females were housed one per cage and given white paper towel as bedding. The control animals received the same liquid diet as the ethanol control animals but were given only five millilitres of the diet, with unlimited access to water and Epol rat cubes. (These control animals were also used in a parallel study by Beyers (1981) on the effects of diphenylhydantoin and phenobarbitone on the fetus). Acetaldehyde in doses of 3.9 milligrams or 11.8 milligrams was added to the liquid portion of the test groups' diet. Unlike the alcohol treated animals these were not averse to taking the liquid diet, so there was no need to withhold the rat cubes.

The mice were given the acetaldehyde for at least two weeks prior to mating and for the duration of gestation.

4.2. RECORDS OF MATERNAL AND FETAL PARAMETERS

Notes on the duration of the experiment, the pre-pregnant maternal weights, the daily dietary intake and the physical states of the mice were taken. Serum acetaldehyde levels were not measured at the time of sacrifice as no suitable assay method was available. Fetal and placental weights, resorptions and obvious external abnormalities were recorded (see tables 4.1., 4.2. and 4.3., pages 141 to 162).

4.3. RESULTS OF CHRONIC ACETALDEHYDE DOSAGE

4.3.1. Maternal findings (see table 4.4.)

There was no significant reduction in the pre-pregnant maternal weights in either of the test groups compared with the controls, indicating that in the test group acetaldehyde intake did not adversely affect their nourishment.

The groups were assessed using the One Way Analysis of Variance Test and significance determined by multiple comparisons among means.

4.3.2. Placental findings (see table 4.4.)

An interesting result was that the placental weights of the test groups were significantly reduced compared with the controls, and the placentas in the acetaldehyde 11.8mg group were significantly lighter than those in the acetaldehyde 3.9mg group. The same statistical test as in 4.3.1. was applied. In this statistical test $p = 0.001$.

4.3.3. Fetal findings (see table 4.5.)

Fetal weights, resorptions, total abnormal live births, alterations in sex ratio and total implants were assessed.

Total fetal abnormalities were identified by:-

- macroscopic examination
- Wilson's sectioning
- heart dissection
- skeletal examination

Wilson's sections, heart sections and skeletons were assessed using the same standards and criteria as described in section 3.3.3.

Incidence of macroscopic abnormalities:

Control group:
- one fetus with exomphalos
- seven monsters/late resorptions
- three dead fetuses

Acetaldehyde 3.9mg group:
- no macroscopically abnormal fetuses
- five monsters/late resorptions
- one dead fetus

TABLE 4.4.

IN VIVO STUDY.

EFFECTS OF CHRONIC ACETALDEHYDE DOSAGE ON MATERNAL AND PLACENTAL WEIGHTS

Dose	Maternal Weight	Placental Weight
ACETALDEHYDE 3.9mg	No significant reduction in weight in the test group (Test. One Way A.O.V.) Control N = 18 Test N = 8	Placental weights were significantly reduced in the test group (Test. One Way A.O.V.) p = 0.001 Control N = 132 Test N = 81
ACETALDEHYDE 11.8mg	No significant reduction in weight in the test group (Test. One Way A.O.V.) Control N = 18 Test N = 9	Placental weights were significantly reduced in the test group (Test. One Way A.O.V.) p = 0.001 Control N = 132 Test N = 70

Placental Weights included only live single fetuses. Twins were excluded.

One Way A.O.V. = One way analysis of variance. Comparison among means.

TABLE 4.5.
IN VIVO STUDY.

RESULTS OF CHRONIC ACETALDEHYDE DOSAGE.

Fetuses assessed at 18 days' gestation

Dose	Fetal Weights Live Births Only	Early resorptions	Abnormal Live Births	Altered Sex Ratio. Only live Fetuses assessed	Total implants
ACETALDEHYDE 3.9 mg	No significant reduction in weight (Test One Way A.O.V.) Control N = 137 Test N = 83	No significant effect Control N = 161 Test N = 100	No significant increase in abnormalities (Fisher Test) Control N = 109 Test N = 73	No significant alteration Control N = 62 Test N = 43	No significant reduction (Test One Way A.O.V.) Control N = 18 Test N = 11
ACETALDEHYDE 11.8 mg	Acetaldehyde fetal weights significantly increased compared with control weights. Control N = 137 Test N = 74 (Test One Way A.O.V.)	No significant effect (Fisher Test) Control N = 165 Test N = 90	No significant increase in abnormalities (Fisher Test) Control N = 109 Test N = 61	No significant alteration Control N = 62 Test N = 39	No significant reduction (Test One Way A.O.V.) Control N = 18 Test N = 10

Statistical tests. Except where otherwise indicated the Chi^2 Test was used (2 tailed test).
One Way A.O.V. = One way analysis of variance. Comparison among means $p = 0.05$
Fisher Test = Fisher Exact Probability Test.
Deaths in utero were assessed using the Chi^2 test but there was no significant increase in deaths in either of the groups studied compared with controls.
Deaths in utero included monsters/late resorptions.

Acetaldehyde 11.8mg group:

- no macroscopically abnormal fetuses
- five monsters/late resorptions
- six dead fetuses

Wilson's sections (see table 4.6.)

No major abnormalities were found on Wilson's sectioning of the test and control fetuses. There were no differences in the ratios of males to females in any of the groups studied. The same standards and criteria for assessment were applied as in section 3.3.3.

Table 4.7. Assessment of Wilson's sections in macroscopically normal live fetuses. Statistical test - Fisher Exact Probability Test.

	Normal	Query abnormal
Controls	50	1
Acetaldehyde 3.9mg	36	1
p = 0.667		
Controls	50	1
Acetaldehyde 11.8mg	28	2
p = 0.308		

The query abnormal fetuses included:

- one control fetus with marginal dilatation of the lateral ventricles of the brain.
- one fetus in the acetaldehyde 3.9mg group with slightly dilated renal calyces and lateral brain ventricles.
- two fetuses in the acetaldehyde 11.8mg group with slightly dilated renal calyces.

Those query abnormal sections which were examined histologically were found to be normal.

Skeletal examination (see table 4.6.)

This was performed using the criteria described in section 3.3.3. The same three grades of abnormality were considered:-

1. any bone invisible or absent
2. any bone except digits absent
3. any bone except digits and/or cervical vertebral bodies absent.

TABLE 4.6.

IN VIVO STUDY

EFFECTS OF CHRONIC ACETALDEHYDE DOSAGE ON FETAL DEVELOPMENT

Fetuses assessed at 18 days' gestation

Group	Wilson Section	Heart Sections		Skeletal Examination*	
	Normal	Normal	Abnormal	Normal	Abnormal
Control	Male 29 Female 33	59	0	53	a.Absent or poorly developed supraoccipital bone. 0 b.Cervical vertebral bodies absent. 1 c.Absent rib (left 9th rib) 1 d.Absent or poorly developed sternal bones. Combination of a. b. and d. above. 2
Acetaldehyde 3.9mg.	Male 22 Female 21	41	0	29	a.Absent or poorly developed supraoccipital bone. 0 b.Cervical vertebral bodies absent. 0 cAbsent or poorly developed sternal bones. 1 Combination of a. b. and c. above plus poorly developed interparietal bones. 1 Combination of a, b and c 1
Acetaldehyde 11.8mg.	Male 19 Female 20	33	0	28	Fused 11th and 12th ribs on left side. (Fig.4.1.) 1 Cervical vertebral bodies absent. 2

*Skeletal Examination. Normal = only digit bones missing. All other bones present.
Only live Fetuses were included in this study.

The incidence of each grade of abnormality was assessed using the Fisher Exact Probability Test. There were no significant differences between test and control groups for any of the three grades of abnormality. The fetuses which were classified as abnormal in grade 2 were included in the total abnormalities shown in table 4.5.

It is possible that most of the abnormalities seen in this study, apart from the fusion of the 11th and 12th ribs in the acetaldehyde 11.8mg group, were due to delays in development rather than actual structural changes (see fig. 4.1.).

Heart dissection

Fifty-nine control, forty-one acetaldehyde 3.9mg and thirty-three acetaldehyde 11.8mg fetuses were examined for cardiac abnormalities using the techniques described in section 2.6.

No abnormalities were found in any of the three groups.

Fetal weights (see table 4.5.)

Fetal weights were significantly increased in the acetaldehyde 11.8mg group compared with the control group. I can offer no explanation for this finding.

4.3.4. Discussion

Kesäniemi and Sippel (1975) treated pregnant Sprague-Dawley rats with intraperitoneal injections of ethanol two grams per kilogram four days before term, and after twenty-five minutes measured the concentrations of ethanol and acetaldehyde in the placentas, fetuses and maternal blood. They found similar concentrations of ethanol (about 50 μmol per mℓ blood/per gram tissue) in the maternal aortic blood, the placental tissue and the fetal tissue. However, the acetaldehyde content of the placenta was only about 25% of that in the maternal aortic blood and there was no acetaldehyde detectable in the fetus. These results indicate that, at least in rats, acetaldehyde formed by maternal metabolism of ethanol is quickly oxidised in the placenta, thus protecting the fetus.

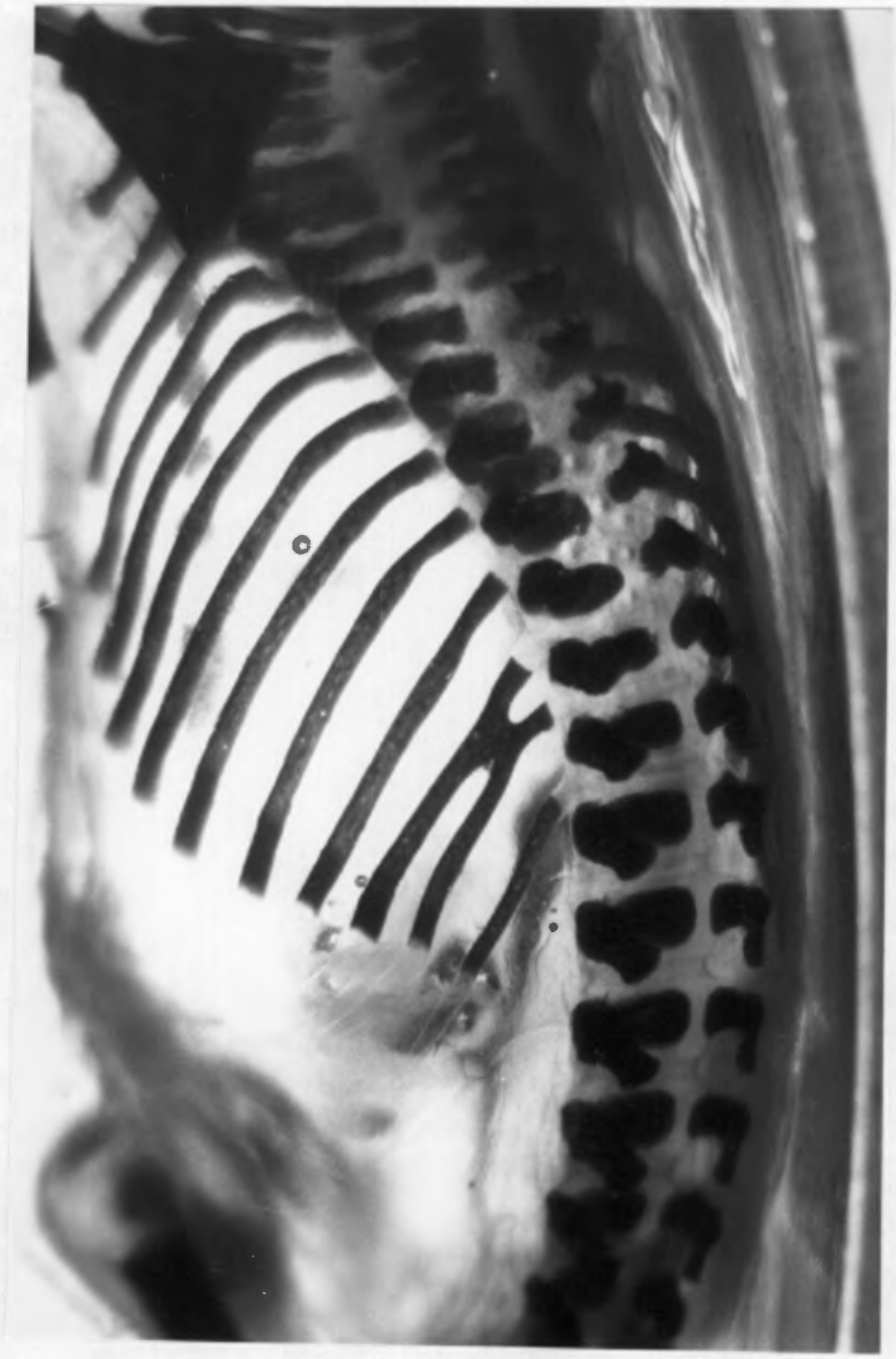

Fig. 4.1.

18-Day fetal skeleton with fused 11th and 12th ribs. Mother treated with acetaldehyde 11.8mg.

Randall et al (1978) fed pregnant mice with liquid diets containing twenty-five percent EDC from gestation days five through ten or twelve through eighteen. They measured the acetaldehyde content of the fetuses on days eleven or nineteen and found it to be eighteen percent (6.7 millimoles per gram) and forty percent (20.7 millimoles per gram), respectively, of that found in the maternal blood. These results indicate that although acetaldehyde appears in the mouse fetus the placenta again forms a protective barrier to some extent.

In the light of these experiments it is not surprising to find that the placentas in the acetaldehyde treated animals were significantly reduced in weight while there were, apparently, no adverse effects on the fetuses. It is possible that the placenta bore the brunt of the acetaldehyde toxicity and whatever small amounts did reach the fetuses were not present in sufficient concentration to produce any measurable effects using my methods of detecting fetal abnormalities.

O'Shea and Kauffmann (1979) injected pregnant mice intravenously with either saline alone (controls) or 1% or 2% acetaldehyde in saline on days seven, eight and nine of gestation and examined the uterine contents on days ten or nineteen of gestation. They found that the acetaldehyde-treated embryos examined on day ten were smaller and showed a higher incidence of developmental delay compared with controls. Anomalies of closure of the cranial and caudal regions of the neural tube were the most commonly noted defects. When examined on day nineteen acetaldehyde -treated fetuses were significantly smaller and weighed less than controls. Superficial examination of fetuses on day nineteen did not reveal any congenital abnormalities either in test or control groups.

It would appear from these findings that some form of repair may have taken place which would account for the differences in morphological findings on days ten and nineteen.

4.3.5. Conclusions

Apart from reduction of placental weights in the treated groups acetaldehyde appeared to have no adverse effects either on the mothers or fetuses studied.

4.4. COMPARISON OF THE ETHANOL AND ACETALDEHYDE CONTROLS

The ethanol control group and the acetaldehyde control group were compared. The ethanol control group received only the 10mℓ liquid diet and water ad libitum, while the acetaldehyde control group received five millilitres of the liquid diet plus rat cubes and water. It was suspected that the liquid diet alone might in itself have adverse effects on the mother and fetus because

(a) it is unnatural for mice to have only liquid food, and

(b) they were often without food for up to twelve hours as many animals consumed most of their diet during the daytime. This is an abnormal eating pattern for mice, which are normally nocturnal animals.

I compared maternal pre-pregnancy weights, fetal weights, placental weights and total implants in the two groups using the Two Sample t Test (unpaired). There was no difference between the maternal weights, but the ethanol control fetuses (on liquid diet only) were significantly lighter ($p < 0.005$) and their placentas significantly heavier ($p < 0.001$) than the acetaldehyde controls (liquid plus solid food). There was no difference in total implants between the two groups. Using the Chi^2 Test or the Fisher Exact Probability Test as applicable, I compared the number of early resorptions, deaths in utero, abnormal live fetuses, and ratio of male to female fetuses in the two groups, and found no significant differences between them.

I conclude from these findings that although the liquid diet alone may have had slight adverse effects on the fetuses, there is no reason to suspect that the liquid diet alone was teratogenic. The differences in the fetal or placental weights in the two groups, although statistically significant, were small.

Table 4.8. Comparison of fetal and placental weights in two control groups.

Group	Fetal weight in grams	Placental weight in grams
Ethanol control group	0.9482 ± 0.1166 Std. error 0.0080	0.1340 ± 0.0211 Std. error 0.0015
Acetaldehyde control group	0.9936 ± 0.1372 Std. error 0.0117	0.1225 ± 0.0271 Std. error 0.0024

When comparing the incidence of congenital abnormalities I found an incidence of 6.7% in the ethanol control group (liquid feed only) and an incidence of 3.7% in the acetaldehyde control group (liquid plus solid food). The 3.7% incidence in the second group is very close to the 3.3% incidence of spontaneous congenital abnormalities found in C3H mice by Heinecke (1972).

It would appear that it is not the liquid diet itself which is harmful but rather the abnormal feeding pattern which this type of diet produces. Some of the animals consumed most or all of their feed during the morning and were then obliged to fast until the next feed. This was particularly evident in the ethanol treated animals.

Periods of fasting early in pregnancy can affect embryonic development in the mouse. Runner and Miller (1956) fasted strain 129 mice for twenty-four, thirty or forty hours starting on the seventh, eighth, ninth or tenth day of gestation and examined the offspring at nineteen days' gestation. They found that fasts of twenty-four or thirty hours caused vertebral and costal deformities and exencephaly; fasts of forty hours were followed by termination of pregnancy. These teratogenic effects of fasting could be almost completely eliminated if fasted mice were given oral supplements of glucose or amino acids. Ellington (1980:385) studied the effects of fasting in pregnant rats and found

significant retardation of development (somite number), and growth (assessed as protein content), of embryos taken from fasted rats at ten and a half days' gestation after fasts of twelve hours or more.

It is important to bear these findings in mind in any in vivo study where a liquid diet is used and this abnormal feeding pattern is observed. The maternal fasting may well contribute to the increased number of abnormalities observed in the ethanol treated animals and their controls.

TABLE 4.1.

ACETALDEHYDE CONTROL GROUP

5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	35	26.1	5.	Healthy	1	-	Monster or late resorption	0	-
					2	0.9583			0.1054
					3	1.0253			0.1055
					4	0.9912			0.1120
					5	1.0023			0.1053
					6	1.0429			0.0985
					7	1.1436			0.1531
					8	1.0008			0.0885
					9	1.0808			0.1109
					10	1.0575			0.0973
2	35	23.4	5.	Healthy	1	0.6085		1	0.1077
					2	1.2200			0.1356
					3	1.1873			0.1526
					4	1.2235			0.1703
					5	1.1263			0.1290

TABLE 4.1. (Continued)

ACETALDEHYDE CONTROL GROUP

5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
2					6	1.1795			0.1221
					7	1.1055			0.1523
3	35	25.5	4.97	Healthy	1	-	Monster or late resorption	2	-
					2	1.1134			0.1473
					3	0.9505			0.1373
					4	1.1183			0.1801
					5	1.1905			0.1453
					6	1.1424			0.1111
4	35	22.4	4.91	Healthy	1	1.1488		0	0.1236
					2	1.1739			0.1230
					3	1.1242			0.1250
					4	1.1314			0.1243
					5	1.1755			0.1700

TABLE 4.1. (Continued)

ACETALDEHYDE CONTROL GROUP

5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
5	42	25.5	5.	Healthy	1	0.9030		1	0.1096
					2	1.0727			0.0943
					3	1.0343			0.1400
					4	1.0124			0.1087
					5	0.9768			0.1023
					6	0.9100			0.0900
					7	0.8813			0.1118
					8	0.9057			0.1060
					9	0.9912			0.1077
					10	1.0232			0.1030
6	42	24.5	4.94	Healthy	1	0.9691		0	0.1286
					2	0.9806			0.1320
					3	0.8767			0.1153
					4	1.0654			0.1145
					5	0.9513			0.1186
					6	0.7956			0.0941

ACETALDEHYDE CONTROL GROUP

TABLE 4.1. (Continued) 5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
6					7	0.9791			0.1145
					8	0.9795			0.0765
					9	0.9158			0.1230
7	42	29.5	5.	Healthy	1	0.9493		0	0.1517
					2	0.9598			0.1041
					3	0.8190			0.1142
					4	0.9364			0.1207
					5	0.7895			0.1068
					6	0.8232			0.1000
					7	0.7954			0.0987
					8	0.8993			0.1266
					9	0.9800			0.0915
8	42	24.1	5.	Healthy	1	-	Monster or late resorption	0	-
					2	1.1662			0.0976
					3	0.9982			0.1078

ACETALDEHYDE CONTROL GROUP

TABLE 4.1. (Continued)

5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
8					4	1.0880			0.1100
					5	1.0752			0.1181
					6	1.0529			0.1070
					7	0.6039			0.0820
					8	0.9974			0.1263
9	49	25.5	5.	Healthy	1	1.0434		0	0.1111
					2	1.0029			0.1011
					3	0.9844			0.1017
					4	0.9677			0.1092
					5	0.8701			0.0844
					6	0.9220			0.0980
					7	0.9250			0.0850
					8	0.8562			0.0781
					9	1.0382			0.1079
					10	0.9549			0.1019

TABLE 4.1. (Continued)

ACETALDEHYDE CONTROL GROUP { 5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
10	49	25.6	4.96	Healthy	1	-	Monster or late resorption	1	-
					2	1.1109			0.1013
					3	0.6814	Dead		(0.3167
					4	1.0732		Twins	(-
					5	0.9802			0.1261
					6	1.2123			0.1932
					7	1.0378			0.1680
					8	1.1017			0.1600
11	49	25.9	4.84	Healthy	1	-	Monster or late resorption	1	-
					2	0.9025			(0.2121
					3	0.8937		Twins	(-
					4	1.1124			0.1557
					5	0.9343			0.1022
					6	1.0800			0.1732
					7	0.7320			0.1099

TABLE 4.1. (Continued)

ACETALDEHYDE CONTROL GROUP

5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
11					8	1.0193			0.1335
					9	1.0583			0.1484
12	49	26.3	4.96	Healthy	1	1.1309		0	0.1491
					2	1.0074			0.1179
					3	0.9302	Dead		0.1106
					4	1.0294			0.1432
					5	1.0329			0.1329
					6	1.0013			0.1258
					7	0.9682			0.1377
					8	0.8760			0.1587
					9	1.0397			0.1596
					10	0.9893			0.1144
13	49	27.9	5.	Healthy	1	1.0817		2	0.2062
					2	0.8834			0.1019
					3	0.9959			0.1487

TABLE 4.1. (Continued)

ACETALDEHYDE CONTROL GROUP

5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
13					4	1.0442			0.1627
					5	1.1600			0.1814
					6	0.7014			0.1385
14	49	25.5	4.86	Healthy	1	1.1275		2	0.1693
					2	1.0292			0.1654
					3	-	Dead		0.1267
					4	-	Monster or late resorption		-
					5	1.0000			0.1454
					6	0.9990			0.1091
					7	1.1459			0.1620
					8	1.1614			0.0961
15	55	27.1	4.95	Healthy	1	0.7293	Exomphalos	0 Twins	(0.3135
					2	0.9271			(-
					3	0.9799			0.1433
					4	0.8797			0.1860

TABLE 4.1. (Continued)

ACETALDEHYDE CONTROL GROUP

5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
15					5	1.0359			0.1257
					6	0.9714			0.1843
					7	0.9417			0.1482
					8	0.9837			0.1555
16	55	23.9	4.96	Healthy	1	1.1269		0	0.1076
					2	1.0342			0.1071
					3	0.8300			0.0832
					4	0.9840			0.1100
					5	0.9721			0.1130
					6	1.0050			0.1094
					7	1.0651			0.1090
17	55	28.5	4.89	Healthy	1	0.9147		3	0.0893
					2	0.9700			0.1061
					3	1.0166			0.1038
					4	0.9090			0.0938
					5	1.0088			0.0900

TABLE 4.1. (Continued)

ACETALDEHYDE CONTROL GROUP

5mℓ of liquid diet daily

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
17					6	0.9169			0.0851
					7	1.0343			0.1005
					8	0.9862			0.0880
18	55	27.7	5.0	Healthy	1	-	Monster or late resorption	1	-
					2	0.9899			0.1211
					3	1.0895			0.1366
					4	0.9440			0.1543
					5	1.0124			0.1325
					6	1.0797			0.1800
					7	1.0599			0.1357
					8	1.0040			0.1000
					9	1.0539			0.1026

TABLE 4.2.

ACETALDEHYDE 3.9mg GROUP

Dose: Acetaldehyde 3.9mg/5mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	36	20.0	4.97	Healthy	1	0.9323		0	0.1679
					2	0.8545			0.1238
					3	1.1032			0.1564
					4	1.0546			0.1518
					5	0.9890			0.1267
					6	1.0465			0.1441
					7	0.9993			0.1206
					8	1.0309			0.1257
2	36	24.5	4.97	Healthy	1	-	Monster or late resorption	0	-
					2	0.8465		Twins	(0.2694
					3	0.9045			(-
					4	0.9496			0.1161
					5	0.9450			0.1170
					6	0.9844			0.1176
					7	0.9651			0.1138
					8	0.9800			0.1383

TABLE 4.2.(Cont.)

ACETALDEHYDE 3.9mg GROUP

Dose: Acetaldehyde 3.9mg/5mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
3	36	23.2	4.92	Healthy	1	1.1287		1	0.1089
					2	1.1276			0.1033
					3	1.1263			0.1299
					4	0.8275			0.0825
					5	0.9622			0.1133
					6	1.1208			0.1399
4	43	25.0	4.96	Healthy	1	1.0203		1	0.0942
					2	-	Monster or late resorption		0.0917
					3	1.0490			0.1053
					4	0.9782			0.0933
					5	1.0078			0.1022
					6	1.0286			0.0914
					7	1.0200			0.0984
5	43	22.5	5.0	Healthy	1	0.9446		0	0.0944
					2	0.8900	Dead		0.1000

TABLE 4.2.(Cont.)

ACETALDEHYDE 3.9mg GROUP

Dose: Acetaldehyde 3.9mg/5mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
5					3	0.9825			0.0976
					4	0.9645			0.0945
					5	0.9747			0.0949
					6	1.0232			0.0901
					7	0.9500			0.1017
					8	0.9691			0.0866
					9	1.0770			0.1057
					10	1.0815			0.1085
6	43	25.4	4.91	Healthy	1	0.9978		1	0.1147
					2	0.9800			0.0715
					3	0.9363			0.0900
					4	0.9526			0.1094
					5	0.9138			0.1146
					6	0.9369			0.1174
					7	1.0562			0.1055
					8	0.9950			0.1217

TABLE 4.2.(Cont.)

ACETALDEHYDE 3.9mg GROUP

Dose: Acetaldehyde 3.9mg/5mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
7	50	30.6	5.0	Healthy	1	0.8730		1	0.1260
					2	-	Monster or late resorption		0.1081
					3	1.0026			0.1500
					4	0.9385			0.1234
					5	1.0125			0.1360
					6	0.7381			0.1673
					7	0.9199			0.1165
					8	0.9631			0.1143
					9	0.9633			0.0968
					10	0.8750			0.1139
8	50	31.1	5.0	Healthy	1	1.0406		2	0.1112
					2	0.8571			0.1097
					3	0.9783			0.1320
					4	0.9020			0.1033
					5	0.8599			0.1060
					6	0.9000			0.1012

TABLE 4.2.(Cont.)

ACETALDEHYDE 3.9mg GROUP

Dose: Acetaldehyde 3.9mg/5mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
8					7	-	Monster or late resorption		-
					8	0.9396			0.1065
					9	0.9901			0.1271
					10	0.9175			0.0858
9	50	25.6	5.0	Healthy	1	-	Monster or late resorption	2	-
					2	1.0599			0.1284
					3	0.9633			0.1109
					4	1.1037			0.1416
					5	0.7249			0.0967
					6	1.0099			0.1388
					7	0.9975			0.1559
10	50	25.6	4.94	Healthy	1	0.9822		1	0.1165
					2	0.9911			0.1338
					3	1.1013			0.1236
					4	1.0722			0.1339

TABLE 4.2.(Cont.)

ACETALDEHYDE 3.9mg GROUP

Dose: Acetaldehyde 3.9mg/5mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
10					5	1.0773			0.1172
					6	1.0568			0.1099
					7	1.0700			0.1347
					8	1.0928			0.1453
11	50	29.8	5.0	Healthy	1	1.0455		1	0.0837
					2	1.0338			0.1296
					3	1.1678			0.1484
					4	1.0482			0.1414
					5	0.9646			0.1420
					6	1.1311			0.1215
					7	1.0227			0.1234

TABLE 4.3.

ACETALDEHYDE 11.8 mg GROUP

Dose: Acetaldehyde 11.8 mg/6 mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
1	31	-	5.81	Healthy	1	1.1575		1	0.1111
					2	0.9673			0.1615
					3	1.1379			0.1476
					4	1.1081			0.0574
					5	1.1553			0.1230
					6	1.0875			0.1083
					7	1.0886			0.1200
2	39	25.6	5.72	Healthy	1	1.1251		0	0.0900
					2	1.0320			0.0935
					3	1.2100			0.1023
					4	1.1732			0.1088
					5	1.2329			0.1317
					6	1.2121			0.1440
					7	1.0169			0.0815
					8	1.2256			0.1132

TABLE 4.3. (Continued)

ACETALDEHYDE 11.8 mg GROUP

Dose: Acetaldehyde 11.8 mg/6 mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
3	39	20.0	5.51	Healthy	1	1.2216		0	0.0981
					2	1.0244			0.0859
					3	1.1632			0.0968
					4	1.1932			0.1212
					5	1.2983			0.1065
					6	1.1902			0.0883
					7	1.1746			0.1083
					8	0.9241	Dead		0.0834
					9	0.9284			0.0838
					10	0.9613			0.0812
4	39	21.3	5.79	Healthy	1	1.1770		0	0.1048
					2	1.0960			0.0996
					3	1.0500			0.0930
					4	1.1320			0.1190
					5	1.0303			0.0776
					6	1.1394			0.0932

TABLE 4.3. (Cont.)

ACETALDEHYDE 11.8mg GROUP

Dose: Acetaldehyde 11.8mg/6 mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
4					7	1.0743			0.0837
					8	1.1500			0.0843
					9	-	Monster or late resorption		0.0976
					10	1.0169			0.0867
5	39	24.2	5.82	Healthy	1	0.9817		Twins	(0.1603
					2	0.7949			(-
					3	1.1100			0.0930
					4	1.1515			0.0876
					5	1.1860			0.1085
					6	1.0813			0.0757
					7	1.1568			0.0943
					8	1.1370			0.0893
					9	-	Monster or late resorption		0.0591
					10	1.1072			0.1000

TABLE 4.3.(Cont.)

ACETALDEHYDE 11.8 mg GROUP

Dose: Acetaldehyde 11.8 mg/6 mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
6	53	20.9	5.46	Healthy	1	1.0400			0.1000
					2	1.0350			0.1100
					3	0.9900			0.1400
					4	1.0250			0.1200
					5	1.0300			0.1050
					6	0.9750			0.1950
					7	1.0100			(-
					8	-	Monster or late resorption	Twins	(0.0800
7	53	23.0	5.35	Healthy	1	1.0753		1	0.1088
					2	0.8107	Dead		0.0818
					3	1.0396			0.1198
					4	0.9665	Dead		0.0118
					5	0.9865			0.0932
					6	0.4806	Dead		0.0794
					7	1.0858			0.1046

TABLE 4.3.(Cont.)

ACETALDEHYDE 11.8 mg GROUP

Dose: Acetaldehyde 11.8mg/6mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
7					8	1.0460			0.1033
					9	1.2185			0.1202
8	53	22.5	5.87	Healthy	1	1.0098		0	0.1136
					2	0.8544	Dead		0.1183
					3	0.8798			0.0793
					4	0.9623	Dead		0.0867
					5	0.8556			0.1300
					6	1.1827			0.1130
					7	0.8235			0.1208
					8	1.1400			0.1087
9	60	23.1	5.47	Healthy	1	-	Monster or late resorption	2	-
					2	-	Monster or late resorption		-
					3	1.0560			0.1072
					4	1.0930			0.0960

TABLE 4.3. (Cont.)

ACETALDEHYDE 11.8 mg GROUP

Dose: Acetaldehyde 11.8 mg/6mℓ Liquid Feed/Mouse/Day

MATERNAL PARAMETERS					FETAL PARAMETERS				
Animal Number	Duration of Experiment in days	Pre Pregnant Weight in grams	Mean Dietary Intake in mℓ	Physical State	Fetus Number	Weight in grams	Obvious External Abnormality or Death	Resorptions	Placental Weight in grams
9					5	0.9854			0.0711
					6	0.9715			0.0905
					7	0.9670			0.0882
10	87	26.1	5.87	Healthy	1	0.9345			0.0941
					2	1.0085			0.1023
					3	0.9879			0.0861
					4	1.2179			0.1517
					5	1.1025			0.1007
					6	1.0472			0.1065
					7	0.8321			0.0900
					8	0.9704			0.0843
					9	-	Monster or late resorption		-

CHAPTER 5

IN VITRO MODEL

An in vitro mouse embryo culture model was established, based on the method described by New (1967; 1978).

5.1. EXPERIMENTAL CONDITIONS

Young adult (six to eight week old) C3H female mice were housed in communal cages, about twenty per cage. They had vermiculite bedding and were fed on Epol rat cubes. Male C3H mice were kept under similar conditions except that they were housed two per cage. The females were placed with the males - five females to two males - for one hour once a week between 7.30 a.m. and 9.30 a.m. Mating was restricted to this short period of time in order to eliminate, as far as possible, age variations in the embryos. Day one of pregnancy was taken as the day following mating. Initially the mice were examined for the presence of a vaginal plug as proof that mating had taken place, but I found this an extremely unreliable method and finally abandoned it, relying rather on relating the mating schedule to diagnosis of pregnancy at eight or nine days' gestation. With experience, it became possible to diagnose this with reasonable accuracy by simple external examination of the mothers (see figure 5.1.).

5.2. EXPLANTATION OF EMBRYOS

The pregnant mice were sacrificed on day eight or nine of gestation by cervical dislocation, washed briefly in seventy percent ethanol, and the uteri removed intact through a midline abdominal incision. The uteri were placed in petri dishes containing sterile Tyrode saline (Tyrode, 1910) at room temperature.

The Tyrode saline solution contained

8.0g NaCl
0.2g KCl
0.2g $CaCl_2$

0.1g Mg $Cl_2.6H_2O$
0.05g $NaH_2PO_4.2H_2O$
1.0g $NaHCO_3$
1.0g Glucose
Water to make one litre

The decidual masses were dissected free from the uterus using number five watchmaker's forceps and placed in a second dish of sterile Tyrode saline. The embryos with visceral yolk sac and ectoplacental cone intact were dissected free from the decidual tissue and Reichert's membrane, under a dissecting microscope (see figure 5.2.1. and 5.2.2.).

5.3. CULTURE CONDITIONS

The embryos were placed two per tube in glass test tubes, capacity ten to fifteen millilitres (see fig. 5.3.), containing two millilitres of sterile rat serum at room temperature. At least one tube in each batch was allocated as a control tube and to the remainder, the appropriate dose of ethanol or acetaldehyde was added. The tubes were gassed with a 95% air / 5% CO_2 mixture for about thirty seconds each, and closed with ground glass stoppers lightly coated with silicone gel. The tubes took approximately forty-five minutes to warm up to 37°C. Problems were encountered with leakage of serum from some of these tubes, and in future work use of a narrow necked tube is proposed.

The sealed tubes were clipped onto a rotatable disc of a design based on that described in the article by Kochhar (1975:276), made by Scientific Industries, Inc., Springfield, Mass. This was mounted in a double-walled perspex incubator with air temperature controlled at 37.0° $\pm$ 0.2°C (figure 5.4.). (On a few occasions the temperature rose briefly to 38° or 39°C because of extremely high ambient temperatures.) The disc accommodated fifteen ten-millilitre tubes and four sixty-millilitre tubes (60mℓ tubes were used when more than thirty embryos were explanted). The large tubes contained six millilitres of serum, the appropriate dose of drug, and six embryos, and were gassed for $\pm$ ninety seconds. Great care was taken to ensure that the disc rotated smoothly and that the embryos were not jolted in any way.

Fig. 5.1.
Diagnosis of Pregnancy.
Pregnant C3H mouse - 9 days' gestation.

Fig. 5.2.1.

9-Day embryo with Reichert's membrane intact. (a) Mag. x 12.

Note: Where quoted magnification factors refer to microscope settings.

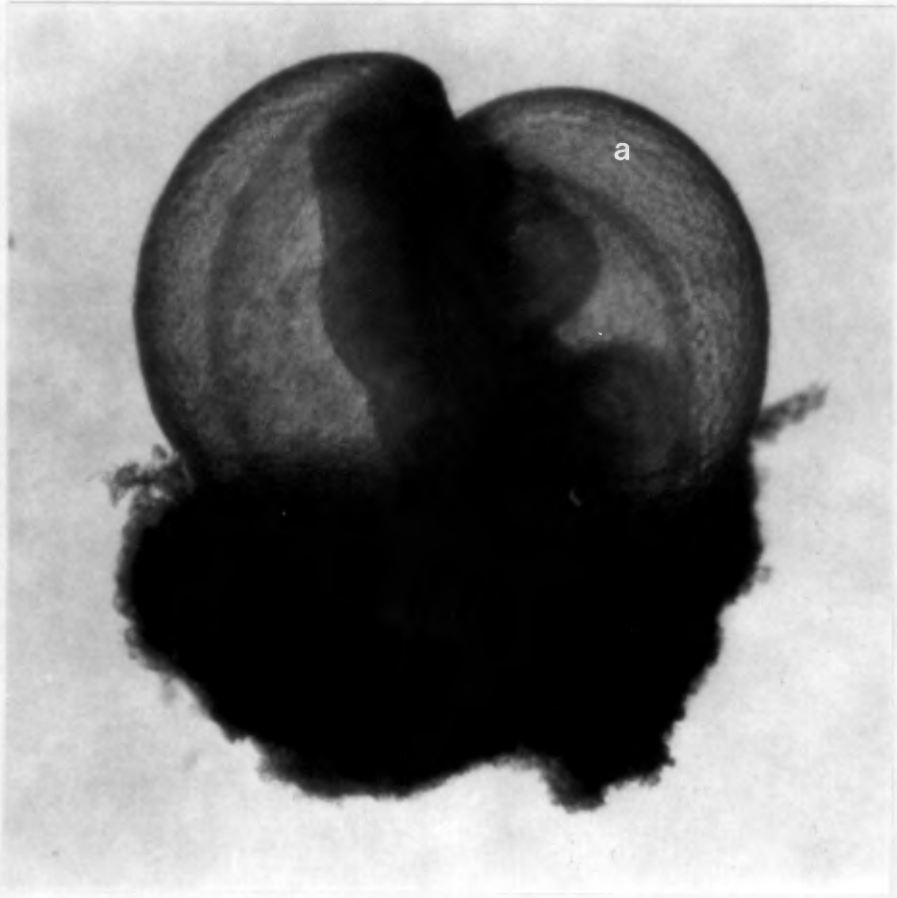

Fig. 5.2.2.

9-Day embryo with visceral yolk sac intact.(a) Reichert's membrane removed.
Mag.x 12.

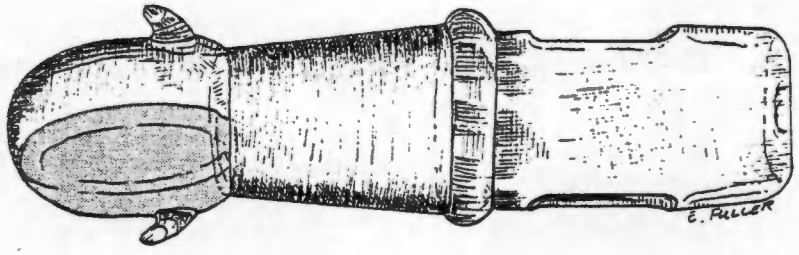

Fig. 5.3. Glass embryo culture tube.

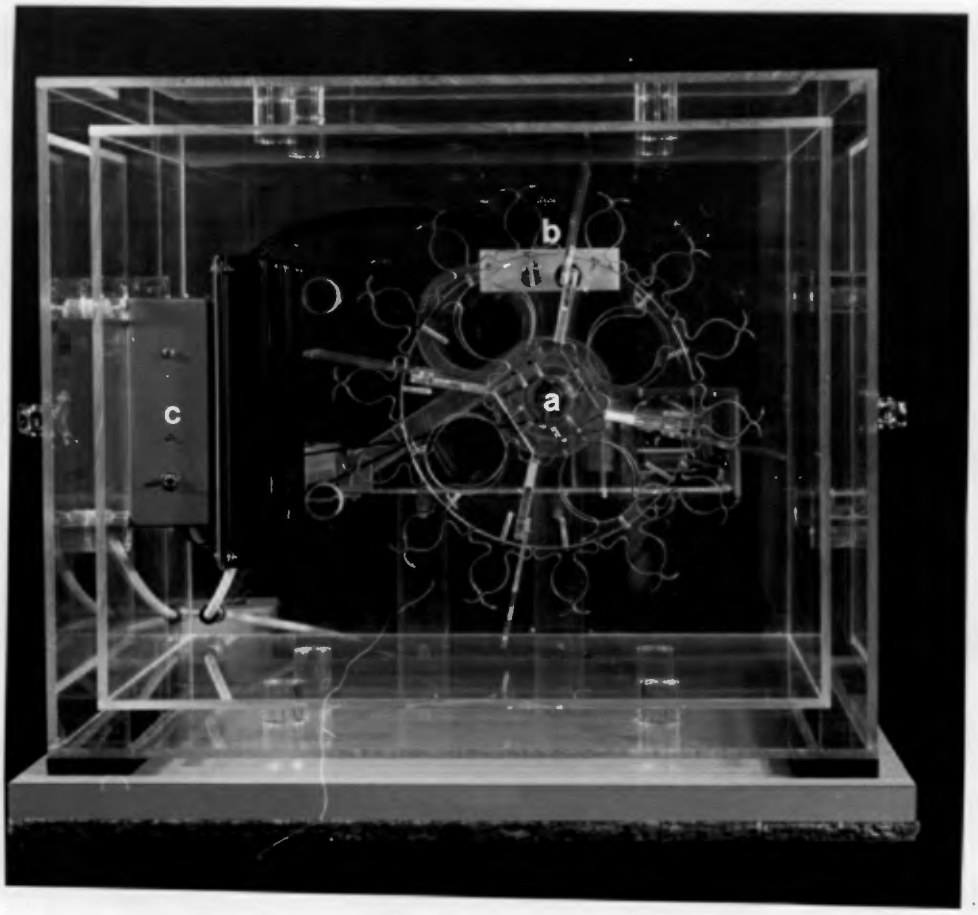

Fig. 5.4. Incubator.

Note - rotating disc with clamps (a)
- temperature sensors (b)
- temperature controller (c)

(Photo: Mrs. E. Fuller)

The glass tubes were round bottomed in order to eliminate turbulence. The disc rotated at a constant speed of thirty-seven revolutions per minute to aid gas/liquid mixture and the temperature was carefully regulated to ensure that the embryos were not exposed to hyper- or hypothermia. Hyperthermia, i.e. temperature in excess of 40°C is known to be teratogenic in rat embryos (Cockroft and New, 1975; 1978). All dissection and manipulation of the embryo took place under a laminar flow hood and all culture glassware was sterilised at 150°C for one and a half hours using dry heat. The ethanol and acetaldehyde were sterilised by serial membrane filtration through Millipore filters RA (1.2µm), AP 15 and GS (0.22µm). The embryos were cultured for twenty-eight hours.

5.4. PREPARATION OF THE RAT SERUM

Whole rat blood was obtained from the animal unit at the University of Cape Town Medical School. Long Evans white rats were sacrificed using ether and blood drawn by cardiac puncture. Usually the blood had clotted by the time I received it. It was then centrifuged and the serum drawn off. It was again centrifuged to precipitate any remaining red cells. All serum was sterilised by serial membrane filtration as described in section 5.3. and stored in sterile vacutainers at -20°C. The serum was not heat inactivated as described by New et al (1976a).

Steele (1972) found that embryo growth was affected by the method of preparation of the serum. If the blood was allowed to clot prior to centrifuging this was termed delayed centrifuged (D.C.) serum. If the blood was immediately centrifuged before clotting had occurred this was termed immediately centrifuged (I.C.) serum.

It has been demonstrated by gel electrophoresis that a protein difference between the two sera exists. After embryos have been in I.C. serum it is depleted of one protein estimated from its band position on the gel to have a molecular weight of ±125 000. Fresh D.C. serum differs from fresh I.C. serum by having a reduced concentration of this protein and by the absence of two

others with molecular weights greater than 200 000 (Klein et al, 1978). The source of the serum is not important i.e. different strains, male, female, pregnant or non-pregnant rats may be used (New, 1966b:1967). Steele observed that when eight and a half day rat embryos were grown in D.C. serum, many of them acquired double hearts and other abnormalities while in I.C. serum they developed normally. Overall growth of primitive streak and early somite stage was significantly less in embryos grown in D.C. serum (Steele and New, 1974). The effects of D.C. serum appear very rapidly. After half an hour, the serum has a tendency to cause double heart formation and by three hours this effect approaches that of standard D.C. serum prepared from blood stored for eighteen hours. (Steele and New, 1974; New et al, 1976a).

The serum which I used should strictly be termed D.C. serum as it was seldom possible to separate all of it within half an hour of the blood being drawn. This was usually done within one to three hours. On no occasion, however, did I observe double heart formation in the mouse embryo.

5.5. CHOICE OF AN APPROPRIATE GAS PHASE

New and Coppola (1970) studied the effects of different concentrations of oxygen on the development of rat embryos in culture. They found that in circulating medium the younger embryos grew best in five percent CO_2 in air while older embryos required much higher concentrations of oxygen. In their study embryos were explanted at 9 1/2, 10 1/2 and 11 1/2 days gestation and cultured for periods of forty or fifty hours. Noting these results I used the five percent CO_2 in air mixture for my mouse embryo culture system.

5.6. CHOICE OF EIGHT AND NINE-DAY EMBRYOS

Mouse embryos undergo organogenesis between 7 1/2 and 15 days gestation, but the most significant change probably occurs between 8 1/2 and 9 days, when the process is greatly accelerated. The contour of the embryo changes and the most devastating congenital abnormalities can be produced in the embryo at this time (Rugh, 1968:116).

The 7 1/2 to 15-day period is also the time of maximum sensitivity to teratogens (Leone and Giavini, 1977). The embryos explanted early in this period (8 or 9 days' gestation) are easy to grow in culture and do not have complex or specialised requirements. Also, as mentioned above, they develop very rapidly during the relatively short culture period of twenty-eight hours (figure 5.5. and 5.6.).

The embryos grew extremely well in our culture system, but development did lag, by a few hours, compared with that in vivo.

New, Coppola and Cockroft (1976b) found that in rat embryos explanted at the headfold stage average rates of protein synthesis and differentiation over forty-eight hours were indistinguishable from those in vivo. The slightly retarded rate of development which I observed may be due to the use of D.C. serum which was not heat inactivated. Another phenomenon which does not occur to the same degree in the rat, was the considerable inter-litter variation. Embryos explanted from the same mother may vary by as much as twelve hours in their development (Theiler, 1972) (see figure 5.7.).

5.7. LABELLING WITH TRITIATED THYMIDINE

After twenty-four hours' incubation the culture tubes were removed and opened under the laminar flow hood and one microcurie of tritiated thymidine introduced (specific activity 5.0 curies per millimole) using a 10µl Hamilton syringe. The tubes were then regassed with the 95% air/5% CO_2 mixture, sealed and returned to the incubator for a further four hours.

5.8. MORPHOLOGICAL ASSESSMENT

Eight- or nine-day embryos referred to below are defined as embryos explanted on the eighth or ninth day of gestation respectively. (Actual age of the embryos would be eight days plus twenty-eight hours or nine days plus twenty-eight hours). After completion of the twenty-eight hour culture period the embryos were removed from their tubes and placed in petri dishes containing normal saline, and observed under the

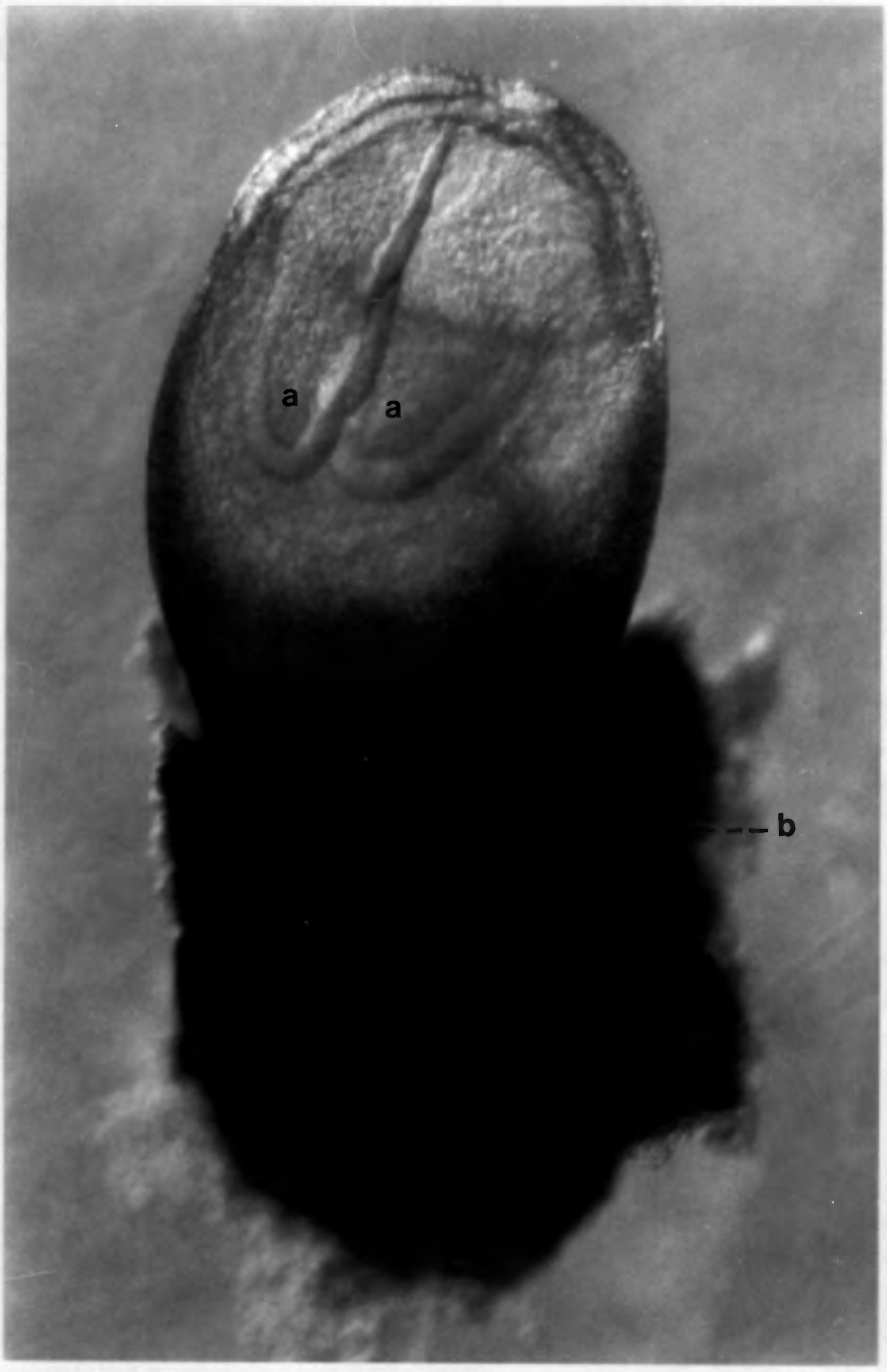

Fig. 5.5.

8-Day embryo with yolk sac intact.

Note - head folds (a)
ectoplacental cone (b)

Mag x 25

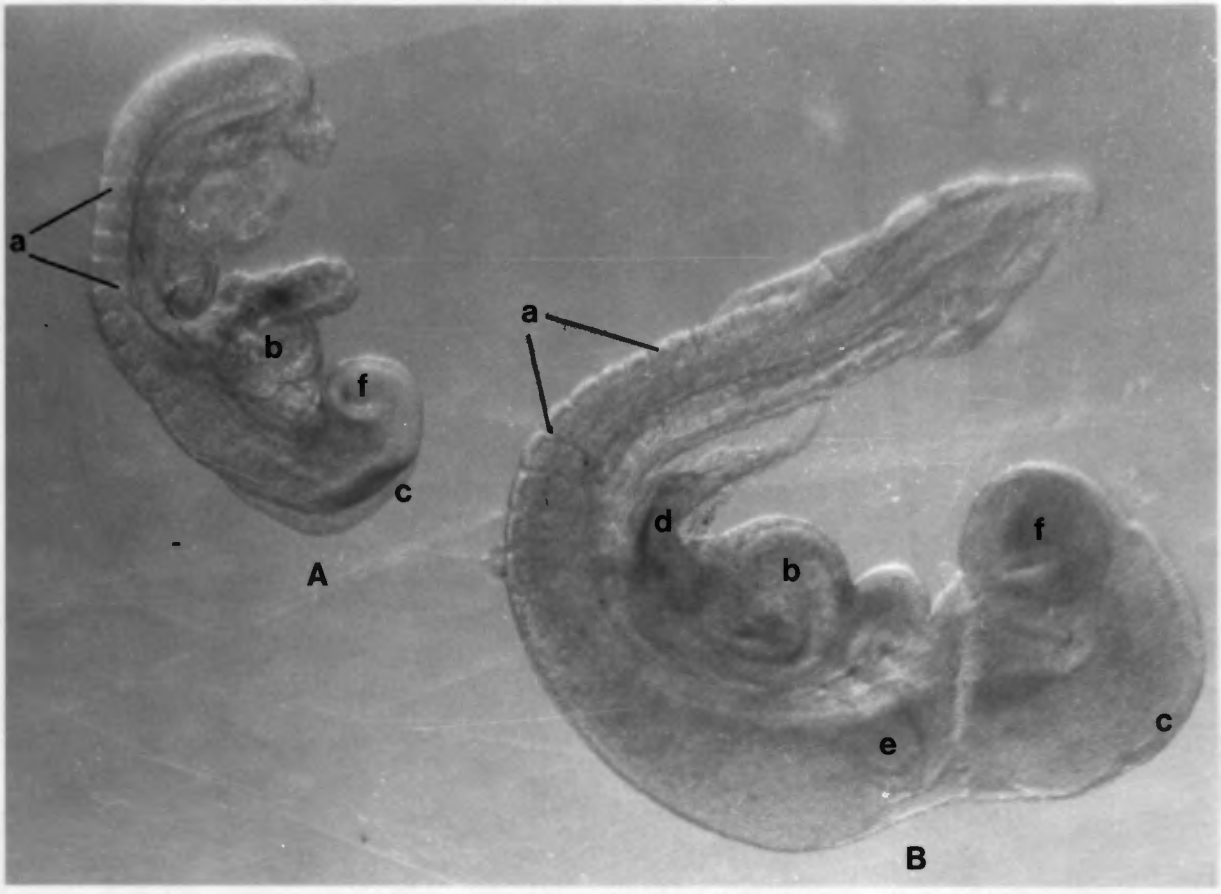

Fig. 5.6.

Embryonic development in vitro.

Embryo A. - explanted at 8½ days' gestation.

Embryo B. - a littermate of embryo A after 28 hours in culture

Note - marked increase in somite number (a)

- heart development (b)
- fusion of the head folds (c)
- development of the fore-limb bud (d)
- otic vesicle (e)
- optic vesicle (f)

Mag x 9.6

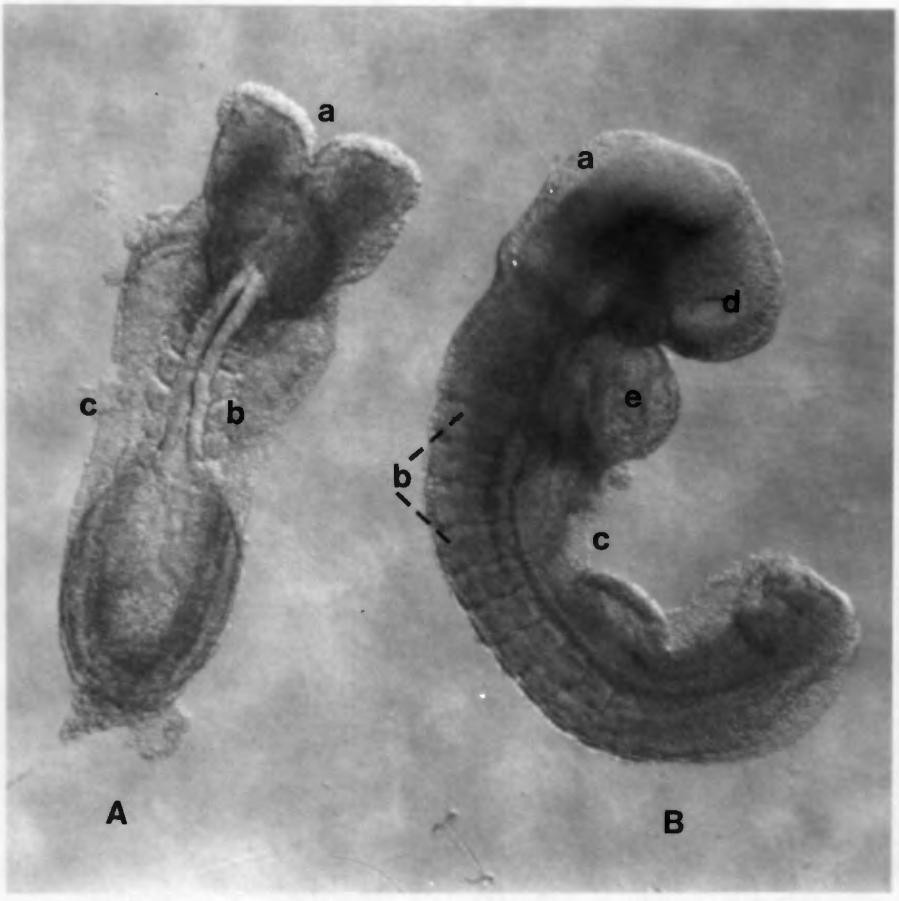

Fig. 5.7. Normal 9-day embryos - variation within a litter.

Note - Embryo A - non fusion of head folds (a)
- 6 pairs of somites (b)
- embryo not yet turned (c)

Embryo B - head folds fusing (a)
- 11 pairs of somites (b)
- embryo turned (c)
- optic vesicle developed (d)
- heart well developed (e)

Mag. x 12

dissecting microscope. General observations were made on the state of the membranes, whether they were opaque or damaged in any way. (For the purpose of statistical analysis all embryos were included whether the membranes were intact or not).

After removal of the membranes the embryos were assessed using the following criteria.

Eight-day embryos

(a) Heart present or absent.
(b) Heart beat present or absent.
(c) Number of somites present.
(d) Central nervous system development according to the size, shape and symmetry of the headfolds.

Nine-day embryos.

(a) Heart present or absent.
(b) Heart beat present or absent.
(c) Number of somites present.
(d) Central nervous system development according to:
  - the size, shape and symmetry of the headfolds
  - the state of fusion of the headfolds.
(e) State of fusion of the neural tube.
(f) Otic and optic vesicles present or absent.
(g) Anterior limb buds present or absent.
(h) Development of the visceral arches.

In the eight-day embryos central nervous system abnormality was defined as small size, abnormal shape or asymmetry of the headfolds. In nine-day embryos central nervous system abnormality was defined as above plus non or irregular fusion of the headfolds (see figures 5.8., 5.9. and 5.10).

Neural tube abnormality was defined as failure of fusion or irregularity of fusion of any part of the neural tube throughout its length (figure 5.11.).

Mandibular and hyoid arches were defined as abnormal in nine-day embryos if their development was asymmetrical or retarded (figure 5.12).

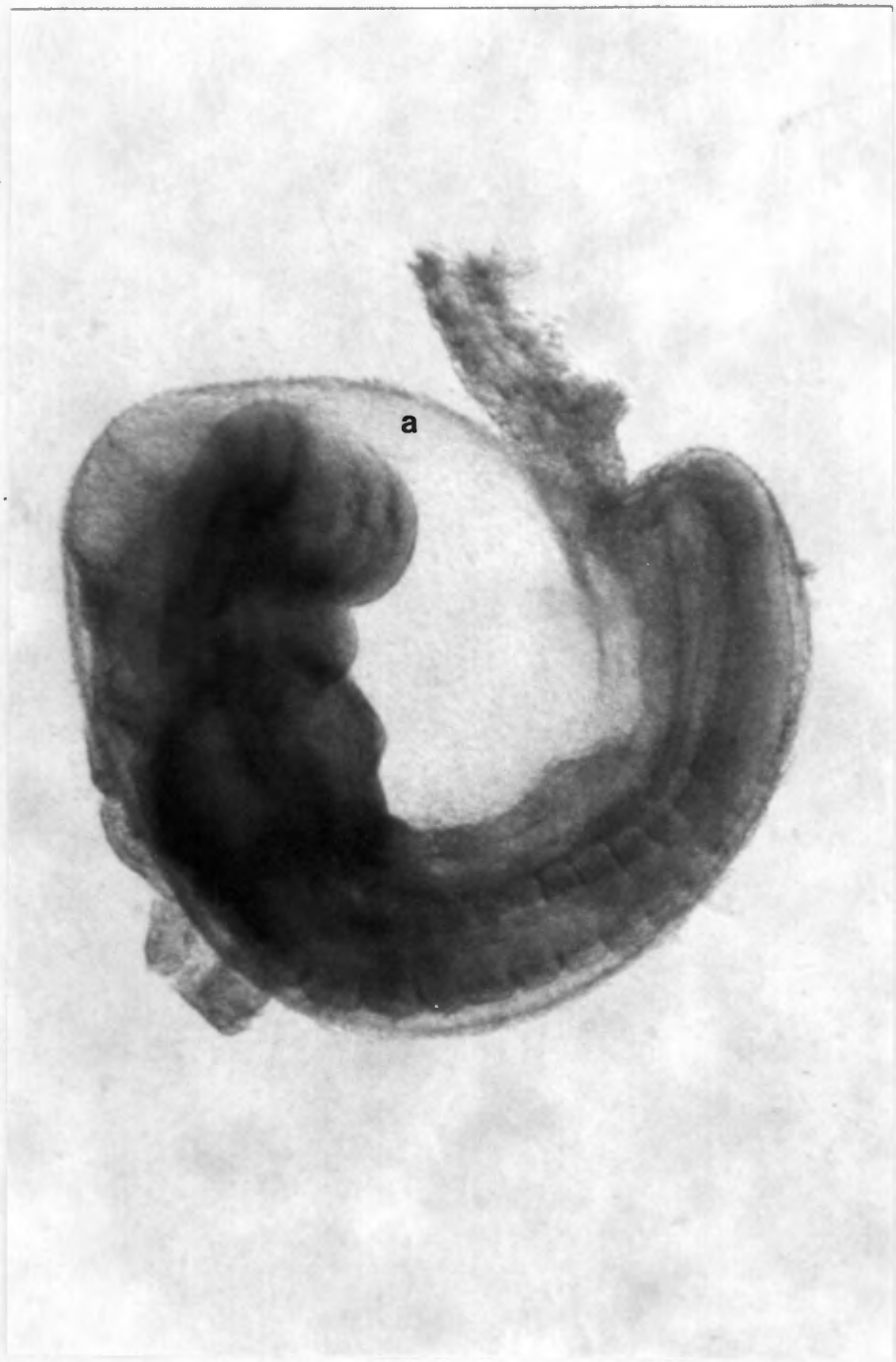

Fig. 5.8. Normal 9-day embryo: amnion intact. (a) Mag. x 12

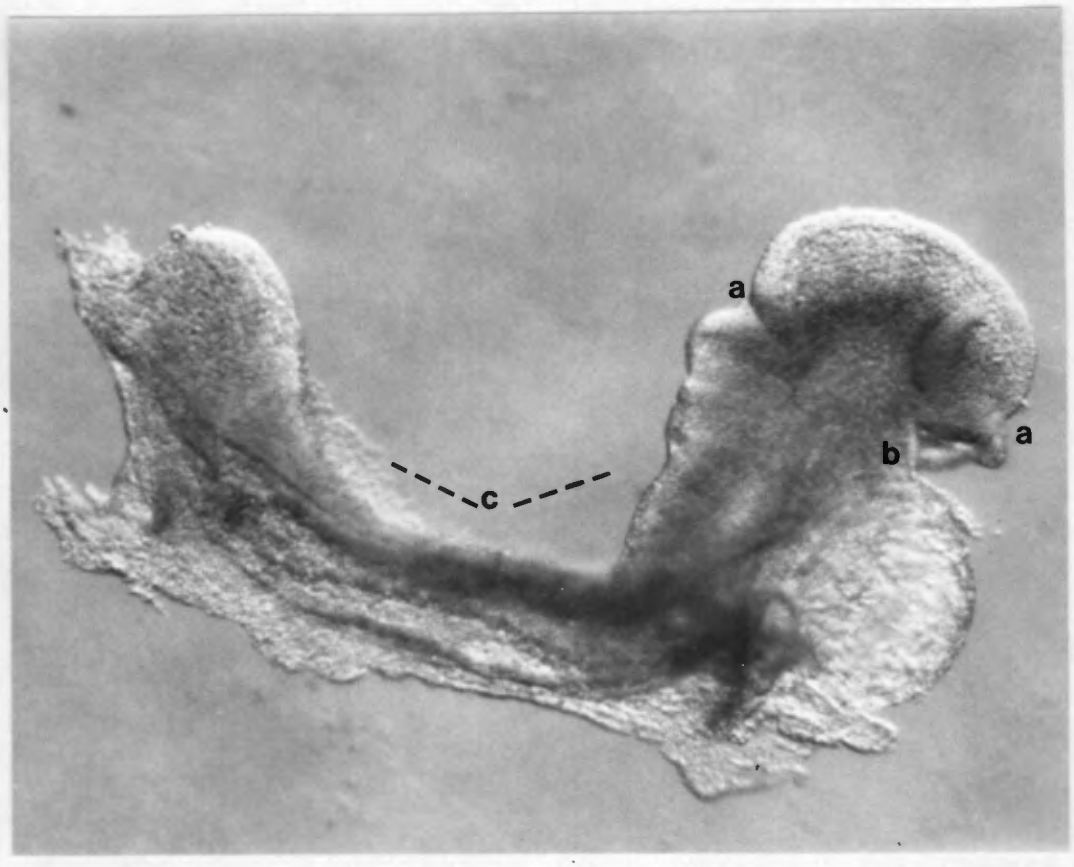

Fig. 5.9.

8-Day embryo - abnormal head folds.

Note - abnormal shape of head folds (a)
- very poor development of visceral arches (b)
- embryo not yet turned (c)

This embryo was explanted at 8 days' gestation and cultured for 28 hours in rat serum containing ethanol 6 000mg/ℓ.

Mag x 25

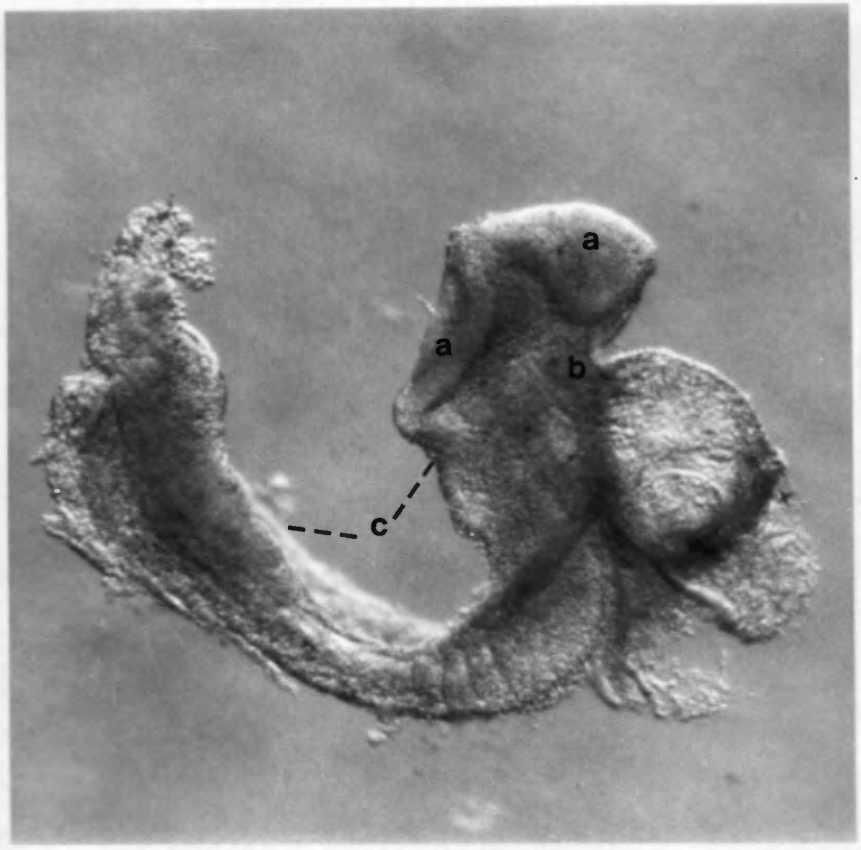

Fig. 5.10.

8-Day embryo - abnormal head folds.

Note - eversion of head folds (a)

- failure of development of visceral arches (b)
- hyper extension of the embryo (c)

This embryo was explanted at 8 days' gestation and cultured for 28 hours in rat serum containing ethanol 1 500mg/ℓ.

Mag x 25

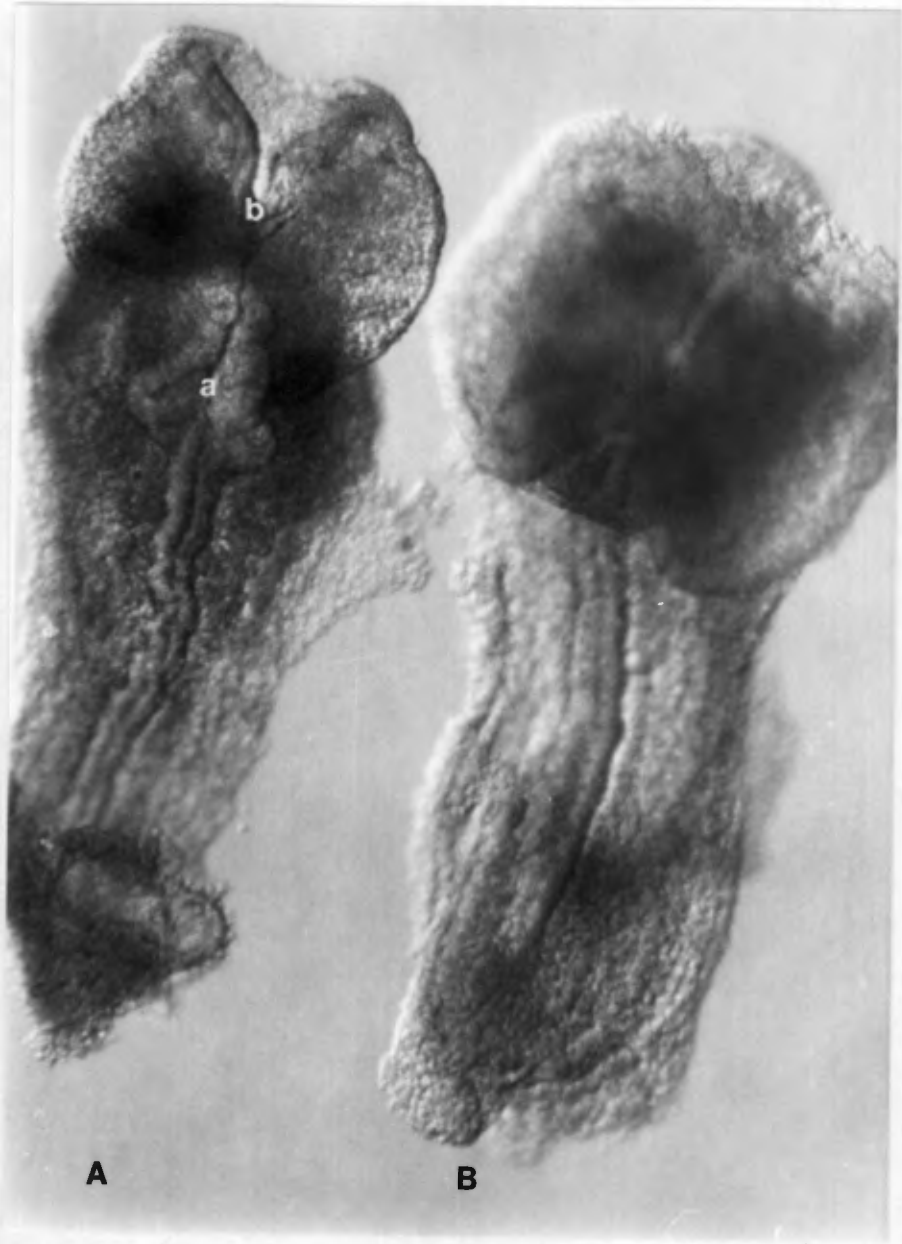

Fig. 5.11.

8-Day embryo - neural tube defect

Embryo A was explanted at 8 days' gestation and cultured for 28 hours in rat serum containing acetaldehyde 7.4mg/ℓ.

Note - loop shaped neural tube defect (a)

- partial fusion of head folds (b)

Embryo B was a control embryo explanted at 8 days' gestation and cultured for 28 hours in rat serum.

Mag. x 25.

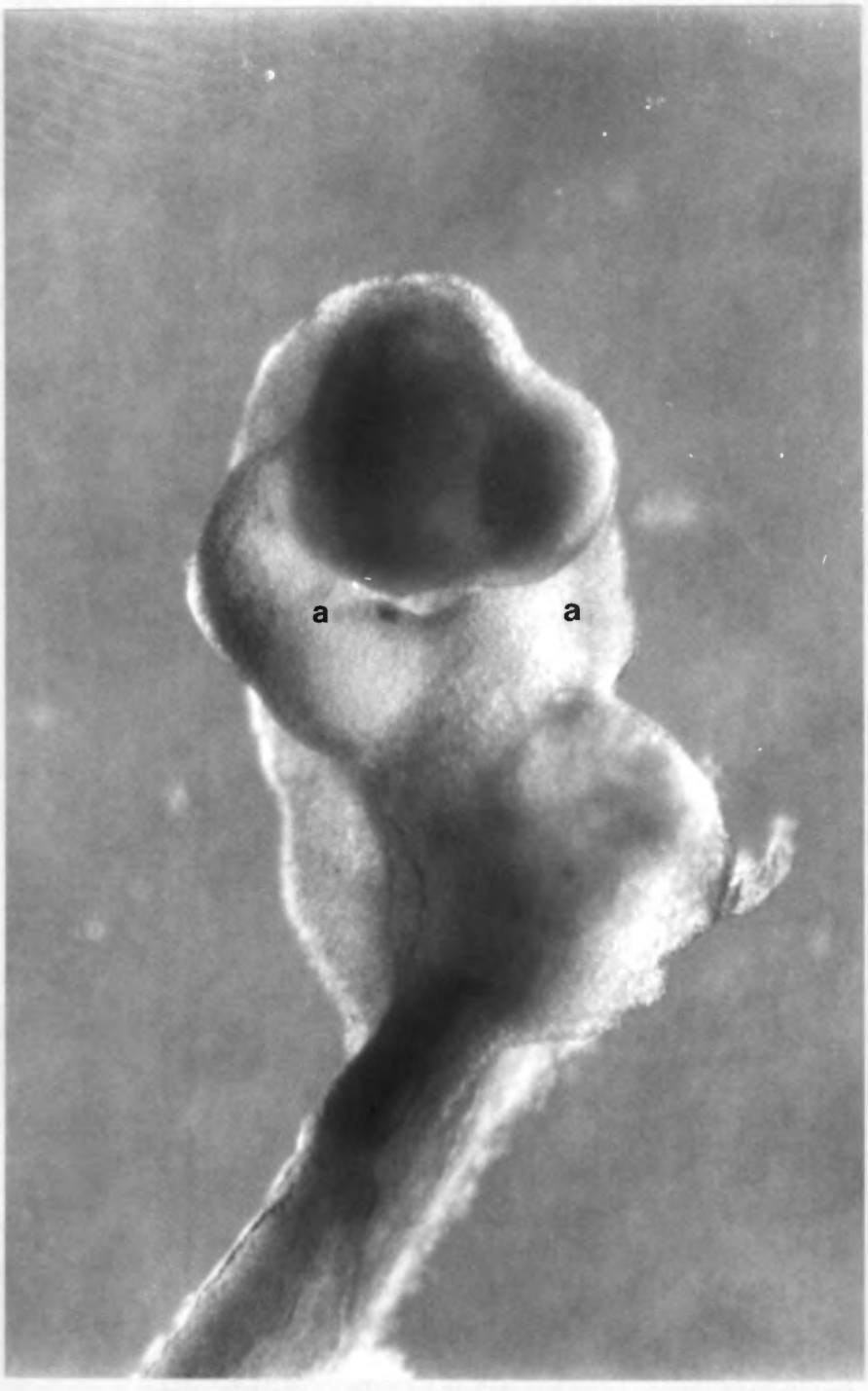

Fig. 5.12.
9-Day embryo - abnormal mandibular arches (a)
This embryo was explanted at 9 days' gestation and cultured for 28 hours in rat serum containing ethanol 3 000mg/ℓ.

5.9. PREPARATION FOR SCINTILLATION COUNTING

After morphological assessment the embryos were placed in five percent acetic ethanol for one hour after which they were fixed in ten percent buffered formalin for a further three to thirty days. They were then dehydrated in

ethanol 50% for half an hour
ethanol 70% for half an hour
ethanol 90% for half an hour

after which they were placed in Soluene - 350 (a tissue solubiliser) and incubated at 45°C in a water bath with shaker to aid dissolution of the tissues. Dissolution time varied with the size of the embryo. After dissolution six millilitres of Dimulume 30 (scintillation fluid) was added to the vials and they were counted in a Beckman LS 8000 scintillation counter.

5.10. MEASUREMENT OF DNA SYNTHESIS

The uptake of tritiated thymidine over a four hour period was used as a measure of DNA synthesis, the thymidine being incorporated into newly synthesized DNA. Treatment of the embryos using the normal histological techniques of fixation and dehydration ensured that all the non-incorporated tritiated thymidine was washed out of the embryo. Fortunately most histological fixatives precipitate DNA, ensuring that the disintegrations per minute measured in the scintillation counter were due to tritiated thymidine incorporated into DNA, and not to any adsorbed material or tritiated water present in the embryo (Rogers, 1979:10).

We counted some samples of our fixatives and ethanol after use and found that most of the adsorbed tritium was removed during fixation (see table 5.1.).

5.11. ADVANTAGES OF AN IN VITRO MODEL

In his survey of in vitro systems and their potential in teratogenicity screening Wilson (1978:140) has enumerated some characteristics of an ideal in vitro test as follows.

TABLE 3.1.

Scintillation counts of solutions into which embryos number 179, 180, 182 and 186 were placed.

Embryo No.	Solution	D.P.M.	D.P.M. of Embryos
179	Acetic Ethanol	559.5	
180	Acetic Ethanol	247.2	
182	Acetic Ethanol	209.0	
186	Acetic Ethanol	0	
179	10% Buffered Formalin	165.2	
180	10% Buffered Formalin	154.3	
182	10% Buffered Formalin	0	
186	10% Buffered Formalin	39.4	
179	Ethanol 50%	0	
180	Ethanol 50%	0	
182	Ethanol 50%	0	
186	Ethanol 50%	0	
179	Ethanol 70%	0	
180	Ethanol 70%	0	
182	Ethanol 70%	0	
186	Ethanol 70%	0	
179	Ethanol 90%	0	
180	Ethanol 90%	0	
182	Ethanol 90%	0	
186	Ethanol 90%	0	
179	Ethanol 95%	0	
180	Ethanol 95%	0	
182	Ethanol 95%	0	
186	Ethanol 95%	0	
179	Ethanol 100%	0	247.01
180	Ethanol 100%	0	400.03
182	Ethanol 100%	0	491.78
186	Ethanol 100%	0	0

It is essential that the model should:

1. use biological subjects available in large numbers
2. involve some aspect of progressive development
3. have relevance to mechanisms of teratogenesis
4. be easily performed and yield interpretable results.

Desirable features are that it should:

1. use an intact organism capable of absorbing, circulating and excreting chemicals
2. give few 'false negatives' (compared with mammals)
3. react to varied types of reagents.

The mouse embryo culture system used in this study fulfils many of these requirements. While it is not yet possible to culture these embryos continuously for the entire duration of organogenesis, techniques are improving and this limitation may be surmountable in the future. At present many useful observations may be made using existing methods.

5.12. ADVANTAGES OF THE IN VITRO MODEL USED IN THIS STUDY

Confusing maternal factors were eradicated, for example differences in maternal metabolism of the drug, maternal health and nutritional status. The absence of maternal and placental influences, while being beneficial in some situations, was of course a disadvantage when attempting to extrapolate findings to man as these factors cannot then be eliminated.

It was possible to introduce specific doses of the test substance or its metabolites directly into the culture medium over a well defined period of time and at an accurately determined stage of embryonic development.

It was possible to label embryos explanted at eight or nine days' gestation with tritiated thymidine. This was not possible in the in vivo situation. Atlas et al (1960) studied DNA synthesis in the developing mouse embryo using tritiated thymidine labelling in vivo. They injected 0.7 microcuries of tritiated thymidine into the peritoneum of pregnant inbred albino mice at various stages of gestation ranging from six to sixteen days. They found that there was no uptake of label into the embryo or its derivatives until the tenth day of gestation, and the embryo proper was first labelled on the eleventh day of development. All these animals were sacrificed twenty-four hours

after the injection and the amount of labelling measured by high resolution autoradiography.

I attempted to label embryos in vivo by intraperitoneal injection of the pregnant mice with 10 microcuries of tritiated thymidine on days nine and ten of gestation. The animals were sacrificed after four hours, the embryos removed and fixed in acetic ethanol and prepared for scintillation counting as described in section 5.7. I found that there was minimal uptake of tritiated thymidine in the nine-day embryos. The counts registered little more than normal background, while ten-day embryos took up a good deal of tritiated thymidine - see table 5.2.
The actual ages of the embryos at the time of sacrifice were 9 1/2 or 10 1/2 days.

Table 5.2. Uptake of tritiated thymidine in vivo: disintegrations per minute

Twenty 9½ day embryos:	
46.87	0.00
20.00	26.86
25.67	87.52
70.05	60.35
65.24	30.53
87.77	70.19
20.17	0.00
89.23	76.05
186.26	73.03
162.44	83.54
Five 10½ day embryos:	
2904.99	
3175.41	
1665.10	
3389.32	
4955.93	

These findings support those of Atlas et al (1960) and indicate that although the tissues surrounding the embryo take up tritium efficiently the label is not detectable in any significant amount in embryos less than ten and a half days old.

It is also worth noting that although the embryos in Atlas' study were exposed to tritiated thymidine for twenty-four hours there was no increase in abnormal mitoses, indicating a lack of interference with growth and differentiation by the tritiated thymidine.

The techniques used in my in vitro study did not prove to be more time consuming or expensive than the in vivo method, making it a viable proposition for teratogenicity testing.

CHAPTER 6

THE EFFECT OF ETHANOL OR ACETALDEHYDE ADMINISTERED IN VITRO

6.1. DOSE OF ETHANOL OR ACETALDEHYDE

The embryos were explanted and cultured under the conditions described in sections 5.1. to 5.5. In the ethanol study 98% ethanol (analytical grade) was added to the 2 millilitres of culture medium in volumes of 4.8 µl, 9.6 µl or 19.2 µl. These amounts of ethanol yielded concentrations of approximately 1500mg/ℓ, 3000mg/ℓ and 6000mg/ℓ respectively. (Assay method described in section 2.7.). The ethanol 1500mg/ℓ dose was based on the serum ethanol concentration achieved in the in vivo experiment. The two higher doses studied here did not correspond to in vivo levels as these could not be achieved without causing maternal toxicity.

In the acetaldehyde study, acetaldehyde in a 1.56% solution was added to each 2 millilitres of culture serum in volumes of 1.2µl, or 3.2µl or 6.4µl. These volumes of acetaldehyde yielded concentrations of 7.4mg/ℓ, 19.7mg/ℓ and 39.4mg/ℓ calculated as outlined below.

1.56% solution of acetaldehyde contains 1.23g/100mℓ of solution (taking acetaldehyde density as 0.788g/mℓ), or 12.3µg/µl of solution. Hence 6.4µl of solution (78.7µg of acetaldehyde) when added to 2mℓ of serum gives a concentration of 39.4mg/ℓ.

The in vitro concentration of 39.4 milligrams per litre was less than one hundredth of the 11.8 milligram dose of acetaldehyde given daily in vivo (which caused no maternal toxicity). In vitro this dose proved to be highly toxic to eight-day embryos, it was halved to the 19.7 milligram per litre concentration and when that also proved toxic, it was again reduced to the 7.4 milligram per litre level.

6.2. ASSESSMENT OF EMBRYOS

All embryos were examined morphologically according to the criteria defined in section 5.8.

Morphological assessment and scintillation counts of eight-and nine-day embryos treated with ethanol or acetaldehyde are listed in tables 6.1. to 6.14. - see pages 205–221.

6.3. RESULTS OF ETHANOL ADMINISTRATION IN VITRO

6.3.1. Eight-day embryos (see table 6.15)

These were explanted at eight days' gestation and cultured for twenty-eight hours as described in sections 5.1. to 5.5.

In the ethanol 1500mg/ℓ group the only effects observed to be significantly different from the control group were an increase in somite count and an increase in the incidence of central nervous system abnormality in the ethanol-treated embryos. Both these effects are difficult to explain. At the next dose (ethanol 3000mg/ℓ) I observed no adverse effects on the test embryo. At the 6000mg/ℓ level the ethanol had marked adverse effects on the heart beat (heart beat was absent in a significant number of the treated embryos), neural tube and central nervous system development (abnormalities occurred in a significant number of test embryos), and DNA synthesis was reduced (see figures 6.1. and 6.2.).

6.3.2. Nine-day embryos (see table 6.16.)

Unlike the eight-day embryos these ethanol-treated embryos showed a definite dose-related effect. The lowest dose of ethanol caused only an increase in the incidence of neural tube abnormalities (see figure 6.3.), and the intermediate (3000mg/ℓ) dose caused an increase in neural tube and central nervous system abnormalities and a decrease in DNA synthesis. The highest dose of ethanol (6000mg/ℓ) caused a significant reduction in somite count, a marked increase in neural tube and central nervous system abnormalities, and a significant decrease in DNA synthesis. Development of the otic vesicle was also slightly impaired (figure 6.4.).

TABLE 6.15.
IN VITRO STUDY

EFFECTS OF ETHANOL ON 8 DAY EMBRYOS

Dose:	Somite Count	Heart Beat - Present Absent	CNS Development	DNA Synthesis	Neural Tube Defects
Ethanol 1500mg/ℓ	Somite count significantly increased in test group. p=0.0009 (Mann-Whitney Test) Control N = 58 Test N = 37	No significant effect on heart beat. Control N = 26 Test N = 36	CNS development significantly abnormal. p=0.02 Control N = 56 Test N = 38	No significant reduction of DNA synthesis. (Mann-Whitney Test) Control N = 39 Test N = 25	No significant increase in neural tube defects Control N = 57 Test N = 38
Ethanol 3000mg/ℓ	No significant effect. (Mann-Whitney Test) Control N = 58 Test N = 39	No significant effect on heart beat. Control N = 26 Test N = 40	CNS development normal. Control N = 56 Test N = 38	No significant reduction of DNA synthesis. (Mann-Whitney Test) Control N = 39 Test N = 27	No significant increase in neural tube defects Control N = 57 Test N = 39
Ethanol 6000mg/ℓ	No significant effect. (Mann-Whitney Test) Control N = 58 Test N = 40	Significant adverse effect on heart beat in test group. p=0.029 Control N = 26 Test N = 40	CNS development significantly abnormal. p=0.003 Control N = 56 Test N = 40	Significant reduction of DNA synthesis in test group. p=0.027 (Mann-Whitney Test) Control N = 39 Test N = 29	Significant increase in neural tube defects p=0.027 Control N = 57 Test N = 39

There was no significant difference in heart development (i.e. heart present or absent) between the test and the control groups.
Statistical Analysis. The Fisher Exact Probability Test was used except where otherwise indicated.

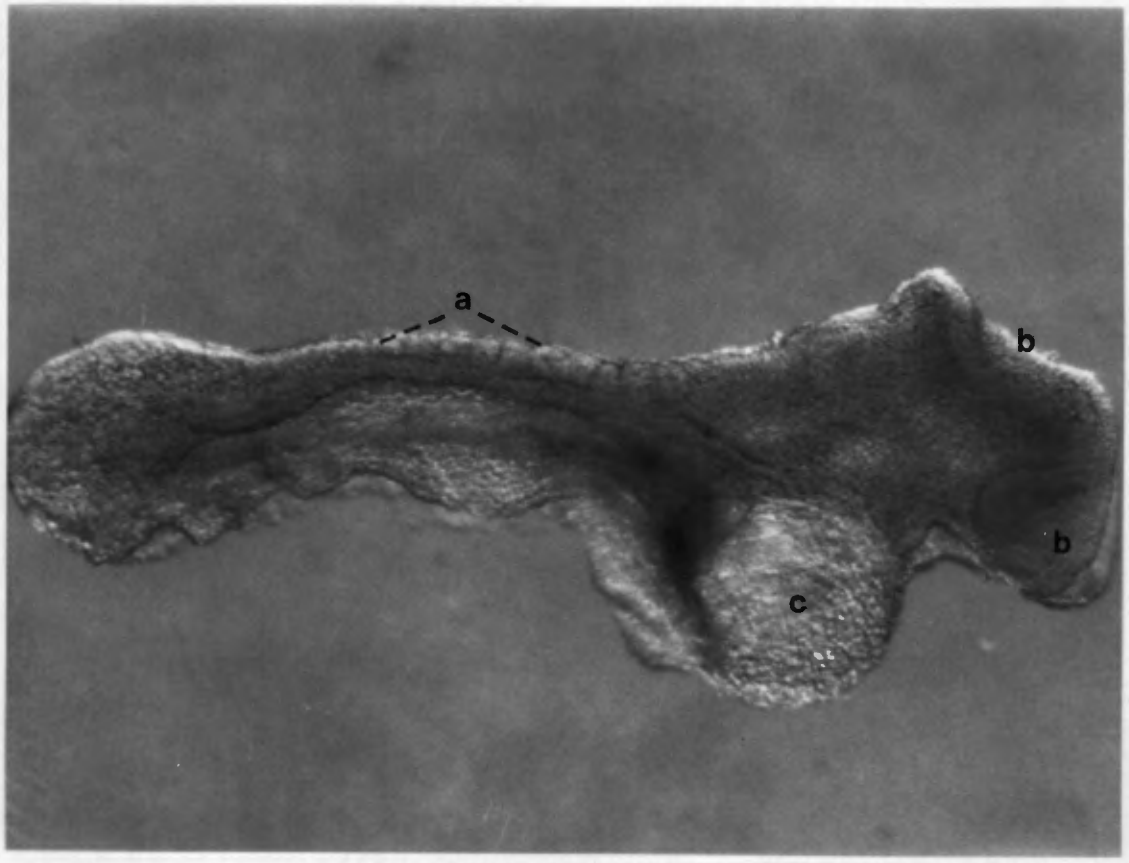

Fig. 6.1.

8-Day embryo - general retardation of development

This embryo was explanted at 8 days' gestation and cultured in rat serum containing ethanol 6 000mg/ℓ.

Note - normal somite development (a)
- abnormal flattened head shape with eversion of the neural folds (b)
- normal heart development (c)

Mag. x 25.

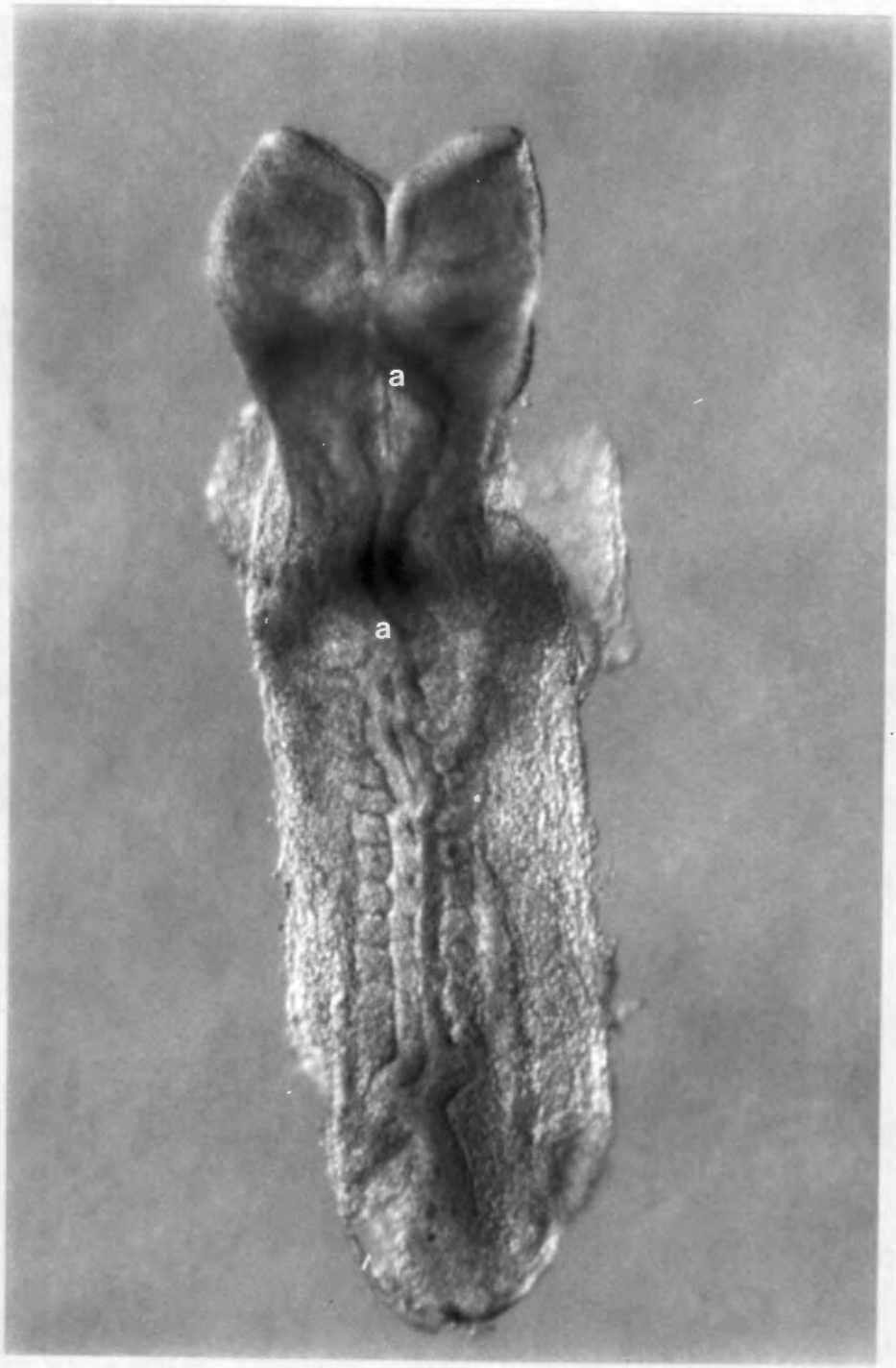

Fig. 6.2.

8-Day embryo - neural tube defect (a)

This embryo was explanted at 8 days' gestation and cultured for 28 hours in rat serum containing ethanol 6 000mg/ℓ.

Mag. x 25.

TABLE 6.16
IN VITRO STUDY

EFFECTS OF ETHANOL ON 9 DAY EMBRYOS

Dose:	Somite Count	Neural Tube Defects	Ear Development	Development of Visceral Arches	CNS. Development	DNA. Synthesis
Ethanol 1500mg/ℓ	No significant effect. (Mann-Whitney Test) Control N = 60 Test N = 26	Significant increase in neural tube defects in test group. p = 0.029 Control N = 60 Test N = 27	No significant effect. Control N = 55 Test N = 27	No significant abnormality. Control N = 57 Test N = 26	CNS development normal. Control N = 49 Test N = 23	No significant reduction in DNA synthesis in test group. (Mann-Whitney Test) Control N = 32 Test N = 18
Ethanol 3000mg/ℓ	No significant effect. (Mann-Whitney Test) Control N = 60 Test N = 48	Significant increase in neural tube defects in test group. p = 0.015 Control N = 60 Test N = 49	No significant effect. Control N = 55 Test N = 40	Significant abnormality in the test group. p = 0.005 Control N = 57 Test N = 43	CNS development significantly abnormal in test group. p=0.00953 Control N = 49 Test N = 42	Significant reduction in DNA synthesis in test group. p=0.006 (Mann-Whitney Test) Control N = 32 Test N = 31
Ethanol 6000mg/ℓ	Somite count significantly reduced in test group. p=0.00003 (Mann-Whitney Test) Control N = 60 Test N = 38	Significant increase in neural tube defects in test group. p = 0.002 Control N = 60 Test N = 38	Ear development slightly reduced in the test group. p=0.054 (Borderline significance) Control N = 55 Test N = 33	No significant abnormality. Control N = 57 Test N = 38	CNS development significantly abnormal in test group. p=0.002 Control N = 49 Test N = 28	Significant reduction in DNA synthesis in test group. p=0.008 (Mann-Whitney Test) Control N = 32 Test N = 23

Eye development, the presence or absence of a heart and heart beat, the contour of the embryos (turned or unturned) and development of the limb buds were also assessed but no significant differences were found between test and control groups. Statistical analysis. The Fisher Exact Probability Test was used except where otherwise indicated.

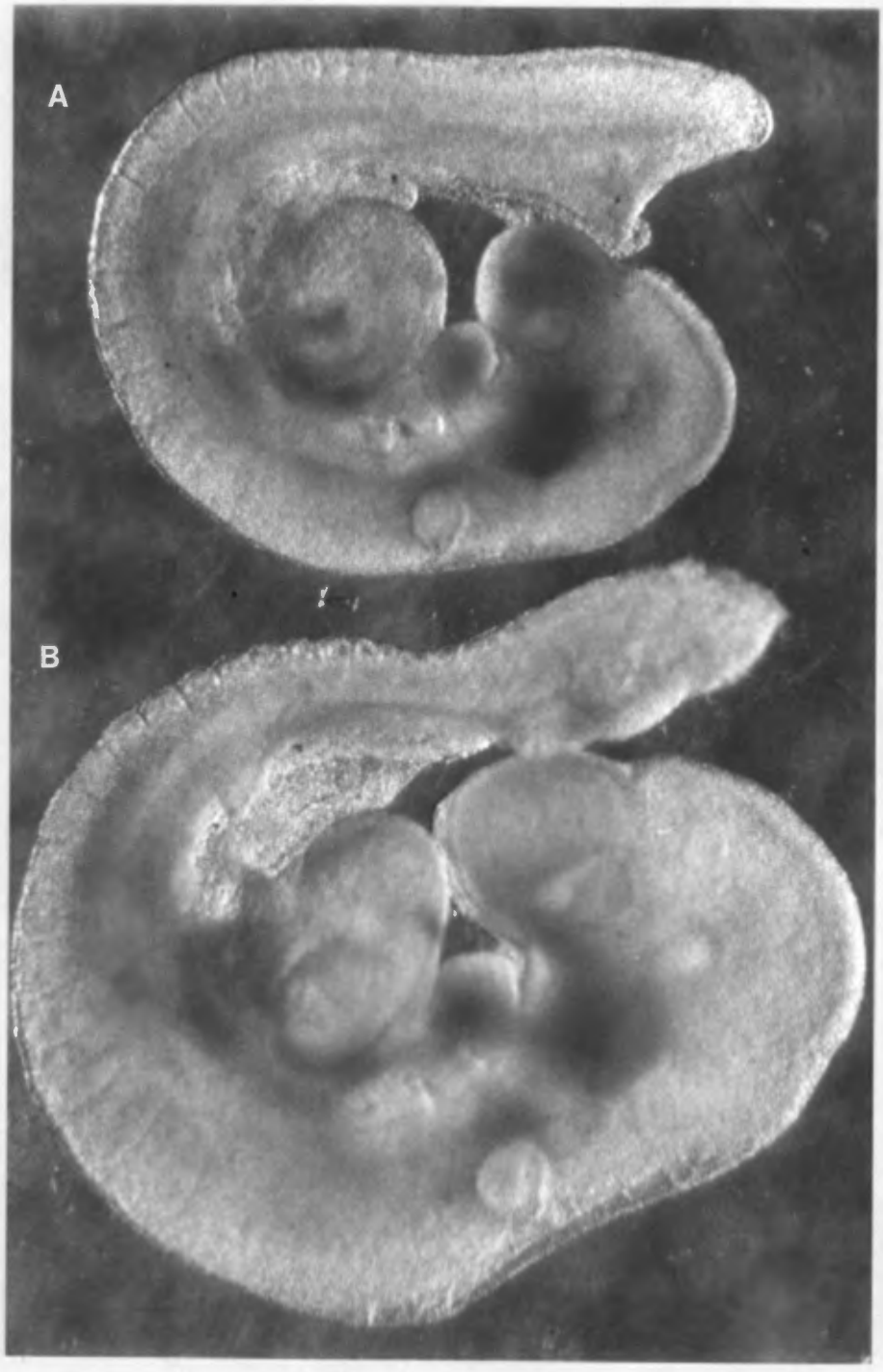

Fig. 6.3.

Comparison of a 9-day ethanol-treated embryo with a control embryo

Embryo A was explanted at 9 days' gestation and cultured for 28 hours in rat serum containing ethanol 1 500mg/ℓ.

Embryo B was explanted at 9 days' gestation and cultured for 28 hours in rat serum.

Note - both embryos were well developed but the ethanol treated embryo was slightly smaller than the control embryo.

- stage of development attained $9^{3}/4$ - 10 days.

Mag. x 12.

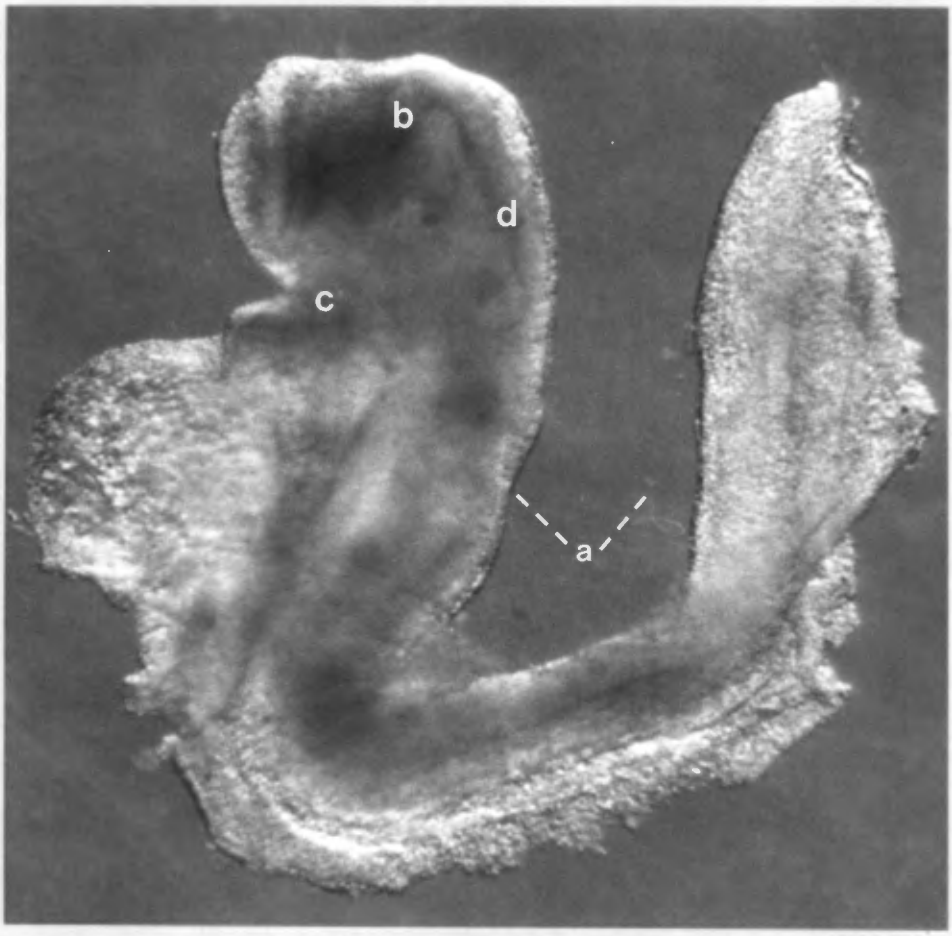

Fig. 6.4.

9-Day embryo - general retardation of development

This embryo was explanted at 9 days' gestation and cultured for 28 hours in rat serum containing ethanol 6 000mg/ℓ.

Note - abnormal contour (a)
- abnormal head shape and poor definition of brain areas (b)
- poor development of the visceral arches (c)
- poor development of the otic vesicle (d)

In his in vitro study Skosyreva (1973) used nine-and ten-day rat embryos (mouse equivalent age about 7 1/2 to 8 days (Rugh, 1968:299)) which he cultured for thirty hours. He added ethanol to the culture medium in concentrations of 100mg/ℓ, 500mg/ℓ, 1000mg/ℓ, 5000mg/ℓ and 10000mg/ℓ . He observed retardation of development at the ethanol 100mg/ℓ dose manifested by a decrease in the number of somites formed, retardation of development of the brain sac, slowing of cardiac systole and under-development of the allantois. The retardation of development included the whole embryo and was not selective. However, distinct embryotoxic effects of ethanol became apparent only at the 500mg/ℓ concentration and toxicity increased with dose. It would appear from this study that rat embryos react to ethanol in a similar way to mouse embryos, but they are more sensitive to ethanol, producing toxic effects at lower doses than those used in this study on mouse embryos.

Brown et al (1979) also cultured rat embryos for 48 hours during the period of organogenesis (9 1/2 to 11 1/2 days) in the presence of ethanol 1500mg/ℓ and 3000mg/ℓ . They demonstrated a significant reduction in embryonic growth and differentiation, total DNA and total protein contents in the 3000mg/ℓ group. No gross structural defects were observed in either treated or control embryos. To assess differentiation and abnormal organogenesis a comprehensive morphological scoring system was used. Retarded development was also demonstrated by a dose dependant reduction in the number of somites developed.

These findings correlate well with my results for nine day mouse embryos treated with similar doses of ethanol.

6.4. RESULTS OF ACETALDEHYDE ADMINISTRATION IN VITRO

6.4.1. Eight-day embryos

As shown in table 6.17., acetaldehyde proved to be toxic to eight-day embryos in all three doses studied. Central nervous system abnormalities were increased and DNA synthesis decreased in all doses. Somite counts were significantly reduced at the 19.7mg/ℓ and 39.4mg/ℓ doses while at the 7.4mg/ℓ and 19.7mg/ℓ doses, the heart beat failed to develop in a large

TABLE 6.17.
IN VITRO STUDY

EFFECTS OF ACETALDEHYDE ON 8 DAY EMBRYOS

Dose	Somite Count	Heart Beat - Present Absent	CNS Development	DNA Synthesis	Neural Tube Defects
ACETALDEHYDE 7.4 mg/ℓ	No significant effect. (Mann-Whitney Test) Control N = 58 Test N = 43	Significant adverse effect on heart beat in test group. p=0.01 Control N = 26 Test N = 43	CNS development significantly abnormal. p=0.024 Control N = 56 Test N = 41	Significant reduction of DNA synthesis in test group. p=0.008 (Mann-Whitney Test) Control N = 39 Test N = 37	No significant increase in neural tube defects Control N = 57 Test N = 42
ACETALDEHYDE 19.7 mg/ℓ	Somite count significantly reduced in test group. p=0.0244 (Mann-Whitney Test) Control N = 58 Test N = 38	Significant adverse effect on heart beat in test group. p=0.002 Control N = 26 Test N = 38	CNS development significantly abnormal. (Borderline significance) p=0.056 Control N = 56 Test N = 38	Significant reduction of DNA synthesis in test group. p=0.0154 (Mann-Whitney Test) Control N = 39 Test N = 29	No significant increase in neural tube defects Control N = 57 Test N = 38
ACETALDEHYDE 39.4 mg/ℓ	Somite count significantly reduced in test group. p<0.00003 (Mann-Whitney Test) Control N = 58 Test N = 26	No significant effect on heart beat. Control N = 26 Test N = 27	CNS development significantly abnormal p=0.0013 Control N = 56 Test N = 18	Significant reduction of DNA synthesis in test group. p<0.0002 (Mann-Whitney Test) Control N = 39 Test N = 20	Significant increase in neural tube defects in test group p=0.045 Control N = 57 Test N = 27

There was no significant difference in the heart development (i.e. heartbeat present or absent) between the test and the control groups.
Statistical analysis. The Fisher Exact Probability Test was used except where otherwise indicated.

number of the test embryos. The incidence of neural tube abnormalities was increased only in the highest dose group (acetaldehyde 39.4mg/ℓ). (See figures 6.5. and 6.6.).

6.4.2. Nine-day embryos

In nine-day embryos the same doses of acetaldehyde were much less damaging (see figures 6.7. and 6.8. and table 6.18.). The results in this group are difficult to interpret. At the highest dose of acetaldehyde the only apparent adverse effect was a reduction in the number of embryos having heart beats at the end of the experiment. This may signify an increase in embryonic deaths in this group. However, the amount of limb development and the somite counts were markedly increased compared with the controls. Paradoxically, this appears to be a beneficial effect, possibly due to some form of malignant response to acetaldehyde causing accelerated development. However, there was no corresponding increase in DNA synthesis. The fact that a statistically significant number of these embryos showed acceleration of development of somite and limb buds but did not have heart beats at the end of the experiment (thus assumed to be dead at the time of assessment), indicates that there must have been some initial growth spurt which was not sustained. If the embryos were already dead or dying at the time the tritiated thymidine was introduced (i.e. after twenty-four hours' culture) this would explain why there was no increase in DNA synthesis (it was measured only over the last four hours of the experiment and not over the whole twenty-eight hour period).

At the intermediate dose of acetaldehyde (19.7mg/ℓ) the incidence of central nervous system abnormalities was increased and DNA synthesis decreased. At the lowest dose the only effect of acetaldehyde was a reduction in the number of embryos having heart beats at the end of the experiment. There was no dose related effect with acetaldehyde at nine days' gestation. Neural tube, ear, eye and heart development were also assessed but there were no differences between test and control groups.

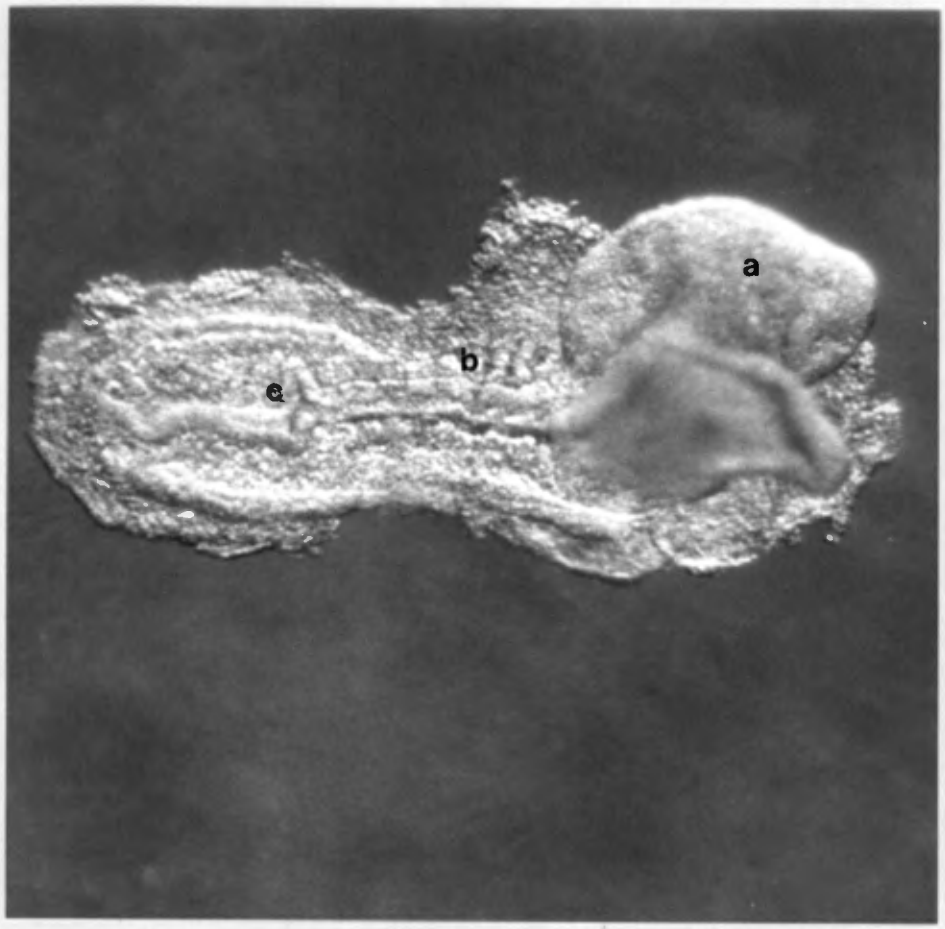

Fig. 6.5.

8-Day embryo treated with acetaldehyde 19.7mg/ℓ

This embryo was explanted at 8 days' gestation and cultured for 28 hours in rat serum containing acetaldehyde 19.7mg/ℓ.

Note - failure of fusion of head folds (a)
- poor somite development (b)
- neural tube defect (c)

Mag. x 25.

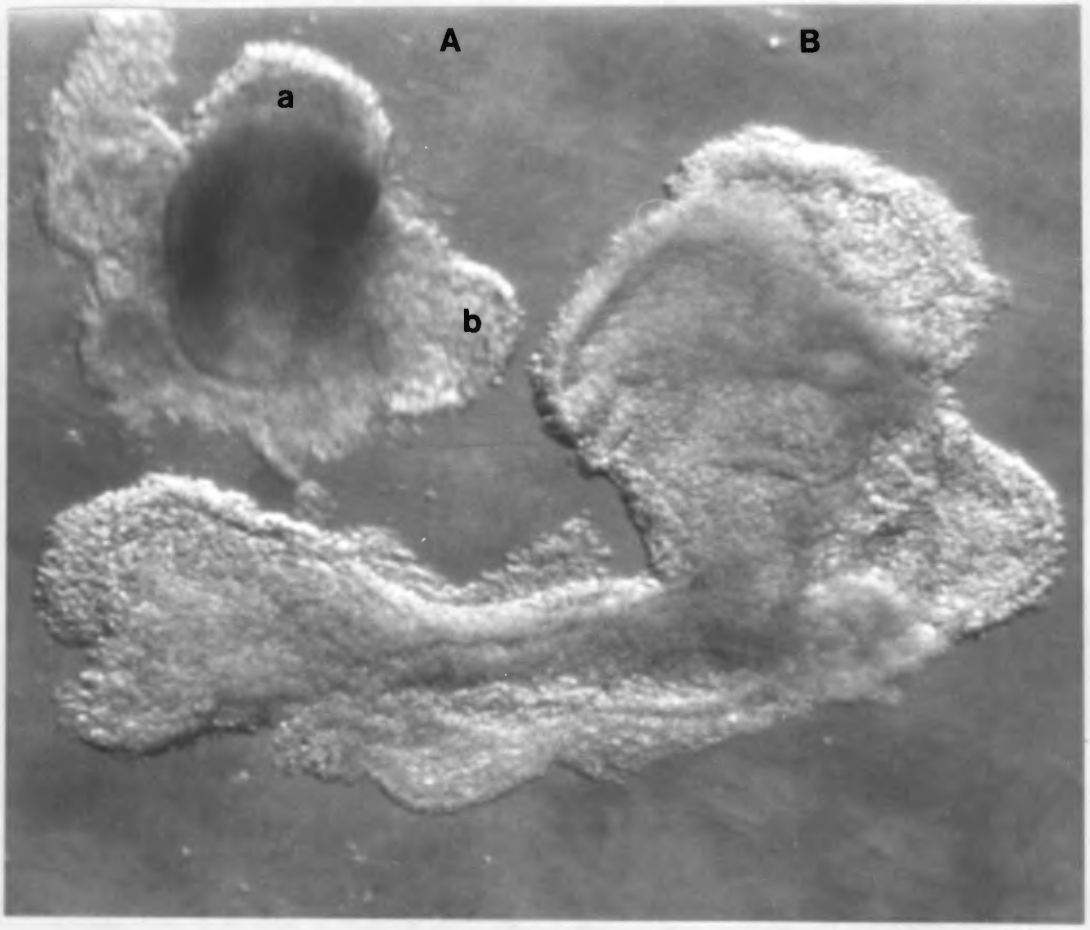

Fig. 6.6.

Comparison of an 8-day control embryo with an acetaldehyde-treated embryo

Embryo A was explanted at 8 days' gestation and cultured for 28 hours in rat serum containing acetaldehyde 39.4mg/ℓ.

Embryo B was explanted at 8 days' gestation and cultured for 28 hours in rat serum.

Note - the extremely poor development of the treated embryo with only the head folds (a) and heart (b) recognisable. Mag. x 25.

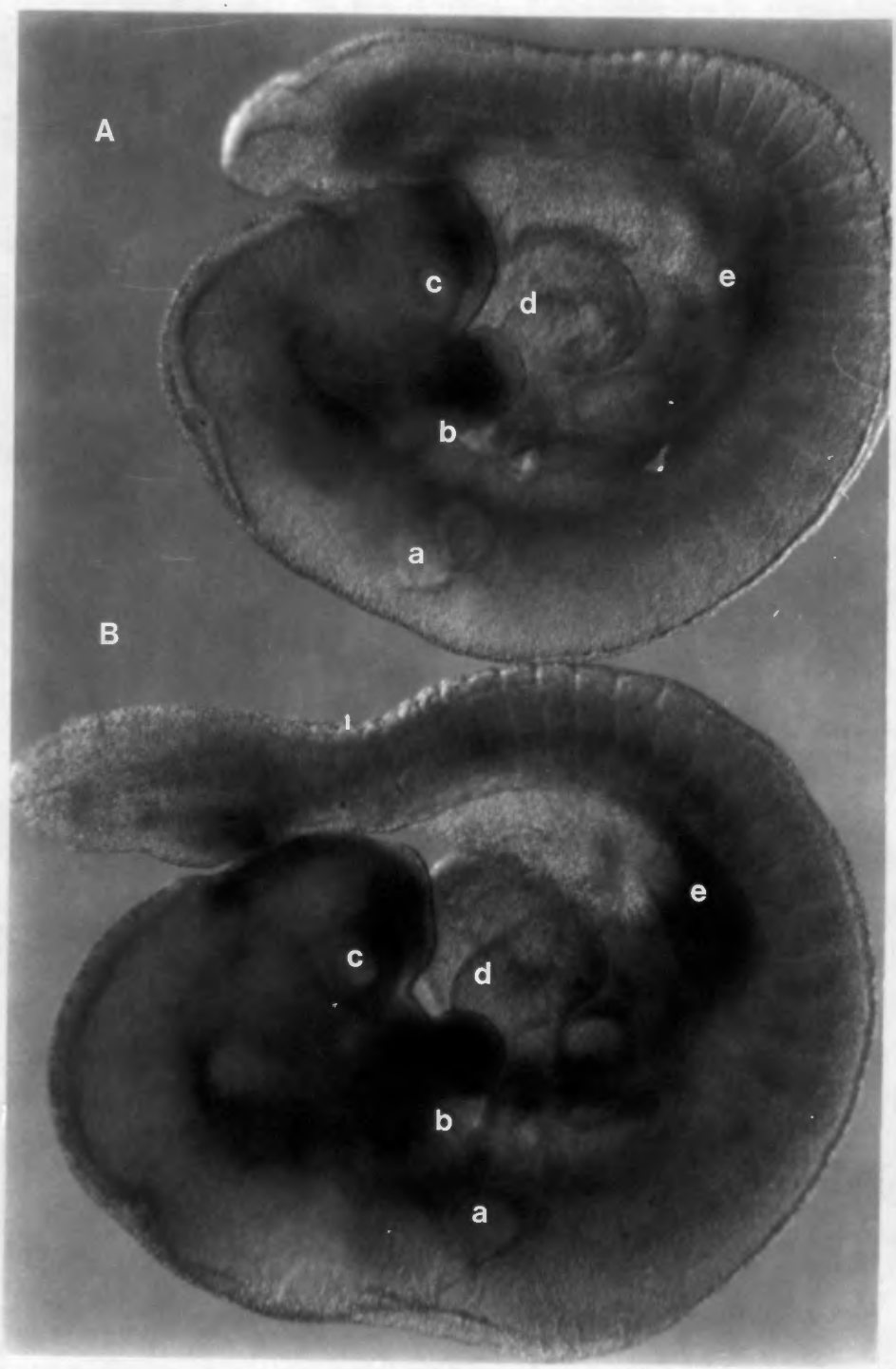

Fig. 6.7.

Comparison of a 9-day control embryo with an acetaldehyde-treated embryo

Embryo A was explanted at 9 days' gestation and cultured for 28 hours in rat serum containing acetaldehyde 7.4mg/ℓ.

Embryo B was explanted at 9 days' gestation and cultured for 28 hours in rat serum.

Both embryos were well developed.

Note - otic vesicle (a) - optic vesicle (c) - limb bud (e)
- visceral arches (b) - heart (d) Mag. x 12.

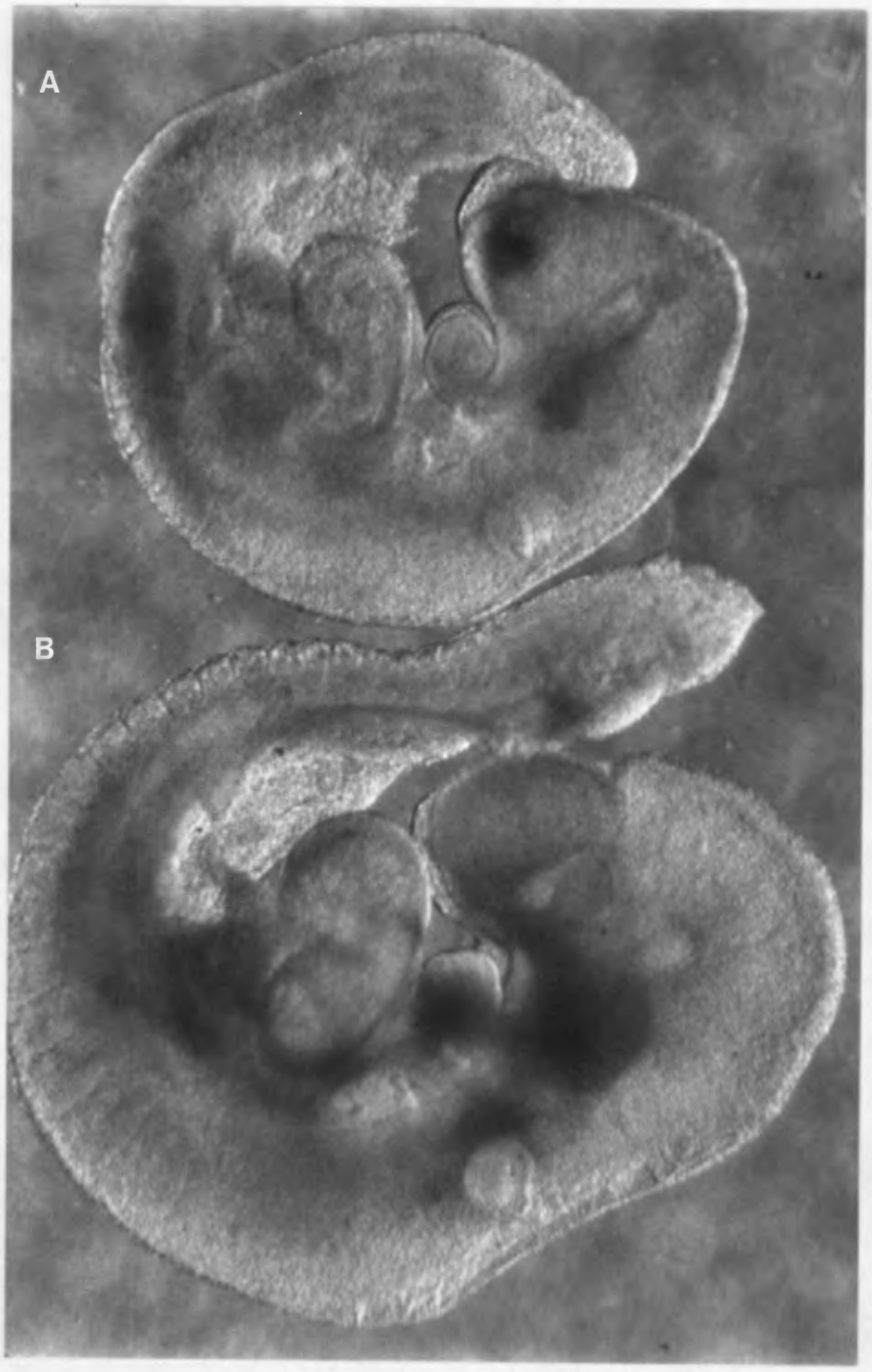

Fig. 6.8.

Comparison of a 9-day control embryo with an acetaldehyde-treated embryo

Embryo A was explanted at 9 days' gestation and cultured for 28 hours in rat serum containing acetaldehyde 39.4mg/ℓ.

Embryo B was explanted at 9 days' gestation and cultured for 28 hours in rat serum.

Both embryos were reasonably well developed although the treated embryo had a slightly abnormal contour and general development appeared retarded. Mag. x 12.

EFFECTS OF ACETALDEHYDE ON 9 DAY EMBRYOS

TABLE 6.18. IN VITRO STUDY

Dose:	Somite Count	Heart Beat – Present Absent	Development of Visceral Arches	Development of limb buds	CNS Development	DNA Synthesis
ACETALDEHYDE 7.4 mg/ℓ	Somite count significantly reduced in test group. (Borderline significance) p=0.0537 (Mann-Whitney Test) Control N = 60 Test N = 36	Significant effect on heart beat. p=0.019 Control N = 58 Test N = 35 (Heart beat absent in test embryos)	No significant abnormality. Control N = 57 Test N = 36	No significant effect. Control N = 56 Test N = 36	CNS development normal. (Fisher Test) Control N = 49 Test N = 32	No significant reduction in DNA synthesis. (Mann-Whitney Test) Control N = 32 Test N = 29
ACETALDEHYDE 19.7mg/ℓ	No significant effect. (Mann-Whitney Test) Control N = 60 Test N = 39	No significant effect on heart beat. Control N = 58 Test N = 37	Significant abnormality in the test group. p = 0.022 Control N = 57 Test N = 37	No significant effect. Control N = 56 Test N = 36	CNS development significantly abnormal in test group. p=0.032 Control N = 49 Test N = 29	Significant reduction in DNA synthesis in test group. p=0.0125 (Mann-Whitney Test) Control N = 32 Test N = 28
ACETALDEHYDE 39.4mg/ℓ	Somite count significantly increased in test group p=0.0024 (Mann-Whitney Test) Control N = 60 Test N = 34	Significant effect on heart beat. p=0.02 Control N = 58 Test N = 32 (Heart beat absent in test embryos)	No significant abnormality. Control N = 57 Test N = 34	Limb development significantly increased in the test group. p=0.000001 Control N = 56 Test N = 33	CNS development normal. Control N = 49 Test N = 31	No significant reduction in DNA synthesis. (Mann-Whitney Test) Control N = 32 Test N = 27

Eye and ear development, the presence or absence of a heart, presence of neural tube defects and the contour of the embryos (turned or unturned) were also assessed but no significant differences were found between test and control groups. Statistical analysis. The Fisher Exact Probability Test was used except where otherwise indicated.

6.5. DISCUSSION

With the exception of the eight-day ethanol 1500mg/ℓ group there was good correlation between abnormal central nervous system development and reduced DNA synthesis which suggests that inhibition of DNA synthesis may be a good indicator of abnormal central nervous system development.

Measurement of DNA synthesis offers a more reliable measure of adverse effects than morphological assessment which is subjective and open to different interpretation by different observers.

Rawat (1975) found that chronic ethanol consumption by pregnant rats resulted in a decrease in the rate of cerebral protein synthesis in the fetus. In this study pregnant albino rats were fed a liquid diet containing 6% (V/V) ethanol or isocaloric sucrose. Chronic ethanol feeding resulted in about 30% decrease in the rate of $[^{14}C]$ leucine incorporation by fetal ribosomes. The inhibitory effect on protein synthesis was found to be concentration-dependant and the capacity of both ribosomes and ph-5 enzymes to synthesize proteins was decreased in brains of fetuses from ethanol-fed mothers.

In the rat the chorioallantoic placenta probably starts functioning just after eleven days' gestation (Beck and Lloyd, 1977) and to a large extent takes over the nutritional functions of the inverted yolk-sac placenta. It is possible that this change in placental function partially accounts for the marked differences between the sensitivities of eight- and nine-day embryos to acetaldehyde, observed in this mouse study.

It is known that the rat placenta can oxidise a large proportion of the acetaldehyde which reaches it, thus preventing most of it from reaching the embryo. This may also account for the apparent reduction in sensitivity to acetaldehyde of the nine-day embryos. Ethanol, however, crosses the placenta freely (see discussion in section 4.3.) and thus would reach the eight- and nine-day embryos with equal facility.

It is important to note that ethanol toxicity in vitro only became significant at ethanol concentrations corresponding to extremely toxic doses to the mothers in the in vivo situation (see section 2.9.), while the maximum acetaldehyde concentration used in vitro was less than one hundredth of the dose which caused no evident toxic effects in the mothers. Taking these facts into account in this in vitro study I would label acetaldehyde as a true teratogen. In other studies it has been found that embryotoxic doses of almost all teratogenic agents are only a fraction of those that produce maternal toxicity (Beck, 1976:54).

Véghelyi and Osztovics (1978) studied the effect of acetaldehyde on cultured lymphocytes and fibroblasts of normal persons and found that acetaldehyde in a concentration of 800µM killed the great majority of cells, while at 400µM it inhibited cell mulitiplications. After seventy-two hours 12% of the cells showed labile chromosomal aberration and sister chromatid exchange (SCE) in the M_2 cells was increased 4 to 6 times. At an acetaldehyde level of 40µM cell progression was still affected and SCE was twice the control rate. Acetaldehyde was thus shown to be both cytotoxic and mutagenic in vitro.

6.6. CONCLUSIONS

In this study ethanol was shown to be embryotoxic only in doses which were highly toxic to the mother and it is probably true to say that almost any drug or chemical agent can be shown to be embryotoxic in very high doses under the appropriate laboratory conditions. But to eliminate the use of drugs or chemicals simply because they can be shown to be embryotoxic at high dosage would be unacceptable.

Acetaldehyde even in the lowest dose studied was very toxic to eight-day embryos and could be labelled as a true teratogen for the following reasons:

- the dose producing toxicity in vitro was only a minute fraction of the maternal non-toxic dose given in vivo
- there was a dose-related response in the eight-day embryos with the severity of the effect increasing with increasing dose
- the marked time specificity of response, i.e. marked

adverse effects at eight days with little effect at nine days is a common finding in teratogenicity tests.

Direct extrapolation of these findings to man, particularly in the case of acetaldehyde, is difficult as the concentrations used were far in excess of the typical levels measured in human chronic alcoholics by Majchrowicz and Mendelson (1970). They studied fifteen male alcoholic volunteers before, during and after a 10 to 15 day period of experimentally induced intoxication and found that blood acetaldehyde concentrations ranged from 0.11 to 0.15 and from 0.04 to 0.08 milligrams per 100 millilitres when blood ethanol concentrations ranged from 1 - 400 milligrams per 100 millilitres after consumption of bourbon or grain ethanol, respectively.

Although the concentrations of acetaldehyde used in vitro in my study were far in excess of the levels found in the human situation described above they were well below the non-toxic doses given to pregnant mice in my in vivo study.

KEY TO TABLES 6.1 to 6.14.

Heart	)	
Heart Beat	)	
Otic Vesicle	)	Plus sign + present
Optic Vesicle	)	Minus sign - absent
Limb Buds	)	
Limb Buds	-	refers to anterior limb buds
CNS	)	N = Normal (Defined in Chapter 5 section 5.8.)
Neural Tube	)	A = Abnormal (Defined in Chapter 5 section 5.8.)
Turned	)	T = Embryo with its dorsal surface arched outwards
Unturned	)	U = Embryo with its ventral surface arched outwards
U/T	)	U/T = Embryo twisted i.e. turning process has begun but is not yet complete. Classified unturned.
D.P.M.	=	Disintegrations per minute.
x or X	=	Not adequately assessed (not included in statistical evaluation)
Visceral Arches	N	= Normal size and shape and development symmetrical
Visceral Arches	A	= Abnormal size, shape and/or asymmetrical development

*Controls to which sterile water was added to the culture medium in volumes equal to the acetaldehyde volumes used.

TABLE 6.1.

Morphological findings and scintillation counts of embryos explanted at eight days' gestation and cultured for twenty-eight hours in rat serum (Control Group)

Embryo Number	Heart	Heart Beat	C.N.S	Neural Tube	Somite Count	D.P.M.
1	+	-	N	N	10	364.3
2	+	+	N	N	12	397.7
3	+	-	A	X	11	517.2
4	+	-	A	X	12	591.2
5	+	+	N	N	0	442.1
6	+	-	A	X	0	462.8
*7	-	-	X	N	0	84.5
*8	+	+	N	N	10	124.7
*9	+	+	N	N	12	599.2
*10	+	+	X	N	5	1331.6
*11	+	+	N	N	0	295.5
*12	+	+	N	N	0	270.6
13	+	+	N	A	11	ARG
*14	+	+	N	N	15	1004.1
*15	+	+	N	A	13	912.4
16	+	-	N	N	7	X
17	+	+	N	N	9	X
*18	+	-	N	N	0	ARG
*19	+	-	N	N	5	390
20	+	-	A	X	0	628.2
21	+	-	A	X	0	199.2
22	+	-	A	X	0	135.1
23	+	-	N	A	3	523.0
24	+	-	N	A	6	ARG
25	+	+	N	N	13	ARG
26	+	+	A	N	11	ARG
27	+	+	A	N	14	ARG
28	+	+	N	N	11	ARG
29	+	-	A	N	0	157.3
30	+	-	A	A	4	116.6
31	x	x	A	A	0	0
32	+	-	A	N	0	114.2
33	+	-	A	X	0	176.0
34	+	+	N	N	9	ARG
35	+	-	N	N	10	ARG
36	+	-	N	N	8	ARG
37	+	-	N	N	5	129.6
38	+	-	N	N	11	77.2
39	+	-	N	N	12	309.5
40	+	-	A	A	0	0
41	+	-	A	A	0	0
42	+	-	A	X	3	0
43	-	-	A	X	0	0
44	+	-	N	A	6	X
45	+	-	N	A	5	X
46	+	+	N	N	12	ARG
47	+	-	N	N	10	738.6
48	+	+	N	N	10	1004.1
49	+	+	N	N	0	119.1
50	+	-	N	N	0	122.5

TABLE 6.1. (Continued)
Morphological findings and scintillation counts of embryos explanted at eight days' gestation and cultured for twenty-eight hours in rat serum (Control Group)

Embryo Number	Heart	Heart Beat	C N S	Neural Tube	Somite Count	D.P.M.
51	+	+	X	N	3	909.0
52	+	+	N	N	7	915.6
53	+	+	N	N	10	1495.1
54	+	+	X	A	6	895.9
55	+	+	N	N	8	ARG
56	+	-	N	N	3	ARG
57	+	-	N	N	3	ARG
58	+	-	N	N	0	ARG

TABLE 6.2.

Morphological findings and scintillation counts of embryos explanted at eight days' gestation and cultured for twenty-eight hours in rat serum containing ethanol 1500mg/ℓ.

Embryo Number	Heart	Heart Beat	C.N.S	Neural Tube	Somite Count	D.P.M.
1	+	+	N	N	10	X
2	+	+	N	N	11	ARG
3	+	-	N	N	3	153.5
4	+	-	N	A	0	102.6
5	+	+	N	N	14	ARG
6	+	+	A	N	15	439.3
7	+	+	A	N	13	542.2
8	+	+	N	A	10	833.8
9	+	+	A	N	16	587.3
10	+	+	A	N	12	538.6
11	+	+	A	N	14	537.7
12	+	+	A	N	13	251.3
13	+	+	A	A	10	261.1
14	+	-	A	N	5	116.7
15	+	+	N	N	10	0
16	+	+	N	N	12	497.2
17	+	+	N	N	10	262.6
18	+	+	N	N	8	280.9
19	+	+	A	N	10	0
20	+	-	A	N	5	X
21	+	+	A	N	13	198.9
22	+	+	A	N	10	ARG
23	+	-	N	A	3	0
24	+	+	A	N	8	199.1
25	+	-	A	N	6	0
26	+	+	A	N	10	337.1
27	+	+	N	N	12	ARG
28	+	-	N	N	10	ARG
29	+	-	A	N	10	ARG
30	+	+	A	N	13	ARG
31	+	+	N	X	10	ARG
32	+	+	N	X	8	ARG
33	+	+	A	N	6	X
34	x	x	A	N	12	X
35	+	+	A	N	X	337.7
36	+	-	A	N	4	300.0
37	+	-	N	N	3	394.5
38	x	x	A	A	0	178.5

TABLE 6.3.

Morphological findings and scintillation counts of embryos explanted at eight days' gestation and cultured for twenty eight-hours in rat serum containing ethanol 3 000mg/ℓ

Embryo Number	Heart	Heart Beat	C.N.S.	Neural Tube	Somite Count	D.P.M.
1	+	-	N	N	10	ARG
2	+	-	N	A	8	681.8
3	+	-	A	X	5	296.2
4	+	-	N	A	4	ARG
5	+	-	N	N	10	612.1
6	+	-	A	A	9	652.0
7	+	+	A	N	6	ARG
8	+	+	A	N	10	ARG
9	+	+	A	N	5	ARG
10	+	-	A	A	6	ARG
11	+	+	N	N	5	349.0
12	+	+	N	N	8	382.0
13	x	-	A	N	0	0
14	+	-	N	N	8	ARG
15	+	-	N	N	10	247.9
16	-	-	N	A	10	ARG
17	+	-	A	X	0	0
18	+	+	N	N	0	X
19	+	+	N	A	4	X
20	+	+	N	A	4	X
21	+	-	A	N	9	X
22	+	-	N	N	6	0
23	+	+	N	N	12	674.4
24	+	+	N	N	12	100.9
25	+	+	N	A	13	1390.8
26	+	+	A	N	13	1444.7
27	+	+	N	N	8	0
28	+	+	N	N	10	0
29	+	-	N	N	4	94.9
30	+	-	N	N	0	60.5
31	+	-	A	N	0	174.5
32	+	-	N	N	4	340.8
33	+	+	N	N	6	X
34	+	-	A	A	0	2942.0
35	+	+	A	N	6	767.0
36	+	+	N	N	10	884.3
37	+	+	X	A	X	1159.5
38	+	+	N	A	6	585.3
39	+	+	X	N	13	1353.9
40	-	-	A	X	0	0

TABLE 6.4.

Morphological findings and scintillation counts of embryos explanted at eight days' gestation and cultured for twenty-eight hours in rat serum containing ethanol 6000mg/ℓ.

Embryo Number	Heart	Heart Beat	C N S	Neural Tube	Somite Count	D.P.M.
1	-	-	A	A	0	293.4
2	+	+	N	A	7	413.2
3	+	-	N	A	7	223.1
4	+	-	A	N	0	ARG
5	+	-	A	A	0	ARG
6	+	-	A	N	5	126.7
7	+	-	A	N	8	129.5
8	+	-	A	X	6	283.8
9	+	-	A	X	3	ARG
10	+	-	A	N	8	220.7
11	+	-	A	X	5	237.5
12	-	-	A	A	0	110.0
13	+	-	N	N	8	421.7
14	+	-	A	A	9	ARG
15	+	-	A	A	11	ARG
16	+	-	A	X	0	270.2
17	-	-	A	X	0	3.80
18	-	-	A	X	0	2.15
19	+	+	A	N	10	ARG
20	+	+	N	A	10	ARG
21	-	-	A	A	3	0
22	+	-	N	N	9	ARG
23	-	-	A	A	0	0
24	-	-	A	A	0	49.8
25	-	-	A	X	4	0
26	+	-	N	N	10	230.7
27	+	-	N	N	7	145.2
28	+	-	A	X	3	395.6
29	+	-	N	A	5	ARG
30	-	-	A	A	4	0
31	+	+	N	N	9	592.6
32	+	+	N	N	6	275.2
33	+	+	N	N	10	339.3
34	+	+	N	A	11	580.9
35	+	+	N	N	12	704.8
36	+	+	A	N	10	402.0
37	+	+	A	N	11	ARG
38	+	+	A	X	0	X
39	-	-	N	X	0	0
40	+	-	A	N	0	0

TABLE 6.5.

Morphological findings and scintillation counts of embryos explanted at eight days' gestation and cultured for twenty-eight hours in rat serum containing acetaldehyde 7.4 mg/ℓ.

Embryo Number	Heart	Heart Beat	C.N.S	Neural Tube	Somite Count	D.P.M.
1	+	-	N	A	8	0
2	+	+	A	A	9	444.1
3	+	-	A	A	4	330.2
4	+	+	N	N	12	413.1
5	-	-	A	A	0	55.0
6	+	-	N	N	4	103.6
7	+	-	N	N	6	208.4
8	-	-	X	A	0	175.3
9	+	+	N	N	11	514.3
10	-	-	A	A	0	0
11	-	-	A	A	0	202.6
12	+	-	A	N	0	95.8
13	+	+	N	N	6	275.5
14	+	+	N	N	7	481.1
15	+	-	N	N	5	0
16	+	-	A	N	12	X
17	+	-	A	N	7	217.1
18	+	-	A	N	14	666.4
19	+	-	A	N	10	125.1
20	+	-	A	N	13	737.1
21	+	-	N	N	3	0
22	+	+	A	A	0	1270.2
23	+	+	A	N	5	573.3
24	+	-	A	N	3	X
25	+	-	A	N	6	ARG
26	+	-	A	N	6	ARG
27	+	-	A	A	0	75.2
28	+	-	A	N	9	423.7
29	+	+	N	N	13	ARG
30	+	-	N	N	11	489.7
31	+	+	N	N	9	297.9
32	+	+	N	N	6	ARG
33	+	-	A	N	10	530.3
34	-	-	N	N	0	0
35	-	-	N	A	0	0
36	-	-	A	A	0	0
37	+	-	N	N	8	0
38	-	-	X	N	0	0
39	+	-	N	N	6	0
40	+	-	N	N	6	0
41	+	-	A	N	8	0
42	+	-	A	N	9	0
43	+	-	A	N	6	0

TABLE 6.6.

Morphological findings and scintillation counts of embryos explanted at eight days' gestation and cultured for twenty-eight hours in rat serum containing acetaldehyde 19.7mg/ℓ.

Embryo Number	Heart	Heart Beat	C.N.S	Neural Tube	Somite Count	D.P.M.
1	+	-	A	N	0	217.1
2	+	+	N	N	0	190.6
3	+	-	N	N	0	1424.4
4	+	+	N	N	7	351.1
5	+	+	N	N	9	349.1
6	+	-	N	N	6	604.9
7	+	+	A	N	6	399.4
8	+	-	A	N	5	112.9
9	+	-	A	N	5	341.4
10	+	-	N	N	0	145.9
11	+	-	N	N	0	194.4
12	+	-	N	N	0	155.4
13	+	+	N	N	6	274.2
14	+	-	A	A	0	0
15	+	-	N	N	6	ARG
16	+	-	N	A	4	ARG
17	+	-	N	N	0	280.0
18	+	-	N	N	0	228.5
19	+	-	A	A	6	570.9
20	+	-	N	A	9	ARG
21	+	-	A	A	4	X
22	+	-	N	N	13	ARG
23	+	-	A	N	0	62.8
24	+	-	N	N	9	ARG
25	-	-	N	N	14	ARG
26	-	-	A	N	3	ARG
27	+	-	A	N	13	ARG
28	+	-	A	A	0	0
29	+	-	A	A	6	102.9
30	+	-	N	N	10	213.3
31	-	-	A	A	0	0
32	-	-	A	A	0	0
33	+	-	A	N	0	60.0
34	+	-	A	N	0	16.6
35	+	-	A	N	0	200.7
36	-	-	A	N	0	32.3
37	+	-	A	N	0	0
38	+	-	A	N	0	0

TABLE 6.7.

Morphological findings and scintillation counts of embryos explanted at eight days' gestation and cultured for twenty-eight hours in rat serum containing acetaldehyde 39.4mg/ℓ.

Embryo Number	Heart	Heart Beat	C.N.S.	Neural Tube	Somite Count	D.P.M.
1	-	-	X	A	0	0
2	+	-	A	N	0	0
3	+	-	A	N	0	0
4	-	-	A	A	0	0
5	+	-	A	N	0	0
6	-	-	X	A	0	0
7	+	+	A	A	0	308.8
8	+	-	A	N	0	8.8
9	+	-	A	N	0	X
10	+	+	A	N	0	ARG
11	+	-	X	A	0	X
12	+	-	X	A	0	0
13	+	+	X	A	0	ARG
14	+	-	X	A	0	0
15	+	+	X	A	0	42.1
16	+	+	X	A	2	319.6
17	-	- +	N	N	0	854.0
18	+	-	X	A	0	X
19	+	+	N	N	0	330.7
20	+	-	N	N	0	24.4
21	-	-	A	A	0	73.0
22	+	-	A	N	0	0
23	+	+	N	N	0	178.1
24	+	+	A	A	0	0
25	+	+	A	A	0	ARG
26	+	+	A	A	0	ARG
27	+	+	A	A	0	273.3

TABLE 6.8.

Morphological findings and scintillation counts of embryos explanted at 9 days' gestation and cultured for 28 hours in rat serum (Control Group).

Embryo Number	Heart	Heart Beat	Otic Vesicle	Optic Vesicle	Limb Buds	CNS	Neural Tube	Turned or Unturned	Somite Count	Arches	D.P.M.
1.	+	+	-	+	-	N	N	U	15	N	ARG
2.	+	+	+	-	-	N	N	X	14	N	ARG
3.	+	+	+	+	-	N	N	T	26	N	ARG
4.	+	+	+	+	-	N	N	U/T	18	N	602.1
5.	+	+	+	+	-	N	N	U/T	20	N	473.5
6.	+	+	-	+	-	N	N	U	22	N	ARG
7.	+	-	+	+	-	N	N	U	25	N	ARG
8.	+	+	+	-	-	N	N	U	16	N	ARG
9.	+	+	+	-	-	A	N	U	16	N	925.3
10	+	+	+	+	-	X	N	T	20	N	991.5
11	+	+	+	+	+	N	N	T	20	N	286.8
12.	+	+	+	+	-	X	N	T	22	N	ARG
13	+	+	+	+	-	N	N	T	25	N	ARG
14	+	+	+	+	-	N	N	T	17	N	ARG
15	+	+	+	+	+	N	N	T	25	N	553.4
16	+	+	+	+	-	A	N	U/T	20	N	ARG
*17	+	+	+	+	-	N	N	T	23	N	ARG
*18	+	+	+	+	+	N	N	T	22	N	ARG
*19	+	+	+	+	-	N	N	U/T	25	N	ARG
*20	+	+	+	+	-	N	N	U/T	25	N	ARG
*21	+	-	+	+	-	N	N	U	17	N	ARG
*22	+	-	+	-	-	X	N	T	17	N	ARG
*23	+	+	+	+	-	N	N	U	20	N	964.1
*24	+	+	+	-	-	A	N	U	13	N	728.5
*25	+	+	+	+	+	N	N	U	20	N	918.9
*26	-	+	+	+	-	A	N	T	23	N	1376.5
*27	+	-	+	+	-	A	N	T	20	N	575.2
*28	+	+	+	-	-	N	N	U	19	N	470.7
*29	+	+	+	+	-	N	N	T	25	N	X
*30	+	+	+	-	-	A	N	U	16	N	135.0
*31	+	+	+	+	-	A	N	U	18	N	1568.0
*32	+	-	+	-	-	N	N	T	20	N	0
*33	+	-	-	-	-	N	N	T	22	N	182.6
*34	+	+	+	+	+	X	N	T	25	N	1990.6
*35	+	-	+	+	+	X	N	T	22	N	235.5
*36	+	-	+	+	+	X	N	T	25	N	169.9
*37	+	+	+	+	-	X	N	U	14	N	247.9
*38	+	+	-	-	-	A	N	U	12	X	0
39	+	-	x	+	-	N	N	U/T	13	N	338.0
40	x	x	+	+	+	X	N	T	27	N	ARG
41	x	x	-	+	-	N	N	T	25	N	ARG
42	+	+	-	+	-	N	N	U	16	N	ARG
43	+	+	+	+	-	N	N	X	20	N	ARG
44	+	+	+	x	-	N	N	T	16	N	530.7
45	+	+	+	-	x	N	N	T	17	N	1033.3
46	+	+	x	x	x	N	N	T	15	N	973.2
47	+	+	x	x	x	N	N	X	20	X	1067.3
48	+	+	x	x	+	N	N	X	26	X	3172.7
49	-	+	x	x	+	N	N	X	28	N	1903.8
50	+	+	+	-	+	N	N	T	20	N	ARG

TABLE 6.8. (Continued)

Morphological findings and scintillation counts of embryos explanted at 9 days' gestation and cultured for 28 hours in rat serum (Control Group).

Embryo Number	Heart	Heart Beat	Otic Vesicle	Optic Vesicle	Limb Buds	CNS	Neural Tube	Turned or Unturned	Somite Count	Arches	D.P.M.
51	+	+	+	-	+	N	N	T	24	N	ARG
52	+	+	+	+	+	N	N	T	25	N	3463.2
53	+	+	+	x	+	N	N	T	25	N	6532.8
54	+	+	+	+	+	N	N	T	28	N	5805.9
55	+	+	+	+	+	N	N	T	26	N	7175.4
56	+	+	+	+	-	N	N	T	18	N	X
57	+	+	+	+	-	N	N	X	20	N	X
58	+	+	+	+	-	N	N	U	24	N	X
59	+	+	+	+	-	N	N	U	22	N	ARG
60	+	+	+	x	x	x	N	U	15	N	ARG

TABLE 6.9.

Morphological findings and scintillation counts of embryos explanted at 9 days' gestation and cultured for 28 hours in rat serum containing ethanol 1 500mg/ℓ.

Embryo Number	Heart	Heart Beat	Otic Vesicle	Optic Vesicle	Limb Buds	CNS	Neural Tube	Turned or Unturned	Somite Count	Arches	D.P.M.
1	+	+	-	+	-	N	N	U	20	N	327.0
2	+	+	-	+	-	N	N	U	16	N	428.8
3	+	-	-	+	-	N	N	U	18	N	387.4
4	+	+	-	+	-	N	N	U	20	N	ARG
5	+	+	+	+	-	A	N	T	18	N	439.7
6	+	+	+	+	X	N	N	U/T	16	N	799.5
7	+	+	+	+	+	N	N	T	22	N	ARG
8	+	+	+	+	+	N	N	T	26	N	ARG
9	+	+	+	+	+	N	N	T	27	N	440.3
10	+	+	+	+	-	N	N	U/T	18	N	1238.7
11	+	-	-	-	-	A	N	U	X	N	O
12	+	+	+	-	-	N	N	U/T	20	N	ARG
13	+	+	+	-	-	N	N	U	19	N	ARG
14	+	+	+	-	-	N	N	T	20	X	ARG
15	+	+	+	+	-	N	N	U/T	18	N	ARG
16	+	+	+	+	-	N	N	U	18	N	522.3
17	+	+	+	+	-	N	N	U	17	N	ARG
18	+	+	+	+	-	N	N	U/T	20	N	1458.0
19	+	+	+	+	+	N	N	T	25	N	761.8
20	+	+	+	+	+	N	N	T	21	N	971.2
21	+	+	+	+	+	N	A	T	25	N	1580.1
22	+	+	+	+	+	N	A	T	22	N	X
23	+	+	+	+	+	N	A	T	27	N	1500.4
24	+	+	+	+	+	N	A	T	27	N	1006.9
25	+	+	+	+	-	X	A	T	22	N	436.1
26	+	+	+	+	-	X	N	U/T	22	N	386.1
27	+	+	+	+	-	X	N	T	24	N	229.7

TABLE 6.10.

Morphological findings and scintillation counts of embryos explanted at 9 days' gestation and cultured for 28 hours in rat serum containing ethanol 3 000mg/ℓ.

Embryo Number	Heart	Heart Beat	Otic Vesicle	Optic Vesicle	Limb Buds	CNS	Neural Tube	Turned or Unturned	Somite Count	Arches	D.P.M.
1	+	+	+	+	-	N	A	U/T	15	N	358.3
2	+	-	+	+	-	A	N	U/T	13	N	ARG
3	+	+	+	+	-	N	N	T	16	N	653.9
4	+	+	+	+	-	N	N	U	20	N	840.2
5	+	+	+	+	-	A	N	T	20	N	646.1
6	+	+	+	+	-	A	N	U	16	N	ARG
7	+	+	+	+	-	N	A	U/T	15	A	ARG
8	+	+	+	+	-	N	N	U/T	15	N	ARG
9	+	+	+	+	-	A	N	U/T	20	N	ARG
10	+	+	+	-	-	N	N	U/T	25	N	ARG
11	+	+	+	-	-	N	N	U	15	N	997.0
12	+	+	+	+	+	N	N	T	23	N	ARG
13	+	+	+	-	-	X	N	U/T	17	N	1304.6
14	X	X	X	+	-	X	N	U/T	19	X	X
15	X	X	X	+	-	X	N	U/T	19	X	X
16	+	+	+	+	+	A	N	X	19	A	1613.5
17	+	+	X	X	-	X	A	U/T	15	N	985.1
18	+	+	+	+	-	A	N	U/T	17	N	ARG
19	+	+	+	+	X	A	N	T	20	N	X
20	+	+	+	+	X	N	A	T	20	N	2155.5
21	+	+	+	+	X	N	N	U/T	20	N	2292.3
22	+	+	+	+	X	X	N	T	20	N	ARG
23	+	+	+	+	X	A	N	X	20	A	4398.8
24	+	+	+	-	X	N	N	T	16	N	1459.8
25	+	+	+	+	X	N	N	T	18	N	768.5
26	+	+	+	+	-	N	A	T	17	N	1801.2
27	+	+	+	+	-	A	A	U/T	18	N	1532.0
28	+	+	+	-	-	A	N	U/T	20	N	1210.0
29	+	+	+	-	-	A	N	T	22	N	1197.5
30	+	+	X	X	X	N	N	U/T	15	N	337.3
31	+	+	X	X	X	X	N	X	15	X	86.9
32	+	+	X	X	+	N	N	T	26	N	1112.2
33	+	+	X	X	+	N	N	T	26	N	3459.0
34	+	+	X	X	+	N	N	T	24	N	3865.1
35	+	+	X	X	+	N	N	T	24	N	1984.5
36	+	+	+	-	-	N	N	T	28	N	ARG
37	+	+	+	-	-	N	N	T	22	N	ARG
38	+	+	+	-	-	A	N	U	20	A	1087.5
39	+	+	+	-	-	X	N	U/T	X	X	1398.0
40	+	+	+	+	+	A	N	X	26	N	6570.1
41	+	+	+	+	+	A	N	X	22	N	4165.2
42	+	+	+	+	+	A	N	U/T	18	N	2539.2
43	+	+	+	+	+	A	A	X	22	A	8606.6
44	+	+	-	-	-	A	N	U	0	A	472.5
45	+	+	+	+	-	N	N	U/T	22	N	2724.2
46	X	X	+	+	-	N	N	X	25	X	X
47	X	X	+	+	+	N	N	X	25	X	X
48	+	+	+	+	-	N	N	U	22	N	X
49	+	+	+	+	-	N	N	U	22	N	X

TABLE 6.11.

Morphological findings and scintillation counts of embryos explanted at 9 days' gestation and cultured for 28 hours in rat serum containing ethanol 6000mg/ℓ.

Embryo Number	Heart	Heart Beat	Otic Vesicle	Optic Vesicle	Limb Buds	CNS	Neural Tube	Turned or Unturned	Somite Count	Arches	D.P.M.
1.	+	+	+	+	-	N	N	U/T	20	N	ARG
2.	+	+	+	+	-	X	A	T	22	N	X
3.	+	+	+	+	+	X	A	T	22	N	X
4.	+	+	+	+	-	X	A	U/T	16	N	X
5.	+	+	+	+	+	N	N	T	19	N	1620.1
6.	+	+	+	x	-	A	A	T	20	N	946.4
7.	+	+	+	x	+	N	N	T	20	N	1069.4
8.	+	+	+	+	+	X	N	T	20	A	ARG
9.	+	+	+	+	+	A	N	T	13	N	ARG
10	+	+	+	+	+	X	N	T	20	N	620.3
11	+	+	+	-	-	A	N	U	13	N	775.5
12.	+	+	+	-	-	A	A	U	10	N	ARG
13	+	+	+	+	-	N	N	U	20	N	522.3
14	+	+	+	-	-	A	N	U	15	N	ARG
15	+	+	+	-	-	X	N	U/T	15	N	324.1
16	x	x	+	-	-	N	N	T	20	N	486.1
17	+	+	+	-	-	N	N	T	18	N	493.3
18	+	+	-	+	-	A	N	U/T	16	N	ARG
19	+	+	-	+	-	N	N	U/T	22	N	543.3
20	+	+	-	-	-	A	N	U/T	13	N	ARG
21	+	+	-	-	-	A	N	U/T	10	N	ARG
22	+	+	-	-	-	N	N	T	18	N	144.6
23	+	+	-	+	-	A	N	U/T	18	N	365.3
24	+	+	x	+	-	A	A	U/T	20	N	ARG
25	+	+	x	+	-	A	N	U/T	18	N	ARG
26	+	+	-	+	-	N	N	U/T	15	N	224.6
27	+	+	-	+	-	N	N	U	15	N	245.8
28	+	+	+	+	-	N	N	U/T	20	N	1414.7
29	+	+	+	+	-	A	X	U/T	20	N	ARG
30	+	+	+	+	-	A	N	U	12	N	ARG
31	+	+	+	+	-	A	N	U/T	8	A	ARG
32	+	+	+	-	-	N	N	T	12	N	0
33	+	+	+	-	-	N	N	U	10	N	0
34	+	-	+	-	-	X	N	T	19	N	0
35	+	-	+	-	-	X	N	T	17	N	0
36	+	-	x	-	-	N	N	U	11	N	0
37	-	-	x	x	-	X	N	T	16	N	0
38	-	-	x	x	-	X	N	T	16	N	0
										N	

TABLE 5.12.

Morphological findings and scintillation counts of embryos explanted at 9 days gestation and cultured for 28 hours in rat serum containing acetaldehyde 7.4mg/ℓ.

Embryo Number	Heart	Heart Beat	Otic Vesicle	Optic Vesicle	Limb Buds	CNS	Neural Tube	Turned or Unturned	Somite Count	Arches	D.P.M.
1.	+	+	+	+	+	A	N	T	22	N	ARG
2.	+	+	+	+	+	N	N	T	28	N	ARG
3.	+	+	+	+	-	X	N	T	20	N	761.1
4.	+	+	+	+	+	N	N	T	27	N	1034.2
5.	+	+	+	+	+	N	N	T	29	N	1315.9
6.	+	+	+	+	+	A	N	T	26	N	2475.6
7.	+	-	+	+	-	N	N	U/T	17	N	0
8.	+	+	+	+	-	N	N	T	19	N	241.9
9.	+	+	+	+	-	N	N	T	25	A	1277.1
10	+	+	+	-	-	N	N	U	16	N	1041.3
11	+	+	+	+	-	N	N	T	22	N	596.5
12.	+	+	+	+	+	N	N	T	30	N	1740.0
13	+	+	+	+	-	N	N	T	25	N	ARG
14	+	+	-	-	-	X	N	T	25	N	708.5
15	+	+	+	+	+	N	N	T	27	N	1486.0
16	+	+	+	+	-	N	N	T	22	N	919.3
17	+	+	+	+	+	N	N	T	28	N	X
18	+	+	+	+	+	N	N	T	24	N	1131.0
19	+	+	+	+	+	N	N	U	23	N	907.8
20	+	+	+	+	+	N	N	T	26	N	716.2
21	+	+	+	+	+	N	N	T	28	N	616.1
22	+	+	+	+	-	X	N	T	20	N	0
23	+	+	+	+	-	N	N	U	18	N	636.0
24	+	+	+	+	-	A	N	U	20	N	142.6
25	+	+	+	+	-	A	N	T	20	N	X
26	+	+	+	+	-	N	N	T	20	N	816.8
27	+	+	+	+	-	N	N	T	16	N	749.5
28	+	+	+	+	-	N	N	U	19	N	ARG
29	+	+	+	+	-	N	N	U	18	N	ARG
30	+	+	-	-	-	A	N	U	13	N	600.4
31	+	+	+	+	-	A	N	U/T	20	N	2318.9
32	x	x	+	+	+	N	N	T	24	N	0
33	+	+	+	+	-	A	N	U	21	N	89.7
34	+	+	+	+	-	A	N	U	18	N	870.2
35	+	+	+	+	-	X	N	T	20	N	1561.6
36	+	+	+	+	+	N	N	T	22	N	1744.0

TABLE 6.13.

Morphological findings and scintillation counts of embryos explanted at 9 days' gestation and cultured for 28 hours in rat serum containing acetaldehyde 19.7mg/ℓ.

Embryo Number	Heart	Heart Beat	Otic Vesicle	Optic Vesicle	Limb Buds	CNS	Neural Tube	Turned or Unturned	Somite Count	Arches	D.P.M.
1.	+	+	+	+	+	X	N	T	26	N	ARG
2.	+	+	+	+	-	N	N	T	22	X	280.4
3.	+	+	+	+	+	N	N	T	28	N	525.4
4.	+	+	+	+	+	N	N	T	28	A	ARG
5.	+	+	+	+	+	X	A	T	25	N	248.1
6.	+	+	+	+	+	N	N	T	30	N	1054.7
7.	+	+	+	+	+	X	A	T	22	N	366.4
8.	+	+	+	+	+	N	N	T	28	N	745.2
9.	+	+	+	+	-	N	N	T	24	N	653.0
10	+	+	+	+	-	N	N	T	24	N	643.5
11	+	+	+	+	-	N	N	T	24	N	ARG
12.	+	+	+	+	-	X	N	T	15	N	X
13	+	+	+	+	+	N	N	T	25	N	ARG
14	+	+	+	-	-	A	N	U	14	N	482.5
15	-	+	+	+	-	N	N	T	23	N	919.2
16	+	+	+	+	-	N	N	U/T	26	N	1056.5
17	+	+	-	+	-	A	X	U/T	19	N	34.3
18	+	+	+	+	-	A	N	T	17	N	0
19	+	+	+	+	+	N	N	T	22	N	1830.7
20	+	+	+	+	-	A	N	T	25	A	X
21	+	+	+	+	-	A	N	T	20	N	591.2
22	+	+	+	-	-	A	X	U/T	12	N	81.0
23	+	+	+	+	-	A	N	U/T	21	N	278.4
24	+	+	+	-	-	A	N	U	9	N	54.8
25	+	+	+	+	-	X	N	U/T	18	N	60.7
26	+	+	+	+	+	N	N	T	26	N	301.0
27	+	+	+	+	+	N	N	T	27	N	2597.3
28	+	+	+	+	+	N	N	T	25	N	1180.7
29	+	+	+	-	+	N	N	U	22	N	859.8
30	+	+	+	x	x	A	N	U	10	N	X
31	+	+	+	x	x	A	N	U	10	N	552.1
32	+	+	+	x	x	X	N	U	13	N	X
33	+	+	+	+	x	X	N	X	19	N	X
34	+	+	+	+	+	N	N	T	24	N	X
35	+	+	+	+	+	N	N	T	24	N	X
36	x	x	-	-	-	X	N	U/T	15	X	0
37	x	x	-	-	-	X	N	U/T	2	X	0
38	+	-	+	-	-	X	N	T	26	N	0
39	x	x	x	-	-	X	N	X	X	X	0
40	+	+	+	-	-	A	N	T	12	A	X

TABLE 6.14.

Morphological findings and scintillation counts of embryos explanted at 9 days' gestation and cultured for 28 hours in rat serum containing acetaldehyde 39.4mg/ℓ.

Embryo Number	Heart	Heart Beat	Otic Vesicle	Optic Vesicle	Limb Buds	CNS	Neural Tube	Turned or Unturned	Somite Count	Arches	D.P.M.
1.	+	+	x	+	+	N	N	T	25	N	428.4
2.	+	+	+	+	+	N	N	T	20	N	328.7
3.	+	+	+	+	+	N	N	U/T	22	N	424.5
4.	+	+	+	+	+	N	N	T	22	N	515.3
5.	+	+	+	+	+	X	N	T	25	N	643.1
6.	+	+	+	+	+	N	N	T	20	N	223.6
7.	+	+	+	+	-	N	N	T	14	N	313.3
8.	+	+	+	+	+	N	N	T	23	N	ARG
9.	+	+	+	+	+	N	N	T	28	N	ARG
10	+	+	+	+	+	N	N	T	25	N	693.9
11	+	+	+	+	+	N	N	T	29	N	798.8
12.	+	+	+	+	+	N	N	T	25	N	1509.9
13	+	+	+	+	+	N	N	T	25	N	2624.4
14	+	+	+	+	+	N	N	T	25	N	447.8
15	+	+	+	+	+	N	N	T	27	N	ARG
16	+	+	+	+	x	N	N	U/T	25	N	1247.5
17	+	+	+	+	+	A	N	T	25	N	ARG
18	+	+	+	+	+	N	N	T	25	N	ARG
19	+	+	+	+	-	N	N	T	22	N	1024.7
20	+	+	+	+	-	A	X	U/T	13	N	85.0
21	+	+	+	+	-	N	N	U	18	N	X
22	+	+	+	+	+	X	N	T	25	N	1139.8
23	+	+	+	+	+	X	A	T	22	N	1175.1
24	+	+	+	+	+	A	N	T	24	N	2261.0
25	+	+	+	+	+	N	N	T	22	N	1578.6
26	+	+	+	+	+	N	N	T	26	N	293.0
27	+	+	+	-	-	A	N	U	9	N	486.7
28	+	+	-	-	-	A	N	U	10	N	1426.5
29	+	+	+	+	+	N	N	T	25	A	254.3
30	+	+	+	+	+	N	N	T	30	N	221.3
31	+	+	+	+	+	N	N	T	26	N	160.8
32	+	+	+	+	+	N	N	T	26	N	59.5
33	+	+	+	+	+	N	N	T	28	N	X
34	+	+	+	+	+	N	N	T	24	N	219.4

CHAPTER 7

THE METABOLISM OF ETHANOL

7.1. ABSORPTION AND DISTRIBUTION OF ETHANOL

The absorption of ethanol is by diffusion, which occurs rapidly in all portions of the gastro-intestinal tract. The rate of absorption is a function of the dose and the concentration of the ethanol being consumed. Also, the initial rate of absorption, the time interval to the peak concentration, and the further course of the blood-alcohol curve are dependant on the amount of filling of the gastro-intestinal tract and its motility. Also important are the degree of vascularisation of the mucous membranes and the concentration and distribution of water in the various organs.

These factors may change during pregnancy, for example gastric emptying is delayed and intestinal motility decreased. Pregnant women may, as a result, have lower peak blood ethanol values which are maintained for a longer period because of secondary reabsorption (Hinckers, 1978). Another cause of the lower blood ethanol level during pregnancy is that after oral ethanol intake it is distributed at a diffusion equilibrium according to the water content of the various compartments (Grüner, 1958:744). During pregnancy there is a total increase of water volume of about 6 litres in all compartments including the fetus, placenta, uterus and amniotic fluid. The pregnant uterus and fetus thus play an important role in the distribution of ethanol. During the various stages of pregnancy the fetal exposure to ethanol varies with the changes in water concentrations within the mother and fetus. Fetal water concentration is very high during early pregnancy while maternal water retention increases during the later stages of pregnancy. Thus maximum exposure to ethanol occurs in the embryo during early pregnancy. Compared with humans, laboratory animals have a higher tolerance for ethanol and lower blood ethanol levels for a given dose because of their long gastro-intestinal transit time which delays absorption. This fact needs to be taken into account when determining dosage in the experimental situation.

7.2. ETHANOL METABOLISM

There is no evidence to suggest that ethanol metabolism is altered in pregnancy. The fetus is capable of metabolising ethanol only after maturation of the liver enzymes, during the latter half of gestation.

In mammals ethanol is predominantly oxidised in the liver in a two step reaction. The main enzyme is alcohol dehydrogenase. This produces acetaldehyde which is broken down to acetate by aldehyde dehydrogenase. For further metabolism acetate must be activated to acetyl coenzyme A. The enzymes responsible for ethanol oxidation are localised in the hepatocyte. Ethanol oxidation results in the production of large amounts of hydrogen, causing a marked increase in the reduction state of the NAD-system in the liver cytosol (Bode, 1978). Some consequences of ethanol oxidation and increased production of reducing equivalents on hepatic metabolism are shown below.

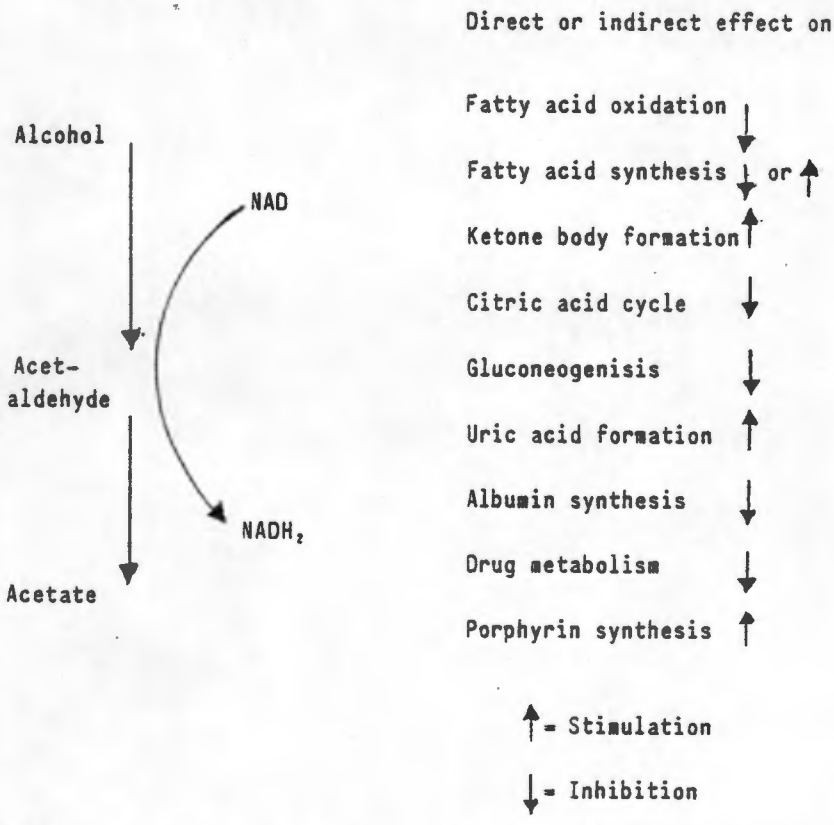

Fig. 7.1. Schematic representation of the effects of alcohol and alcohol metabolism on some pathways of intermediary metabolism in the liver.

(Bode, 1978:125).

Alcohol dehydrogenase is the most important enzyme for ethanol oxidation (von Wartburg, 1971:63; Theorell, 1965:553; Büttner, 1965:300). It exists in multiple molecular forms, and in human

liver seven iso-enzymes have been identified. The hepatic iso-enzyme patterns are different in the fetal, neonatal, childhood and adult periods of life (Murray and Motulsky, 1971:71). In addition to heterogenicity, human liver alcohol dehydrogenase shows polymorphism (Schenker et al, 1971:271; Smith et al, 1971:251). An atypical variant enzyme has been found in human liver with a specific activity approximately five times greater than normal. It is found in 4% to 20% of Europeans (von Wartburg and Schürch, 1968:936) and is very common in Japan (Ogata and Mizohata, 1971:3). Atypical individuals may oxidise ethanol at a faster rate in the initial phase before the reoxidation of liver alcohol dehydrogenase (LADH) becomes the rate limiting step. This causes higher initial blood acetaldehyde levels than in normal subjects and coincides with the high frequency of signs of ethanol intolerance in Oriental populations (Hollstedt, 1981:7). Whether these elevated blood acetaldehyde levels are of clinical importance with regard to chronic ethanol damage or the fetal alcohol syndrome remains to be determined.

Pikkarainen and Räihä (1967) studied the development of alcohol dehydrogenase activity in human liver and found that alcohol dehydrogenase activity was detectable in two-month-old fetuses but in amounts of three percent to four percent of adult activity. The activity increases linearly and adult levels are found in five year olds. It would appear from these results that the human fetus has a much lower capacity to oxidise ethanol.

7.3. MICROSOMAL ETHANOL OXIDATION AND CATALASE

A microsomal ethanol oxidising system (MEOS) which is distinguishable from alcohol dehydrogenase has been described (Orme-Johnson and Ziegler, 1965:78; Lieber and de Carli, 1968:917). It is possible that the MEOS is mainly responsible for non-alcohol dehydrogenase oxidation (Lieber, 1973:821) but other investigators have questioned the existence of unique microsomal ethanol oxidising systems (Thurman and Scholz, 1973:441; Thurman et al, 1974:287; Carter and Isselbacher, 1971:282; Isselbacher and Carter, 1974:493).

7.4. ENZYMES OF ALDEHYDE METABOLISM

Two types of enzyme can be distinguished in vitro:

1. aldehyde dehydrogenase using NAD as coenzyme, and
2. aldehyde oxidase which produces hydrogen peroxide (Bode, 1978:127). Aldehyde dehydrogenases have been found in many tissues with various substrate specificity (von Wartburg, 1971:63; Deitrich, 1972:7232; Lindros, 1974:417).

7.5. FACTORS INFLUENCING ETHANOL OXIDATION RATES IN MAN (Bode, 1978)

1. Prolonged fasting decreases ethanol metabolism in man.
2. Fructose increases ethanol oxidation.
3. Protein restriction inhibits blood ethanol clearance.
4. Chronic intake of ethanol accelerates blood ethanol clearance during consumption or immediately after ethanol withdrawal but this value returns towards normal after two to three weeks of abstinence (Mendelson, 1968:319; Mezey and Tobon, 1971:707; Bode et al, 1977).
5. Drugs. In recent studies of chronic alcoholics (Mezey and Robles, 1974:248) and healthy volunteers (Bode and Bode, 1977) an increase in the rate of blood ethanol elimination was shown after pre-treatment with phenobarbital.
6. Liver disease itself affects ethanol oxidation rate only in patients having severely disturbed liver function (Mallach et al, 1972:732).

7.6. GENETIC ASPECTS OF ETHANOL METABOLISM

Genetic factors may influence both the metabolism of ethanol and the effects of ethanol on psychophysiological function. Recent reports point to individual and racial differences in the biological response to ethanol administration in man (Hollstedt, 1981:7).

Alcoholism appears to be a multifactorial genetically influenced disorder. (Cloninger et al, 1972). There are many factors that might individually or in combination underlie a genetic influence in higher risk individuals. These may include a possible unique

reaction to a single dose of ethanol, differences in the metabolism of ethanol, and differential susceptibility to the consequences of long-term exposure to ethanol (Omenn, 1975:12).

In their study Schuckitt and Rayses (1979:56) showed that blood acetaldehyde concentrations were significantly elevated after moderate ethanol intake by twenty healthy males (non alcoholics), whose parents or siblings were alcoholics, compared with matched controls having no familial alcoholism. These findings have clinical significance. The increased acetaldehyde concentrations could mediate the short-term effects of ethanol, causing an altered state of intoxication. It is also possible that those individuals who are predisposed to alcoholism are more vulnerable to organ damage from acetaldehyde. This higher acetaldehyde plateau may have an effect on the development of ethanol addiction (Schuckitt and Rayses, 1979). It is also possible that the higher acetaldehyde level following ethanol intake found in relatives of alcoholics and also in abstinent alcoholics may have some bearing on the fetal alcohol syndrome assuming that acetaldehyde is the main teratogen in this syndrome. It should be possible to measure acetaldehyde levels after ethanol intake in women contemplating pregnancy and to predict which women are likely to be the most at risk of having infants with the fetal alcohol syndrome even with moderate ethanol intake. It would be particularly important to screen those women who have a family history of alcoholism even if they themselves are not alcoholics.

CHAPTER 8

THE FETAL ALCOHOL SYNDROME : PROGRESS IN UNDERSTANDING ITS PATHOGENESIS

For many centuries it has been known that alcohol affects the fetus. However the Fetal Alcohol Syndrome (F.A.S.), now well known as a clinical entity, was described by Jones and co-workers only as recently as 1973 (Jones et al, 1973b). The most commonly occurring features are typical facial characteristics, central nervous system dysfunction and pre- and post-natal growth deficiency, although a wide range of abnormalities involving almost every organ system has been reported in connection with the syndrome. It is estimated that a third to half of the offspring of chronic alcoholic mothers exhibit the F.A.S. to some degree (Hanson et al, 1976; Corrigan, 1976; Jones et al, 1976).

The pathogenesis of the syndrome has not been properly elucidated. Many questions have been raised as to whether the damage is due directly to alcohol, or indirectly, for example, to the metabolic products of alcohol, the effects of alcoholism on maternal health and nutrition, or the often associated nicotine or drug abuse. The type of beverage abused may be important, and genetic factors may have a role. These questions are difficult to answer using purely clinical studies. Two liver enzyme systems, alcohol dehydrogenase and the microsomal ethanol oxidising system, are responsible for ethanol metabolism in man. It has been shown that atypical alcohol dehydrogenase enzymes occur in a small proportion of Caucasians and a high proportion of Orientals (von Wartburg and Schürch, 1968:936; Ogata and Mizohata, 1971:3). Atypical individuals may oxidise ethanol at a faster rate, causing higher initial blood acetaldehyde levels than in normal subjects. Genetic factors may influence both the metabolism of ethanol and the effects of ethanol on psycho-physiological function (Hollstedt, 1981:7). In the light of these findings it would be interesting to compare the incidence of the F.A.S. in different ethnic groups.

Animal models have been established in a variety of species. Of the rodent models the mouse appears to give the most consistent results. Most strains of mice studied so far have demonstrated a characteristic dose response effect, ranging from no effect at low levels of ethanol intake to lethal effects at high levels, although the safe range of dosage has differed between strains. These models are of value in that it is possible to eliminate many of the factors which confuse the picture in human studies.

Animal experiments have confirmed that ethanol is a potent neurotoxin, and also that the F.A.S. is caused by the intake of ethanol and is not due to complications such as maternal malnutrition. Genetic factors seem to be important too, and studies using different strains of mice have shown that they have differing degrees of susceptibility to ethanol. Chernoff (1977) showed that CBA mice were more sensitive to the induction of the syndrome than C3H mice. He suggested that the difference in susceptibility was due to the rate of ethanol metabolism. The two liver enzyme systems mentioned above also exist in the mouse, and the alcohol dehydrogenase system is known to be under genetic control.

The limitations of in vivo animal studies are that even with control of the environment, genetic factors, and dietary intake, it is not possible to eliminate all of the unwanted side effects of maternal or placental metabolism of ethanol and acetaldehyde. Maternal metabolism of ethanol produces confounding factors, for example hypoglycaemia, hypothermia, and indirect malnutrition by action on the gut and on protein, vitamin and mineral metabolism (Kissin and Begleiter, 1972). Kesäniemi and Sippel (1975) treated pregnant rats four days before term with intraperitoneal injections of ethanol and measured the concentrations of ethanol and acetaldehyde in the placentas, fetuses and maternal blood. They showed that while ethanol passed freely through the placenta, acetaldehyde was quickly oxidised in it - there was no acetaldehyde detectable in the fetus. In a study using pregnant mice Randall et al (1978) showed that while the placenta formed a partial barrier to the passage of acetaldehyde, some did reach the fetus, the

acetaldehyde content of the latter varying with the stage of gestation. It appeared from their study that the placenta was efficient in handling the acetaldehyde early in gestation, and that late in pregnancy this efficiency had dropped by more than fifty percent. While the placenta may have some protective effect with regard to acetaldehyde it is important to remember that in both man and mouse, differentiation, and, to a lesser extent, organogenesis, are well advanced before the placenta starts functioning.

In vitro models, also, are of importance in elucidating the mechanisms of ethanol-induced damage. Using the whole embryo culture system described it is possible to eliminate maternal and placental factors and to study the direct effects of ethanol or acetaldehyde in known concentrations at specific stages of development. It is also possible to label the embryo in vitro at an early stage of its development (9 and 10 days' gestation) and thus study the effects of teratogens on DNA synthesis. In the in vivo system radiolabelling of DNA has been achieved only after 10 days' gestation (Atlas et al, 1960), and again maternal and placental factors play a role in the uptake of label.

The main disadvantage of the in vitro system is that it is possible to culture the embryos only over a limited period of their development, with acute doses of the test substance. Long term studies are not feasible, and as the system itself is artificial it is remote from the human situation. However the in vitro method has merit in that it provides information which would be difficult to obtain using conventional in vivo models or clinical studies, and it can be a valuable tool in teratogenicity screening tests.

It is worth noting that use of an in vitro embryo culture model in combination with an in vivo model overcomes most of the limitations of each system. Following up this advantage I applied both techniques in parallel studies on C3H mice.

Using the in vivo model with long term dosage of ethanol, I found that even the lowest doses studied produced adverse effects on mouse fetuses. The harmful effects increased in severity with increasing dosage, and occurred in spite of good maternal

nutrition as evidenced by maternal weights at the time of sacrifice (there were no significant weight differences between ethanol-treated and control mice). Chronic dosage of acetaldehyde did not produce any adverse effects on the fetuses or mothers, other than significant reduction in placental weights. It is likely that most of the acetaldehyde was oxidised in the placenta and that the amounts reaching the embryo were not sufficient to cause any obvious teratogenic effects.

The Fetal Alcohol Syndrome appears to be the result of exposure throughout prenatal development, therefore intermittent exposure during a limited period of development would be expected to cause only a part of the total syndrome. While evidence from the mouse supports this expectation, human data on the effects of binge drinking is scarce (Chernoff, 1979). My mouse experiments with the acute "binge" dosage of ethanol did not produce statistically significant harmful effects on the fetuses although the placental weights were affected. This lack of adverse effects should be viewed with caution as only four days during gestation and one dose of ethanol were tested. The in vitro acetaldehyde work on 8-day and 9-day embryos indicated that timing of exposure may be of critical importance. Further studies, both animal and clinical, are needed to determine the effects of intermittent ethanol exposure on prenatal development.

The in vitro results were particularly interesting. The failure of ethanol to produce any teratogenic effects on the embryos except at dose levels which would be highly toxic - if not lethal - in the in vivo situation, indicates that acetaldehyde may well be the teratogen in the F.A.S. The finding that acetaldehyde given at 8 days' gestation was very toxic to the embryos even in the smallest dose studied adds weight to this argument. The effect of acetaldehyde was very time-specific, with little evidence of toxicity in embryos treated at 9 days' gestation.

In both the ethanol and acetaldehyde studies in vitro, the developing nervous system appeared to be particularly sensitive, and inhibition of DNA synthesis correlated well with the development of central nervous system abnormality. It may be

possible to demonstrate this relationship between reduced DNA synthesis and abnormal nervous system development using other neurotoxic drugs, and, if the correlation is consistent, to use this as a screening test for new drugs which have central nervous system effects.

Our attempt at quantitating DNA synthesis by means of auto-radiography produced inconclusive results. I believe this was largely due to three factors: the variability of uptake of the label, the small number of embryos studied in each group, and the technical difficulties encountered in preparing comparable sections for grain counting. However this work showed that the embryos were capable of incorporating tritiated thymidine into the cells and that uptake of label occurred in a variety of tissues (heart, brain and somites). It is reasonable to assume that incorporation of tritiated thymidine indicates active synthesis of new DNA. However Atlas et al (1960) have pointed out that the grain count, in the absence of dilution by division, reflects only the rate of incorporation of tritiated thymidine into the nucleus, if the availability time of tritiated thymidine is constant (as it was in my study). With varying nuclear sizes, differing distances from nuclear source to film, and the possibility of dilution of label by cell division, even the crudest interpretation based on grain counts may be suspect. These researchers were also unable to find any correlation between uptake of label and state of differentiation of the embryo in general.

From my own experiments and study of the literature I would agree with Chernoff's statement (1979):

> An understanding of the methods by which alcohol induces teratogenesis is important from a preventative point of view. By understanding the biochemical basis for initiation of teratogenesis, it should be possible to learn the basis for the difference in susceptibility observed in the syndrome. Knowing the basis of this difference, a quantitative test could be developed, allowing a woman to test her individual susceptibility of having a child with the syndrome before she conceives.....

Rapidly advancing technology and research methods should make this statement a reality in the near future.

The in vitro whole embryo culture system lends itself to more sophisticated analysis than I have used. The protein mapping techniques of Klose (1975) and the technique of cutting ultrathin frozen sections of unstained unfixed material described by Appleton (1978), in conjunction with X-ray micro-analysis, auto-radiography and biochemistry may well provide more answers concerning the mechanisms of normal embryonic development and the effect of teratogens upon it.

Recent clinical and animal studies in vitro and in vivo have contributed substantially to the body of knowledge already in existence. From my findings I would postulate that the action of ethanol on the fetus is an indirect one, mediated by acetaldehyde, and that acetaldehyde is the true teratogen in the F.A.S. Other investigators, using different experimental techniques, have also implicated acetaldehyde, for instance O'Shea and Kauffmann (1979), and Veghelyi and Osztovics (1978).

There is still much work to be done, but cause for satisfaction does exist, as we have progressed along the road to understanding the pathogenesis of the Fetal Alcohol Syndrome. It will be important in future to focus on those approaches which have proved most reliable in studying the syndrome. In this regard application of the in vivo and in vitro models in combination, using the drug, and where possible, its main metabolite, offers great potential.

APPENDIX 1

AUTORADIOGRAPHY METHOD

1. Embryos cultured in vitro as described in Chapters 5 and 6 were used in this study. At least five embryos from each group were set aside for autoradiography and in addition there were ten unlabelled autoradiography controls (five embryos explanted at eight days' gestation and cultured for twenty-eight hours and five embryos explanted at nine days' gestation and cultured for twenty-eight hours).

2. The embryos were fixed in acetic ethanol (5% glacial acetic acid in absolute ethanol) for one hour and then placed in 6% saline buffered formalin where they were kept until dehydration and embedding could be done. The embryos were dehydrated in:-
50% ethanol for 30 minutes
70% ethanol for 30 minutes
90% ethanol for 30 minutes
95% ethanol for 30 minutes
100% ethanol for 1 hour (Appleton, 1972).

After dehydration the embryos were embedded in Spurr's resin using the following regime:-
pure acetone for 1/2 hour
2 parts acetone 1 part resin for 1 hour
1 part acetone 1 part resin for 1 hour
pure resin for $\pm$ 4 hours
They were then embedded in gelatin capsules and polymerised at 70°C overnight (Geurnicke, 1981).
The resin embedding medium contained:
E.R.L. 4206 - 10 grams
D.E.R. 736 - 6 grams
N.S.A. - 26 grams
S-1 - 0.4 grams

3. Preparation of the glass slides

Microscope slides supplied by T and C Scientific Supplies (Pty) Ltd. were used. These showed negligible background activity in the autoradiographs. The slides were washed, rinsed thoroughly in distilled water and finally stored in 100% ethanol. Before use the slides were dried and subbed with egg albumin composed of egg white 10mℓ, glycerol 10mℓ and water 10mℓ which had been filtered using the millipore filtration system described in section 5.3., and stored at 4°C until used.

4. Preparation of autoradiographs

The autoradiographs were prepared in a fully equipped darkroom. The dipping method was chosen (Rogers, 1979) and Ilford K_2 emulsion used. The emulsion was prepared in a 1:1 solution with distilled water and maintained at 45°C. The slides were dipped, drained and allowed to dry overnight in the dark room, then packed in lightproof boxes and stored at 4°C for a minimum of three weeks.

The autoradiographs were developed according to the following schedule: the slides were immersed in developer for 2 minutes, in the stop bath for 15 seconds, in the fixer for 4 minutes, and finally washed in running tap water for one hour.

5. Staining procedure

The slides were stained using Giemsa's stain (Dacie and Lewis, 1975) as follows:
they were placed in phosphate buffer for 30 minutes at 60°C, then immersed in Giemsa's stain (1:10 solution with buffer) for 10 to 15 minutes at 60°C and rinsed in distilled water. The slides were allowed to dry and cover slips were attached using Pe X mounting medium.

6. Grain counting

The stained sections were observed under oil immersion and the number of black grains per square centimetre were counted. Each square centimetre comprised a grid of one hundred squares. This was done in the head region, the heart and the somites.

The following rules were applied:-

a. Eight-day embryos.

Head area: densely stained tissue was counted usually in the forebrain or neural fold. Randomly selected areas were counted and if there was not sufficient tissue available the counts were corrected to three hundred squares (see fig. A1).
Heart: the whole heart was counted.
Somites: the number of grains in two whole somites was counted (see fig. A2).

b. Nine-day embryos.

Head area: the densely stained tissue in the region of the myelencephalon, metencephalon or telencephalon was counted. This included the marginal, mantle and ependymal cell layer but not the mesenchymal and ectodermal areas. Three areas - three hundred squares were counted.
Heart: randomly selected areas were counted to a total of one hundred squares.
Somites: the total number of grains in two whole somites was counted.

The grains were counted in an attempt to quantitate the uptake of tritiated thymidine in the various regions, and to determine whether ethanol or acetaldehyde significantly reduced the uptake in any particular area. It would be reasonable to expect, in view of my findings in sections 6.3.1. and 6.3.2. that uptake would be reduced in the head areas of treated embryos. Unfortunately there was such a large variation in counts in all the sections (both in the treated embryos and the controls) that it proved impossible to produce any sort of meaningful result although statistical tests were applied.

7. Results. (see tables A1 and A2).

The Fisher Exact Probability Test was used and the test and control embryos divided up, for each region assessed, into those embryos with counts less than ten and those with counts greater than ten for a comparable area.

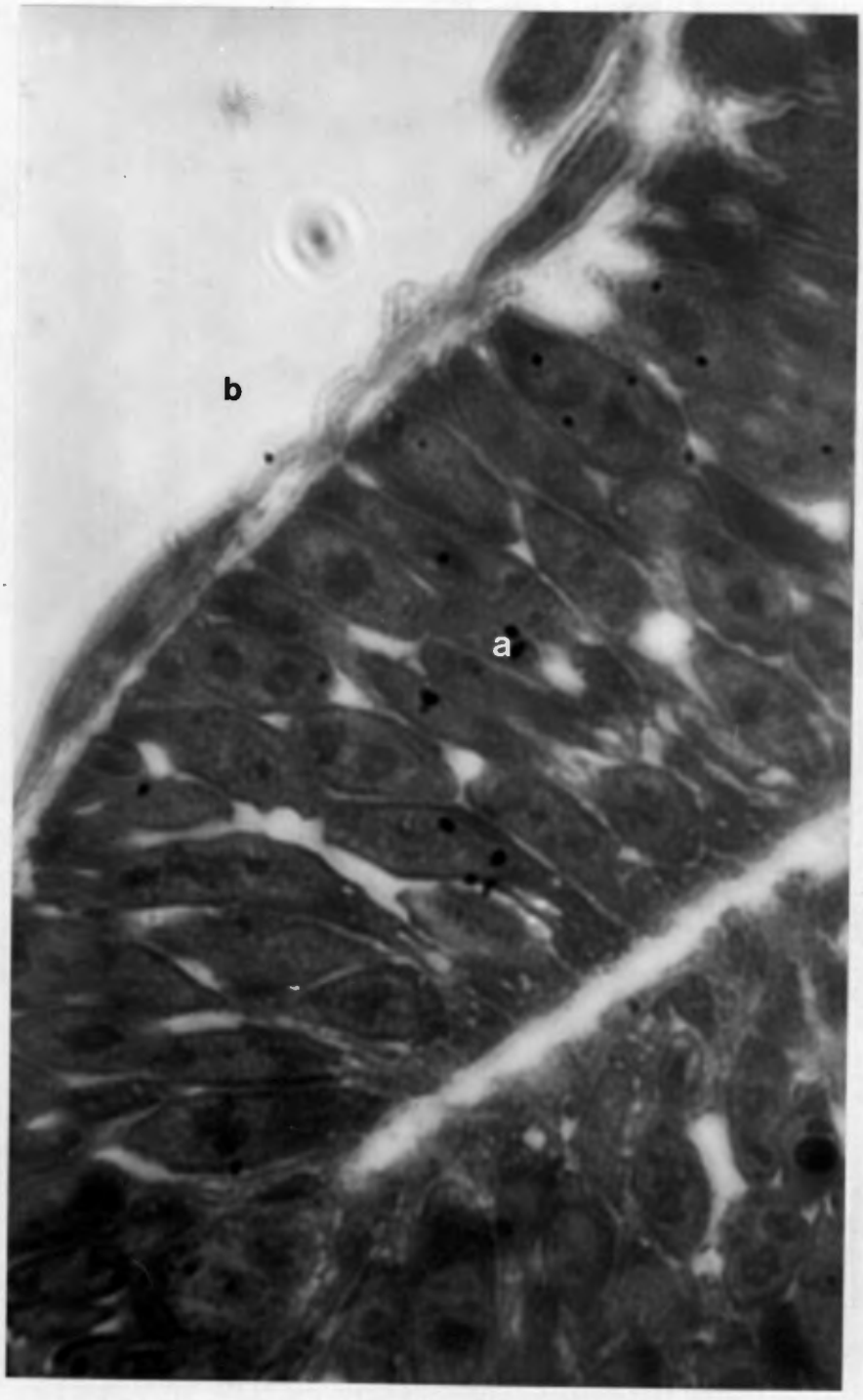

Figure A1.

Autoradiograph of brain tissue from an embryo explanted at 8 days' gestation and cultured for 28 hours in rat serum containing ethanol 1500mg/ℓ and labelled with tritiated thymidine.

Note - silver grains (a)

- background free of label (b)

Mag. x 162.5

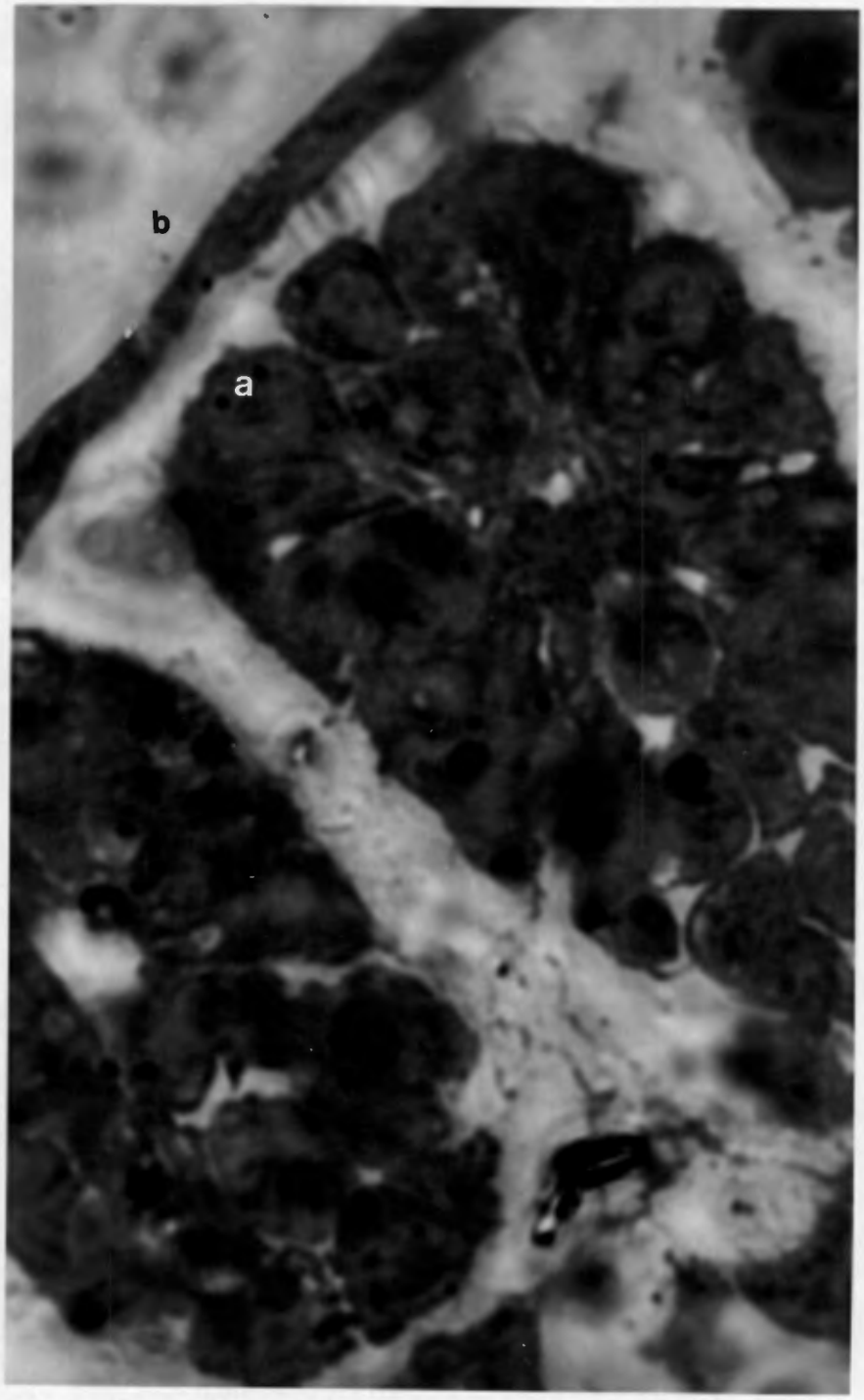

Figure A2.

Autoradiograph of somite tissue from an embryo explanted at 8 days' gestation and cultured for 28 hours in rat serum containing ethanol 1500mg/ℓ and labelled with tritiated thymidine.

Note - silver grains (a)

- background free of label (b)

Mag. x 162.5

TABLE A1

Results of grain counts in embryos explanted at eight days' gestation and cultured for 28 hours.

Head area: Counts corrected to 300 squares.

Heart: Whole heart counted. – means that the heart was not visible on the section.

Somites: Two whole somites were counted. The number of the somite counted is shown in brackets. If designated (a) or (b) it means that the number of the somite could not be determined because the section was incomplete. - means that no somites were visible in the section.

Embryo treatment	Counts		
	Head	Heart	Somite
Control	116	150	(1) 12 (3) 22
Control	14	–	–
Sterile Water Control	3	0	–
Ethanol 1 500mg/ℓ	40	2	(1) 2 (3) 1
Ethanol 1 500mg/ℓ	160	0	(1) 12 (3) 12
Ethanol 1 500mg/ℓ	116	74	(2) 16 (3) 26
Ethanol 3 000mg/ℓ	32	53	(1) 15 (3) 23
Ethanol 6 000mg/ℓ	1 369	112	(2) 153 (6) 155
Ethanol 6 000mg/ℓ	2 126	160	(1) 142 (2) 65
Ethanol 6 000mg/ℓ	9	–	–
Acetaldehyde 7.4mg/ℓ	130	10	–
Acetaldehyde 7.4mg/ℓ	48	7	(2) 31 (3) 18
Acetaldehyde 7.4mg/ℓ	6	3	(a) 2 (b) 1
Acetaldehyde 7.4mg/ℓ	8	0	(a) 0 (b) 2
Acetaldehyde 19.7mg/ℓ	102	6	–
Acetaldehyde 19.7mg/ℓ	408	50	–

TABLE A1 (Continued)

Embryo treatment	Counts		
	Head	Heart	Somite
Acetaldehyde 19.7mg/ℓ	7	2	-
Acetaldehyde 19.7mg/ℓ	4	1	-
Acetaldehyde 19.7mg/ℓ	0	0	-
Acetaldehyde 39.4mg/ℓ	0	-	-

TABLE A2

Results of grain counts in embryos explanted at nine days' gestation and cultured for 28 hours.

Head area: 300 squares counted.

Heart: 100 squares counted. - means that the heart was not visible on the section.

Somites: Two whole somites were counted. The number of the somite counted is shown in brackets. If designated (a) or (b) it means that the number of the somite could not be determined because the section was incomplete. – means that no somites were visible in the section.

Embryo treatment	Counts		
	Head	Heart	Somite
Control	53	5	-
	7	0	(a) 0 (b) 3
	11	0	(a) 0 (b) 0
	39	9	(a) 1 (b) 0
	45	0	(a) - (b) -
Autoradiography Control	13	10	(a) 30 (b) 20
Ethanol 1 500mg/ℓ	537	167	(a)177 (b)139
	1	0	(a) 3 (b) 0
	426	220	(a)139 (b)172
	17	35	(a) 60 (b) 30
	204	12	(a) 14 (b) 12
Ethanol 3 000mg/ℓ	124	-	-
	32	17	(3) 20 (4) 32
	82	17	(a) 30 (b) 36
	49	-	(a) 2 (b) 0
	43	5	(a) 9 (b) 7
Ethanol 6 000mg/ℓ	117	53	(b) 64 (9) 46

TABLE A2 (Continued)

Embryo treatment	Counts		
	Head	Heart	Somite
Ethanol 6 000mg/ℓ	153	75	(5) 185 (6) 82
	6	12	(a) 2 (b) 4
	10	9	(a) 0 (b) 0
Acetaldehyde 7.4mg/ℓ	0	0	-
	0	0	-
	0	0	(a) 0 (b) 0
	0	0	(a) 0 (b) 0
Acetaldehyde 19.7mg/ℓ	27	1	-
Acetaldehyde 39.4mg/ℓ	4	1	-
	33	2	(a) 30 (b) 3
	71	18	(a) 4 (b) 4

Findings.

Eight-day embryos

There were no significant differences between test and control embryos in any of the areas counted.

Nine-day embryos.

Acetaldehyde 7.4mg/ℓ caused a decrease in the head area counts $p = 0.015$.

Ethanol 1500mg/ℓ caused an increase in the heart counts $p = 0.045$ and an increase in the somite counts $p = 0.002$.

Ethanol 3000mg/ℓ caused an increase in the somite counts $p = 0.023$.

Ethanol 6000mg/ℓ caused an increase in the heart counts $p = 0.024$ and an increase in the somite counts $p = 0.023$.

As can be seen from tables A1 and A2 the numbers of embryos in each group were very small and it seems that no real significance can be attached to these findings. There is certainly no correlation between these results and the uptake of tritium measured by liquid scintillation counting or the level of development (assessed morphologically) attained by the embryos in the different groups. This failure of correlation between uptake of label measured by autoradiography and levels of morphological development attained was also shown by Atlas et al (1960:171).

8. Problems encountered

(i) There was a very high failure rate in the preparation of sections, due to loss of embryos or damage during dehydration and embedding, or failure to produce comparable sections. In some groups no usable slides were produced.

(ii) Orientation in the resin proved to be very difficult because of the fragility of the embryos.

(iii) Although we attempted to cut sagittal sections at all times this was not easy as many of the embryos were in the process of turning (altering their curvature from the ventral surface arched outwards to the dorsal surface arched outwards) and thus had a corkscrew shape.

(iv) It was not possible to count exactly comparable areas in test and control embryos because of the different levels of development achieved. This was particularly true of the eight-day acetaldehyde treated embryos which were so malformed that obtaining even one good section was difficult.

9. CONCLUSIONS

Although uptake of label was good and the preparation of the autoradiographs successful in that there was little background activity, for the purpose of quantitating tritiated thymidine uptake and correlating it with morphological development in eight- and nine-day mouse embryos in vitro, this method was unsuitable. However it was useful in that it showed that the label was taken up intracellularly and that there was little background activity. This indicates that the results obtained by liquid scintillation counting were real, probably not due to contamination or adsorbed label.

APPENDIX 2.

STATISTICAL TESTS USED.

The following tests were used as applicable in this study:

1. Chi^2 Test (Segal, 1956a).
2. Fisher Exact Probability Test (Segal, 1956b). This test was used in place of the Chi^2 Test if the smallest expected frequency was less than five.
3. Mann-Whitney Test (Segal, 1956c).
4. One Way Analysis of Variance Test with multiple comparisons among means (Rimm et al, 1980).
5. Two Sample t Test - unpaired - (Hine and Wetherill, 1975).

All p values quoted were for 2 tailed tests and a p value of less than 0.05 was regarded as statistically significant.

All the calculations except the Chi^2 Test were carried out using a Hewlett-Packard desk top calculator model HP9825A. For the Chi^2 Test I used a programmable Hewlett-Packard calculator model HP33E.

APPENDIX 3

EQUIPMENT AND MATERIALS USED, AND SUPPLIERS

Equipment and materials mentioned in Chapter 2.

C3H and ICR mice	U.C.T. Animal Unit H.D.O.T. Building U.C.T. Medical School Anzio Road Observatory 7925 R.S.A.
Vermiculite	U.C.T. Animal Unit H.D.O.T. Building U.C.T. Medical School Anzio Road Observatory 7925 R.S.A.
Epol rat cubes	Epol (Pty) Limited Animal feed manufacturers P O Box 497 Cape Town 8000 R.S.A.
Biocide "D" detergent disinfectant	Biocide Products P O Box 208 Maitland Cape R.S.A.
Dial-O-Gram Scale Manufactured by Ohaus Scale Corporation	Laboratory stock - supplier not known.
Sobee and Nutrament T	Bristol Mead Johnson Pharmaceutical Division of the B-M Group P O Box 2515 Johannesburg 2000 R.S.A.

Feeding tubes	Hagu (Pty) Limited Representing Labotec (Pty) Ltd P O Box 773 Cape Town 8000 R.S.A.
Alizarin Red S. Manufactured by: B.D.H. Laboratory Reagents B.D.H. Chemicals Ltd, Poole, England	Hickman & Kleber (Pty) Ltd P O Box 83 Bellville 7530 R.S.A.
Wild M5. dissecting microscope	Wild & Leitz R.S.A. (Pty) Ltd P O Box 1135 Rhine Road 8050 R.S.A.

Additional equipment and materials mentioned in Chapter 3.

Sartorius chemical balance	Zeiss West Germany Optical Instruments (Pty) Ltd. P O Box 4051, Cape Town. 8000. R.S.A.
Ethanol (Analytical grade)	T and C Scientific Supplies P O Box 2953 Cape Town 8000 R.S.A.

Additional equipment and materials mentioned in Chapter 4.

Acetaldehyde. (Analytical grade).	T. and C. Scientific Supplies (Pty) Ltd. P O Box 2953, Cape Town, 8000. R.S.A.
Nikon M-35A camera	Research Instrumentation (Pty) Ltd P O Box 19 OBSERVATORY 7935 R.S.A.

Additional equipment and materials mentioned in Chapter 5.

Glass test tubes	Made by the University glass-blower Dept. of Physical Chemistry University of Cape Town University Private Bag Rondebosch 7700 R.S.A.
24/29 Quickfit stoppers	Hickman & Kleber (Pty) Ltd P O Box 83 Bellville 7530 R.S.A.
Silicone stopcock grease Manufactured by: Dow Corning Corporation Midland, Michigan, U.S.A.	Laboratory stock.

Rotator-incubator	Dept. of Biomedical Engineering University of Cape Town 33 Mostert Road Observatory 7925 R.S.A.
Temperature controller	Bridge Designs P O Box 117 Newlands 7725 R.S.A.
Laminar flow hood	Hagu (Pty) Limited Representing: Labotec (Pty) Ltd P O Box 773 Cape Town 8000 R.S.A.
Millipore filters	Millipore S.A. (Pty) Ltd P O Box 1561 Bellville 7530 R.S.A.
B.D. Sterile vacutainers Manufactured by: Division of Becton, Dickenson & Co. Rutherford, New Jersey	Research Surgical (Pty) Ltd P O Box 19 Observatory 7935 R.S.A.
5% CO_2 in Air	Afrox Limited P O Box 332 Cape Town 8000 R.S.A.
Tritiated thymidine	Weil Organisation (Pty) Ltd P O Box 298 Claremont 7735 R.S.A.

Soluene 350	Packard Instruments (Pty) Ltd P O Box 400 Bellville 7530 R.S.A.
Dimilume 30	Packard Instruments (Pty) Ltd P O Box 400 Bellville 7530 R.S.A.
Beckman LS8000 Scintillation Counter	Beckman Instruments (Pty) Ltd P O Box 963 Cape Town 8000 R.S.A.

Additional equipment and materials mentioned in Appendix 1.

Spurr's resin	Wirsan Scientific P O Box 31032 Braamfontein Transvaal. R.S.A.
L.K.B. Ultratome 3	Beckman Instruments P O Box 963 Cape Town 8000 R.S.A.
Glass slides	T and C Scientific Supplies P O Box 2953 Cape Town 8000 R.S.A.
Ilford K.2 Emulsion	Ciba Geigy P O Box 224 Cape Town 8000 R.S.A.
Wild M.20 Microscope c counting grid	Wild and Leitz R.S.A. P O Box 1135 Rhine Road 8050 R.S.A.
Kodak D-19 Developer Powder	Kodak S.A. (Pty) Ltd P O Box 735 Cape Town 8000 R.S.A.
Kodak indicator stop bath cat. 3156759	Kodak S.A. (Pty) Ltd - address as above
Kodak unifix powder cat. 9130071	Kodak S.A. (Pty) Ltd - address as above

Zeiss Photomicroscope	Zeiss West Germany Optical Instruments (Pty) Ltd Zeiss Scientific Equipment P O Box 4051 CAPE TOWN 8000 R.S.A.

Except where otherwise indicated all chemicals and reagents used were standard laboratory stock.

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