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ALTERED CARBOHYDRATE METABOLISM IN DIELDRIN-INDUCED
NEONATAL INVIABILITY

by

Ⓢ John Carlo Costella

A Thesis
submitted to the Faculty of Graduate Studies
through the Department of
Biology in Partial Fulfillment
of the requirements for the Degree
of Master of Science at
The University of Windsor

Windsor, Ontario, Canada

1979

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ABSTRACT

Altered Carbohydrate Metabolism in Dieldrin-Induced
Neonatal Inviability

by

John Carlo Costella

Prenatal exposure to dieldrin causes neonatal inviability. Two of the possible causes, altered carbohydrate metabolism and central nervous system hyperstimulation, were studied in CD-1 mice.

The twenty-four hour oral LD₅₀ for dieldrin was measured in 5-day old pups that had been fasted for 24 hrs prior to treatment. The LD₅₀ 27 mg/kg, was 1/5 the maximum dose a fetus could receive at a maternal dose of 2 mg/kg, p.o. daily, from days 6 to 18 of gestation. Pups dying of dieldrin intoxication convulsed and tremored, whereas those prenatally exposed did not. The inviability does not occur as a result of acute toxicity.

Carbohydrate parameters were measured in caesarean-delivered pups at delivery and 19 and 24 hrs later. The dams received dieldrin (2 mg/kg, p.o.) over days 6 to 18 of gestation. Treated pups had similar glycogen concentrations in the liver, skeletal muscle and heart at birth (control levels: 70.99 ± 4.5 mg/g, 6.45 ± 1.11 mg/g, 9.66 ± 1.02 mg; respectively). The rate of depletion however was increased in the treated pups. Liver glycogen was reduced by 38% (controls, 18.84 ± 3.30 mg/g) at 19 hrs and by 74% (controls, 4.53 ± 1.32 mg/g) at 24 hours. Cardiac muscle glycogen increased in the first 19 hours, but there were no differences between groups (control, 11.55 ± 1.29 mg/g). By 24 hours,

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a 50% (control 11.03 ± 1.01 mg/g) reduction was seen in the cardiac glycogen stores of treated pups. Skeletal muscle glycogen decreased slightly in the first 19 hours, but no difference was noted between groups (controls, $4.46 \pm .38$ mg/g). A 50% reduction of muscle glycogen (controls, $5.36 \pm .56$ mg/g) was seen in treated animals by twenty-four hours. Dieldrin induces hypoglycemia. Treated animals showed 30% (controls, 0.95 ± 0.23 mg/ml), 44% (controls, $0.940 \pm .132$ mg/ml), and 100% (controls, $.322 \pm .100$ mg/ml) reductions of blood glucose at birth, 19 and 24 hrs, respectively. Prenatal dieldrin did not affect blood lactate, pyruvate, or urea, or liver lactic dehydrogenase or fructose-1,6-dephosphatase.

The dieldrin-induced inviability is not caused by CNS stimulation. The condition, however, is associated with an enhanced carbohydrate utilization characterized by hypoglycemia, and increased glycogenolysis. Prenatal dieldrin does not appear to affect gluconeogenesis.

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INTRODUCTION

The chronic ingestion of low doses of organochlorine insecticides can affect mammalian reproduction in three ways: a) decreased fertility, b) decreased fecundity, and c) increased neonatal mortality.

The latter is the most important effect. Effects on fertility and fecundity only occur at doses which cause mortality of the pregnant females themselves; in contrast, increased neonatal mortality occurs at much lower doses. For example, dietary dieldrin, at 5.0 ppm, significantly decreases the number of mouse pups surviving to weaning; fertility and fecundity, however, are affected only at 20 and 25 ppm, where there is considerable female mortality (Virgo and Bellward, 1975). Similar results were obtained by Clement and Okey (1974), who showed that o,p'-DDT reduced rat fertility only at doses approaching the acutely toxic level.

Increased neonatal mortality at low doses of insecticide has also been shown for DDT (Fahim et al., 1970; Gilbert, 1969; Aulerich et al., 1971), dieldrin (Murphy and Korschgen, 1970; Aulerich et al., 1971; Clark et al., 1978), endrin (Morris, 1968), kepone (Huber, 1965), methoxychlor (Harris et al., 1974) and mirex (Ware and Good, 1967). As a point of interest, Aulerich and Ringer (1977) have found that polychlorinated biphenyls increase kit mortality in ranch mink.

Virgo and Bellward (1977) have shown that within the litter of a dieldrin-fed mouse the pups are affected in utero such that their viability as neonates is decreased. This inviability has been shown to be an "all or none phenomenon," in that either all pups die or a

normal number survive (Virgo and Bellward, 1975). Even when foster-nursed, the inviability was found to persist. Although proximate causes of mortality exist, i.e., pup killing and pup neglect (Virgo and Bellward, 1975) they can only alter the rate at which the predestined mortality occurs, they cannot alter the final outcome, that is death. Thus, the most important cause of pup mortality is neonatal inviability.

No studies have yet been undertaken with regard to the mechanism of this inviability. One possible cause, may be stimulation of the central nervous system (CNS), since dieldrin is a CNS stimulant, as are most of the organochlorine insecticides. However, unlike DDT, which causes a marked stimulation of the sympathetic nervous system that leads to respiratory depression and hyperthermia (Henderson and Woolley, 1970), dieldrin activates the parasympathetic system causing vasodepression and bradycardia (Gowdey, 1954) that leads to circulatory collapse and death.

Backstrom et al. (1965) demonstrated that dieldrin crosses the placenta, and Hathaway et al. (1967) found that dieldrin is secreted from the uterine walls and absorbed by free and implanted blastocysts. DDT (Hart et al., 1972) and polychlorinated biphenyls (Masuda et al., 1978) have been shown to cross the placenta and enter the fetus. Polishuk et al. (1977) found the concentration of total DDT, dieldrin and heptachlor epoxide is higher in fetal blood, and the placenta, than in maternal blood. It is apparent, therefore, that fetuses can be exposed to organochlorine insecticides in utero.

It is possible then, that the pups of dieldrin treated mice are poisoned by the insecticide. Several workers have determined the acute toxicity of

organochlorine insecticides in neonates. Henderson and Woolley (1969) found that 728 mg/kg was the LD₅₀ for 10-day old rats treated with DDT; in comparison the LD₅₀ for 60-day old rats was only 250 mg/kg. These results agree with those of Lu et al. (1965) who studied DDT and dieldrin in newborn rats. With dieldrin, the LD₅₀ for a newborn rat (under 24 hrs of age) was 168 mg/kg as compared to 37 mg/kg for the adult animal. The LD₅₀ for DDT was greater than 4,000 mg/kg in newborns, as opposed to 195 mg/kg in the adult. Hudson et al. (1972) found a similar pattern in mallard ducks exposed to various organochlorines. Henderson and Woolley (1970) postulated that this decreased sensitivity is due to an innate insensitivity of the neonatal CNS coupled with a reduced ability to extract the compound from the plasma; the latter effect is due to the low lipid concentrations in the neonatal central nervous system.

Another possible cause of the inviability is disrupted carbohydrate metabolism. Carbohydrate metabolism plays an extremely important role in energy production during the perinatal period.

The mammalian fetus receives a constant supply of glucose from the mother. At the time of birth however, this supply is cut off and the neonate must use endogenous sources of energy until the processes of lactation and digestion are established.

Normal carbohydrate metabolism in the perinatal period has been reviewed by Dawes and Shelley (1968), Ballard (1970), and Adam (1971). In the last days of gestation, the fetus accumulates large amounts of glycogen in various tissues, especially the liver, the skeletal musculature, and the heart. At birth, there occurs a marked hypoglycemia and hypoxia which stimulates the rapid mobilization of liver glycogen,

and the utilization of the other stores. Simultaneously, changes are seen in the enzymes associated with glycogen metabolism, i.e., glycogen synthetase and phosphorylase. Thus, glycogen serves as an immediate energy source in this critical period; it seems probable that if its storage or mobilization is disrupted, the normal development of the neonate could be impaired.

Disruption of glycogen storage is known to be affected by chemical agents. Maternally administered pentobarbital decreases the levels of glycogen in the fetal liver (Delphia et al., 1967), and in fetal liver (Delphia and Singh, 1972). More recently, the same effect has been found in fetal liver with maternally administered epinephrine (Delphia, 1975). Although the liver glycogen levels are important for the maintenance of blood glucose, cardiac glycogen may be even more crucial to survival. Hoerter (1976) showed that maintenance of contractile and intracellular activity in fetal rabbit heart was directly related to the glycogen levels in this tissue. In fact, it has been shown that the ability of the neonatal animal (Dawes and Shelley, 1968) to withstand hypoxia is directly related to the glycogen stores in the heart. Should dieldrin affect either the storage or the mobilization of glycogen in neonates, the alterations could precipitate cardiac failure and circulatory collapse.

Glycogen metabolism is affected, by organochlorine insecticides, in adult livers. Kacew and Singhal (1973) showed that prolonged administration (45 days) of sublethal amounts of various insecticides, i.e., DDT, α -chlordane, heptachlor and endrin, caused a marked fall in liver glycogen in male rats. Similar results were also found with a

single, acute dose of p,p'-DDT (Kacew and Singhal, 1973a). However, Bhatia et al. (1973) have found an increase in liver glycogen, stimulation of glycogen synthetase, and inhibition of glycogen phosphorylase following a single, acute dose of dieldrin. The discrepancy may be attributable to the compound used in these studies since animals and route of administration were identical in both cases. There have been no conflicting reports with regard to chronic, low dose treatment. All studies report a decrease in liver glycogen following chronic administration of an insecticide. Singhal and Kacew (1976) believe that the effects, seen on carbohydrate metabolism, are a result of modulation, by the insecticides, of the cyclic AMP-adenylcyclase-protein kinase system.

Despite the large amount of glycogen found in the liver at birth, these stores cannot maintain blood glucose for more than six to twelve hours as this is when the liver glycogen levels are practically exhausted as has been demonstrated in neonatal rat (Gain and Watts, 1976) and neonatal pig (Elliott and Lodge, 1976). In fact, blood glucose levels will begin to fall even in the presence of liver glycogen. Gluconeogenesis has been shown to be the most important process for glucose homeostasis in the neonatal period; its inhibition causes marked hypoglycemia in suckling rats (Ferre et al., 1977).

The liver and the kidney are the principal tissues involved in gluconeogenesis and potential precursors include lactate, glycerol, and the so-called glucogenic amino acids. Both lactate, as a result of, anaerobic metabolism, and amino acids have been shown to rise in the immediate postnatal period (Girard et al., 1975; Snell and Walker, 1973).

It is possible that these serve as the principal substrates since some animals, such as rats and mice, have low, endogenous triglyceride stores (Hahn, 1970) and are therefore unlikely to have enough glycerol for gluconeogenic purposes. However, as nursing commences, the glycerol obtained via the milk could then become a major substrate for gluconeogenesis. Should dieldrin exposure prenatally affect gluconeogenesis, it would ultimately impair the glucose homeostasis of the newborn.

Organochlorine insecticides have been shown to affect gluconeogenesis in adult animals. Kacew and Singhal (1972, 1973, 1973b) have demonstrated that exposure to both acute and chronic doses of various organochlorine insecticides markedly increases the activities of the four, rate-limiting enzymes of the gluconeogenic pathway (phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase (PC), fructose-1, 6-diphosphatase (FDPase) and glucose-6-phosphatase (G-6-Pase)). Bhatia et al. (1973) also found that the activation of PEPCK, PC, and FDPase occurred following acute exposure to dieldrin; however, the activity of G-6-Pase was significantly reduced. Story and Freedland (1978) have studied the effects of DDT on gluconeogenesis in hepatocytes isolated from starved rats. They demonstrated that DDT had no effect on gluconeogenic rates from glycerol, alanine and fructose but it decreased gluconeogenesis from lactate; this was attributed, in part, to lowered PEPCK activity in DDT treated hepatocytes.

As mentioned previously, lactate and amino acids serve as the main precursors for gluconeogenesis. Blood urea, an indicator of amino acid metabolism, was found to be elevated after chronic exposure

to various organochlorine insecticides (Karew and Singhal 1973, 1973b) indicating an increased amino acid catabolism, presumably for gluconeogenic purposes. In the gluconeogenic scheme, lactate is converted to pyruvate by way of lactate dehydrogenase (LDH) and the pyruvate continues on to glucose. It has been shown that LDH activity can be inhibited both in vivo and in vitro by mirex (Abston and Yarbrough, 1974; Hendrickson and Bowden, 1975; Abston and Yarbrough, 1976; Robinson and Yarbrough, 1978); kepone (Hendrickson and Bowden, 1975); and by dieldrin, aldrin, endrin, and chlordane (Hendrickson and Bowden, 1976). The latter study showed that for these cyclodiene compounds, those possessing an epoxide group (i.e., dieldrin and endrin) were twice as inhibitory compared to their non-epoxide analogue.

It is apparent that carbohydrate metabolism plays a crucial role in the period between birth and suckling. Energy deficiencies during this period could disturb developing systems and ultimately cause the death of the animal. Such deficiencies could arise directly, from inadequate stores of glycogen or from an altered utilization of the stores. Furthermore, inviable pups may have deficient quantities of gluconeogenic precursors or they may be unable to metabolize these.

This study was undertaken with the following specific goals in mind:

- 1) to determine the acute toxicity of dieldrin in neonatal mice; and
- 2) to determine the effect of dieldrin, ingested by the pregnant female, on neonatal carbohydrate metabolism.

MATERIALS AND METHODS

A - General Materials and Methods

The mice were of the CD-1 strain and were purchased at 8-10 weeks of age from Canada Breeding Farms and Laboratories (St. Constant, Quebec). The timed-pregnant animals were nulliparous. The mice were caged in pairs, except for those giving birth which were housed singly. The animals were kept under a constant photoperiod of 14 hours of light (lights on at 0600 hrs) in quarters maintained at 21°C. Bedding material was hardwood shavings ("Beta Chip," Northeastern Products, Warrensburg, N.Y.). Purina Lab Chow and tap water were provided ad libitum.

All chemicals used throughout this study were analytical reagent (AR) grade or better. The reader is directed to Appendix A for information on the chemicals and suppliers. Technical dieldrin,* a gift of Shell Canada (Toronto), was administered in corn oil by oral intubation. Animals received a daily dosage of the test solution in a volume which ranged from 25-60 μ l. Control animals received a corresponding volume of corn oil.

B - Acute Toxicity of Dieldrin to Neonates

All animals used in this experiment were produced in a breeding colony of CD-1 mice maintained in our animal quarters. Virgin females

* Technical Dieldrin-86.1% HEOD- 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene.

(8-10 wks.) were housed in pairs with fertile males and inspected daily at 0800 hrs. and at 1600 hrs. for copulation plugs. Females with plugs were housed singly and allowed to gestate without further treatment. Dams were permitted to deliver naturally and nurse their litters until day 4 (day 0 equals birth date) postpartum at which time the pups were isolated from the female. Preliminary studies showed that neonates of 5 days of age or greater had to be employed to avoid deaths from mechanical traumatization to the esophagus. Thus, dieldrin was administered 24 hrs after isolation in 10 μ l of corn oil, via a 24 gauge stainless steel feeding tube attached to a microliter syringe. The pups were allowed to suckle on the feeding tube until the whole volume was at least within the oral cavity. The mortality was then recorded at 24 hr intervals until complete mortality was seen.

Pups from each litter were divided equally among the different dosage groups thereby minimizing any differences in sensitivity between litters. Range finding experiments indicated that the LD₅₀ was approximately 30 mg/kg. Thus, the final experiment utilized 7 doses of the pesticide- 2, 25, 37.5, 50, 62.5, 75, and 250 mg/kg. Between 60-80 pups were treated at the various doses. The mortality at 24 hrs was used to calculate the lethal dose (LD₅₀) according to the method of Litchfield and Wilcoxon (1949).

C - Dieldrin-Induced Neonatal Inviability

This study employed 40 timed-pregnant mice which were divided into five equal groups. One group served as controls, receiving corn oil, while the other four received dieldrin: 0.5, 1.0, 2.0, or 4.0 mg/kg.

Treatment began on day 6 of pregnancy and continued, daily, until day 18. The animals were allowed to deliver and the litters isolated, from the females, at birth. Pregnant females were checked every 8 hours for litters. The isolated pups were kept in the animal quarters at 21°C and the mortality recorded every 24 hours until 100% mortality had occurred at all doses.

Female body weight on day 6 and on day 19 was recorded. The female was decapitated on day 19, the liver excised, blotted dry and weighed. Litter sizes were also recorded.

D - Factors Affecting Pup Mortality

1) Temperature

Twenty timed-pregnant CD-1 mice were divided into two equal groups. One group served as controls, receiving corn oil, while the other was treated with dieldrin (2 mg/kg). The treatment schedule was the same as the inviability study. Females were allowed to deliver naturally and the pups isolated at birth. In this study, however, the animals were placed in an incubator (Blue M Electric Company, Blue Island, Illinois) with a 100-watt incandescent bulb as the heat source. The temperature in the incubator was kept at 25°C. The mortality was recorded at 24 hrs and thereafter at 8 hr intervals, until all pups died. These data were then compared to those obtained at 21°C to determine whether there was an effect of temperature on the mortality of neonates.

2) Mode of Parturition

In the biochemical studies it was necessary to obtain pups of

equivalent age and caeserean-delivery was chosen as the method to achieve this. Thus, the effects of caeserean-delivery on the survival of the neonate had to be determined.

Nine timed-pregnant mice were divided into two groups, one group serving as control and the other receiving 2 mg/kg dieldrin. At 2300 hrs on day 18 the females were decapitated. The uterus was exposed by midline incision and both horns reflected outside the abdominal cavity. Each pup was removed from the uterus and all the extraembryonic membranes. The umbilical cord was cut and the pup blotted dry. Following delivery of the litter, which took less than three minutes, the animals were transferred to clean cages and placed in the incubator at 25°C. Mortality was determined at 1, 12, 19, 24, and 32 hrs post delivery. The mortality was compared to that of naturally delivered pups maintained at 25°C to determine the effects of mode of parturition on mortality.

E - Carbohydrate Metabolism in the Dieldrin-Induced Inviabile (DII)
Mouse Pup

1) General

Animals and treatment regime were as described above. Pregnant females were decapitated and the pups delivered by caeserean section late on day 18, commencing at 2100 hrs. Pups were observed at birth (0 time) to determine the status of animals at this time; at 19 hrs postpartum, which is an arbitrarily chosen time, in which there exists no mortality differences between control and DII pups; and finally at 24 hrs where there exists a significant mortality of inviable pups with respect to controls. At these times the pups were decapitated for biochemical analysis. Pups that were not killed at birth were kept in

clean cages at the appropriate temperature until required. Table I summarizes the parameters that were determined.

TABLE I - THE BIOCHEMICAL PARAMETERS DETERMINED
IN THE INVIABLE NEONATES

<u>Tissue</u>	<u>Parameters</u>
Liver	Glycogen Lactic Dehydrogenase Fructose-1,6-Diphosphatase
Skeletal Muscle (Gastrocnemius)	Glycogen
Cardiac Muscle	Glycogen
Blood	Glucose Lactate Pyruvate Urea

The trunk blood from all decapitated pups in a litter was pooled in order to obtain sufficient quantities for the various determinations. For glycogen determinations, the tissues from three pups, chosen randomly from each litter, were assayed individually and the mean of the three observations used as the value for that litter. This procedure was followed for all of the tissues, except cardiac glycogen, where the ventricles from the 3 pups were pooled to estimate the cardiac glycogen. All tissue weights were recorded in order to express the final results as a fraction of tissue weight. Enzyme determinations were conducted

on liver tissue pooled from three randomly chosen pups. Thus, the sample unit for all determinations was the female (\equiv litter) and not the individual pup.

2) Isolation and Quantification of Glycogen

The isolation and quantification of glycogen was by the method of Seifter et al. (1950).

(i) Reagents and Chemicals

Anthrone reagent (0.2% w/v) was prepared by dissolving 0.2 g anthrone (Fisher) in 100 ml of aqueous 95% H_2SO_4 prepared from AR grade concentrated sulfuric acid (Fisher); 95% and 60% (v/v) solutions of ethanol were also prepared. Standards were prepared, from dextrose (Fisher) as the assay measures the amount of glucose in the reaction medium, in 0.25% benzoic acid (Fisher) so as to prevent bacterial contamination. A saturated solution of Na_2SO_4 (Fisher) and a 30% KOH (w/v) solution were prepared in distilled water.

(ii) Isolation of the Glycogen

Into 17x120 mm conical, Pyrex centrifuge tubes were pipetted 1 ml of 30% KOH and 50 μ l. of saturated Na_2SO_4 solution. The tissues from decapitated pups were excised rapidly, blotted dry and weighed on a Roller-Smith Torsion Balance (Biolar Corp., North Grafton, Massachusetts). Whole livers (50-100 mg) were assayed, pooled ventricles (20-25 mg) were used for cardiac levels, and both gastrocnemii were excised and pooled (5-15 mg) for skeletal muscle determinations. A glass rod was used to transfer the weighed tissues to the centrifuge tubes, after which the

tubes, with the glass rod in place, were placed in a boiling water bath for digestion of the tissues. The time required for digestion (10-30 minutes) was dependent upon the weight of the tissue sample. After digestion, the tubes were cooled to room temperature and to each was added 1.5 ml of 95% ethanol. The resultant precipitate was stirred with the glass rod which was removed after the glycogen had been washed from it with 4 ml of 60% ethanol. The tubes were mixed on a Vortex Genie mixer for 10 sec (setting 6) and transferred to a 75°C water bath for 10 min. Following this, the samples were cooled to allow recrystallization of the glycogen and then centrifuged at 400xg (IEC Model K Centrifuge) for 10 min to precipitate the glycogen. The supernatant was then discarded and the pellet resuspended in distilled H₂O. A sufficient quantity of water was added to the precipitates to bring each to a specific final volume:

liver - 10 ml

skeletal muscle - 5 ml

ventricles - 5 ml

For skeletal and cardiac muscle a 2.0 ml aliquot was used for analysis. The liver solution aliquot ranged from 50 to 500 µl depending upon the glycogen concentration. These aliquots were diluted to 2.0 ml with distilled water for the assay. The 2.0 ml quantities were pipetted into 20x150 mm Pyrex tubes and placed in an ice bath. Four ml of the anthrone reagent were added very slowly to each tube. The samples were swirled while the anthrone was added to ensure proper mixing and cooling of the tubes. The tubes were then placed in a boiling water bath for 10 minutes and then cooled to room temperature. The absorbance of the

samples were then measured against a reagent blank at 620 m μ with a Perkin-Elmer Model 575 Double Beam Spectrophotometer. Unknown concentrations of glycogen were calculated from a standard curve.

3) Determination of Plasma Glucose

Plasma glucose was determined by the method of Kingsley and Getchell (1960).

(i) Reagents

Peroxidase buffer reagent was prepared as follows: 5 mg. horse-radish peroxidase (Sigma) was added to 59 ml of 0.1N NaOH and 125 ml 0.1M KH_2PO_4 , dissolved and the solution diluted to 500 ml with distilled water. To this volume was added 5.0 ml of 1% o-dianisidine (w/v) (Sigma). This reagent was stable for several weeks if mixed thoroughly and kept in a dark bottle at 4°C. Glucose oxidase (Sigma) was prepared daily by adding 100 mg glucose oxidase to 10 ml of water. This was shaken vigorously for 30 sec and the resultant clear liquid was decanted and used for assay. D-glucose standards were prepared in 0.25% benzoic acid and stored at room temperature. Fifty percent sulfuric acid was prepared from reagent grade H_2SO_4 .

(ii) Procedure

The trunk blood from decapitated pups was collected in heparinized capillary tubes. The tubes were sealed and centrifuged in a micro-capillary centrifuge (IEC Model B) for three minutes after which the tubes were fractured at the cell-plasma interface and the plasma was pooled into 6x50 mm culture tubes. The plasma was stored at -20° C until analysis.

Into 13x100 mm test tubes were added 4.5 ml of peroxidase buffer reagent. To each tube was added 20 μ l of plasma; the reagent blank which received any plasma, also received 1.5 ml of 50% H_2SO_4 . The glucose oxidase (0.5 ml) was then added and the tube contents mixed thoroughly. The tubes next were placed in a water bath at 37°C for 30 min after which 1.5 ml of 50% H_2SO_4 were added to stop the reaction. The tubes were placed in an ice water bath to cool. The absorbance of the samples were then measured against the reagent blank at 530 m μ with a double beam spectrophotometer (Perkin-Elmer Model 575). The colour for both the glycogen and glucose assays was stable for hours following incubation. Unknown concentrations were calculated from a standard curve.

4) Determination of Blood Urea

Blood urea was measured according to the method of Jung et al. (1975).

(i) Reagents

O-phthalaldehyde reagent (200 mg/ml): To 800 ml. of water were added 74 ml. concentrated H_2SO_4 . When this was cool, 200 mg o-phthalaldehyde (Sigma) and 1.0 ml of 30% Brij-35 (Fisher) were added. The solution was diluted to 1 l with distilled water. The solution is stable for years at room temperature if stored in dark glass bottles.

N-(1-naphthyl)ethylenediamine (NED) Reagent: To 600 ml of distilled water were added 222 ml conc. H_2SO_4 and 5 g boric acid (Fisher). The solution was allowed to cool and 600 mg of NED dihydrochloride (Sigma) and 1.0 ml of 30% Brij-35. The solution was made up to 1 l to give a final NED concentration of 431 mg/ml. NED

reagent is also stable for years if stored at room temperature in dark glass.

Urea standards were prepared in 5 mM H_2SO_4 and stored at room temperature.

(ii) Procedure

Blood was obtained as described above. Plasma samples were kept frozen until analyzed.

Into 13x100 mm. culture tubes was placed 2.5 ml. of o-phthalaldehyde reagent. To this was added 50 μ l of plasma; the blank received water. The NED reagent, 2.5 ml, was then added and the tubes mixed. The samples were then incubated for 30 min at 37°C after which they were cooled to room temperature and the absorbance measured against the reagent blank at 470 m μ (Perkin-Elmer Model 575). Unknown concentrations were calculated from a standard curve.

5) Determination of Lactate and Pyruvate

Both metabolites were assayed by the method of Olsen (1971).

(1) Reagents and Chemicals

Perchloric acid (Fisher) for blood deproteinization was prepared at a concentration of 5.1% (w/v). Standards of sodium pyruvate (Sigma) and lithium-L-lactate (Sigma) were prepared in distilled water and stored at 4°C. Fresh standards were prepared weekly.

Lactic dehydrogenase (LDH) (Sigma) was prepared in two different concentrations by dilution of the stock solution with 2.2 M ammonium sulfate (Fisher). For lactate determination the concentration of LDH used was 5 mg protein/ml; the pyruvate assay employed LDH at a

concentration of 0.01 mg protein/ml.

The following solutions were prepared just prior to use.

Lactate analysis - 2 mg NAD and 10 μ l LDH (5 mg/ml) per ml. of hydrazine buffer (see Appendix B). Pyruvate analysis - 5 μ l. of 2 mM NADH and 20 μ l LDH (0.01 mg/ml) per ml phosphate buffer (see Appendix B).

(ii) Procedure - Blood and Standard Preparation

Immediately after collection, 200 μ l of whole blood were deproteinized with an equal volume of ice cold perchloric acid (5.1% w/v). Standards were diluted with acid in a similar manner. All tubes (12x75 mm test tubes) were then centrifugated at 3,000xg for 15 min at 4°C (Sorvall Model RC2B). The resulting supernatants were centrifuged again at 3,000xg for 15 min and the final supernatant used in both the lactate and the pyruvate assays. Blood samples were analyzed on the day they were obtained.

(iii) Lactate Quantification

The incubation was conducted in 17x120 mm conical Pyrex centrifuge tubes. To each tube was added 1.0 ml of the NAD-LDH reagent solution. Fifty microliters of the supernatants were then transferred to the tubes, mixed and incubated at room temperature for 30 min. Following incubation the mixture was diluted with 10 ml of 0.07 M HCl. The tubes were mixed, transferred to 13x100 mm test tube and the fluorescence measured with a Turner Model 430 Spectrofluorometer. The excitation wavelength was 340 nm, the emission wavelength was 460 nm; bandwidths were 60 nm and the sensitivity range was $\times 10$ - $\times 30$.

The lactate standard curve was a straight line but the slope varied from day to day. Thus, five standards were run with the unknown samples at all times and the concentration of unknown calculated by the ratios method employing each of the five standards and averaging the five resultant values for the unknown.

(iv) Pyruvate Analysis

The pyruvate assay was conducted in 13x100 mm test tubes. To each tube was added 2.0 ml of the NADH-LDH reagent solution. A 100 μ l aliquot of deproteinized blood was then added, mixed and incubated for 30 min at room temperature. Following incubation the fluorescence was measured, as per lactate, but without dilution of the reaction mixture. Unknown concentrations were calculated by the ratios method as described above.

6) Determination of Fructose-1, 6-Diphosphatase

The enzyme was assayed by the method of Latzko and Gibb (1974).

(i) Chemicals and Reagents

- a) Tris buffer - pH 7.5 (see Appendix B)
- b) Magnesium chloride - $6\text{H}_2\text{O}$ (0.5M) (CANLAB)
- 1.02 g /100 ml H_2O
- c) Nicotinamide adenine dinucleotide phosphate (NADP) (Sigma)
- 8.5 mg NADP- Na_2H /ml H_2O
- d) Fructose-1, 6-diphosphate (50 mM) (Sigma)
- 27.9 mg/ml H_2O
- e) Phosphoglucose isomerase (PGI) (Sigma)
- 1.0 mg protein/ml

- f) Glucose-6-Phosphate Dehydrogenase (G-6-PDH) (Sigma)
- 0.5 mg protein/ml
- g) 2-Mercaptoethanol (Sigma)
- 70 μ l stock (16 M) in 5 ml. H₂O

(ii) Tissue Preparation

Three pups were chosen at random from the litter, decapitated and the livers excised and transferred to ice-cold Tris buffer (0-4°C). The tissue was then blotted dry, weighed and a 1:20 w/v homogenate prepared in Tris buffer by use of a Tekmar Model SDT Tissue Homogenizer at a setting of 7. Tissues were kept cold (0-4°C) throughout all procedures. The homogenate was centrifuged at 7,000xg (Sorvall Model RC2B) for 15 min at 4°C. The resulting supernatant, which was assayed for FDPase activity, could be stored for up to 48 hrs without loss in activity.

(iii) FDPase Quantification

Into 10x75 mm test tubes were pipetted the following final concentrations of reagents - Tris buffer 0.1 M, MgCl₂·6H₂O 10 mM, NADP 0.5 mM, 2-mercaptoethanol 5mM, PGI 10 μ g protein/ml, G-6-PDH 5 μ g protein/ml, and enough water and sample to bring the final volume to 1.0 ml.

Samples were kept at 4°C until assayed at which time they were placed at room temperature for 20 min prior to assay. The preparation was then pipetted into the incubation mixture and mixed thoroughly. The amount of sample was adjusted to give an absorbance change of not more than 0.100A/min. The change in the absorbance was measured against

water at 340 nm with a Beckman Model 25 Double Beam Spectrophotometer. The absorbance was recorded every minute for five minutes and the average $\Delta E/\text{min}$ calculated. The volume activity (U/l) was calculated according to Latzko and Gibbs (1974) with the final activity expressed as units per gram of liver. One unit of activity is that amount of enzyme that would cause an absorbance change of 1.0A per minute at room temperature and pH 9.0 (Pontremoli et al., 1965).

7) Lactic Dehydrogenase Determination

Lactic dehydrogenase (LDH) was assayed according to the spectrophotometric assay of Bergmeyer et al. (1965).

(i) Reagents

- a) Phosphate-pyruvate solution (PPS) pH 7.5 - see Appendix B.
- b) NADH prepared by dissolving 10 mg NADH (Sigma) in 1.5 ml PPS.

(ii) Tissue Preparation

The livers from three randomly chosen pups were excised and pooled in ice-cold (0-4°C) PPS. The livers were then blotted dry, weighed and transferred to a volume of PPS which would yield a 10% homogenate. Homogenization was done, using a Tekmar Model SDT tissue homogenizer at a setting of 7, in 17x100 mm Corex centrifuge tubes. The homogenate was centrifuged at 7,000xg for 15 min at 4°C (Sorvall Model RC2B) and the resulting supernatant used for LDH determination. Enzyme preparations were stored for no longer than 24 hrs prior to use at 4°C.

(iii) LDH Assay

Into 13x100 mm test tubes were pipetted 2.85 ml of PPS and 50 μ l of NADH solution. The homogenates were then diluted 1:200 (i.e., 50 μ l of homogenate in 10 ml of PPS) to bring the enzyme activity into the measurable range ($\Delta E/\text{min} \times .020A/\text{min.}$). A 100 μ l volume was then added to the tube, mixed and the absorbance measured every minute for five minutes in a Beckman Model 25 Spectrophotometer. The average $\Delta E/\text{min}$ was then used to calculate the enzyme activity as described by Bergmeyer et al, (1965). Enzyme activity is expressed, in units per gram wet weight; with the unit defined as the amount of enzyme which converts 1 umole of substrate per minute at room temperature and pH 7.5.

F - Statistical Analysis of Data

All the data have been analyzed statistically. Continuous, quantitative data, i.e., biochemical parameters, were analyzed by a one-way analysis of variance. When more than one dose was included, the treatments were compared to control by Dunnett's d' Test. Discrete qualitative data, i.e., mortality data, were analyzed by X^2 , corrected for continuity by Yates' procedure. The procedures and tables used are described in Steel and Torrie (1960). The standard error of the mean has been recorded for all mean values.

RESULTS

A - Acute Toxicity of Dieldrin to 5-Day Old Mice

Five day old mice are very sensitive to acute doses of dieldrin. The mortality caused by a single dose of dieldrin in neonatal mice has been summarized in Table II.

TABLE II - THE MORTALITY OF 5-DAY OLD MICE
TREATED WITH A SINGLE DOSE OF DIELDRIN

Time (Hrs.)	% Alive				
	0	24	48	72	96
Dose (mg/kg)					
2	100	100	84.61	50.00	19.23
25	100	51.61	19.35	9.68	0
37.5	100	40.62	12.50	3.12	0
50	100	32.56	1.16	0	0
62.5	100	28.79	6.06	1.51	0
75	100	23.08	4.61	0	-
250	100	0	-	-	-

It is apparent that the mortality is dose related: 2 mg/kg causes no mortality at 24 hrs while 250 mg/kg causes 100% mortality. Table III presents the data used in calculating the LD₅₀ according to the method of Litchfield and Wilcox (1949).

The LD₅₀ for a 5-day old mouse pup was calculated, from Table III to be 27 mg/kg. The upper and lower 95% confidence limits are 21.1 mg/kg and 35.5 mg/kg, respectively.

In the chronic, low dose experiments pregnant mice receive a daily, per os, treatment with 2 mg/kg dieldrin from day 6 to day 18

TABLE III - MORTALITY DATA USED FOR THE LD₅₀ DETERMINATION

* Statistically significant mortality from the no effect dose (2 mg/kg) by X² with Yates' correction for continuity, p < .05

Dose	Number Alive @ 24 Hours Dose Total	% Alive
2	26/26	100
25	32/62*	51.6
37.5	26/64*	40.6
50	28/86*	32.6
62.5	19/66*	28.8
75	15/65*	23.1
250	0/30*	0.0

of pregnancy. Theoretically, assuming that all pesticide administered to the female crosses the placenta and is taken up equally by the fetuses (ca. 10) the in utero exposure could result in a cumulative dose of dieldrin equal to 117 mg/kg.

After the first 24 hrs all surviving pups, except those treated with 2 mg/kg, exhibited tremoring and convulsions indicative of central nervous system stimulation.

B - Dieldrin-Induced Neonatal Inviability in the CD-1 Mouse

Prenatal dieldrin was found to cause inviability in this strain of mouse. The inviability occurred at doses which had little or no toxic effect in the pregnant female.

The effects of prenatal dieldrin on maternal parameters are presented in Table IV.

TABLE IV - EFFECTS OF DIELDRIN ADMINISTERED DAILY, PER OS, FROM DAYS 6-18 OF GESTATION ON LITTER SIZE, MATERNAL WEIGHT GAIN AND LIVER WEIGHT. (x ± s.e.). SAMPLE SIZE IN BRACKETS

Dose	Litter Size	% Change (a) in Body Weight	Liver Weight, (mg L/gBW) (b)
Control	11.5 ± .19(8)	13.11 ± .72(8)	63.89 ± 1.75(8)
0.5 mg/kg	11.5 ± .60(8)	18.44 ± 2.27(8)	67.11 ± 1.37(8)
1.0 mg/kg	9.62 ± 1.13(8)	14.85 ± 2.27(8)	77.51 ± 5.9*(8)
2.0 mg/kg	11.37 ± .73(8)	16.7 ± 1.69(8)	80.22 ± 1.68*(8)
4.0 mg/kg	10.62 ± .86(8)	7.88 ± 1.66(7)	101.12 ± 5.39*(7)

a) $\frac{\text{Day 19 Wt} - \text{Day 6 Wt}}{\text{Day 19 Wt}} \times 100\%$ - data shown not statistically significant by 1-way anova following arcsin transformation.

b) mg Liver/g Body Weight = mg L/gBW

* Statistically significant from control (p < .05) by Dunnett's d' Test.

The litter size and maternal weight gain were unaffected by any of the dosages used. Liver weights, however, were increased, when compared to controls, except 0.5 mg/kg. Dam mortality was seen only at 4.0 mg/kg where 30% of the 10 females died.

The mortality of the pups isolated from the females is presented in Figure 1. It is apparent that inviability was induced in this strain and that it is dose related. The most rapid mortality was seen at 4.0 mg/kg and it decreased at the lower doses. The pups did not show any physical signs of toxicity (i.e., tremors and convulsions) as was seen in the acute toxicity study.

The earliest, statistically significant mortality was at 24 hours and was caused by 4.0 mg/kg dieldrin. This dose was, however, not used in the other studies because of the high dam mortality. Rather, a dose of 2 mg/kg dieldrin was chosen because it induced the next most severe inviability and caused no dam mortality.

C - Two Factors Affecting Pup Survival

(1) Effects of Temperature

Increases in the ambient temperature significantly reduced the survival of isolated pups. The mortality rate of isolated pups maintained at 25°C were compared to those kept at 21°C and the results presented in Figure 2. It is readily apparent that the elevated temperature increases the mortality rate not only of inviable pups but also of controls. The maximum longevity in both groups was decreased by 47% and 50% in control and inviable animals, respectively. This result indicates that temperature affects both control and

FIGURE 1 - Mortality pattern in isolated pups of dams treated with various doses of dieldrin, per os, from days 6-18. Approximately 70-90 pups were observed at each of the treatments. Data were analyzed by χ^2 (2x2 contingency tables) with Yates' correction for continuity. * indicates statistical significance ($p \leq .01$) when compared to control animals.

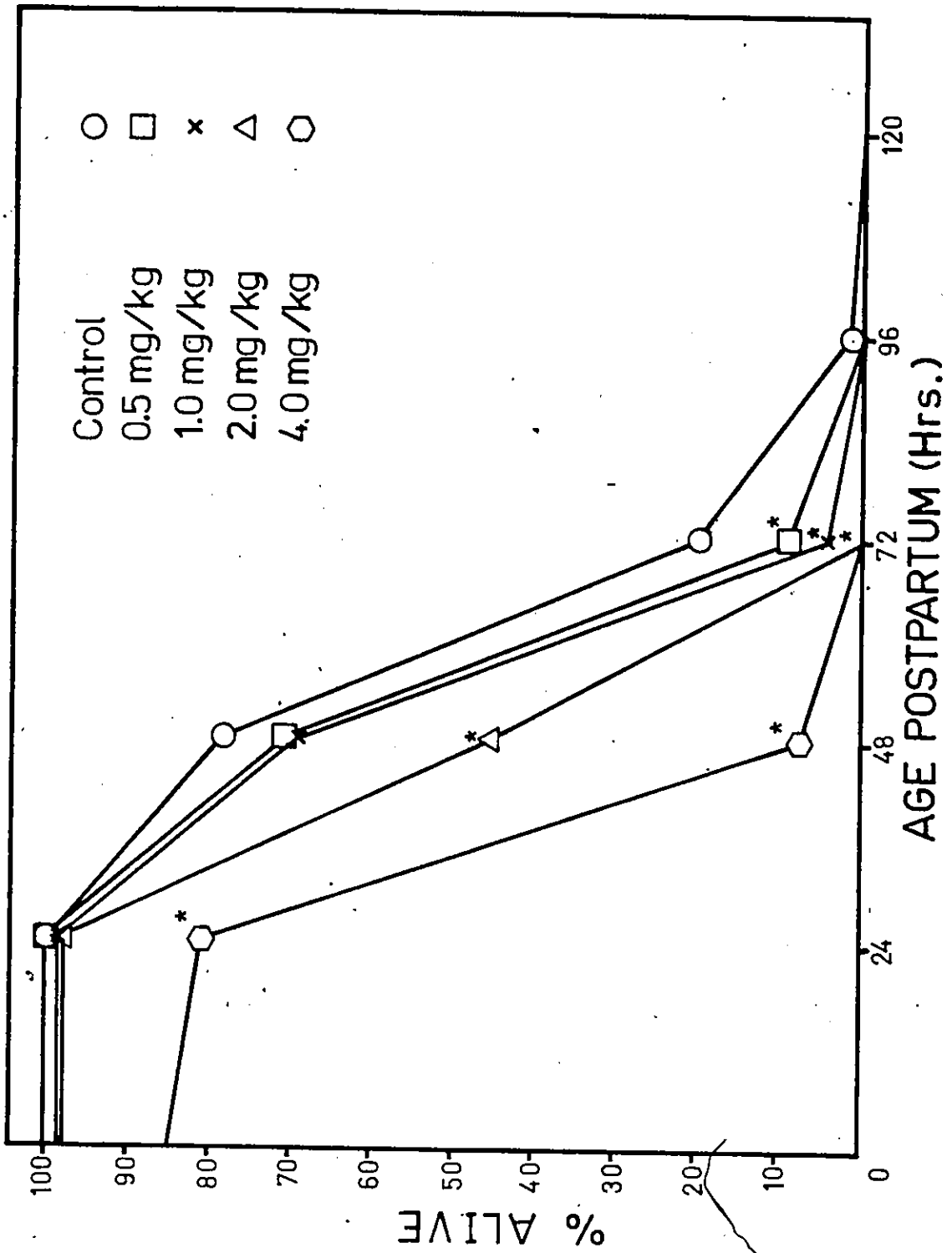
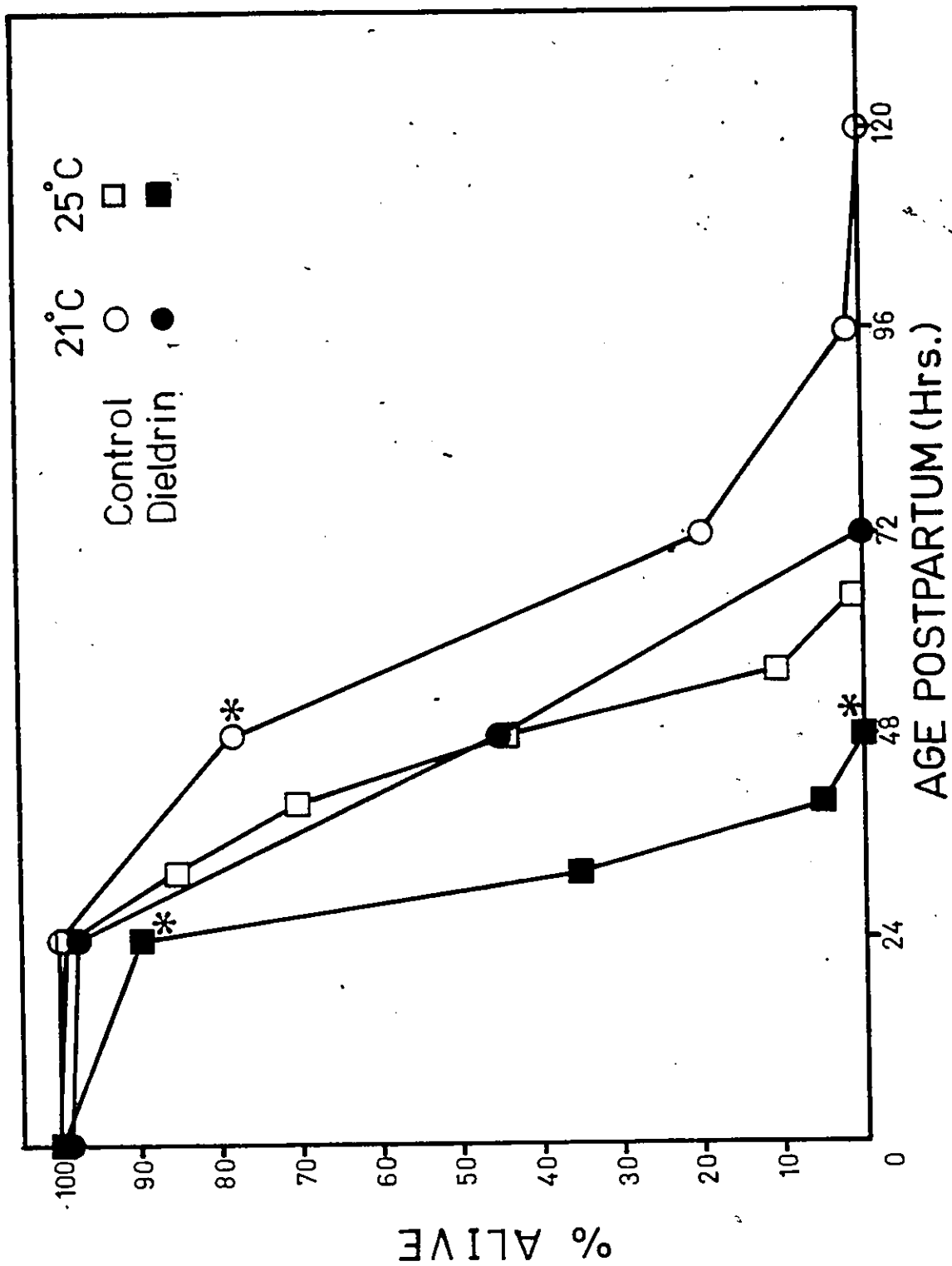


FIGURE 2 - Comparison of isolated pup mortality maintained at 21°C and 25°C. Comparisons were made between controls and between treated pups at comparable times. χ^2 , for a 2x2 contingency table with Yates' correction for continuity. Sample sizes range from 70-100 pups at each temperature and treatment. * shows statistically significant differences between groups at $p \leq 0.025$.



inviably equally and does not potentiate the inviability. In general, pups isolated at both temperatures felt cold and were unresponsive to physical stimuli.

(ii) Effects of Mode of Parturition

This experiment showed that the mode of delivery affects the survival of the pups. The mortality of vaginally-delivered pups was compared to that of caesarean-delivered pups. All studies were carried out at 25°C. The data obtained from the temperature effects experiment was used to make the comparison in this study.

Figure 3 presents the mortality of caesarean-delivered pups. The mortality does not differ between control and inviable pups until 24 hrs post-delivery where significantly more treated pups are dead. Interestingly, the maximum longevity is almost the same for control and inviable pups, although not all control pups are dead (2.8%) at 32 hours. It appears that caesarean delivery shortens the onset of mortality and increases the mortality rate in inviable pups when compared to controls.

Figure 4 presents the effects of mode of parturition on pup survival. Caesarean delivery shortens the maximum longevity in both control and treated pups. The control animals appeared to be more affected as there was a 50% reduction in maximum longevity as compared to a 33% decrease in the inviable pups.

Caesarean-delivery has been found to decrease the maximum longevity in both control and treated animals when compared to vaginally-delivered pups. Furthermore, this method (caesarean) appears to affect the survival rates of control pups more than that

FIGURE 3 - Mortality of isolated pups delivered by caesarean section and maintained at 25°C. Treated pups were delivered from females receiving 2 mg/kg dieldrin daily, per os, day 6-18 of gestation. Sample sizes were 37 and 48 for control and treated, respectively. X^2 analysis (2x2 contingency table) with Yates' correction showed statistical significance (*) at $p \leq .005$.

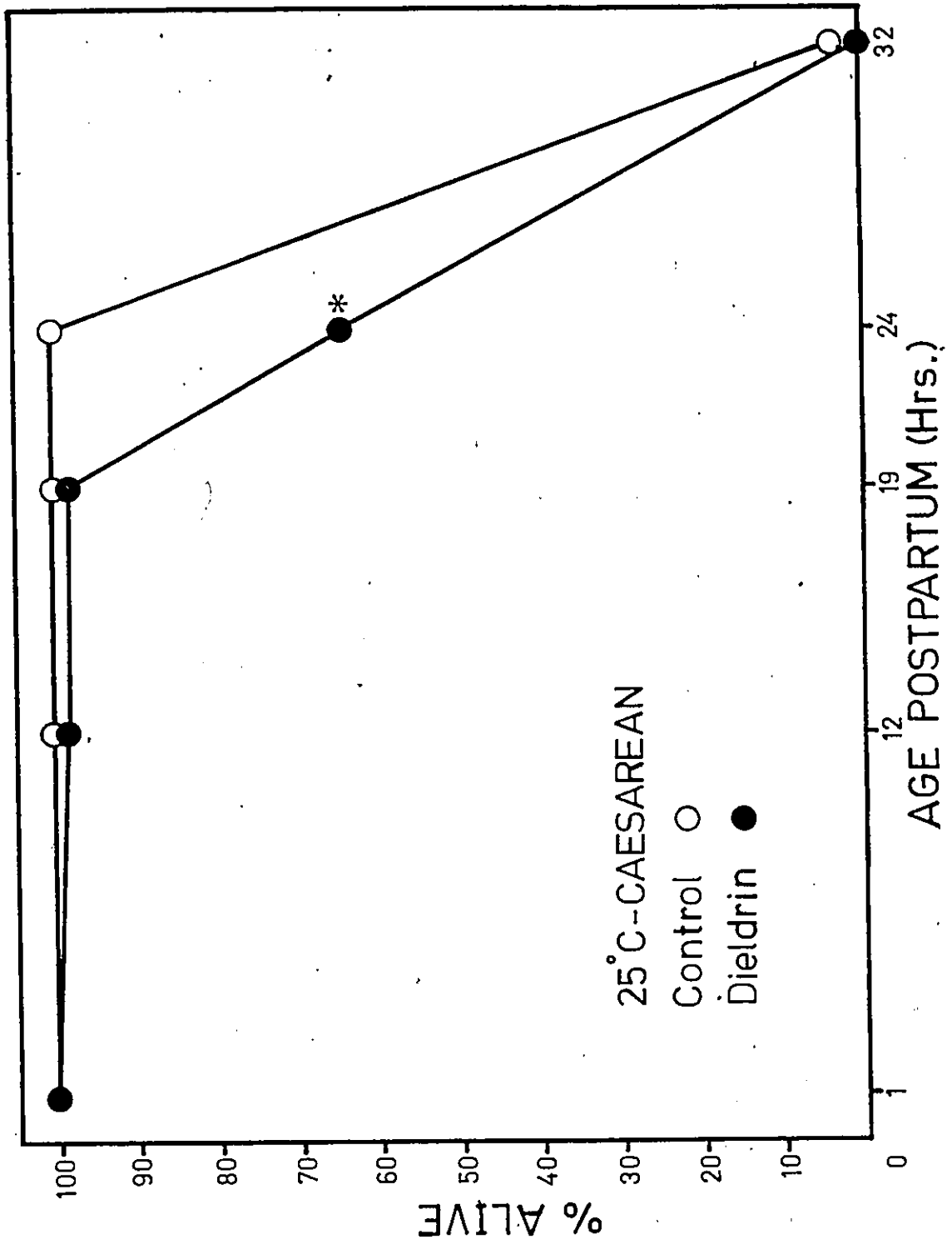
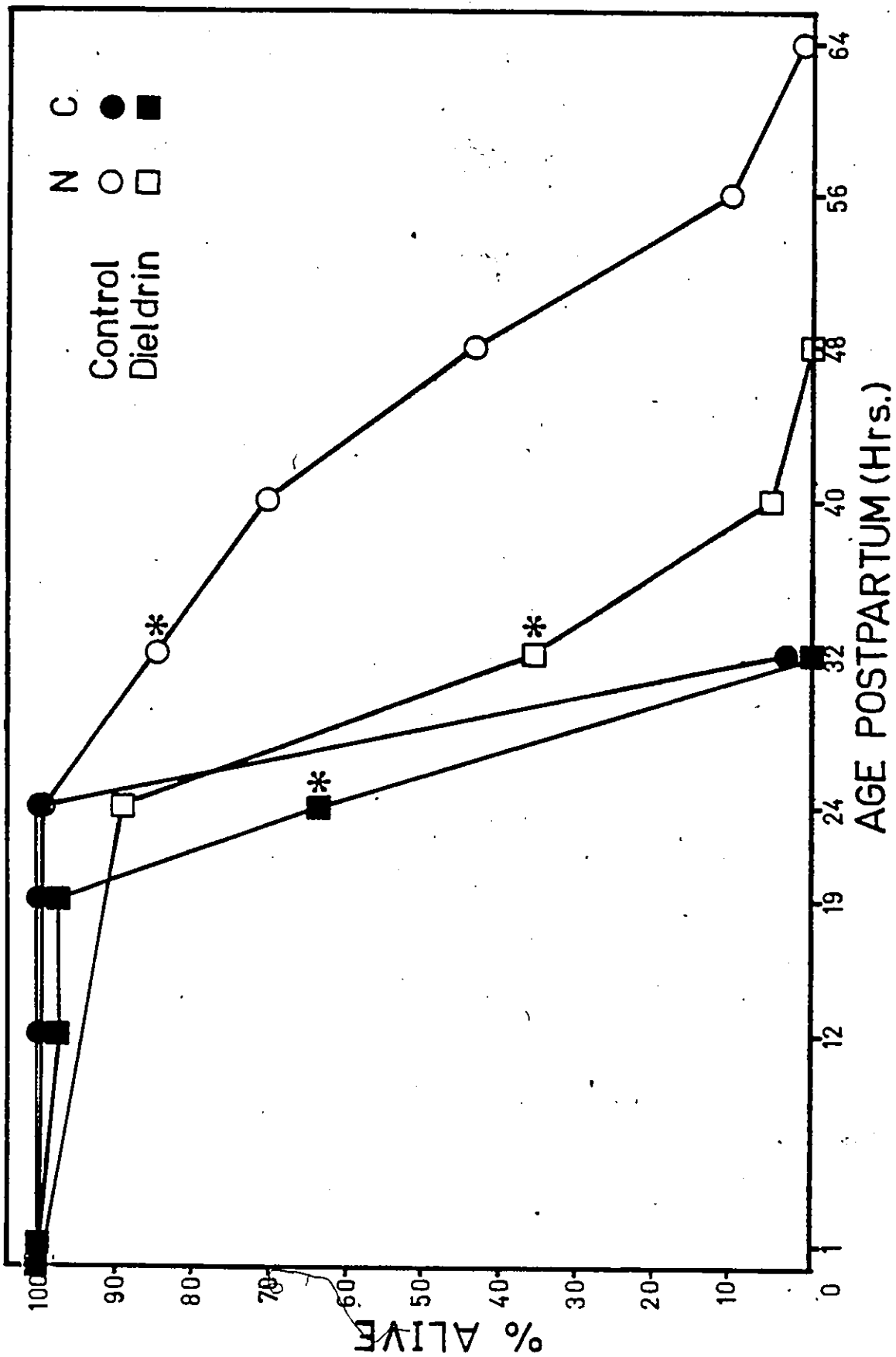


FIGURE 4 - Comparison of the mortality of isolated pups delivered naturally or by caesarean section and maintained at 25°C. Inviabile pups were born of females treated daily, from day 6-18 of gestation with 2 mg/kg dieldrin, per os. Data was analyzed at comparable times between controls and between treated pups. Statistical analysis was by χ^2 (2x2 contingency table) with Yates' correction for continuity and significance (*) at the level of $p \leq .005$ is indicated. N = natural delivery; C = caesarean delivery.



of inviable pups.

D - Carbohydrate Metabolism in Pups of Dieldrin Treated CD-1 Mice

Aspects of carbohydrate metabolism were measured at 0, 19, and 24 hrs post-delivery. Prenatal dieldrin exposure was found to alter carbohydrate metabolism in inviable pups.

The glycogen concentration in liver, skeletal muscle and heart are presented in Figures 5-7. Hepatic glycogen (Figure 5) is rapidly depleted in the first 19 hrs indicating that these stores are readily mobilized at birth. Treated pups mobilize the stores at a faster rate than controls, because there is a significantly greater reduction in the stores by 24 hours.

Skeletal muscle glycogen (Figure 6) also decreases from delivery to 19 hours. The glycogen in the skeletal muscle shows an apparent increase at 24 hours, however, the concentration is not statistically different from the 19 hr. values (df 1, 17; $F = 1.596$; $p > 0.05$). The treated pups showed a significant decrease at 24 hrs (df 1, 19; $F = 16.22$; $p < .005$); since the control values do not differ between 19 and 24 hours, the difference between control and treated pups must be due to the decrease noted at this time.

The concentration of cardiac glycogen (Figure 7) increased slightly in the first 19 hrs in both control and treated mice, but decrease by 24 hrs when the levels in the inviable pups are significantly ($p < .005$) decreased. Cardiac glycogen levels, in the treated mice, were approximately 50% of control values at 24 hours.

Glucose and urea concentrations in the plasma are presented in Figure 8. In viable pups appear to be hypoglycemic during the period

FIGURE 5 - Changes in liver glycogen during the first 24 hrs of life in the caesarean-delivered, isolated pups of dieldrin-treated females (2 mg/kg daily, per os, from day 6-18 of gestation). (n = 10-13 at each point.) Data analyzed by a one-way analysis of variance; (a) df-1, 17; F = 3.11, p = 0.10; (b) df-1, 19; F = 8.46, p < .01.

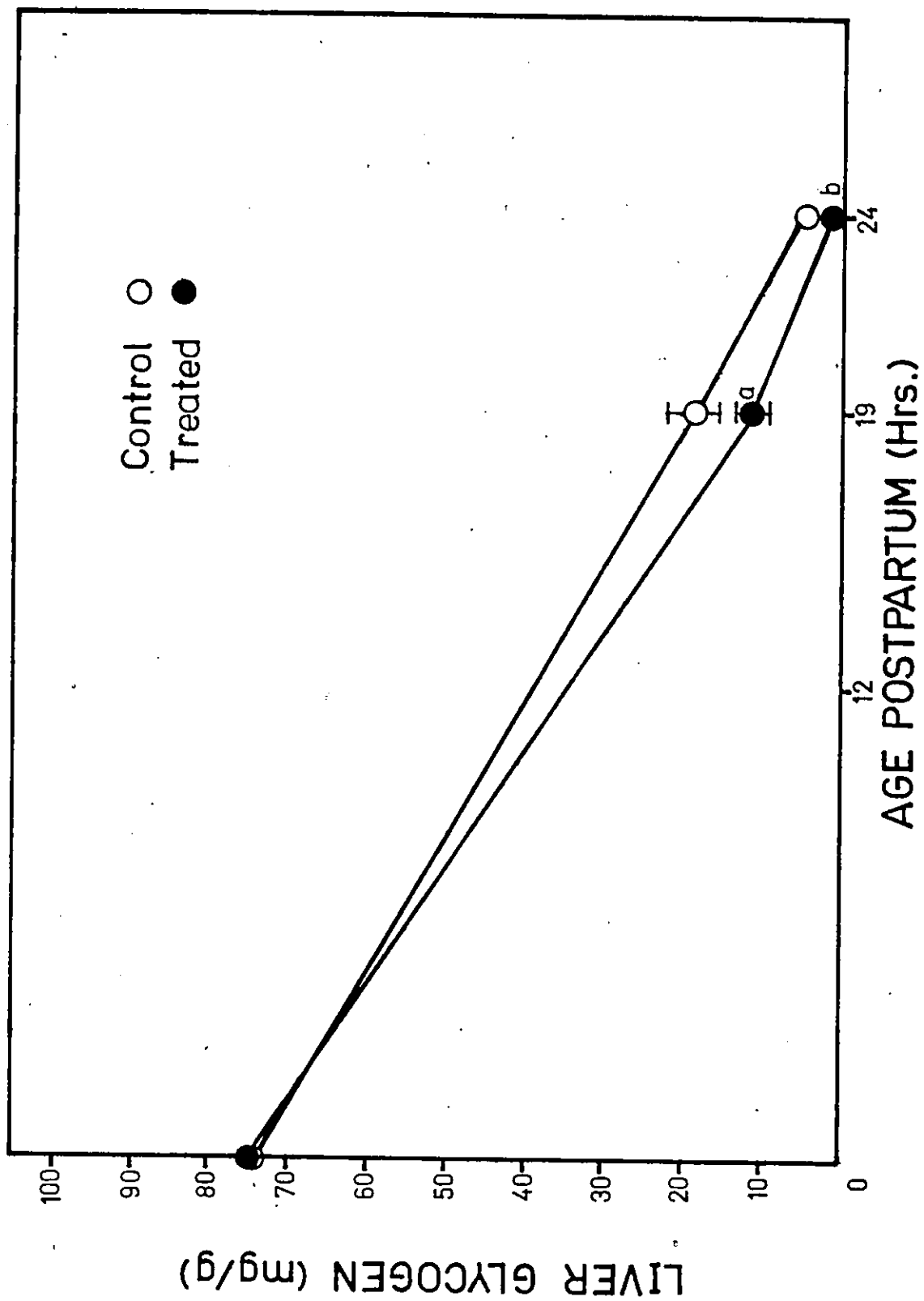


FIGURE 6 - Changes in muscle glycogen concentration, during the first 24 hours of life, in the caesarean-delivered, isolated pups of dieldrin-treated mice (2 mg/kg daily, per os, from day 6-18 of gestation). (n = 10-13 at each point.) Data analyzed by a one-way analysis of variance; (a) df-1, 19; F = 28.79; p < .005.

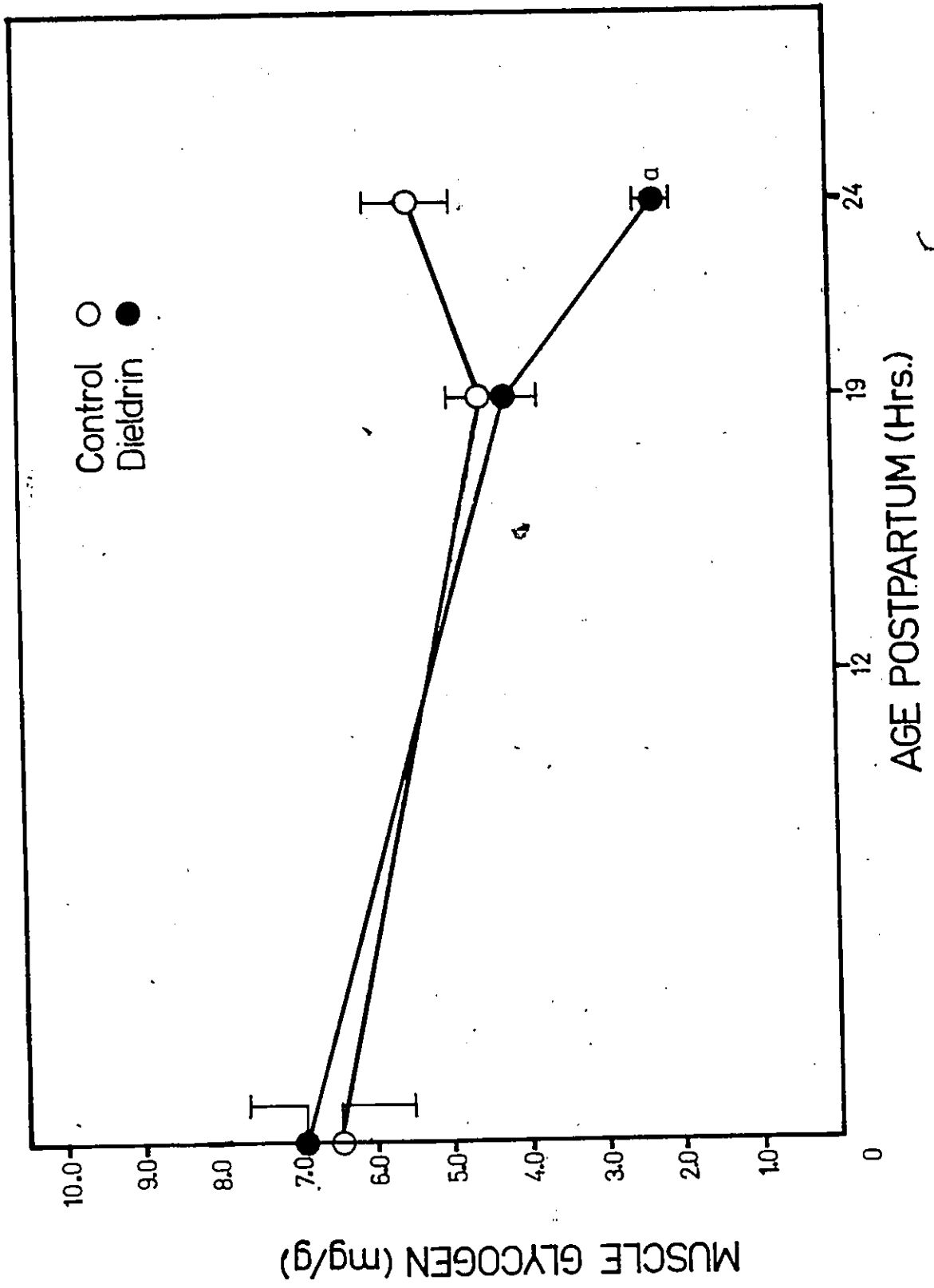


FIGURE 7 - Changes in cardiac glycogen concentration, during the first 24 hours of life, in the caesarean-delivered, isolated pups of dieldrin-treated mice (2 mg/kg daily, per os, from day 6-18 of gestation). (n = 10-13 at each point.) Data analyzed by one way analysis of variance; (a) df 1, 19; F = 21.16; p < .005.

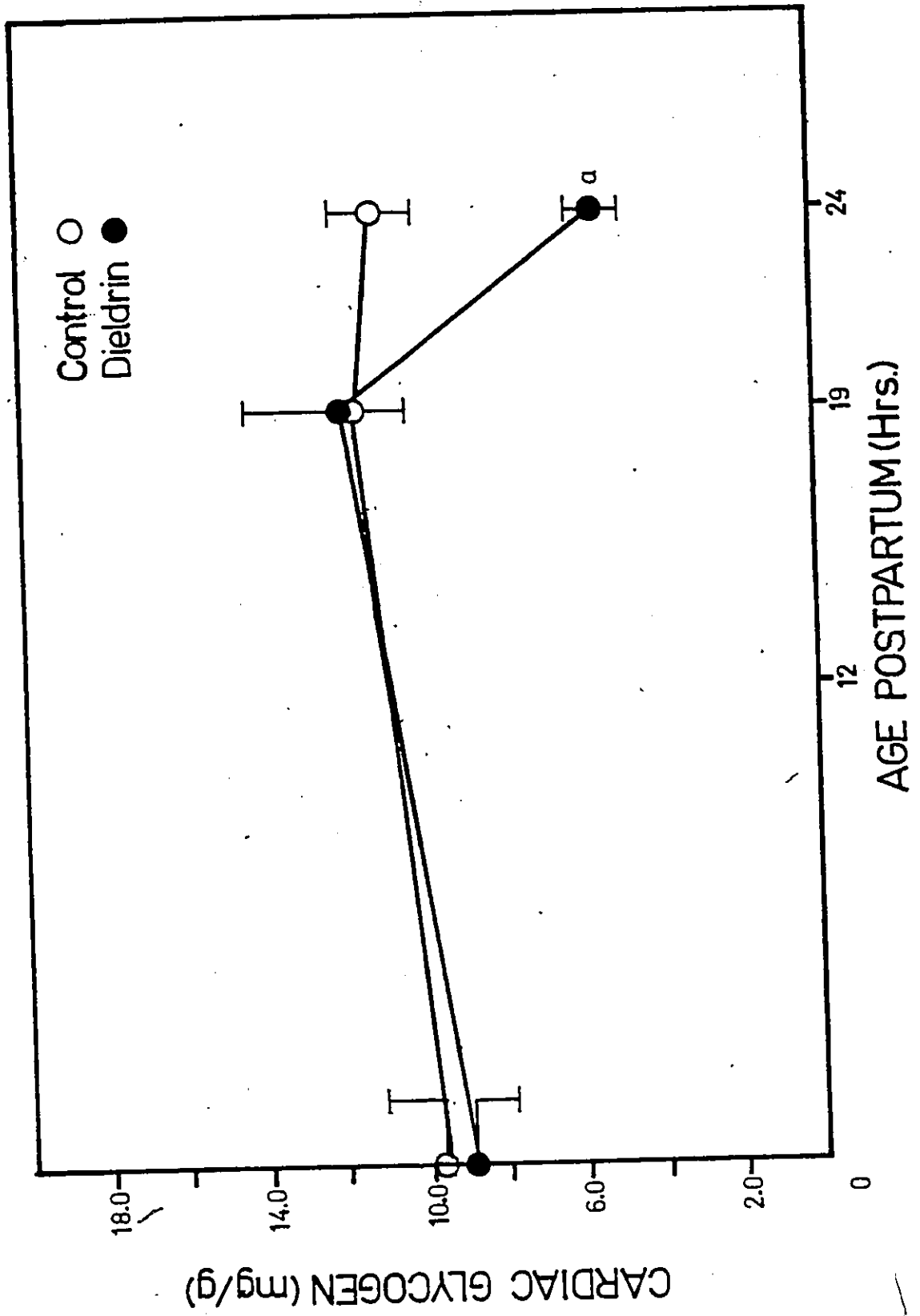
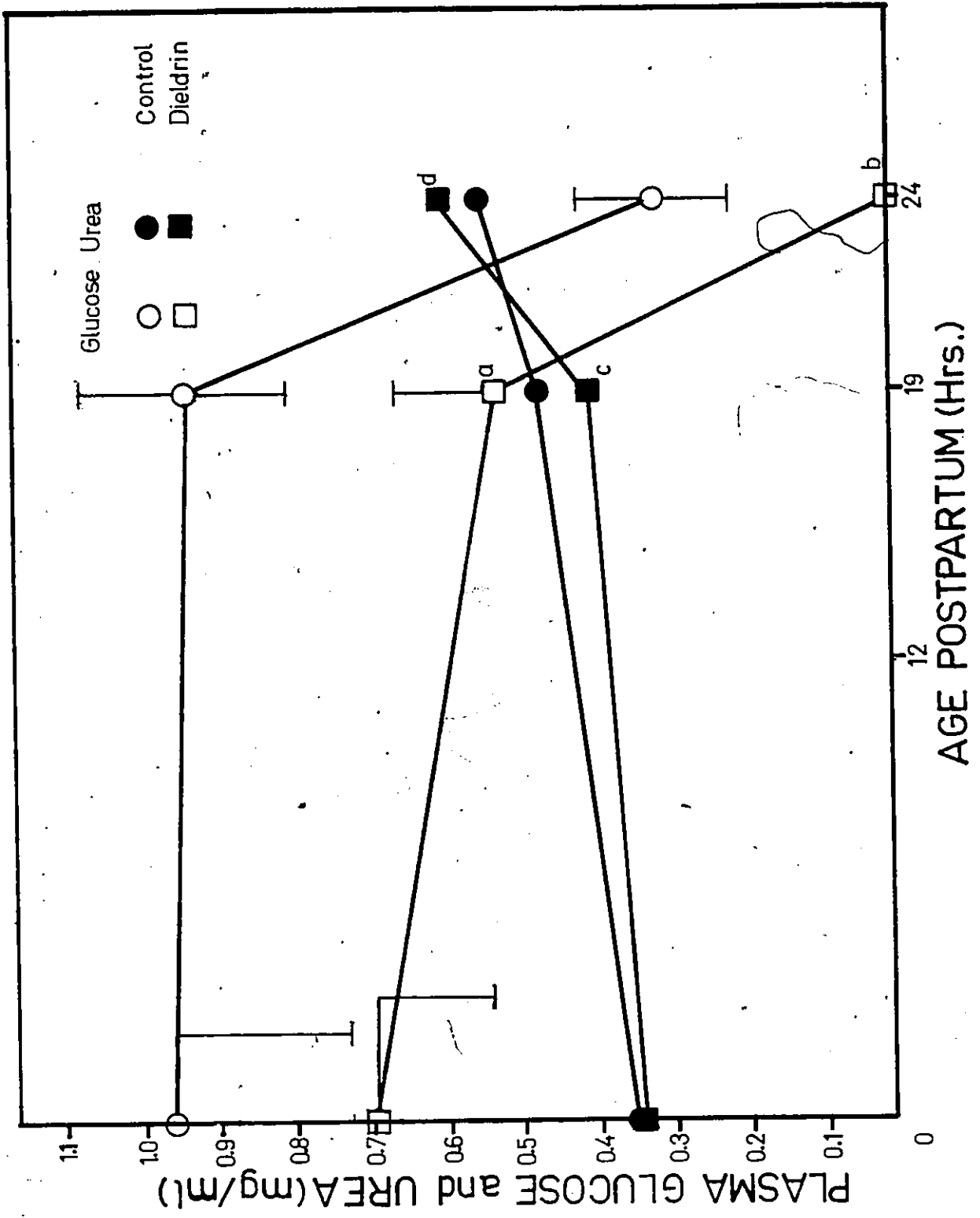


FIGURE 8 - Changes in the concentration of plasma glucose and urea, during the first 24 hours of life, in caesarean-delivered, isolated pups of dieldrin-treated mice (2 mg/kg daily, per os, from day 6-18 of gestation). Each point is the mean of 11-13 observations. The data was analyzed by a one-way analysis of variance:

- (a) $df = 1, 17$; $F = 4.34$; $p = 0.054$.
- (b) $df = 1, 16$; $F = 13.02$; $p < 0.005$.
- (c) $df = 1, 13$; $F = 4.05$; $p = 0.07$.
- (d) $df = 1, 13$; $F = 3.18$; $p = 0.10$.



studied. These animals are born with reduced plasma glucose levels, the reduction is not significant because of the large individual variation, and the hypoglycemia becomes more pronounced ($p = 0.54$ at 19 hrs) with time. At 24 hrs the treated animals had no detectable plasma glucose ($p < .005$). Plasma urea, which is an index of amino acid metabolism, was not significantly different in the treated mice at birth. There was a general increase in plasma urea levels in both control and treated animals, but there were no differences in the two groups at any time indicating that dieldrin did not stimulate amino acid catabolism.

Dieldrin had no effect on the pup weight during the 24 hour period examined but there was a significant reduction in liver weight at both 19 and 24 hrs as shown in Table V.

TABLE V - CHANGES IN PUP WEIGHT AND LIVER WEIGHT,
DURING THE FIRST DAY OF LIFE, IN CAESAREAN-
DELIVERED PUPS OF DIELDRIN TREATED MICE
(2 mg/kg daily, per os, day 6-18 of gestation)
 $\bar{X} \pm SE (n)$

	<u>Control</u>	<u>Treated</u>
0-hrs pup weight (g)	1.44 \pm .06(13)	1.38 \pm .06(14)
Liver weight (mg L/gBW)	56.17 \pm 1.31(13)	55.35 \pm 1.76(14)
19 hrs pup weight (g)	1.37 \pm .02(9)	1.33 \pm .03(10)
Liver weight (mg L/gBW)	48.19 \pm 1.32(9)	41.35 \pm .73(10) ^a
24 hrs pup weight (g)	1.34 \pm .02(10)	1.32 \pm .05(11)
Liver weight (mg L/gBW)	41.27 \pm 1.29(10)	36.58 \pm .90(11) ^b

Data analyzed by a one-way analysis of variance. At birth there was no difference in either parameter.

(a) $df = 1, 17$; $F = 19.34$; $p < .005$.

(b) $df = 1, 19$; $F = 9.19$; $p < .01$.

The reduction in liver weight, over the 24 hr period, was 26% and 34% in control and treated mice, respectively. This reduction can be attributed in part to the depletion of liver glycogen, which at birth has been reported to account for up to 10% of the liver wet weight (Dawes and Shelley, 1968). It can only be speculated, that the remainder of the loss resulted from depletion of other stores (i.e., lipid) or the destruction of proteins for energy purposes.

Prenatal dieldrin did not alter the levels of precursors and enzymes of the gluconeogenic pathway. Table VI presents the changes in the parameters measured as indicators of gluconeogenesis. Inviabile pups did not show any change in blood lactate and pyruvate, when compared to controls, indicating that these gluconeogenic substrates were unaffected. Similarly, the plasma urea levels were unaltered indicating that gluconeogenesis from amino acids was the same in treated and control pups. The activities of LDH and FDPase were not significantly different in either group, therefore, inviable pups have similar enzymatic capabilities as controls. LDH activity recorded for livers are in agreement with those of Wroblewski (1958), for canine liver, following proper conversion to common units. Fructose-1, 6-dephosphatase activity, also agrees with those cited by Latzko and Gibb (1974) for rat liver.

The results indicate that prenatal dieldrin exposure does not affect pup weight during the first 24 hours of life but a significant reduction in liver weight is seen during this time. At birth, the glycogen stores are not significantly different between control and inviable pups. This implies that the glyconogenic capacity in fetuses

TABLE VI - BLOOD LACTATE, PYRUVATE, LIVER LACTIC DEHYDROGENASE (LDH), AND FRUCTOSE-1, 6-DIPHOSPHATASE (FDPase), DURING THE FIRST 24 HRS OF LIFE, IN CAESAREAN-DELIVERED, ISOLATED PUPS OF DIELDRIN-TREATED MICE

	Blood Lactate (mM)	Blood Pyruvate (mM)	LDH u/g	FDPase (μmole/min/g)
0 time				
Control	11.42 ± .86(4)	0.338 ± .152(4)	180.32 ± 6.84(3)	5.19 ± .18(5)
Treated	11.29 ± .84(4)	0.265 ± .092(4)	184.41 ± 22.62(3)	5.11 ± .27(5)
19 hrs.				
Control	1.79 ± .21(4)	0.056 ± .007(4)	215.86 ± 12.5(4)	5.95 ± .83(4)
Treated	2.07 ± .38(4)	0.058 ± .009(4)	190.57 ± 10.19(4)	8.04 ± .82(4)
24 hrs				
Control	2.08 ± .45(5)	0.050 ± .004(4)	259.22 ± 3.48(3)	5.92 ± .30(3)
Treated	1.87 ± .36(5)	0.058 ± .009(4)	240.00 ± 18.71(4)	5.73 ± .43(4)

No significant differences noted.

is unaltered by prenatal exposure to dieldrin. At birth, inviable neonates have a reduction in the plasma glucose when compared to controls. Furthermore, plasma lactate, pyruvate, urea, and the activities of hepatic LDH and FDPase are unaffected. In the post-natal period, there is a depletion in the glycogen stores, with inviable neonates showing a more rapid depletion rate than controls in liver, skeletal muscle and heart. The hypoglycemia which is present at birth persists and becomes more pronounced with time. Gluconeogenic parameters (i.e., lactate, pyruvate, LDH and FDPase) show no significant changes, indicating a similar gluconeogenic capacity as controls.

DISCUSSION

A - Acute Toxicity of Dieldrin to Neonatal Mice

Acute toxicity does not appear to be the mechanism involved in the neonatal inviability. All acutely treated animals exhibited tremors and convulsions, classic signs of central nervous system intoxication. However, inviable pups do not exhibit any of these symptoms, and for this reason it is believed that the mechanism of the inviability lies outside the central nervous system.

Young mammals are generally less sensitive to the effects of CNS stimulants than are adults. Goldenthal (1971) has compiled the LD₅₀ values for various CNS stimulants, including d-amphetamine, picrotoxin, and strychnine sulfate, and has found the LD₅₀ of these compounds to be significantly higher in newborns. This insensitivity is particularly dramatic in the case of DDT, where the LD₅₀ is greater than 4,000 mg/kg in newborn rats and only 195 mg/kg in adults (Lu et al., 1965). These workers also reported an LD₅₀ of 168 mg/kg for newborns treated with dieldrin and this is four times greater than the LD₅₀ in adult rats.

The results of the dieldrin acute toxicity experiment show that 5-day old mice have an LD₅₀ of 27 mg/kg. Interestingly, this is less than the 37 mg/kg reported by Hodge et al. (1967) as the LD₅₀ for adult mice. This discrepancy with earlier reports may be a reflection of species differences. All earlier studies employed rats, whereas this study used the mouse. There have been no other reports comparing neonatal and adult toxicity of central nervous system stimulants in the mouse. However, the possibility of a species difference gains support from the

work of Hodge et al. (1967), who showed species differences in the LD₅₀'s of aldrin and dieldrin for mammalian and avian species. Gingell and Wallclave (1974) studied the acute toxicity of DDT to mice and hamsters, and found the mouse to be much the more sensitive. Whereas, a dose of 310 mg/kg was fatal to mice, no hamsters died even at a dose of 1600 mg/kg. The differences in sensitivity are apparently caused by differences in the permeability of the blood/brain barriers; at equivalent doses, the concentrations of DDT in the mouse brain were two times greater than in the hamster brain.

The central nervous system of the 5-day old CD-1 mouse is more sensitive to an acute oral dose of dieldrin than that of the adults. However, it is unlikely that acute toxicity is involved in the inviability since inviable pups do not exhibit tremors or convulsions prior to death, as do the acutely poisoned pups.

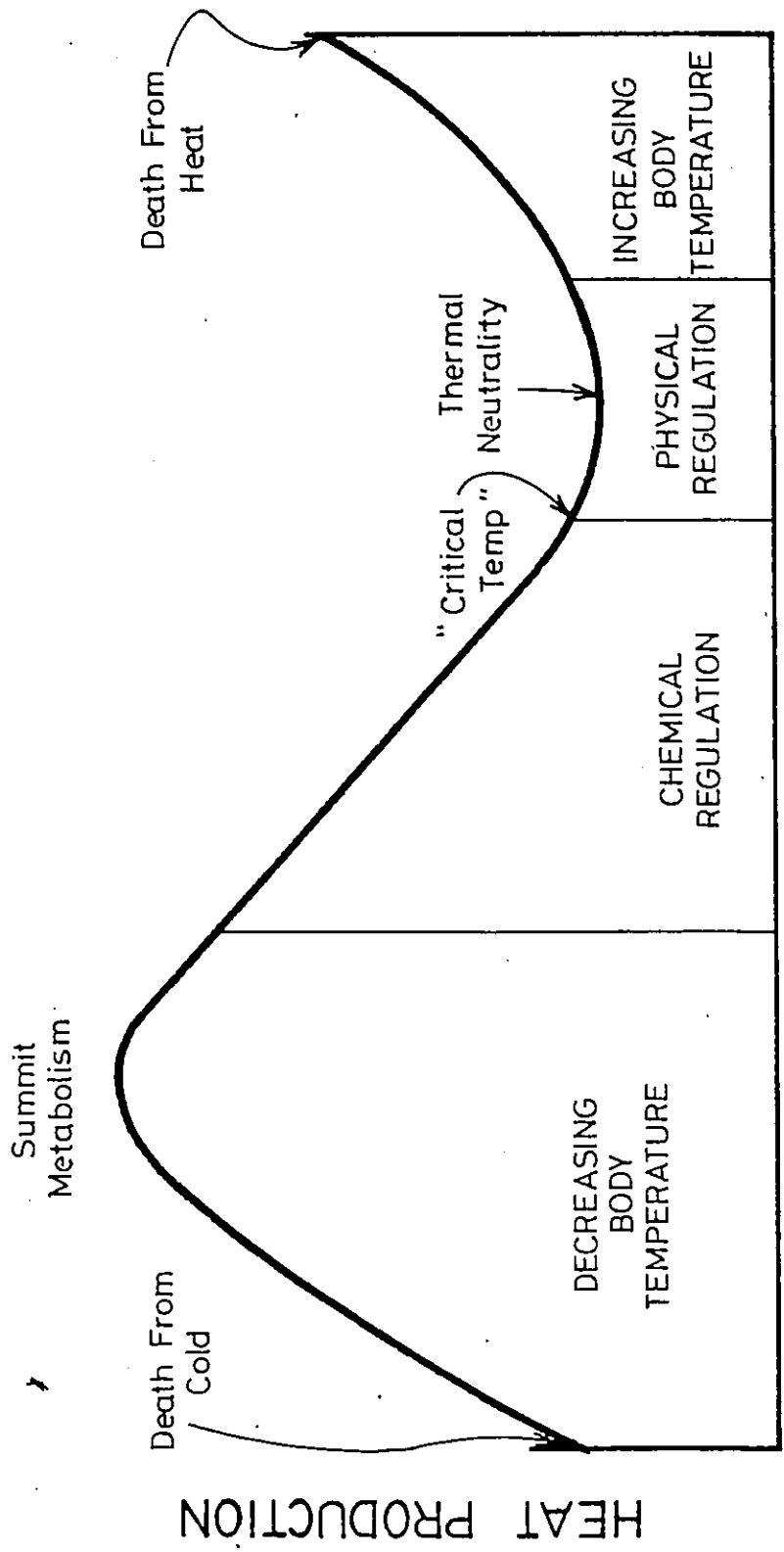
B - Two Factors Affecting Survival in Isolated Pups

(i) Temperature

The body temperature of all mammals remains, within remarkably narrow limits, throughout life. The metabolic response to changes in the ambient temperature is basically the same for all mammals and has been summarized in Figure 9. The thermoregulatory capacities of mammals is limited by thermal insulation, and particularly by the area of exposed surface. Thus, the smaller the animal, the larger the rate of O₂ consumption in a thermoneutral environment, the higher the critical temperature and, within limits, the greater the increase in metabolic rate on exposure to cold (Smolens and Kime, 1978).

THE HOMEOTHERMIC MODEL

adapted from Sinclair (1976)



ENVIRONMENTAL TEMPERATURE

FIGURE 9

A newborn rat weighs about 5 gms. It cannot increase its heat production upon cold exposure before it has suckled. But, within 24 hours, it can double its heat production if the environmental temperature falls 2°C, from 36°C (the lower end of the thermoneutral range) to 34°C (Taylor, 1960). This same study reported that the summit metabolism occurs at 30°C. The newborn mouse is smaller than the rat (ca. 1 gm) but it, too, responds to cold exposure, on the day of birth, by an increase in heat production (Cassin, 1963). This worker showed the neutrothermal zone to be between 35-37°C and that summit metabolism occurred at 30°C.

The animals used in this study were maintained at environmental temperatures of 21°C and 25°C. From the homeothermic model it is seen that both temperatures lie below the summit metabolism temperature of 30°C. These animals were in the area where body temperature decreases; in agreement with the observation that neonates felt cold. Animals at 21°C, perhaps survived longer than those at 25°C because their metabolic rate was lower and therefore the depletion of energy stores was slower, conferring an increased longevity.

(ii) Mode of Parturition

Naturally-delivered pups survive longer than caesarean-delivered neonates. The possible explanation is that vaginally-delivered pups benefitted by having had a chance to nurse. In the experiment where the females delivered vaginally, they were checked for litters only every 8 hours. In this time period, newborns had the chance to nurse, as evidenced by the fact that milk was visible in their digestive tracts.

The milk obtained, therefore, could have provided an extra energy source which would prolong survival.

Another possible reason for the difference in mortality is the transient hypoglycemia which is seen at birth (Coke et al., 1971; Girard et al., 1973a; Snell and Walker, 1973a). Gain and Watts (1976) state that, in rats, this hypoglycemia is seen only in caesarean-delivered newborns. If this also occurs in the mouse, then the caesarean-delivered neonates will mobilize glycogen to compensate for the hypoglycemia and perhaps decrease their body reserves earlier, which would increase mortality.

A final cause of the increased mortality is the caesarean-delivery itself. Caesarean section can be considered premature delivery, because it is impossible to determine the exact time at which natural birth would occur. As such, some pups will be born in an immature state, with immature "life-systems."

In conclusion, this study showed that mortality rate was increased in caesarean-delivered pups compared to vaginally-delivered pups. Several suggestions were put forth that possibly account for the difference; these were: milk obtained by naturally-delivered pups yielding them an extra-energy source, a transient hypoglycemia as a result of caesarean section, and finally that caesarean pups are simply premature and immature.

C - Prenatal Dieldrin Exposure and Altered Carbohydrate Metabolism in Neonatal Mice

Carbohydrate metabolism plays an extremely important role during the neonatal period. The mammalian fetus receives a constant supply of

glucose from the mother; after birth, however, the neonate must depend upon endogenous energy sources until lactation and digestion are established. The newborn accomplishes glucose homeostasis, during this transition period, by two means — glycogenolysis and gluconeogenesis.

Numerous workers have studied glycogen metabolism, at the time of birth, emphasizing the activities of the two key enzymes — glycogen synthetase and glycogen phosphorylase. Both enzymes have an active and an inactive form designated synthetase I (active) and synthetase D; phosphorylase a (active) and phosphorylase b (Mayes, 1973). It was thought for some time, that the dynamics of glycogen metabolism in the fetal period involved glycogen synthesis, with an almost complete absence of degradation. This was followed by a reversal of those activities in the immediate post partum period (Mersmann et al., 1972; Devos and Hers, 1974). More recently, Watts and Gain (1976) have reiterated the statement of Bocek et al. (1969) that it is the ratio of phosphorylase to synthetase that controls glycogen metabolism. The former workers found a close relationship between the ratio of phosphorylase a/synthetase I and the glycogen content of the liver. An increase or decrease in this ratio would result in glycogenolysis or glycogenesis, respectively.

The whole process of glycogen metabolism is under strict hormonal control. Numerous workers have stressed the importance of corticosteroids and insulin in the deposition of glycogen (Manns and Brockman, 1969; Avdalovic et al., 1970; Burton et al., 1970; Greengard and Dewey, 1970; Vaillant and Jost, 1971; Monder and Coufalik, 1972; Eisen et al., 1973; Pines et al., 1975; Liggins, 1976; Barnes et al., 1977). Both hormones

appear to act on glycogen synthetase with the corticosteroids serving to initiate the synthesis of new enzyme and insulin stimulating the conversion of the D form to the I form.

At birth, stimulation, either hypoglycemia or hypoxia, causes the release of glucagon which induces the mobilization of glycogen. Glucagon has been shown to increase immediately after birth (DiMarco, et al., 1978). The presence of glucagon, in itself, does not appear to account for the glycogen mobilization, although Schwartz and Rall (1973) have demonstrated reductions in fetal glycogen upon administration of exogenous glucagon. The fetal rat liver appears insensitive to the glycogenolytic effect of glucagon, presumably due to a lack of glucagon receptors (Vinicor, 1976). Therefore, in the neonatal period, glycogen depletion is the result of an increase in both glucagon levels and tissue glucagon receptors. The glucagon is believed to increase intracellular levels of c-AMP which activates pre-existing phosphorylase b and at the same time inactivates synthetase I (Hunter, 1969; Greengard and Dewey, 1970; Snell and Walker, 1973; Pines et al., 1975; Biondi and Viola-Magni, 1977). The stimulation of the sympathetic nervous system, in response to the stress of birth, has also been implicated in the activation of phosphorylase (Shimazy and Amakawa, 1975; Németh, 1977).

Prenatal diieldrin did not affect the deposition of glycogen in the fetus. It did, however, enhance glycogenolysis in the neonate.

At birth, inviable pups have identical glycogen stores as controls. This indicates that the glycogenic capacity, in the late fetal stages, is not affected in the inviable pup. Reductions in fetal liver and heart glycogen have been reported following the maternal administration

of pentobarbital (Delphia et al., 1967; Delphia and Singh, 1972). The reason for the difference is not known, but may be related to the compounds used.

All of the glycogen storage sites showed significantly decreased stores 24 hrs post-delivery, just prior to death. Singhal and Kacew (1976) have also noted decreases in liver glycogen following chronic or acute exposure to organochlorine insecticides. A decreased liver glycogen in association with increased glycogen phosphorylase activity, has recently been found, following acute or chronic exposure to the chlorinated organophosphate insecticide, dichlorvos (Teichert-Kuliszewska and Szymczyk, 1979). Thus, the enhanced glycogenolysis seen in inviable pups is probably due to increased phosphorylase activity.

The possible consequences of this increased glycogen depletion become readily apparent when the use of these stores is considered. Hepatic glycogen maintains blood glucose levels, because this is the only tissue, other than kidney, which has glucose-6-phosphatase. The implications of an increased glycogen depletion will be discussed later in conjunction with hypoglycemia and CNS function.

Skeletal muscle glycogen is required for metabolic energy during muscle function. However, inviable pups, as well as control pups, do not display any physical activity which could account for the depletion. In viable pups may have an increased metabolic rate which might account for the difference. The stores are probably not utilized for heat production as in shivering, since Bruck (1970) has stated that although shivering is developed in newborn animals it is not used even under severe cold stress.

Cardiac glycogen, which is greatly reduced by 24 hrs, may play a crucial role in the survival of the neonate. The contractile and intracellular activity of the fetal heart is directly related to the levels of glycogen (Hoerter, 1976). Furthermore, the ability of the neonatal animal to survive hypoxia is directly related to the glycogen stores in the heart (Dawes and Shelley, 1968). Although there is still a moderate quantity of cardiac glycogen left at 24 hrs, dieldrin pups could in fact be dying of circulatory collapse because of the enhanced glycogen utilization in the fasted state.

The mechanism of the enhanced glycogenolysis remains to be determined. Singhal and Kacew (1976) believe the effect is a direct result of the action of the pesticide on the cells to stimulate the cyclic AMP-adenylcyclase-protein kinase system. Kacew and Singhal (1973a) found glycogen depletion, following acute exposure to p,p'-DDT, in adrenalectomized animals. This suggests that adrenal mediation, either cortical or medullary, is not involved in the response. Glucagon, which is released during hypoglycemic states, has not been studied during pesticide exposure and as such its function remains obscure.

Hyperglycemia has been reported in adults exposed to various insecticides (Bhatea et al., 1973; Kacew et al., 1973; Teichert-Kuliszewska and Szymczyk, 1979). However, it was found that treated animals were consistently hypoglycemic. This hypoglycemia became more pronounced with time until at 24 hrs there was no detectable blood glucose. The fact that the hypoglycemia becomes worse, despite the depletion of a large quantity of liver glycogen, indicates another possible cause of the inviability. At 24 hrs it is conceivable that the blood glucose

should fall since liver stores are essentially gone. Several explanations could account for the hypoglycemia seen at earlier times. It is possible that the inviable pups have such an increased demand for glucose that the glycogen stores cannot meet it. The increased glucose demand could arise as a result of an increased energy requirement or it could be due to an inefficient production of high-energy phosphates.

Organochlorine insecticides have been shown to alter body temperature (Henderson and Woolley, 1970; Hrdina et al., 1974). The alteration in body temperature varies depending upon the compound used and the age of the animal. However, regardless of whether hypothermia or hyperthermia is elicited, the end result would be increased oxygen consumption. Since glucose is the major substrate at birth, the increased demand for substrate, as a result of the birth process and the temperature altering ability of insecticides, could produce the hypoglycemia noted.

An inefficient production of high energy phosphates could arise if oxidative phosphorylation were decreased. Bergen (1971) has shown that dieldrin inhibited electron transport in the cytochrome B area of the electron chain. If it blocked electron transport in inviable pups, ATP production would be limited to two moles produced per mole of glucose. Such a severe reduction in ATP synthesis could result in increased glucose demand and hypoglycemia could result.

The consequences of hypoglycemia directly involve the central nervous system. Glucose is the only nutrient that can be utilized by the brain in sufficient quantities to meet its requirements (Guyton, 1976). Therefore, it is necessary to maintain the blood glucose concentration that will provide this necessary nutrition. In the human, the clinical manifestations

have been extensively studied. Guyton (1976) states that as blood sugar levels fall into the range of 50 to 70 mg percent, the CNS usually becomes quite excitable and is manifested as nervousness, trembling and sweating. As the glucose continues to fall to 20 to 50 mg percent convulsions and unconsciousness occur. Below 20 mg percent a state of coma remains and CNS function is depressed. Goodner (1973) has written that if hypoglycemia remains uncorrected for longer than one or two hours, irreversible lesions in the brain may lead to death. The cause of death in hypoglycemia, Banting (1963) has noted as respiratory failure. It is highly likely, therefore, that by 24 hrs the total absence of plasma glucose causes brain damage probably leading to death by respiratory failure in inviable pups.

In the immediate postnatal period there is a dramatic increase in the levels of the rate-limiting gluconeogenic enzymes -- glucose-6-phosphatase (G-6-Pase), fructose-1,6-diphosphatase (FDPase), pyruvate carboxylase (PC), and phosphoenolpyruvate carboxykinase (PEPCK). In general, gluconeogenesis does not occur, in utero, because of unfavourable energy conditions and the absence of PEPCK; the other enzymes are present in measurable quantities. The ontogeny of gluconeogenesis has been studied extensively in the rat (Yeung and Oliver, 1967; Phillipidis and Ballard, 1970; Snell and Walker, 1973; Girard et al., 1973; Girard et al., 1975; Beaudry et al., 1977); the guinea pig (Bartels, 1974; Robinson, 1976; Raghunathan and Arinze, 1977); and the sheep (Stevenson et al., 1976; Warnes et al., 1977a, 1977b). Despite species differences in the development of the gluconeogenic enzymes, i.e. PEPCK activity is present in guinea pig and sheep prior to birth, no

gluconeogenesis is seen. The appearance of gluconeogenesis after birth may in fact result from the appearance of a well-oxygenated environment (Ballard, 1971; Warnes et al., 1977b) which yields a favourable nucleoside triphosphate level and an oxidized cytosol redox state; which are requirements for activity in the gluconeogenic pathway.

The stimulus for the onset of gluconeogenesis is not yet fully understood. Both glucagon and catecholamines, released in response to hypoglycemia and hypoxia, have been implicated (Yeung and Oliver, 1968; Adam, 1971; Ayuso-Parilla et al., 1977; Beaudry et al., 1977).

It appears that dieldrin-induced neonatal inviability is not associated with an altered gluconeogenic pathway. Organochlorine insecticides have been shown to affect gluconeogenesis, but reports from the literature are contradictory. Singhal and Kacew (1976) have reported elevations of all key gluconeogenic enzymes after chronic or acute exposure to organochlorine insecticides. Bhatia et al. (1973) have reported a similar elevation of all enzymes except G-6-Pase which was decreased following acute exposure to dieldrin. Byard (1975) found inhibition of G-6-Pase activity following exposure to mirex and Sein and Chu (1979) noted no inhibition with DDT. Story and Freedland (1978) demonstrated that DDT decreased gluconeogenic rates from lactate; which was in part attributable to decreased PEPCK activity.

Prenatal dieldrin had no effect on the activity of FDPase in inviable pups. Effects on gluconeogenesis may perhaps require a higher dose of pesticide. This study had a total cumulative dose of dieldrin of 24 mg/kg in the 12-day treatment period. Kacew et al. (1973) administered cumulative dosages of up to 225 mg/kg over their 45-day

treatment period. The inhibition of LDH, as reported by several workers (Hendrickson and Bowden, 1976; Robinson and Yarbrough, 1978), was not seen. The dose, may in fact, have been too low. In conjunction with the lack of an effect on liver LDH were the unaffected levels of lactate and pyruvate. In contrast, blood lactate and pyruvate were elevated in rats treated with a single, high dose of dieldrin (Bhatia et al., 1972). The workers accounted for this elevation by the increase in physical activity of dieldrin-treated animals. In viable pups showed no appreciable activity and therefore, blood lactate and pyruvate would be expected not to change.

Blood urea is elevated following acute or chronic exposure to insecticides (Bhatia et al., 1973; Kacew et al., 1973), indicating increased amino acid catabolism. Blood urea levels are unaltered in viable pups. Although levels of amino acids in blood, have been shown to increase immediately after birth (Girard et al., 1975), they do not contribute a large amount to gluconeogenesis since their catabolism is low in the first 5 days postnatally (Snell and Walker, 1973). For this reason then, an elevation in blood urea would not be expected.

Prenatal dieldrin exposure did not affect the activities of liver FDPase and LDH. Furthermore, the levels of blood lactate and pyruvate in viable pups were unchanged from control pups; as well the levels of blood urea indicated that amino acid catabolism was not different. It is suggested, therefore, that dieldrin-induced neonatal inviability is not associated with an altered gluconeogenic pathway.

CONCLUSIONS

It is unlikely that dieldrin stimulation of the central nervous system is responsible for the inviability. Pups treated with an acute dose of dieldrin died manifesting the classic symptoms of CNS poisoning -- tremors and convulsions. However, inviable pups die without exhibiting these symptoms. For this reason then, the mechanism of the inviability probably lies outside the central nervous system.

The dieldrin-induced inviable pups had normal levels of glycogen at delivery, but these stores are depleted, postnatally, at a faster rate, than those in control mice. This increased depletion may be critical in the heart where maintenance of cardiac contractility is dependent upon the levels of cardiac glycogen.

The inviable pup is born hypoglycemic and this condition worsens with time. It may account for the rapid depletion of liver glycogen. The hypoglycemia may be caused by an increased energy demand or an inhibition of ATP production. The prolonged hypoglycemia may cause brain damage and death.

Dieldrin exposure, prenatally, has no effect on gluconeogenesis since levels of enzymes, precursors, and metabolites associated with the pathway are not significantly changed.

In summary, dieldrin-induced neonatal inviability is not caused by hyperstimulation of the CNS; it is associated with increased glycogenolysis, hypoglycemia, and a normal gluconeogenic pathway.

APPENDIX A

Chemicals and Suppliers

Ammonium Sulfate - Fisher

Anthrone - Fisher

Benzoic Acid - Fisher

Brij-35 - Fisher - a surfactant containing polyoxyethylene lauryl alcohol in a 30% w/v solution.

Boric Acid - Fisher

Dextrose - Fisher

O-Dianisidine-DiHCl - Sigma - 3,3'-dimethoxybenzidine

EDTA-Na₂ - Fisher

Fructose-1,6-diPhosphate-Na₃ - Sigma

Glucose Oxidase - Sigma - 17,200 units/gm solid

Glucose-6-Phosphate Dehydrogenase - Sigma - 330 units/mg Protein

Horseradish Peroxidase - Sigma - 87 units/mg Protein

Hydrazine Hydrate (85%) - Fisher

Hydrazine Sulfate - Fisher

Hydrochloric Acid - Mallinckrodt

Lactic Dehydrogenase - Sigma - 660 units/mg Protein

Lithium-L-Lactate - Sigma

Magnesium Chloride-6 Hydrate - Canlab

2-Mercaptoethanol - Sigma

NAD - Sigma

NADH - Sigma

NADP - Sigma

N-(1-naphthyl) Ethylenediamine Dihydrochloride - Sigma
Perchloric Acid (70%) - Fisher
Phosphoglucose Isomerase - Sigma - 550 units/mg protein
O-Phthalaldehyde - Sigma
Potassium Hydroxide - Canlab
Potassium Phosphate (K_2HPO_4)-Fisher
Potassium Phosphate (KH_2PO_4) - Fisher
Sodium Hydroxide - Fisher
Sodium Pyruvate - Sigma
Sodium Sulfate - Fisher
Sulfuric Acid - Fisher
Trizma Base - Sigma - Tris(hydroxymethyl) aminomethane
Urea - Fisher

Addresses

Canlab - Canadian Laboratory Supplies, Toronto, Canada.
Fisher - Fisher Scientific Co., Montreal, Canada.
Mallinckrodt - Mallinckrodt Inc., St. Louis, Missouri
Sigma - Sigma Chemical Co., St. Louis, Missouri.

APPENDIX B

Preparation of Buffers

Hydrazine Buffer (1.1 M; pH 9.0)

1.0 g Na₂ EDTA (Fisher) plus 6.5 g Hydrazine Sulfate (Fisher) and 29.5 ml of 85% Hydrazine Hydrate Solution (Fisher). Dissolve in distilled water and make up to 500 ml. Adjust pH to 9.0 with 5N HCl (Mallinckrodt).

Phosphate Buffer (1 M; pH 7.0)

1.0 g Na₂ EDTA plus 60.45 g K₂H PO₄ and 20.40 g KH₂ PO₄ add contents to a beaker full of water and dissolve. Make up to 500 ml and adjust pH with 5N HCl.

Tris Buffer (1 M; pH 7.5)

Dissolve 121 g of Tris in 800 ml of H₂O and adjust to pH with 5N HCl. Dilute to 1000 ml and recheck pH. Adjust the pH if required.

Phosphate Pyruvate Solution (PPS)

-0.005 M Phosphate Buffer pH 7.5; 3.1×10^{-4} M pyruvate.
4.37 g K₂H PO₄ plus 0.56 g KH₂PO₄ and 18.75 mg Sodium Pyruvate. Dissolve in distilled water and make up to 500 ml. Adjust pH with 5N HCl.

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