DEDICATION

To my wife, to our children, and to our parents.

ACKNOWLEDGMENTS

I am deeply indebted to Dr. Quentin N. LaHam who directed these studies. Having presented me with a carte blanche, he gave me all his silent authorization. One can only be acquainted with him after having worked with him and having personally fulfilled the demands that are required for success.

I am very grateful to Dr. André DesMarais, for his guidance and encouragement during my undergraduate years.

I wish to thank Drs. E.O. Dodson, D.J. Hobden, and Nancy McAllister for criticism of the thesis itself.

I would like to thank Mrs. Monika Tocchi, Mrs. Connie Graveline, Mrs. Alice Tomlinson, Mr. Jacques Hélie, Miss Nicole Leclair and Mr. Gilles Robitaille for their technical assistance.

I would especially like to thank Mr. George Ben-Tchavtchavadze for the excellent photographs.

This study was made possible by National Research Council grant A-1019.

ABSTRACT

Organophosphates are shown to be teratogens, producing aeopthalmia, acrania, micromelia, hydrocephaly, and hyperplasia of the maxilla in hatched chicks. The resultant teratisms could not be consistently reproduced. Explanations for this inconsistency are given, along with possible means of obtaining reproducible teratisms.

After biochemical, histochemical and prophylactic treatment, and the injection of 1,2 propanediol-1-C^{14} into the yolk sac of eight-day chick embryos, specific toxicity of 1,2 propanediol was attributed to a transition of functional activity in the embryo. The results are discussed in reference to Claude Bernard's concept of "foie transitoire".

RESUME

Les études sur les effets tératogènes des organophosphates, ont permis d'observer des aeopthalmies, des acranies, des micromélies, des hydrocéphalies, et des hyperplasies de maxillaires de poussins. Ces tératismes n'ont pu être reproduits avec régularité. L'auteur a proposé des explications sur les causes probables de ces irrégularités ainsi que des mesures pouvant permettre la reproductibilité des résultats.

A la suite de tests biochimiques, histochimiques et prophylactiques, et l'injection de 1,2 propanediol-1-C^{14} dans le sac vitellin d'embryons de poulet de huit jours, les effets toxiques du 1,2 propanediol ont été attribués à

une transition embryonnaire de l'activité fonctionnelle. Les résultats ont été discutés à la lumière du concept du "foie transitoire" de Claude Bernard.

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Part I

Teratological Effects of Organophosphates on the Developing Chick Embryo

INTRODUCTION

"..... understanding of the "abnormal" will become but an extension of our insight into the "normal", while, pari passu, the study of the "abnormal" will contribute to the deeping of that very insight" (Weiss 1961).

Teratisms have challenged embryologists for many years, but the recent thalidomide tragedy (Somers 1963) has brought renewed interest in them. A large number of known teratogenic compounds has been catalogued (Wilson 1964) and the malformations have been extensively described. Teratisms can be caused by physical agents, diseases, vitamin and endocrine deficiencies or excesses, and many toxic chemicals. The embryo responds in a similar manner to all teratogens, and there is little relationship between the chemical structure or type of physical stress and the resultant defects. In spite of the scientific and technological advances which have occurred, there has been little advance in understanding how teratogens disrupt the normal process of embryogenesis.

The organophosphates, which are widely employed as pesticides, are also potent teratogens. The use of organophosphates as pesticides was proposed by Willy Lange in 1939 (Holmstedt 1963) but little interest was shown until the end of the Second World War when organophosphates were released

for scientific research. This led to their use as pesticides, and shortly thereafter literature on the mechanisms of the toxic effects of these anticholinesterase agents began to appear (Whittaker 1951, Casida 1964, Krupka 1964, Nachmansohn 1966, Nastuk 1966, Aldridge and Barnes 1966 a,b).

Acetylcholinesterase is important in the neuromuscular junction, the sympathetic synapsis, the parasympathetic neuro-effectors, and the sympathetic innervation of the adrenal and sweat glands. The cholinergic system involves the transfer of acetylcholine from the presynaptic to the postsynaptic unit in axon transmission or to the muscle unit in neuromuscular transmission. If cholinesterase is not present to hydrolyse acetylcholine, acetylcholine persists and the response to a single stimulus is much prolonged. Anticholinesterase agents such as the organophosphates exhibit various degrees of toxicity but all produce fasciculation, pupil contraction, constriction of the bronchioles, muscle twitch, fibrillation and muscular weakness followed by prostration and convulsions, respiratory failure, and asphyxiation.

The organophosphates can also be toxic in ways other than the inhibition of acetylcholinesterase. Khera et al (1965, 1966) have shown that the injection of organophosphates into the yolk sac of ducks eggs can result in teratisms in ducklings. Rogers et al (1964) and McLaughlin et al (1963) have produced similar teratisms in chicks.

The oxidation and metabolism of the organophosphates is much better known than their interference with cellular processes (Millen and Woollam 1963, Heath 1966, and Casida 1956).

In this study the teratogenic effects of organophosphates were explored. It was first necessary to determine the stage in chick embryonic development during which the organophosphates produced teratisms, then studies could be made to discover which biochemical processes were disturbed.

Materials and Methods

Incubation

Fertile White Leghorn eggs purchased from a commercial hatchery* were incubated in a Jamesway single stage incubator-hatcher Model 252B according to the recommendation of the manufacturer. Two hours were allowed for the eggs to achieve incubation temperature. This procedure gave development conforming to the stages of Hamburger and Hamilton (Hamilton 1965).

Preparation of Organophosphates

a) Water Soluble: A stock solution of Phosphamidon (2-chloro-2-diethylcarbamoyl-1-methylvinyl-dimethyl phosphate) was prepared containing 5 mg/ml in distilled water. Prior to inoculation, the stock solution was diluted to the concentrations required for injection of 0.1 ml of the solution per egg. 15 ml bacteriological test tubes fitted with Millipore Swinny adapters were autoclaved for 15 minutes. The prepared inoculi were filtered into the test tubes using a 10 cc syringe. A distilled water control was similarly prepared.

b) Water Insoluble: An alternative aseptic technique was used for these organophosphates, because the viscosity of 1,2 propanediol precludes the use of a Millipore filter.

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* Pioneer Hy-Line Corn Company, Chatham, Ontario.

Screw-cap bacteriological test tubes and 1, 5 and 10 ml pipettes were sterilized in the autoclave. 1,2 propanediol, the solvent for the water insoluble organophosphates, possesses antimicrobial properties and does not need sterilization. This was confirmed by sterilizing 1,2 propanediol with dry heat and also by autoclaving. No difference between the sterilized and the unsterilized solvent was observed when plated on a nutrient agar medium. The 1,2 propanediol was guaranteed by the manufacturer to be better than 99% pure.

Stock solutions in 1,2 propanediol at a concentration of 1 mg/ml were prepared for the following organophosphates: Dylox (0,0 dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate), Baytex (Bay 29493; 0,0-dimethyl-0-[4-(methylthio)-m-toyl] phosphorothioate, Sumithion (0,0 dimethyl-(3-methyl-4-nitrophenyl) phosphorothioate), and Parathion (0,0 diethyl-p-nitrophenyl thiophosphate). The stock solutions were diluted to the final concentrations required for injection of 0.1 ml per egg.

Concentrations Inoculated

Batches of eggs at known stages of development were injected with organophosphates and the ensuing mortality determined. Separate mortality determinations were made for each day of the first 10 or 12 days of development for each organophosphate concentration tested. Thirty eggs were injected each day for each concentration and allowed to continue development until hatching or death. The mortality results are given on the basis of day of injection causing death, not on day of death.

1. Phosphamidon was injected for the first twelve days of incubation in amounts of 10.0 µg and 100.0 µg per egg.

2. The above experiment was repeated with the addition of a third series which received a concentration of 500 µg/egg.

3. Due to the inconsistency of the results two additional runs were made with 100 and 500 µg of Phosphamidon per egg using embryos one to ten days in ovo.

4. Parathion, Dylox, Baytex, and Sumithion were each injected at 10.0 and 100 µg/egg using embryos of one to ten days of development.

5. A final series was treated with 500 µg of Baytex per egg for embryos from one to ten days old.

Inoculation of Eggs

All eggs were candled prior to inoculation to determine the position of air sac and embryo, and these positions were pencilled on the shell. Eggs with displaced air sac, tremulous air sac, cracked shells, improperly calcified shells or blood clots were discarded. A modification of the technique of McLaughlin et al (1963) was employed to inoculate the eggs.

In the later stage embryos (4-12 days), ten eggs at a time were arranged in a rack with the air sac region up and the marked position of the embryo facing the inoculator (Plate I). The air sac region was washed three times with

cotton gauze soaked in 70% ethanol. A small hole was pierced in the middle of the outlined air sac with a sterile teasing needle and the hole wiped with the gauze to remove any pieces of broken shell.

Inoculation was done with a sterile disposable tuberculin syringe fitted with a 1" x 20G needle. This ensured that the inoculant was deposited in the centre of the yolk. The eggs were injected from a vertical position with the needle at a slight angle away from the embryo.

The 1-3 day old eggs were rotated several times on their long axis, placed horizontally in the inoculation rack, and the needle introduced in a horizontal position.

After inoculation the holes were sealed with a drop of warm paraffin wax and the eggs returned to the incubator. The elapsed time from removal to return to the incubator was about 15 minutes.

Thirty eggs of each age were inoculated for each concentration used. The eggs were candled every three days after treatment to follow their development.

Histological and Histochemical Methods

The following methods were used as described by Pearse (1960); Koelle's thiocholine method for cholinesterase sites, Gomori's myristylcholine technique for motor end-plates, a modified azo dye method for alkaline phosphatase, and a general stain with haematoxylin, phloxin and orange G (HPO). These methods were used for study of the thigh muscles of pre- and post-hatched chicks.

Results

Phosphamidon

a) Mortality

The results of the first experiment indicated that with the 100 µg dose there were two periods of high susceptibility (fig. 1); one following injections on the second day (100% mortality) and other following injections on the eighth day of incubation (95% mortality). With the 10 µg dose, the mortality rate rapidly declined after the first day. The control eggs inoculated with distilled water showed unexpectedly high mortality for injections on the first five days, but mortality then dropped to 0.5% per day for the remainder of the experiment. This high level of mortality in the control approximated that in the chicks treated with 10 µg of Phosphamidon.

The results of subsequent experiments were not consistent (fig. 2-4). The 100 µg/egg dose of Phosphamidon (fig. 1), which appeared to establish a dose-response relationship, in repeat experiments (fig. 2) was less toxic than the 10 µg/egg dose, and the peaks of mortality on the second and eighth day were no longer obtained. The 10 µg/egg series now showed a high mortality on the third day of incubation (fig. 2).

Phosphamidon at 500 µg/egg fluctuated in toxicity for the first seven days then increased in toxicity (fig. 2,3). A further series with 500 µg/egg gave results similar to those obtained with the 100 µg/egg series (fig. 4).

b) Teratology

The frequency and types of deformities observed were as inconsistant as the mortality curves. The most frequent defect in surviving hatched chicks is referred to as a neurotoxic syndrome. A very wide gait with hyperextension of one or both hind limbs causes the chick to rest on its sternum. The chick cannot stand and, therefore, shuffles around on its sternum (Plate II, fig. 1,2). The chick in fig. 1 shows lateral extension of the right hind limb and flexure of the left tarsus. The control chick shows the normal stance at the same age.

Fig. 2 shows chicks with hyperextension of one or both legs. 10-20% of hatched treated chicks show these hind limb defects; the remainder had normal posture. Phosphamidon at 100 µg/egg produced other teratisms at a frequency of 2-5%. They are: hydrocephaly (Plate III, fig. 1), massive distortion of the maxilla with no eye development (Plate III, fig. 2), and a crossed beak with no development of the left eye (Plate IV, fig. 1).

Even at 10 µg/egg, Phosphamidon caused teratisms. The chick shown in Plate IV, fig. 2 failed to develop a right eye, and twisting of the maxilla has occurred. A dose of 500 µg/egg produced a micromelia syndrome in one chick. (Plate V).

c) Histological and Histochemical Examination

Histological examination of the motor end-plates in

the thigh muscle of normal embryos with Gomori's myristylcholine technique gave the following picture: motor end-plates that reacted with myristylcholine were present in abundance from the seventeenth day of incubation to hatching. After hatching, localization with this method revealed a gradual decline in stained end-plates until the twelfth day, after which the number of marked end-plates was negligible. The myristylcholine sites were absent in the neurotoxic chicks.

In studies of normal embryos with Koelle's thiocholine method, no acetylcholine or butyrylcholine-esterase sites were found in the thigh muscle until hatching day. Acetylcholinesterase sites were few in number at first, but the numbers of sites increased through the sixth day. The butyrylcholinesterase sites were always few in number, and on the seventh day after hatching the test failed to demonstrate any of them.

Examination of the thigh muscle of a six day old chick with severe neurotoxic syndrome showed a few acetylcholinesterase sites of weak concentration (Plate VI, fig. 1) and no butyrylcholinesterase sites. The control chick of the same age possessed many more acetylcholinesterase sites of heavier concentration but no butyrylcholinesterase sites (Plate VI, fig. 2).

The HPO stain on the same thigh muscle demonstrated degeneration of the muscle units in the affected chick (Plate VII, fig. 1), while the muscle of the control chick

was normal (Plate VII, fig. 2). The alkaline phosphatase test showed a weaker concentration of the enzyme in the treated chick.

Water Insoluble Organophosphates

a) Mortality

When Parathion was inoculated at 10 µg/egg and 100 µg/egg (fig. 5), a very high but irregular mortality was noted. The 10 µg dose was much more toxic than the 100 µg dose for the first two days, then declined to a toxicity below that of the 100 µg injections. The two concentrations converged on day seven, rose together on day 8, and while the 100 µg toxicity continued its increase, the 10 µg toxicity fell.

The control (1,2 propanediol) showed high toxicity on the first day of incubation (76%) which descended to a negligible percent by day seven but rose to 100% mortality on the next day. This was followed by a drop to no mortalities by the twelfth day of incubation. At first, the high eighth day mortality of the control eggs was thought to be an experimental error. Hoping to correct this supposed mistake and to obtain a consistant teratogenic effect, eggs were treated with Dylox, Baytex or Sumithion in concentrations equivalent to that of Parathion. The composite chart (fig. 6) illustrates the range of toxicity of the three organophosphates and of the control injections with the solvent 1,2 propanediol. It was evident that the glycol

solvent itself was nocuous and that the 100% mortality on the eighth day due to 1,2 propanediol was not an experimental error.

As a final confirmation of the unpredictability of organophosphates dissolved in 1,2 propanediol, Baytex was injected at a concentration of 500 µg/egg. The mortality (fig. 7) was again erratic when compared to the previous inoculations.

b) Teratology

Parathion is known to be more toxic than Phosphamidon yet no teratisms were obtained with it. Examination of all the dead embryos showed that death was due to severe hemorrhage.

Sumithion in a 10 µg dose per egg may have an immediate effect, or the effect may be delayed. The embryos in Plate VIII, fig. 1,2 were inoculated on the second day of development, but each embryo progressed to a different stage before dying. The chick shown in fig. 1 died almost immediately, while that in figure 2 died on about day nine. An embryo (Plate IX, fig. 1) treated on the third day of incubation with this same concentration (10 µg/egg) appears to have died on day five.

The embryos in Plate IX, fig. 2 and Plate X, fig. 1 were inoculated with 100 µg of Sumithion on days three and four respectively. These embryos had advanced to different stages of embryogenesis before dying, but the exact ages cannot be decided because of the gross distortion to the organs.

The embryos in Plate X, fig. 2 and Plate XI, fig. 1,2 were inoculated on the first, second, and third day respectively with 10 µg of Baytex. The chicks inoculated on days one and three (Plate X, fig. 2 and Plate XI, fig. 2) lived to term but failed to hatch, while the embryo treated on the second day (Plate XI, fig. 1) died soon after inoculation.

Teratisms varied from hyperplasia of the mandible with distorted eye development (Plate X, fig. 2) to a massive disturbance of the developing embryo (Plate XI, fig. 1) and acrania (Plate XI, fig. 2). Other eggs injected with 10 µg of Baytex on days one, two, and three (Plate XII, fig. 1,2 and Plate XIII, fig. 1) further illustrate the irregularity with which the organophosphates affect the embryos. The developmental events have been affected differently in each of these chicks.

The chick in Plate XIII figure 2 was treated with 10 µg of Dylox on the seventh day. This teratism represents a combination of malformations observed in Plate X, fig. 2 and Plate XI, fig. 2. The cranium and the eyes have failed to develop, while the mandible shows hyperplasia.

The frequency of teratogenesis in all experiments ranged from 6-10% of total eggs injected. When 1,2 propanediol was the solvent, many embryos died shortly after the injection. These were not malformed but showed signs of massive hemorrhage.

Discussion

Dosage and Timing

In these experiments, wide variation in toxicity and teratogenesis are the most prominent features of the results. Smithells (1966) has pointed out that lethal agents, chemical or physical, can produce malformations provided that the dosage and timing are appropriate. In cleidoic eggs the dosage and the timing are affected by the yolk mass and the yolk sac membrane, both formidable obstacles to the entry of injected toxins into the embryonic system. The properties of the solvent are also important in transmission of the toxic substance to the embryo.

The yolk can give significant protection to the developing embryo by diluting a chemical to a less toxic level. Also Walker (1967) has shown that the density of the solvent employed for injections into the yolk sac either aids or hinders the compound in reaching the embryonic system. Thus, propylene glycol tends to remain at the site of injection since its density (1.036) is similar to that of yolk (1.035), while compounds dissolved in corn oil (density 0.92), for instance, rise to a position immediately under the embryo and are more quickly absorbed.

The last and most significant of the obstacles to be traversed by the injected compound is the yolk sac membrane. The toxic substance may first be degraded by enzymes utilized by the yolk sac membrane itself, or left to

lie in the yolk until the carbohydrate, lipid or protein cycle of utilization begins in the embryo. Since it is known that there are cycles of utilization of the yolk ingredients by the embryo, selective permeability may inhibit movement through the membrane.

Needham's calculations indicate that during the first week of development much of the material derived from the yolk is incorporated into the yolk sac membrane for its own development (Romanoff 1960). Therefore relatively small amounts of toxins would be passed on to the embryo.

The size of the building blocks presented to the embryo for its synthetic activities by the yolk sac membrane is unknown (Williams 1967). With overlapping cycles of carbohydrate, lipid and protein metabolism in the chick embryo, the absorption of the injected compound by the yolk sac membrane may be governed by the membrane and metabolic cycle in progress at the moment. Thus the toxic material could be held at the membrane until the appropriate cycle began. Or all or part of the toxic material could be held back by selective permeability until it degenerates.

Another feature of the yolk sac membrane is its function as a transition liver. We do know that there are digestive enzymes present in the yolk sac membrane but what types and how they function is not known. The degree to which the liver and yolk sac membrane are analogous is unknown, but if the yolk sac membrane functions as a liver,

it could degrade all or part of the toxin.

With a significant delay, the compound's target organ may well progress from a susceptible to an immune stage and leave the compound unable to express any teratogenic potential. Consistant deformities in the hind limbs of chicks are produced by Malathion (LaHam and Greenberg 1969), when the solvent rises through the yolk to the embryo, the concentration is higher than those used here, and injections are at susceptible periods in embryogenesis.

Teratisms

The postural defects produced in teratogenic experiments must not be confused with a disability caused by improper care of the hatching chicks. If eggs which are about to hatch are widely spaced in the hatching trays, the chicks develop a condition similar to the neurotoxic syndrome. This condition results from the newly hatched chicks' inability to obtain traction on the broken egg shells in order to stand.

Various postural defects were obtained with Phosphamidon, but it was not verified whether the etiology was the same as that described by Davies (1963). The histological results in his hens, in which the clinical signs were well developed, showed demyelination of the sciatic nerves, the spinal cord, and the medulla.

Leg and foot deformities have also been observed by Khera et al (1965) in ducklings after treatment of 13 day

old embryos with 100 µg/egg of the organophosphates EPN and Systox. In a further study (1966) the authors failed to show a demyelination of the peripheral nerves and the spinal cord. It was assumed that the foot deformities were associated with muscular dystrophy of the thigh and shank muscles.

The histological examination of the thigh muscles of the neurotoxic chicks using a HPO stain demonstrated degeneration of the muscle fibers. A histochemical investigation of several dehydrogenase enzymes of the thigh muscle by LaHam et al (1968) also indicates degeneration, as do the present results on alkaline phosphatase, acetylcholinesterase and myristylcholine.

The events which culminate in these limb deformities must have begun with action of the toxins on the tissues close to or at the motor end-plates (Khera et al 1966). Denz (1951) inoculated anticholinesterase drugs into adult rats and could not demonstrate any pathological lesions of the motor end-plates. Harris (1954) reached the similar conclusion that morphological alterations were not present in the motor end-plates of animals subjected to neurotoxic drugs. But in view of the role of acetylcholinesterase in transmission of the nerve impulse, disruption of its function is obviously involved.

Since teratisms occurred only during the first week of chick embryogenesis, it would appear that the anticholines-

terase agent could enter into and damage the embryo at any time during this period. In all of the teratisms observed, the affected organs were either the cranium (leading to aeopthalmia, acrania, or both) or the hind limbs.

Mortality

Two critical periods in normal development have been observed by commercial hatcheries (Hamilton 1952), one at the three to four day period (3-4% mortality), and the other just prior to hatch on the nineteenth to twentieth day (4-15% mortality). For the remainder of the incubation period the daily rate of mortality is less than one percent per day. Higher mortalities are the result of the substances injected. The amount of toxin reaching the embryo depends on the timing and dose, as discussed above. High amounts caused death, while sublethal amounts sometimes produced teratisms.

The one event which was always reproducible was the high toxicity of 1,2 propanediol on the eighth day of chick embryogenesis. There was a sudden elevation in mortality on the eighth day followed immediately by zero mortality. Why a glycogenic glycol should become so nocuous at the end of the first week of chick development will be further investigated in part two.

Part II

Toxicity of 1,2 Propanediol on the Developing Chick Embryo

Introduction

The acute toxicity of 1,2 propanediol on the eighth day of incubation was intriguing, because it was difficult to see why it was so lethal. The glycol is a simple three carbon compound with no specific inhibitory properties. Metabolic and biochemical studies had suggested that it was innocuous, acceptable, glycogenic, and a product of biological systems (see Appendix). On the second to sixth days of incubation the lethality of 1,2 propanediol was almost zero. The toxicity study in our laboratory showed that some process on the eighth day had been drastically affected. It also seemed that much of the teratogenic expression of the water-insoluble organophosphates was being masked by the effect of 1,2 propanediol.

A literature review indicated that this phenomenon had never been observed before. This study was undertaken to clarify the action of 1,2 propanediol and its specific effects on chick embryo development on the eighth day.

By the eighth day, organogenesis is almost complete and initial functional activity has commenced (Willier 1954). The heart and the circulatory system in the embryo are well established. By the end of the chick's fifth day

of incubation, the definitive vessels of the yolk sac membrane have differentiated (Romanoff 1960 b). Mature erythrocytes have started to develop by day six and have reached their plateau by day eight.

The endocrine organs will soon begin to assume their secretory functions (Willier 1954). The mesonephric kidney is functional, but the metanephric kidney will not commence its activities until the eleventh day (Romanoff 1960 c, Hamilton 1965, and Gibley and Chang 1967). In the eighth-day chick embryo, the hepatocytes have begun to store glycogen (Zaccheo and Grossi 1967, O'Connor 1953, Zwilling 1951). At this time, the glycogen body in the lumbosacral region of the spinal cord begins to differentiate (Romanoff 1960 a). This body stores glycogen, but it is not known to have any other function.

Three critical events on the eighth day of development might be associated with the acute toxicity of 1,2 propanediol. First, Boell (1955) presented a semilogarithmic plot of oxygen consumption during chick development which showed an inflection point between the seventh and eighth days. The respiratory rate continued to increase but at a lower rate than that previously. If Novikoff and Potter's (Boell 1955) results with PNA (pentose nucleic acids) and DNA are similarly plotted, a change in the slope of the straight line also occurs on the eighth day. Boell's conclusion from this evidence was that there was a transition in the chemical growth of the chick.

Second, Claude Bernard's hypothesis of the "foie transitoire" (Zwilling 1951, Bellairs 1963, and Thomas and Just 1964), states that the yolk sac membrane stores glycogen until this function can be transferred to the embryonic liver.

The final possibility is that 1,2 propanediol interferes with the development of the glycogen body.

Thus the problem was approached with these three considerations in mind: a chemical transition in embryonic development, Claude Bernard's transition liver, and the lumbosacral glycogen body. The study of the eight-day chick embryo was made with histochemical and biochemical methods. To gain further insight into the problem, 1,2 propanediol-1-C^{14} was administered and traced, and attempts were made to counteract the effects of 1,2 propanediol with amino acids, vitamins, hormones, and other chemicals.

Materials and Methods

Fertile White Leghorn eggs purchased from a commercial hatchery were set in a Jamesway single stage incubator as previously described. The guaranteed fertility of the eggs was 85-90%. Periodically five eggs were staged to verify that the eggs were on the developmental schedule described by Hamilton (1965).

On each of the first ten days of development, thirty eggs were inoculated with 0.1 ml of 99% pure 1,2 propanediol purchased from Kodak Distillation Products, Rochester, New York. The method of inoculation was described in the methods section of Part I. To confirm the toxicity of 1,2 propanediol, four separate experiments were performed. The controls were injected with 0.1 ml of distilled water.

Histological Study

Embryos which died on days 7,8 and 9 were placed with their yolk sac membranes in Bouin's fixative.

For pathological examination, the embryos were stained with hematoxylin, phloxin, and orange G(HPO). The carbohydrate content of these embryos and their yolk sac membranes was investigated with the AB-PAS (alcine blue-periodic acid Schiff's) procedure (Histochemistry 1962).

Any variation in lipid distribution in day 8 embryos was studied after staining with Sudan Black B (Culling 1963 a). In order to section these young embryos, 5.0 mm. sections of the embryos were fixed in neutral formol-calcium

for four hours at 4°C. then transferred to a gum sucrose solution at the same temperature (Pearse et al 1963, and LaHam et al 1969).

The connective tissue matrix was stained with a Mallory stain (Culling 1963 b). The following enzymes were identified histochemically using methods described by Pearse (1960): cytochrome oxidase (Burstone's method), glutamic dehydrogenase (GDH), α-glycerophosphate dehydrogenase (α-GPDH), malic dehydrogenase (MDH), lactic dehydrogenase (LDH) and succinic dehydrogenase (SDH). These enzymes were selected to observe if any metabolic errors occured in glycolysis, Kreb's cycle or oxidative phosphorylation.

Biochemical Study

1,2 propanediol is known to be glycogenic. To determine whether injection of 1,2 propanediol had altered the glycogen content of the yolk sac membrane, a quantitative enzymatic determination (Johnson and Fusaro 1966) was made on the yolk sac membrane of live embryos from days seven, eight, and nine and dead embryos from day eight. These results were analyzed statistically by analysis of variance and by Duncan's multiple range test (Steele and Torrie 1960).

The quantity of cytochrome oxidase in the yolk sac membrane was investigated using the method of Pearl et al (1963). The calculations were expressed as change in optical density per 30 minutes incubation per µg of protein.

Triplicate readings on the three samples from days 6, 7, 8, and 9 were made and the results analyzed statistically (Snedecor 1959). The amount of protein was determined by the method of Lowry et al (Damn 1966).

Since the main pathway in 1,2 propanediol degradation is towards lactic acid, lactate was determined quantitatively on eight day embryos and yolk sac membranes. Each of the five samples from dead embryos was read three times. There were only four samples of embryos which survived the glycol injection. The results were analyzed statistically employing the analysis of variance for unequal samples (Steele and Torrie 1960).

Prophylactic Investigation

The purpose of antidote experiments was to further understand the metabolism of the eight-day embryos and to find out how 1,2 propanediol interfered with this metabolism (see Appendix. Metabolic pathway of 1,2 propanediol).

The experiments included 30 eight day embryos in each sample. The method of injection was as previously described, and the eggs were candled 24 hours after treatment. The compounds injected were amino acids, vitamins, and hormones, and the solvent was 1,2 propanediol.

In the metabolism of acetone, radioactive C^{14} is transferred from 1,2 propanediol to serine, methionine, and choline (Sakami 1950 a, b). If 1,2 propanediol interferes with amino acid metabolism, then replacement of the amino

acids would prevent death. Since the action of 1,2 propanediol is so rapid, the amino acids (Nutritional Biochemical Corp.) were dissolved in 0.5 ml of 1.0 N sodium hydroxide, then made up to 10.0 ml with 1,2 propanediol. The control injections were 0.5 ml of sodium hydroxide in 1,2 propanediol. Table 1 lists the amino acids used with the injected amounts expressed as mg/egg.

Transmethylation from 1,2 propanediol is involved in the metabolism of acetone to the amino acids and excess 1,2 propanediol may inhibit transmethylation. Therefore folic acid (Nutritional Biochemical Corp.) was administered to the embryos to stop this possible inhibition. A stock solution of 10.0 mg/ml was prepared with 0.5 ml of distilled water to dissolve the vitamin and 9.5 ml of 1,2 propanediol added. The stock solution was serially diluted to obtain the concentrations listed in Table 2.

The most obvious gross effect of 1,2 propanediol was massive hemorrhage. Ascorbic acid is involved in maintaining the integrity of blood vessels walls, and in the production of adrenocortical hormones. This vitamin was prepared in a stock solution of 10.0 mg/ml in 1,2 propanediol, serially diluted, and inoculated in the amount specified in Table 2.

Vitamin B_{12} is necessary for the transfer of methyl groups and to maintain hormone balance. To ascertain if 1,2 propanediol interfered with either of the processes, vitamin B_{12} (Sigma) was prepared and inoculated as above. The amounts injected are listed in Table 2.

TABLE 1

Amino acids	Amount Injected (mg /egg)
Alanine	1.0, 2.5
Arginine*	1.0, 0.1
Asparagine	1.0, 0.1
Glycine	1.0, 0.1
Glutamine	1.0, 0.1
Histidine*	1.0, 0.1
Isoleucine*	1.0, 0.1
Leucine*	1.0, 0.1
Lysine*	1.0, 0.1
Methionine*	5.0, 1.0, 0.6
Serine	5.0, 1.0, 0.6
Valine*	1.0 mg.
serine+methionine+alanine	2 mg of each
alanine+valine+leucine+isoleucine	0.5 of each
phenylalanine+tyrosine	0.5 of each
glutamic acid+aspartic acid	0.5 of each
arginine+lysine+histidine	0.5 of each
glutamine+asparagine	0.5 of each

* essential to young hatched chicks

Pathological examination revealed that 1,2 propanediol caused extensive damage to the central nervous system. Normal development of C.N.S. requires pantothenic acid. Therefore injections of this vitamin were made to find out if 1,2 propanediol interfered with the action of pantothenic acid. The preparation for this injection was the same as before, and the amounts injected are listed in Table 2.

Nicotinamide adenine dinucleotide (NAD) is the hydrogen carrier for the breakdown of 1,2 propanediol to lactic acid. To find out if an insufficient amount of NAD is the cause of death, nicotinic acid, and nicotinamide were prepared as above and injected in the amounts indicated in Table 2.

Experimental investigations by several authors suggest that hormonal activity in the chick embryo begins soon after the eighth day. To test possible hormone imbalance as the result of 1,2 propanediol administration, the following hormones were injected: corticosterone, adrenal corticotrophic hormone (ACTH), adrenaline, cortisone, and insulin. The latter three are stimulators of glycogenesis. Corticosterone and insulin are soluble in 1,2 propanediol. Adrenalin, cortisone, and ACTH were dissolved in minimal volumes of hydrochloric acid (2N), ethanol, and distilled water respectively, then brought to a 10.0 ml volume with 1,2 propanediol. The amounts injected are listed in Table 2.

To find out if 1,2 propanediol interfered with enzymes from the liver or yolk sac membrane, four yolk sac membranes from six day-old embryos and four livers from twelve

TABLE 2

Vitamins	Amount Injected (µg/egg)
Folic acid	1.0, 0.5, 0.25, 0.1, 0.05, 0.01, 0.001
Ascorbic acid	10, 5, 0.5, 0.05, 0.01, 0.001, 0.0001
Vitamin B_{12}	1.0, 0.5, 0.25
Pantothenic acid	5.0, 1.0, 0.5, 0.05
Nicotinic acid	5.0, 2.5, 1.0
Nicotinamide	5.0, 2.5, 1.0

Hormones	Amount Injected (µg/egg)
Corticosterone	100, 25, 10, 1, 0.1, 0.01
ACTH	1.25, 0.125, 0.0125
Adrenalin	100.0, 1.0, 0.1, 0.01
Cortisone	100,
Insulin	10, 1.0, 1000, 500, 250, 125, 100, 10, 5, 1.0, 0.5.

day old embryos were placed in 5.0 ml of 0.1 M phosphate buffer pH 7.0 at 4°C. The samples were homogenized immediately in separate glass homogenizers and centrifuged at 15,000 r.p.m. for 20 minutes in a Sorval centrifuge. The supernatants were passed through sterile millipore filters and frozen until required. On day eight, one group of eggs received 0.1 ml of the supernatant of the yolk sac membrane, and the other 0.1 ml of the liver supernatant. One series, comprising both yolk sac membrane and liver injected eggs, received 1,2 propanediol immediately. Another series was injected with 1,2 propanediol four hours after the injection of yolk sac membrane or liver supernatants.

The number of liver mitochondria in rats increased when hyperthyroidism was induced (Enster 1965). To test the possibility of producing a precocious increase in embryonic liver mitochondria, fertile eggs were treated 2,3 and 4 days in advance of day eight with 3, 3', 5 triiodo-L-thyronine (T_3). As blood vessels on these days were much too small to receive an injection, two other methods of inoculation were employed. T_3 was either deposited on the air sac membrane with a ½" x 26G needle or a small hole was drilled in the side of the shell and the material injected under the shell membrane. A stock solution of 10.0 µg/ml of T_3 in 0.9% sodium chloride was sterilized by filtering through a sterile millipore filter and serially diluted under aseptic conditions. The prepared solutions were injected at 0.1 ml per egg in the following amounts:

1.0 µg; 100.0, 50.0, 10.0, 5.0, and 0.1 nanograms per egg.

General Experiments

To confirm whether the high mortality on the eighth day could be the result of shock, eight day old embryos were inoculated with a needle dipped in 1,2 propanediol, but no material was injected.

The toxicity of 1,2 propanediol-1-phosphate (Nutritional Biochemical Corp.) was tested by injections of 100.0 mg/egg into the yolk sac of eight day embryos.

The dose response of day eight embryos to 1,2 propanediol was determined with injections of the following amounts per egg: 78.0, 52.0 and 26.0 mg/egg. This experiment was performed twice.

To test the sensitivity of the eighth day embryo to glucose and some products of glycolysis, glucose was injected into the yolk sac at 75.0, 50.0, 25.0, and 12.5 mg/egg. Sodium lactate or pyruvic acid were injected into the yolk sac in the following amounts: 89.3, 59.5, 29.8, 5.0, and 1.0 mg/egg respectively.

Tracer Experiment

1,2 propanediol-1-C^{14} with a specific activity of 48 Mc/mM (International Chemical and Nuclear Corporation, California) was diluted and injected into groups of ten eggs on days 6, 7, 8, 9, and 10 of incubation. 1.0 µc/egg was administered in 1,2 propanediol at a total volume of

0.1 ml. Four hours after treatment, the yolk, the yolk sac membranes, and the embryos were removed from five eggs. The yolk sac membranes and the embryos were very briefly washed in 0.9% sodium chloride, blotted dry, weighed, and frozen with liquid air. The yolk was shaken in its container for several minutes with the aid of a vortex shaker, then a 1.0 ml aliquot was removed, weighed in a 15.0 ml. centrifuge tube, and stored in the freezer.

The extraction of the radioactive material from the yolk sac membrane and the embryos was done by the same procedure. The sample was placed in a glass homogenizer with 3.0 ml of distilled water. After thorough homogenizing, the liquid was transferred to a 15.0 ml centrifuge tube. The glass homogenizer was rinsed twice with 2.0 ml of distilled water and the wash added to the centrifuge tube. 1.0 ml of 30.0% trichloroacetic acid was added to the combined homogenate, the tube was shaken with a vortex shaker and allowed to stand for ten minutes.

The tube was centrifuged in an International H N at full speed for ten minutes. The supernatant was decanted into a 25.0 ml volumetric flask. The liquid in the flask was frozen with liquid air in a thin layer about half way up the bulb of the flask. Once frozen, the flask was attached to a Virtis Freeze-Dryer to remove the water. The flask was removed, 1.0 ml of 1,2 propanediol added, and the inner walls of the flask rinsed with the glycol.

The contents of the flask were poured into a scintillation counting vial, and to this was added 15.0 ml of a scintillation mixture which consisted of: ethylene glycol monomethyl ether 1.0 liter,toluene 2.0 liters, ethanol 1.5 liters, 2,5-diphenyloxazole (PPO) 10.0 g, and 1,4-bis-2-(5phenyloxazolyl)-benzene (POPOP) 0.2 g.

The vials were counted in a Nuclear Chicago Scintillation counter model number 8401, system 703, for which the counting efficiency was 45%. The final results were expressed as counts per minute per gram wet weight of the original sample (c.p.m./g.).

To the thawed aliquot of yolk was added 5.0 ml of distilled water, and the test tube was shaken well with the vortex shaker. 1.0 ml of 30.0% trichloroacetic acid was added, and the tube was shaken again with the vortex shaker and allowed to stand for ten minutes. After centrifuging, the precipitate was washed twice with 2.0 ml of distilled water, and the washings were combined with the first supernatant in a 25.0 ml volumetric flask. The procedure from this point was the same as that for the yolk sac membrane and the embryo.

The precipitate from each of these samples was treated in the following manner. To a 20.0 mg sample of the precipitate weighed out in a counting vial, 2.0 ml of hydroxide of Hyamine (Nuclear Chicago) was added. The counting vial was placed in an oven at 55°C for 24 hours to help dissolve the precipitate. The presence of red blood cells can pro-

duce a coloured solution. Very few of the samples were coloured, but those that were, were replaced with colourless samples. After the precipitate had dissolved, 10 drops of 30.0% hydrogen peroxide were added to remove any unobservable colour. 15.0 ml of the scintillation mixture was added and the solution was acidified with hydrochloric acid. Radioactivity was counted in the same manner as above.

An analysis of variance, supplemented by Duncan's multiple range test, was carried out on the results from live samples from days 6, 7, and 10 and the dead samples from day eight. Samples from day 9 were too small for statistical analysis.

RESULTS

Injection of 1,2 propanediol on the first day of incubation results in a high mortality. Mortality from injection on day two is less and the figure drops gradually to that for sixth day injections (fig. 8). Mortality from injections on day seven is higher, and very much higher still for injections on day eight, and returns to the day seven level for injections on day nine. From day nine to day fifteen embryos survived the glycol injections. The day ten to day fifteen results have been confirmed by Khera (1968).

Pathological Study

When the dead embryos were removed for pathological examination, it was found that dark red hemorrhages covered the entire body (Plate XIV, fig. 1). In some even the amniotic fluid was reddish. Hemorrhaging so severe would by itself cause death.

The control embryos showed no bleeding except that which resulted from handling (Plate XIV, fig. 2).

1,2 propanediol acted very rapidly. When the eggs were candled one hour after treatment, the number of dead embryos was approximately 40%. By the fourth hour, those embryos which were going to die were already dead, while those which were still alive lived to hatching.

The microscopic examination fully confirms the macroscopic observation of massive hemorrhage. In a longitudinal

section through the head (Plate XV, fig. 1) all blood vessels are engorged with blood cells as compared to the control (Plate XV, fig. 2). There is engorgement of vessels of the epidermis, the tongue, the neck region, spinal cord, and ocular region. The appearance of the section would suggest that the purpose of the experiment was to map the circulatory system.

A similar section through the body (Plate XVI, fig. 1 and 2) shows the same. The heart is packed with blood cells, and vessels in the epidermis and other organs are full.

In the liver (Plate XVII, fig. 1) the sinusoids are packed with blood cells. The arrangement of the cells as seen in the control (Plate XVII, fig. 2), has been disturbed in the experimental embryos.

The kidney shows similar conditions (Plate XVIII, fig. 1 and 2). In the treated animal the spaces between the tubules and Bowman's capsules are wider than normal and are filled with blood. It appears that in both the liver and the kidney the volume of blood introduced is greater than the capacity, and the organ itself has burst. Yet examination of the cells of the organs themselves shows no breakdown of cell membranes.

In the spinal cord there was extrusion of cellular debris into the neural canal, necrotic ependymal cells, and loss of the distinct demarkation between the mantle and marginal layers (Plate XIX, fig. 1 and 2). The cell

membranes in the liver and kidney appeared intact but the ependymal cells of the spinal cord have definitely ruptured their cell membranes (Plate XX, fig. 1 and 2), forcing cellular debris into the neural canal. Fingerlike projections from the ependymal cells into the neural canal are easily discernable.

Even in serial cross sections it is impossible to identify organs such as the adrenals, pituitary, and thyroid because they are obliterated by engorged blood vessels.

The carbohydrate content of the yolk sac membranes and the embryos increases during days 7, 8, and 9 as shown by the PAS reaction. No difference was observed between treated and non-treated embryos in the carbohydrate content of the yolk sac membranes and embryos.

The only change observed in lipid content was in the spinal cord. There was an accumulation of lipids around the periphery of the marginal layer (Plate XXI, fig. 1 and 2), and some of the debris in the neural canal was also lipid in nature (Plate XXII, fig. 1 and 2). The type of lipid was not identified.

Study of sections stained with Mallory's stain for connective tissue shows that the cells of the liver, kidney (Plate XXIII, fig. 1 and 2), and spinal cord are now prodominently basophilic with what appears to be a loss of fibrin, glia fibers and acidophil granules. There are acidophilic and basophilic droplets in the neural canal

of the 1,2 propanediol treated embryos (Plate XXIV, fig. 1 and 2). It appears that destruction of the architecture of the cell has exposed the collagen and reticular fibers for staining. The liver, kidney, and spinal cord of controls are primarily acidophilic.

Burtone's histochemical test for cytochrome oxidase showed a slight difference in colour reaction time in the yolk sac membranes from days 6-9. Day six yolk sac membranes produced the affirmative red colour very quickly while day nine yolk sac membranes were not as quick to react. After 30 minutes the reaction was stopped, the difference in colour was discernable under the microscope, but it does not show in the photographs.

The dehydrogenase enzymes did not appear to change during the seventh, eighth, and nineth days.

Biochemical Study

The quantitative biochemical examination of glycogen in the yolk sac membrane revealed a ten fold drop in the glycogen of the surviving treated eighth day embryos (Table 3). The control seventh day embryos possessed a mean glycogen value of 1.57 µg/mg of yolk sac membrane tissue, which differs little from that of the control eighth day, 1.31 µg/mg. The control nineth day embryos have increased their yolk sac membrane glycogen to a mean value of 2.42 µg/mg of tissue. Yolk sac membranes from dead eighth day embryos have a mean value of 1.08 µg/mg,

TABLE 3

Amount of glycogen (µg/mg) in yolk sac membrane of untreated (C), treated but survived (X), and dead (D) embryos on day 7, 8 and 9.

Group	No. Embryos	Mean Glycogen
C7	4	1.57
C8	4	1.31
X8	4	0.13
D8	4	1.08
C9	4	2.42

Underscoring indicates no significant difference according to Duncan's test of significance at 5% level.

X8 D8 C8 C7 C9

TABLE 4

Cytochrome oxidase (Δ O.D./30 min./µg protein) in yolk sac membrane of 6, 7, 8 and 9 day old chick embryos.

Day of incubation	Mean Cytochrome Oxidase
6	5.8×10^{-4}
7	7.4×10^{-4}
8	5.4×10^{-4}
9	4.9×10^{-4}

while live but treated eighth day yolk sac membranes have a much lower value (0.13 µg/mg). An analysis of variance supplemented by Duncan's multiple range test shows that at the 5.0% level, the nineth day controls differ from all other groups, seventh day controls are significantly different from live and dead treated eighth day embryos, while there is no significant difference between seventh day controls and eighth day controls. Eighth day controls differ from treated live eighth day yolk sac membranes but not from treated dead eighth day yolk sac membranes. Live and dead treated eighth day yolk sac membranes are not significantly different.

An apparent increase in cytochrome oxidase activity of day seven yolk sac membranes was observed (Table 4). The change in optical density per 30 minutes per µg protein (Δ O.D./30 min./µg protein) on day seven is 7.4×10^{-4} compared with 5.8×10^{-4}, 5.4×10^{-4}, and 4.9×10^{-4} on days six, eight, and nine respectively. The student's "t" test for groups of unequal size shows no difference between the activity on all days.

The lactic acid analysis of the bodies of eight day controls, alive but treated, and dead treated eighth day embryos reveal no statistically significant difference between the live treated and dead treated eighth day embryos at the 5% level. While there is a difference between dead treated nineth day (720.3 mg/g) and eighth day controls (237.6 mg/g), there is no difference between dead treated

eighth day embryos (664.6 mg/g) and eighth day controls (Table 5). For the yolk sac membranes of these same groups, the value of 929.6 mg/g for the dead treated eighth day embryos is different from all other groups. The lactic acid of the yolk sac membranes of the live treated eighth day embryos, 624.4 mg/g, is not different from the eighth day control, 564.1 mg/g, at the 5.0% level (Table 6).

Prophylactic Investigation

Whether injected singly, in classified groups of acidic, basic, and neutral amino acids, or other mixtures, amino acids in the quantities used did not prevent death from 1,2 propanediol. The same is true for the vitamins. The attempt to lessen or prevent the hemorrhaging with ascorbic acid and vitamin B_{12} failed, as did pantothenic acid for normal C.N.S. development, folic acid for transmethylation, and nicotinic acid and nicotinamide for NAD production.

The injections of ACTH and corticosterone did not change the affect of 1,2 propanediol upon the eight day embryos. The glycogen stimulators, insulin, cortisone, and adrenalin did appear to mitigate the effect of the glycol. But,when the tests were repeated the mortalities were similar to those from 1,2 propanediol alone. (Table 7). The analysis of variance shows no significant difference at the 5.0% level.

TABLE 5

Amount of lactic acid (mg/g) in the body of live untreated (C8), dead (D8), and live treated (X8) eight day embryos.

Group	No. Embryos	Mean Lactic Acid
C8	4	237.6
D8	5	664.6
X8	5	720.3

Underscoring indicates no significant difference according to Duncan's test of significance at 5% level.

C8 D8 X8

TABLE 6

Amount of lactic acid (mg/g) in yolk sac membrane of live untreated (C8), dead (D8), and live treated (X8) eight day embryos.

Group	No. Embryos	Mean Lactic Acid
C8	4	564.1
X8	5	624.4
D8	5	929.6

Underscoring according to Duncan's test of significance at 5% level.

C8 X8 D8

The crude enzymes extracted from the sixth day yolk sac membrane and the twelfth day embryonic livers did not affect mortality due to 1,2 propanediol.

It was hoped that by fortifying the embryonic liver, we could make the embryos better able to withstand 1,2-propanediol injection on the eighth day. When 1.0 μg/egg of T_3 was either deposited on the air sac membrane or beneath the shell membrane, approximately 50.0% of the embryos died before the 1,2 propanediol treatment. The best survival was obtained by placing the T_3 upon the air sac membrane in a concentration less than 10.0 nanograms. The mortality for this latter treatment before 1,2 propanediol injection on the eighth day was never more than one death per group (i.e. 3.3%). This prophylactic treatment did not reduce mortality when 1,2 propanediol was introduced into the yolk sac on the eighth day.

General Experiments

High mortality on the eighth day was not a result of the shock of injection. Of sixty eggs inoculated, but not injected with a needle dipped in 1,2 propanediol, only one death was observed. The symptoms and percent mortality of 1,2 propanediol-1-phosphate on day eight are the same as those of 1,2 propanediol. The dose response experiment with 1,2 propanediol at 78, 52 and 26 mg/egg gave a mortality of 48.3, 25.0, and 6.7% respectively.

TABLE 7

Percent mortality when glycogenic hormones were injected at 0.1 mg/egg with 1,2 propanediol on the eighth day.

	Test 1	Test 2	Test 3	Test 4	Test 5
Insulin	63.3	36.6	33.3	86.6	76.6
Cortisone	3.3	30.0	33.3	63.3	66.6
Adrenalin	20.0	37.9	40.0	93.3	83.3
Control	70.0	70.0	83.3	80.0	80.0

Glucose at 75.0-12.5 mg/egg had no effect upon treated eight day embryos. Sodium lactate injection produced mortalities which varied with the amount injected. With 89.3 mg/egg, the mortality was 83.3%, with 59.5 mg/egg 50.0%, and with 29.8 mg/egg 33.3%. Pyruvic acid injection into the yolk sac of eighth day embryos resulted in 70.0 and 60.0% mortality for amounts of 5.0 and 1.0 mg/egg. Upon examining the embryos which had died from sodium lactate and pyruvic acid injections, the hemorrhage signs were present, but they were not as severe as the hemorrhage due to 1,2 propanediol.

Tracer Experiment

The experimental results from the supernatant fractions from embryos injected with 1,2 propanediol-1-C^{14} are illustrated graphically in figures9, 10, and 11, and tabulated in Tables 8, 9, and 10. In both the live embryos and their yolk sac membranes, there is a gradual increase in 1,2 propanediol absorption on day six and seven, followed by a tremendous increase on day eight. The quantity of glycol in the embryos and yolk sac membranes of live specimens decreases on day nine, and it tends to remain at this value on day ten. For the dead embryos the count/min/g of tissue on days eight and nine are higher than for the live embryos; 4,383, and 4,833 as compared to 3,701 and 3,022 respectively.

TABLE 8

Activity (count/min/g) of 1,2 propanediol-1-C^{14} in the supernatant fraction from live (X) and dead (D) embryos on day 6, 7, 8, 9, and 10.

Day of incubation		No. Embryos	Range	Mean Activity
6		5	744 2,089	1,377
7		5	920 2,385	1,549
8	D	5	3,724 5,688	4,383
	X	3	2,228 5,916	3,701
9	D	2	4,056 5,610	4,833
	X	3	2,929 3,153	3,022
10		5	2,070 3,370	2,806

Under scoring indicates no significant difference according to Duncan's test of significance at 5% level (for samples of five embryos only)

6 7 10 D8

TABLE 9

Activity (count/min/g) of 1,2 propanediol-1-C^{14} in the supernatant fraction of yolk sac membrane of live (X) and dead (D) embryos on day 6, 7, 8, 9, and 10.

Day of incubation		No. Embryos	Range	Mean Activity
6		5	526 1,204	783
7		5	784 1,915	1,247
8	D	5	1,484 2,838	2,043
	X	3	2,467 3,071	2,767
9	D	2	2,081 2,568	2,324
	X	3	1,999 2,185	2,104
10		5	1,836 2,960	2,165

Under scoring indicates no significant difference according to Duncan's test of significance at 5% level.

6	7	D8	10

TABLE 10

Activity (count/min/g) of 1,2 propanediol-1-C^{14} in the supernatant fraction of yolk from live (X) and dead (D) embryos on day 6, 7, 8, 9, and 10.

Day of incubation		No. Fractions	Range	Mean Activity	
6		5	3,222 4,296	3,563	No statistical difference according to the analysis of variance.
7		5	3,286 5,469	4,100	
8	D	5	1,537 3,861	2,695	
	X	3	2,289 5,121	3,637	
9	D	2	2,542 3,893	4,718	
	X	3	2,636 2,848	2,741	
10		5	2,677 6,231	4,465	

Duncan's multiple range test shows the value for the supernatant fraction from the dead eighth day embryos to be significantly different from all other values at the 5.0% level. Also, the counts for supernatant fractions of tenth day embryos are different from those from embryos of days six and seven, while the count/min/g of sixth and seventh day embryos are not significantly different.

The sample of yolk sac membrane from the dead eighth day embryos (2,403 count/min/g) is lower than that from the surviving embryos (2,767 count/min/g) for the same day. On day nine, the yolk sac membrane values for both live and dead embryos were similar (2,324 and 2,104 count/min/g). From the yolk sac membrane, the count/min/g for day ten membranes (2,165) is different from those from day six (783.0) and day seven (1,247) but not from those from day eight (2,043). Count/min/g of eighth day membranes are different from those from day seven and day six, but there is no statistical difference between results from days six and seven.

An analysis of variance for the supernatant fractions of the yolk samples indicates no difference among the samples at the 5.0% level.

Distribution of 1,2 propanediol-1-C^{14} in the precipitated fractions from the embryo, yolk sac membrane, and yolk is represented graphically in figures 9, 10, and 11, and tabulated in Tables 11, 12, and 13. In the graph, the

activity in the precipitate from the embryos shows the greatest variation. Following a gradual increase from day six to seven, there is a tremendous rise in activity on day eight, after which the count/min/g declined. The precipitate fractions from the yolk sac membrane and yolk show a steadily increasing activity on each day over the period investigated.

Statistically, the mean count/min/g from the precipitate of the dead embryos is different from those from the live embryos of days 6, 7, and 10. The count from the tenth day embryos (2,081) is different from that of day six embryos (1,041) but not different from that of day seven embryos (1,223). There is no difference between sixth and seventh day embryos.

In the yolk sac membrane precipitate, the average count/min/g on day ten (3,675) is different at the 5.0% level from those of day 6, 7, and 8, which gave values of 780, 2085, and 1790 count/min/g respectively. The yolk sac membrane mean value from day seven is different from that of day six, but not from that of day eight. There is a difference between counts of yolk sac membranes of dead eighth day embryos and live sixth day embryos.

Count per minute per gram of sample from the yolk precipitate show no statistical difference at the 5.0% level.

TABLE 11

Activity (count/min/g) of 1,2 propanediol-1-C^{14} in the precipitate fraction from live (X) and dead (D) embryos on days 6, 7, 8, 9, and 10.

Day of incubation	No. Embryos	Range	Mean Activity
6	5	640 1,650	1,041
7	5	430 2,870	1,224
8 D	5	4,920 5,825	5,309
8 X	3	2,330 6,520	4,266
9 D	2	3,750 6,540	5,145
9 X	3	2,785 3,990	3,451
10	5	1,710 2,425	2,081

Underscoring indicates no significant difference according to Duncan's test of significance at 5% level (for samples of five embryos only).

6 7 10 D8

TABLE 12

Activity (count/min/g) of 1,2 propanediol-1-C^{14} in the precipitate fraction of yolk sac membrane from live (X) and dead (D) embryos on days 6, 7, 8, 9, and 10.

Day of incubation	No. Embryos	Range	Mean Activity
6	5	20 280	156
7	5	135 605	417
D	5	385	
8		525	358
X	3	650 750	688
D	2	385	
9		595	490
X	3	410 530	486
10	5	620 965	735

Underscoring indicates no significant difference according to Duncan's test of significance at 5% level.

6 D8 7 10

TABLE 13

Activity (count/min/g) of 1,2 propanediol-1-C^{14} in the precipitate fraction of yolk from live (X) and dead (D) embryos on days 6, 7, 8, 9, and 10.

Day of incubation		No. Embryos	Range	Mean Activity	
6	X	5	55 1,245	317	
7	X	5	1,075 1,835	1,423	No statistical difference at 5% level.
8	D	5	425 940	633	
	X	3	690 1,405	962	
9	D	2	730 770	750	
	X	3	515 860	678	
10	X	5	750 2,185	1,201	

DISCUSSION

Early toxicity studies of 1,2 propanediol showed a minimal lethal effect. This caused acceptance of this glycol as an innocuous solvent. McLaughlin et al (1963) reported negligible deaths with propylene glycol, when it was injected prior to incubation at 57.0 mg/egg. A dose of 104.0 mg/egg in the present experiment resulted in high mortality on the first day of incubation but became ineffective by day six. High susceptibility during the early days of development is related to the general sensitivity of the chick embryo during organogenesis. The sudden increase in deaths on the eighth day followed by no deaths after day nine was unexplained in the literature.

Eighth day Mortality

A massive hemorrhage over the entire embryo points to the cause of death. Microscopic studies reveal that the entire vascular system, along with the heart, liver, and kidneys, is engorged with red blood cells. This hemorrhage was so extensive that the smaller organs were completely obliterated. While the arrangement of cells in the liver and kidney had been disrupted, there did not appear to be any damage to individual cell membranes in these organs.

At this period of development most of the red blood cells are formed by the yolk sac. There is little or no storage of red blood cells in the spleen. Therefore, the large accumulation of blood in the tissues must be a result of interference with outgoing blood vessels. This conclusion gains support from the observations of Gebhardt (1968). He observed that the action of 1,2 propanediol was lethal on the fourth day of chick development and within a few hours large hemorrhages were observed in the extraembryonic blood vessels. If the damage is blockage in the efferent vascular system, then pressure build up would result in engorgement of all vessels and organs. With no outflow in the liver and kidneys pressure from incoming blood would increase culminating in the destruction of the vascular network of these organs.

Investigators have demonstrated that mortalities in rats and rabbits do result from 1,2 propanediol administration, but no organ or system was particularly affected. In pathological examinations the kidneys, heart, spleen and liver appeared normal. (Seidenfeld and Hanzlik 1932, Weatherby and Hoog 1938, and Newman et al 1940). The central nervous system, which escaped investigation in all these studies, was in our embryological study damaged extensively. In the spinal cord, there is not only a loss of the red staining acidophilic material as observed in Mallory's procedure but also cytolysis of the ependymal cells. The distinctive border between the mantle and the marginal layer has also been lost.

It is not possible to decide which is affected first, the nervous system or the vascular system. Does damage to the nervous system cause damage to the vessels, are the vessels damaged first and the nervous system secondarily effected, or are both systems damaged directly by 1,2 propanediol?

The site of action of most of the toxic agents which cause death is the central nervous system (Modell 1967). In our experiments damage to the central nervous system could be the primary cause of death followed by hemorrhage and destruction of the liver and kidneys.

Baer et al (1968) have demonstrated by in vitro studies that phospholipids, which are analogues of α-lecithins and α-cephalins, can be synthesized from 1,2 propanediol. The D-propylene glycol-α-phosphorylcholine is very hemolytic while the D-propylene glycol-α-phosphorylethanolamine is not. Phospholipids or phosphatides are the main cellular lipid component of the cell membrane. The embryonic central nervous system may be undergoing very rapid biochemical development on the eighth day. If the presence of D-propylene glycol-α-phosphorylcholine interfered with this development, cellular destruction might result.

The Sudan Black B stain indicates the presence of a type of lipid debris in the neural canal of dead treated eighth day embryos with no alteration of lipids anywhere else. This lipid could be D-propylene glycol-α-phospho-

rylcholine or cell membrane fragments.

1,2-propanediol itself could cause death of the embryo. It has been shown in the isotope experiment that the uptake of 1,2-propanediol-1-C^{14} into the embryos and yolk sac membrane is very high on day eight. It is possible that both 1,2-propanediol itself and the phospholipid form act at the same time on the embryo.

Negative Experiments

Florence Moog (1943) has demonstrated histochemically the presence of cytochrome oxidase in the 1-4 day chick embryo. Histochemical investigation in our experiment showed a quicker reaction in the sixth day yolk sac membrane, but biochemical investigation showed that there was no statistical difference in the activity from the sixth to the nineth day. It thus appears that there is no change in activity in the cytochrome oxidase system during this period.

Since there is no difference in the amounts of lactic acid in those embryos which survived and those which died from the glycol injections, death due to high concentrations of lactic acid in the embryos can be eliminated. That high lactic acid concentration can cause death has been shown by injecting sodium lactate alone. Yolk sac membranes from dead embryos treated with 1,2-propanediol have significantly higher concentration of lactic acid than do the control and surviving embryos. The death

of the yolk sac membrane system, therefore, might be caused by this high lactic acid concentration.

The death of the yolk sac membrane by the injection of 1,2-propanediol contributes two points to be considered. First, it is known that 1,2-propanediol is metabolized to lactic acid (see Appendix). The oxidation of the glycol to lactic acid by the yolk sac membrane may result in this significantly high amount of lactic acid and produce hemolysis of the erythrocytes. For it has been observed that the injection of sodium lactate alone produces symptoms similar to that of 1,2-propanediol. If this is the case, then the yolk sac membrane becomes an active metabolizing organ. Second, the injection of 1,2-propanediol itself may have resulted in the death of the yolk sac membrane function and this death is expressed by a high amount of lactic acid. When animals die because of many known reasons, there is a high amount of lactic acid in their tissue but the death is not attributed to lactic acid accumulations.

Though attempts at prophylactic therapy were extensive, none were successful. Not all possibilities were examined, but disturbance due to interference of the glycol with transmethylation, ACTH, corticosterone, amino acid metabolism, ascorbic acid, pantothenic acid, vitamin B_{12}, nicotinic acid, and nicotinamide may be eliminated, provided that these substances entered the embryo.

The hormones, insulin, adrenalin, and cortisone, presented both favourable and unfavourable results. The favourable results can be attributed to their common property, glycogenic stimulation. It is perplexing that the first three trials in this series appeared successful and the final two failed. A possible explanation is that the eggs on the first trials were not exactly at the day eight stage. The last two trials were staged with greater accuracy. At any rate, interference by 1,2-propanediol with the actions of these hormones was not the cause of death when 1,2-propanediol was injected.

The idea of causing a precocious increase in liver mitochondria with T_3 appeared very promising, but there are many difficulties in assuring that the T_3 does reach the liver. Inoculations into the chorioallantoic system were impossible because of the small size of these vessels on days 4, 5, and 6 of incubation. Injections were not made into the yolk sac, since it was felt that the yolk sac membrane would bind much of the T_3. The best way of carrying T_3 to the embryonic liver appeared to be by depositing the solution close to the chorio-allantoic veins, either under the shell membrane or on the air sac membrane. In order to have a good survival of embryos for day eight treatment with 1,2-propanediol, not more than 10 nanograms must be placed on the air sac membrane. Although no beneficial results were obtained, this idea must not be dis-

carded until it has been determined whether or not the T_3 actually reached the liver.

Death on the eighth day due to shock can be rejected, because the embryos were not disturbed by the puncture of a needle dipped in 1,2-propanediol. Glucose injections do not affect the embryos, which indicates that the volume of material injected was not the cause of death. The phosphorylated form of 1,2-propanediol produces the same mortality and effects as does the free glycol. Pyruvic acid does affect the embryos unfavourably. It may be concluded from these tests that the eighth day chick embryo is especially sensitive to injections of some materials.

Transition State

The explanation for eighth day mortality may involve what Willier (1954) called the "phase of initial functional activity", that is, that period after organogenesis in which the organs in the chick embryos begin to assume the functions of the adult organs. While the embryo is in a stage of differentiation and organogenesis, it is reasonable to assume that the embryo per se must be receiving its building units from a metabolically active source or sources. One would not expect an embryonic organ to attain metabolic activity until the entities for that organ have been developed to carry out this function. This phase of prefabricated supply must continue until the embryo is ready to assume a functionally active role.

Claude Bernard's "foie transitoire" was the first indication of a transition phase for avian development. He hypothesized that the yolk sac membrane operates as the glycogen storage area until the liver can assume this function (Zwilling 1951, Bellairs 1963, and Thommes and Just 1964). An electron microscopic investigation supports the glycogenic function of the yolk sac membrane (Bellairs 1963).

Several lines of evidence indicate that the hepatocytes begin to synthesize and store glycogen on the eighth day (Zwilling 1951, Willier 1954, O'Connor 1953, and Zaccheo and Grossi 1967). Thommes and Just (1964) observed that the level of glycogen in the yolk sac membrane remains constant during the third to fourteenth day. When these authors expressed their results as a glycogen decrease after glucagon treatment, they obtained a maximum response on day eight. This is comparable to the statistically significant decrease in glycogen of the yolk sac membrane in the embryos which survived in 1,2-propanediol injection. The yolk sac membranes from days 7, 8, and 9 were examined, and these results indicate an increase in glycogen during these days. The sensitivity of glycogen determination in our study is much greater than in that of Thommes and Just, which could account for the observed increase. Zaccheo and Grossi (1967) observed by immunochemical techniques that the hepatocytes acquire the capacity for albumin synthesis, glycogen storage, and lysosomal differentiation between the sixth and eighth days of incubation.

The glycolytic pathway enzymes increase in activity in the chick embryo liver after the eighth day. The following enzymes: hexokinase, phosphofructokinase, triose phosphate isomerase, phosphoglycerate kinase, pyruvate kinase, and phosphopyruvate carboxylase were shown to increase (Rinaudo 1966). During embryogenesis new enzymes appear in liver tissues with the result that certain subtle shifts occur during liver formation with some enzymes increasing while others decrease (Conklin 1966). Major changes in LDH isozymes take place during the sixth to the twelfth day (Conklin 1964). A sudden synthesis in LDH and MDH occurs in the nineth to the twelfth day chick embryo (Solomon 1958), although this was not detected in our less sensitive tests. The changes in enzyme patterns have been further confirmed by Solomon (1959) and Nebel and Conklin (1964).

Other metabolic changes which are known to occur on the eighth day include the following: nucleic acid metabolism shows an intense activity in early stages of chick embryo development but especially in days 7-10 of incubation (Barbiroli et al 1965). DNA decreases from the third to the eighth day but increases from the eighth day until the 13th day after which it gradually falls. RNA follows a similar course (Shimabayashi and Iwamoto 1964).

A microspectrophotometric observation (Thorell and Raunich 1966) on erythrocyte development in the chick embryo demonstrated that the RNA to hemoglobin ratio changes

from 6.1 at 36 hrs. to 0.7 at 144 hrs. (6th day). High RNA in the cytoplasm is a general cytochemical characteristic of intense protein synthesis. This change in RNA/Hb ratio can be regarded as a change in biochemical activity during ontogenesis.

Active protein synthesis of the chick embryo commences on day eight. Sensitivity to the antiviral action of interferon becomes evident only on the eighth day, then rapidly increases (Isaacs 1966). Boell's (1955) presentation of an inflection point of oxygen consumption on the eighth day further shows a transition in the growth of the chick embryo. Needham (1933) reported that most of the material absorbed from the yolk during the first week of incubation is employed for the growth of the yolk sac membrane.

The results of the 1,2-propanediol-1-C^{14} experiment add to the above evidence. The courses followed by the embryo and the yolk sac membrane are similar. Prior to the eighth day of incubation, the uptake of the glycol is low but increasing. Then on the eighth day 1,2-propanediol-1-C^{14} enters the embryo at a very high rate. By the tenth day the count/min/g have decreased but remain at a higher level than that prior to day eight. The precipitate fractions from the embryos demonstrated that much of the glycol has been incorporated into the embryo. In the precipitate from the yolk sac membrane, the data reveal that over this period the amount of 1,2-propanediol incorporated into its structure increases.

Therefore, the transition for Claude Bernard's "foie transitoire" is probably on the eighth day of chick embryo incubation. Before this day the embryo is primarily in a state of organogenesis. The organs along with their biochemical architecture are developing to a functional metabolic role. With the completion of organogenesis, especially in the liver, the embryonic organs are ready to assume their adult functions.

The high death rate in days 1-4 of incubation is attributed to the sensitivity of the developing systems, both extra-embryonic and embryonic. During the period of 1-6 days the yolk sac membrane has consumed much of the yolk products and assumed the glycogenic storage role. Only a sufficient amount of nutrients is passed onto the embryo to meet requirements. When the transition occurs on the eighth day, the yolk sac membrane becomes more permeable and the liver begins its functional activity. The liver is unable to metabolize the sudden surge of glycol with the result that hemolysis occurs. The glycol is metabolized by the liver in ensuing days and mortality drops dramatically.

Teratogenesis

Many of the teratisms in chicks result from treatment prior to the eighth day. The agents employed vary widely, but the teratologic expression is the same. These teratisms are always associated with cranial or limb develop-

ment. Although the sites of action of the agents are unknown, it has been assumed by investigators that the sites are different because of the difference in metabolism of the agents.

Is it possible to have a common site of damage for the similar teratogenic expression? The 1,2-propanediol experiment, along with other experiments, illustrates that the yolk sac membrane is not only a functional organ but also a barrier for the period of organogenesis. A defective yolk sac membrane can be responsible for a defective embryo (Knowlton 1967). Thus it would be possible for teratisms to be produced indirectly after yolk sac membrane damage. Research with radioactive compounds would help pin point the actual site of teratogenesis.

Many research workers in embryology make random selections as to what day they will do their biochemical or teratological studies. In order to obtain a clearer picture of the event under study a wider period must be considered. The pattern determined for a three day period may not be the same for the fourth day.

The transition state may not be confined to birds. The prenatal growth process is the same for birds and mammals, with time and weight being the only difference (Laird 1966).

Pregnant mothers are advised by doctors to be cautious of coming into contact with German measles during the first

three months, as the disease causes teratisms during this period. Micromelic babies also resulted when mothers took thalidomide to relieve nausea in the first three months of pregnancy.

We must await further evidence from teratological studies in mammals before we can determine to what degree these experiments in teratogenesis can be extrapolated to the placenta. If we consider Anna Laird's (1966) statistical comparison of embryonic development of birds and mammals, we can reasonably expect the problems in teratogenesis to be very similar.

CONCLUSION

Although no evidence was presented for the biochemical pathways involved in the production of teratisms, the experiments with 1,2-propanediol do provide evidence for a transition on the eighth day in chick embryo development. The organophosphates Phosphamidon, Dylox, Baytex, and Sumithion are teratogens, although their effect was erratic in these experiments.

The problem of inconsistent teratisms with the organophosphates rests with overcoming three important factors. First is the injection of the appropriate concentration. A concentration that is too high would produce outright death of the embryos, and a concentration that is too low would be innocuous or might be rendered innocuous by enzymatic systems. Second, the solvent selected must not be toxic in itself and must carry the teratogen to the yolk sac membrane for immediate absorption. Finally, the time of the administration of the teratogen must coincide with the susceptibility of the target organ. It is a well known fact that the techniques and methods for one species may not be appropriate to another species. The testing of teratogens must not be limited to one or two randomly chosen days in embryogenesis but must encompass a greater consecutive period.

Since the completion of the present study and largely as a result of it, LaHam and Greenberg (1969), produced

consistent teratisms in chick embryos with Malathion. The resulting malformations are micromelia of the hind limbs, cranial distortion, and a general reduction in body growth.

The results from the toxicity experiments with 1,2-propanediol suggest that the glycol has masked the teratogenic properties of the water insoluble organophosphates. Death in the embryos treated with 1,2-propanediol occurred in a matter of hours. Thus masking can be considered demonstrated, although some teratisms were obtained.

The exact cause of death in the chick embryo due to 1,2-propanediol administration is not clear. It was demonstrated in the tracer experiment that the glycol enters the embryo on day eight at a tremendous rate. The ependymal cells of the central nervous system appear to have taken the brunt of the injection. These cells are actually destroyed, whereas the cellular disturbance in the liver and kidney appear to be a physical displacement. Haemorrhaging of the embryo can be explained by the hemolytic property of 1,2-propanediol.

The yolk sac membrane, as well as digesting, acts as a protective barrier for the developing embryo. This poses the problem of indirect or direct production of teratisms. Does the teratogen affect the yolk sac membrane, does it escape degradation by the enzymatic apparatus of the yolk sac membrane and affect the erythrocytes or does

it by-pass the yolk sac membrane and the erythrocytes and attack a specific organ? These problems suggest more experiments with tracer teratogens.

The toxicity of 1,2-propanediol due to an imbalance in hormones, amino acids, and certain vitamins was tested and eliminated.

Claude Bernard called the yolk sac membrane the "foie transitoire". In the light of present knowledge this term is very appropriate. The one answer which these experiments do give is the day of transition. From the evidence compiled by other investigators plus those of the author, it can be stated that in the chick embryo the "chemical transition" occurs on the eighth day of development.

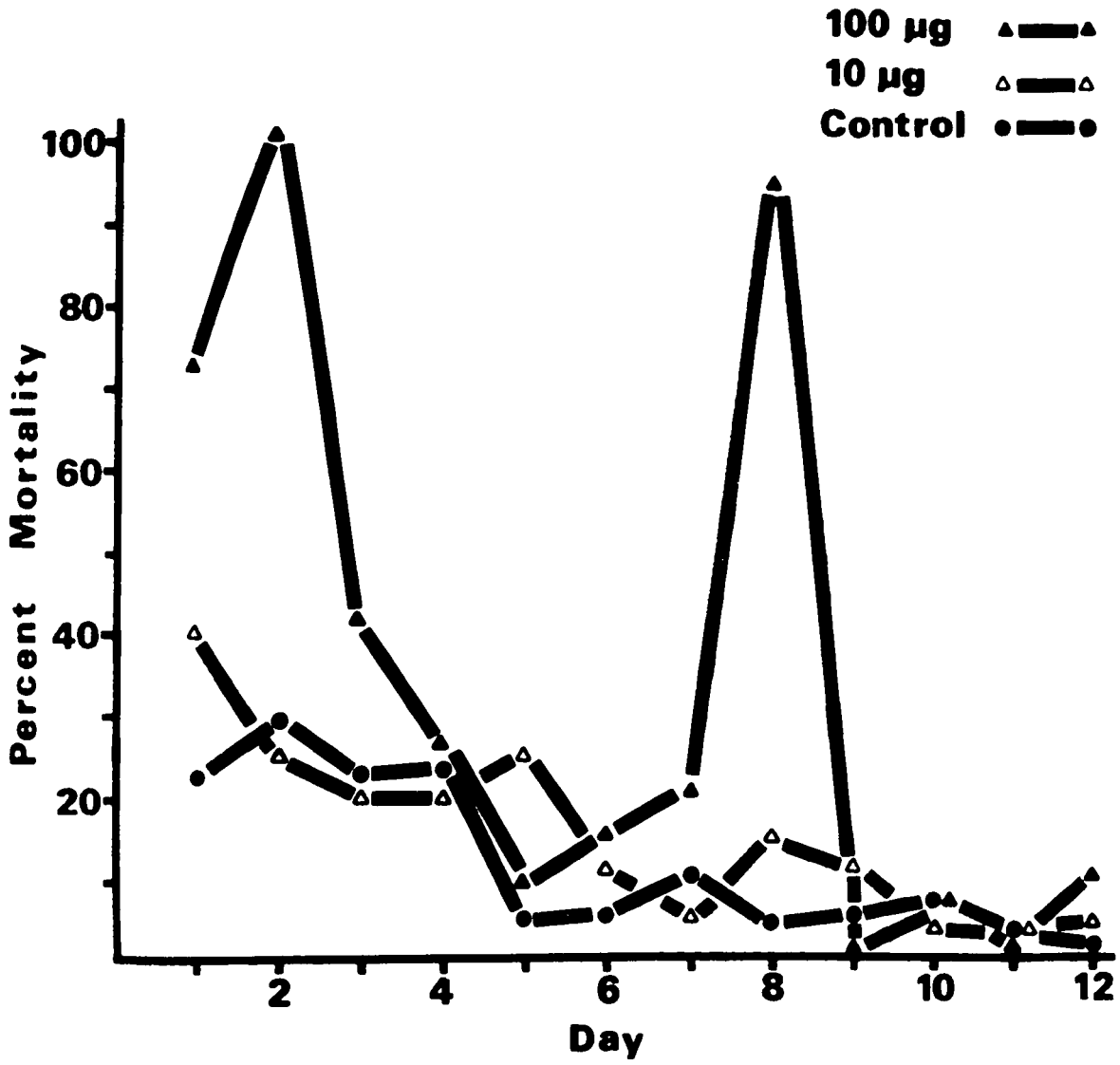


Fig. 1 Toxicity of Phosphamidon during the first twelve days of chick embryo development, with distilled water as the control.

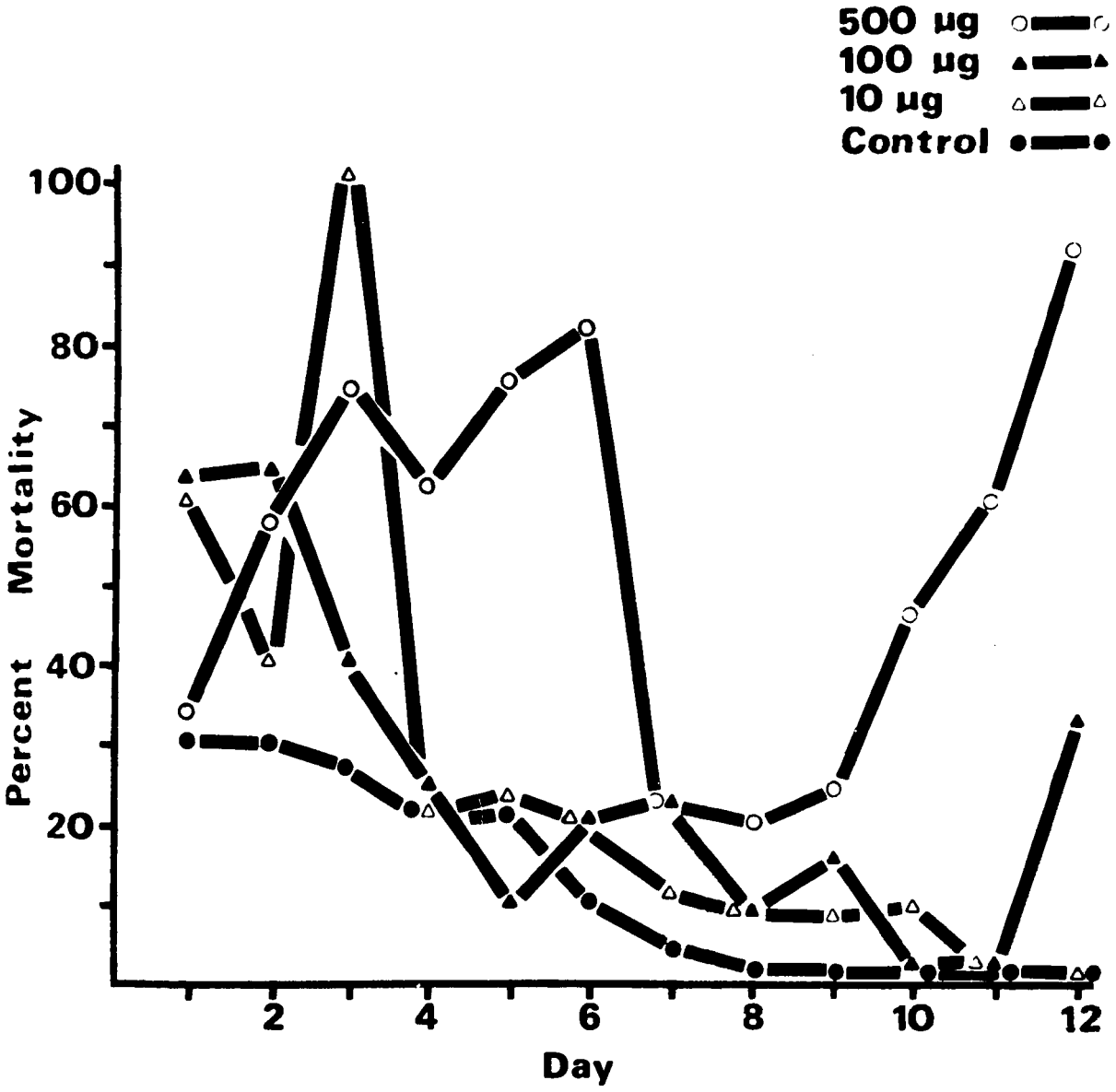


Fig. 2 Toxicity of Phosphamidon injected into the yolk sac during each of the first twelve days of chick embryo development, with distilled water as the control.

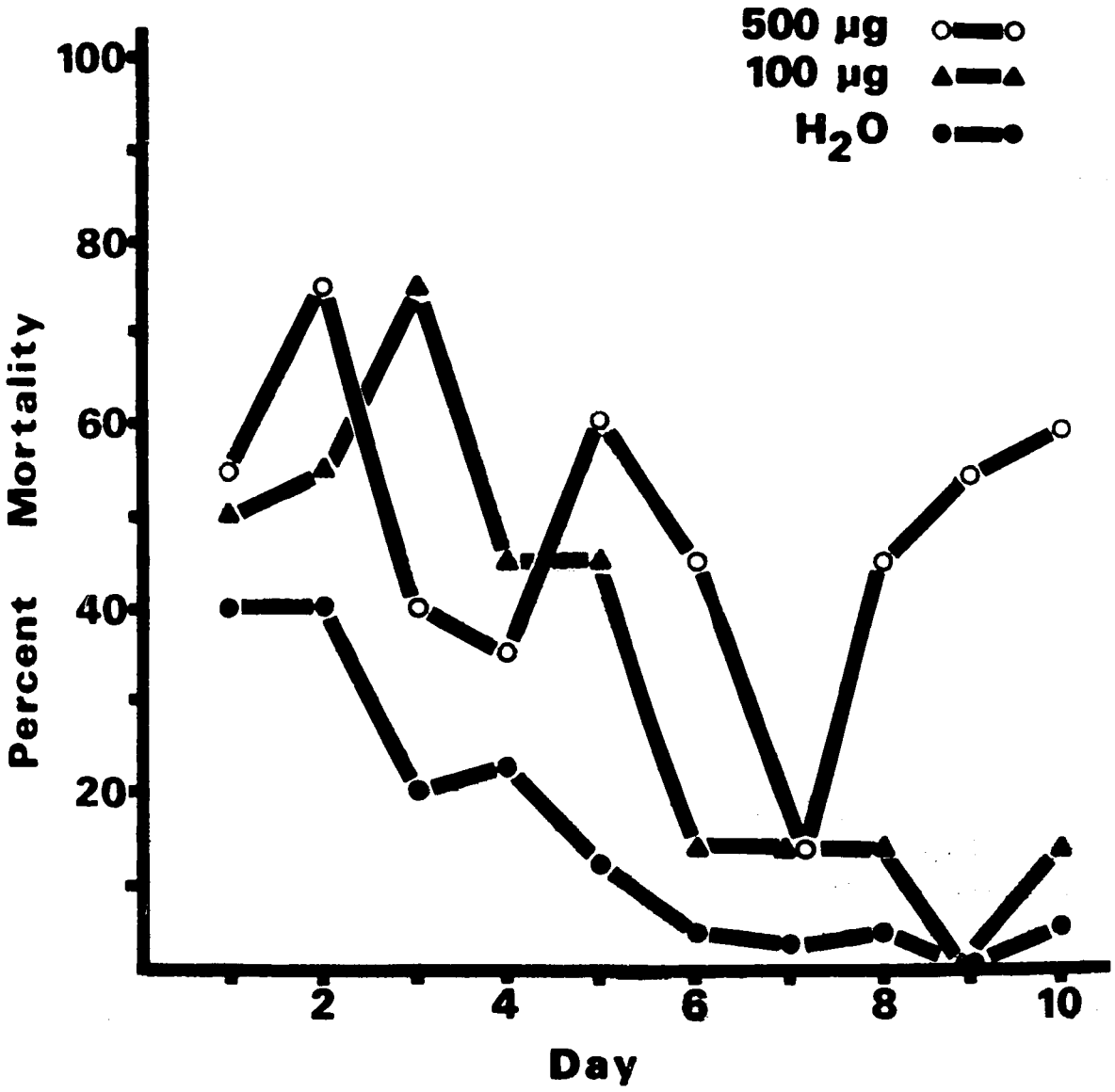


Fig. 3 Toxicity of Phosphamidon during the first ten days of incubation of the chick embryo.

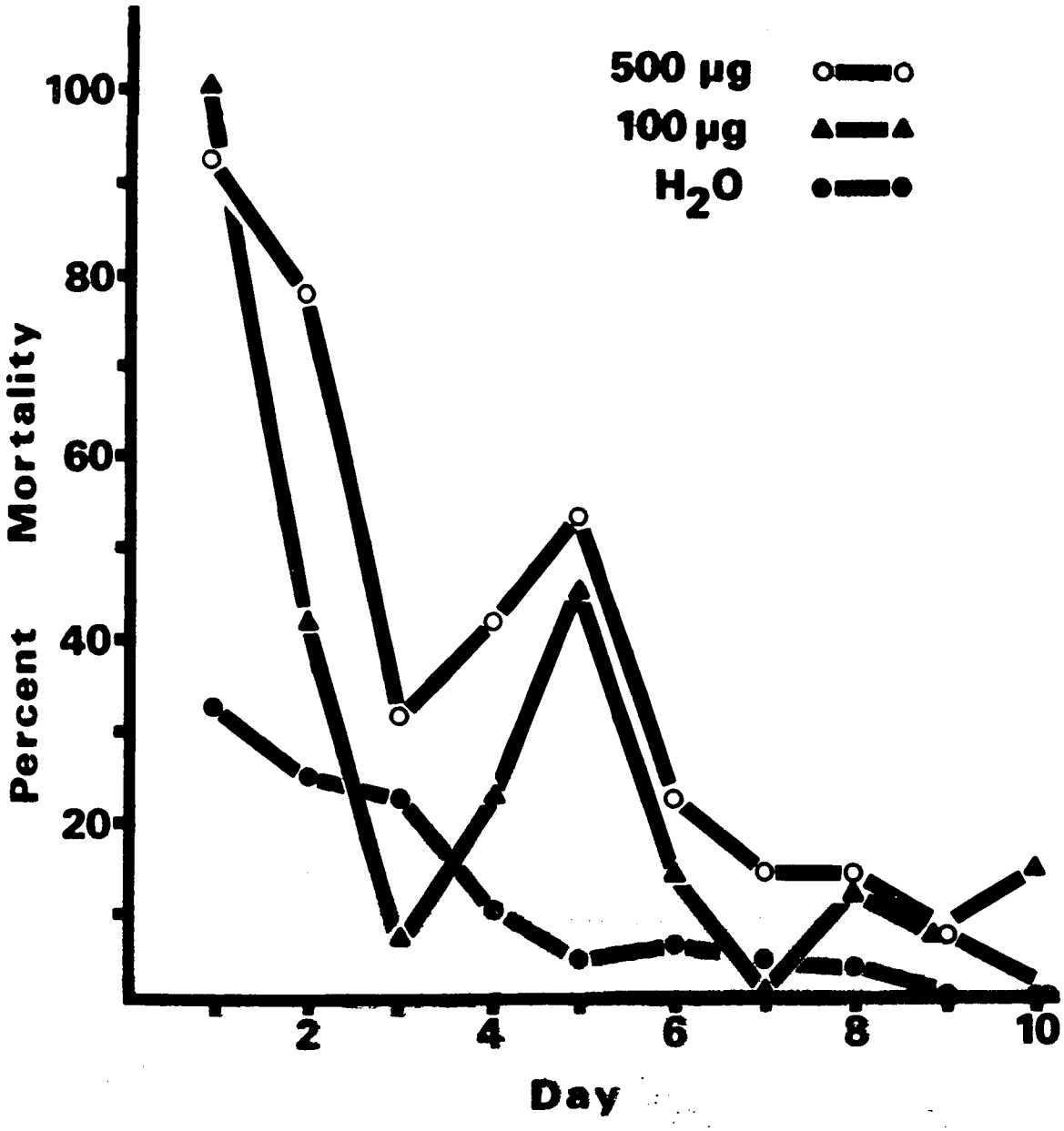


Fig. 4 Toxicity of Phosphamidon during the first ten days of incubation in chick embryo development.

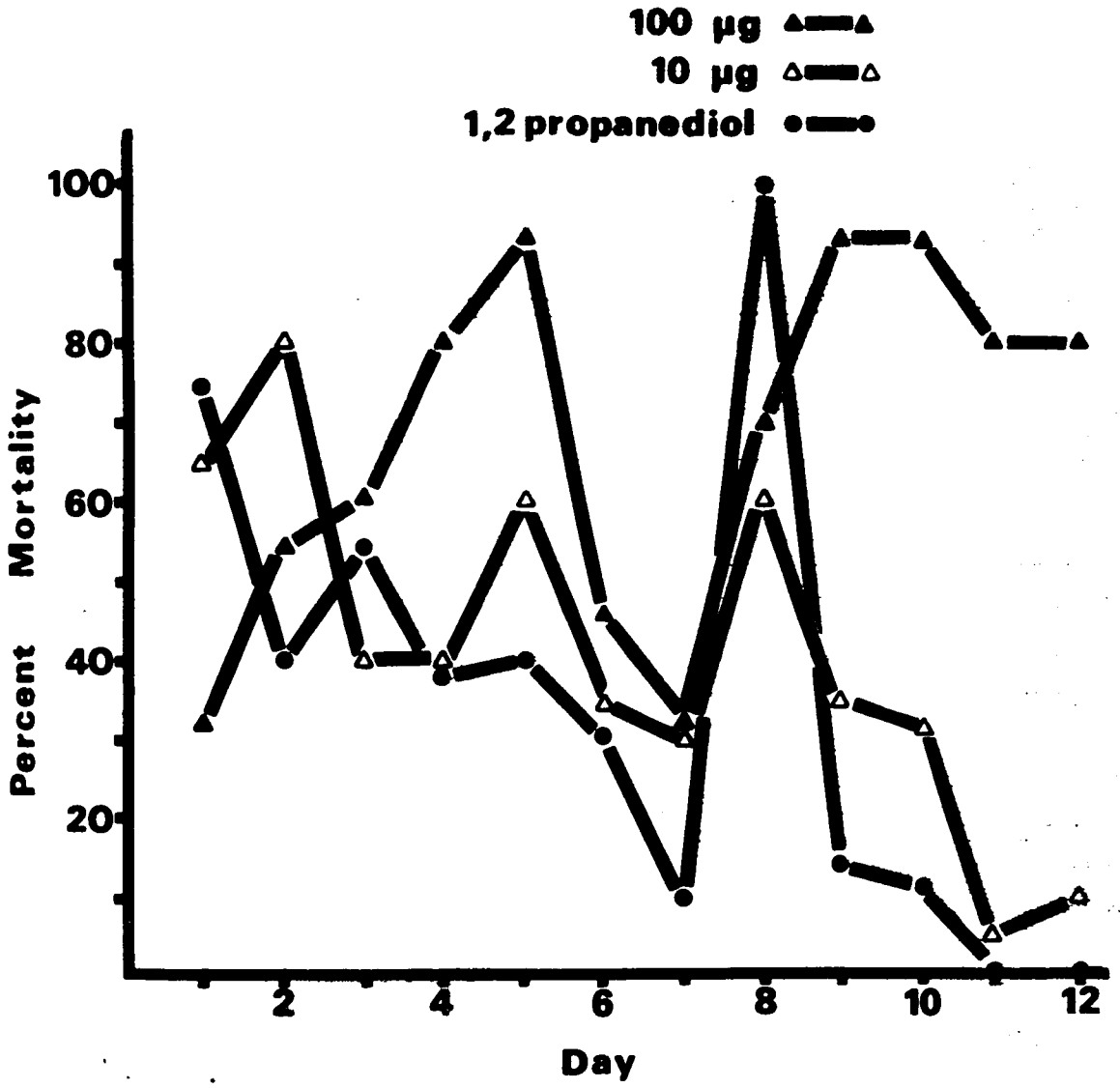


Fig. 5 Toxicity of Parathion during the first twelve days of chick embryo development.

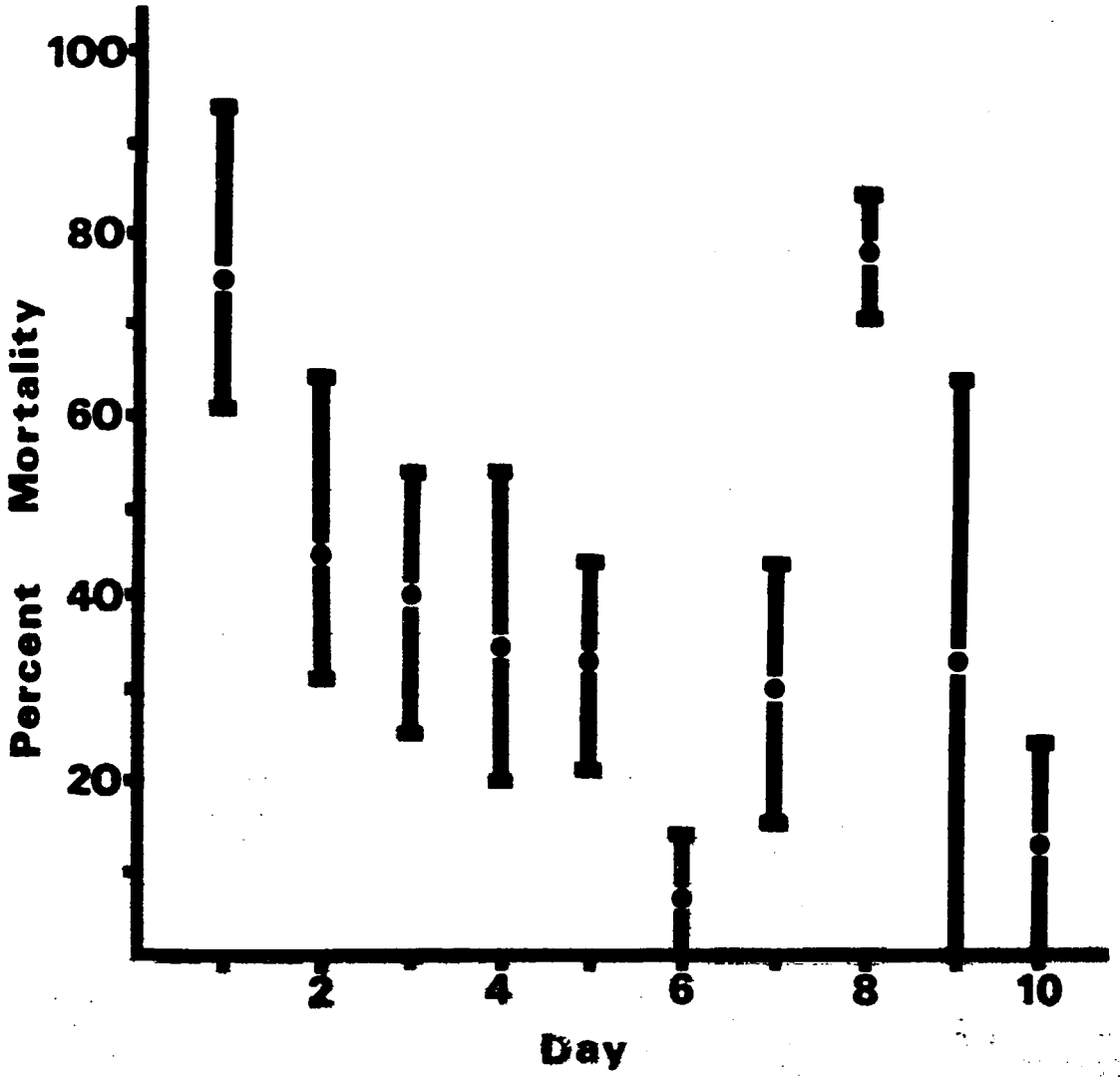


Fig. 6 Composite graph showing range of toxicity of Dylox, Baytex, Sumithion, and 1,2 propanediol in chick embryo development.

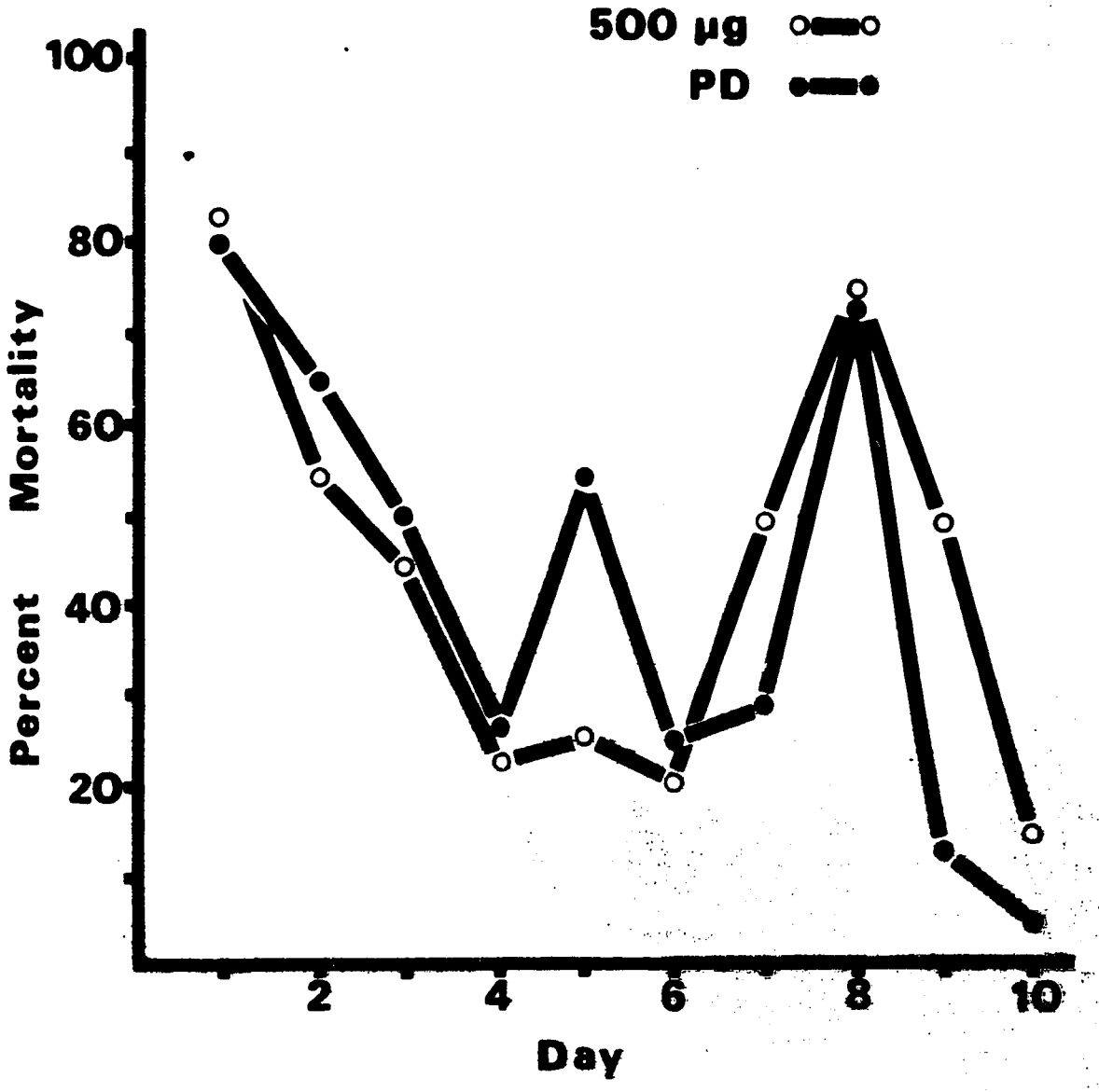


Fig. 7 Toxicity of Baytex during first ten days of incubation in chick embryo development. PD: 1,2 propanediol.

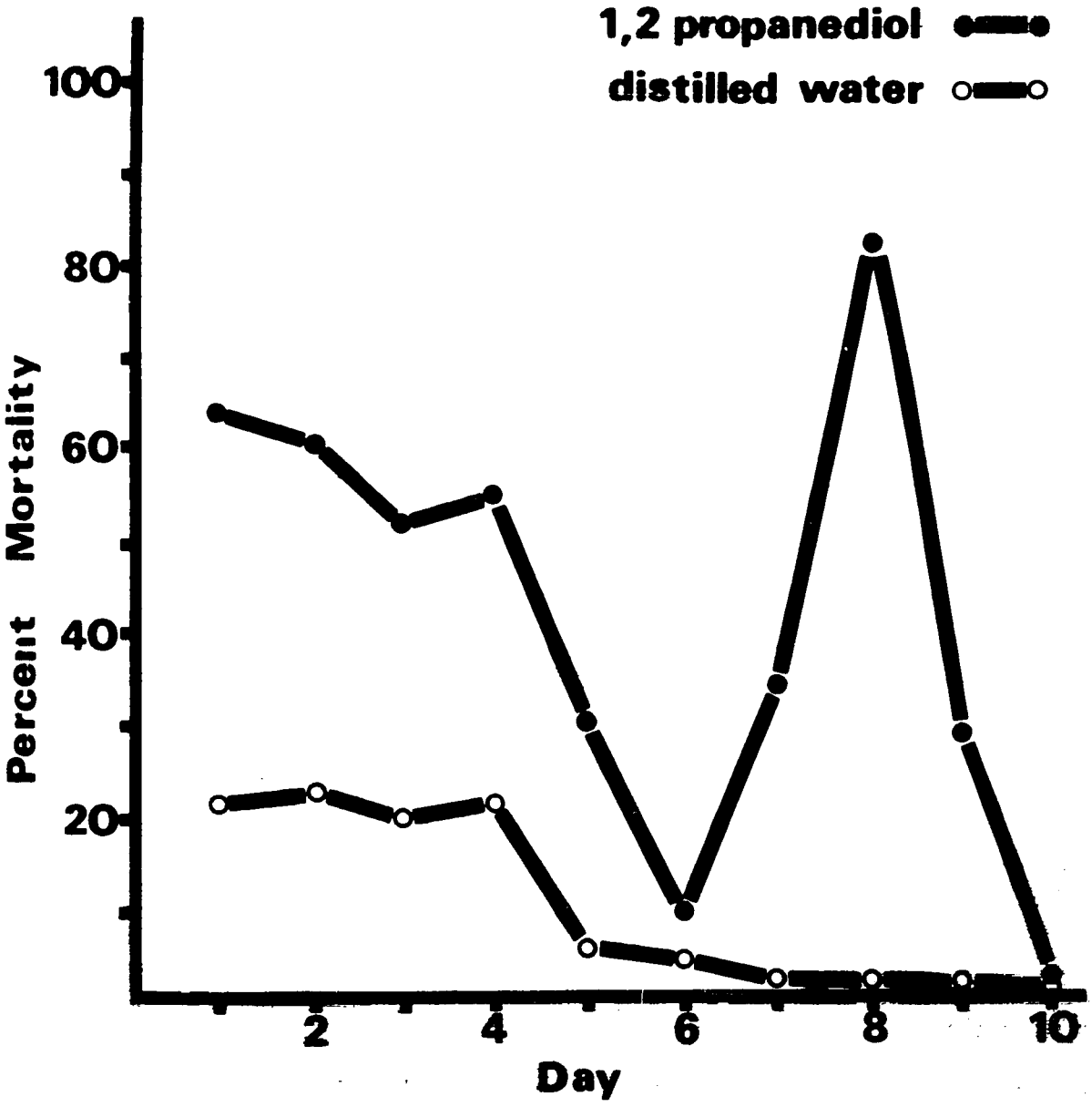


Fig. 8 Toxicity of 1,2 propanediol during the first ten days of chick embryo development.

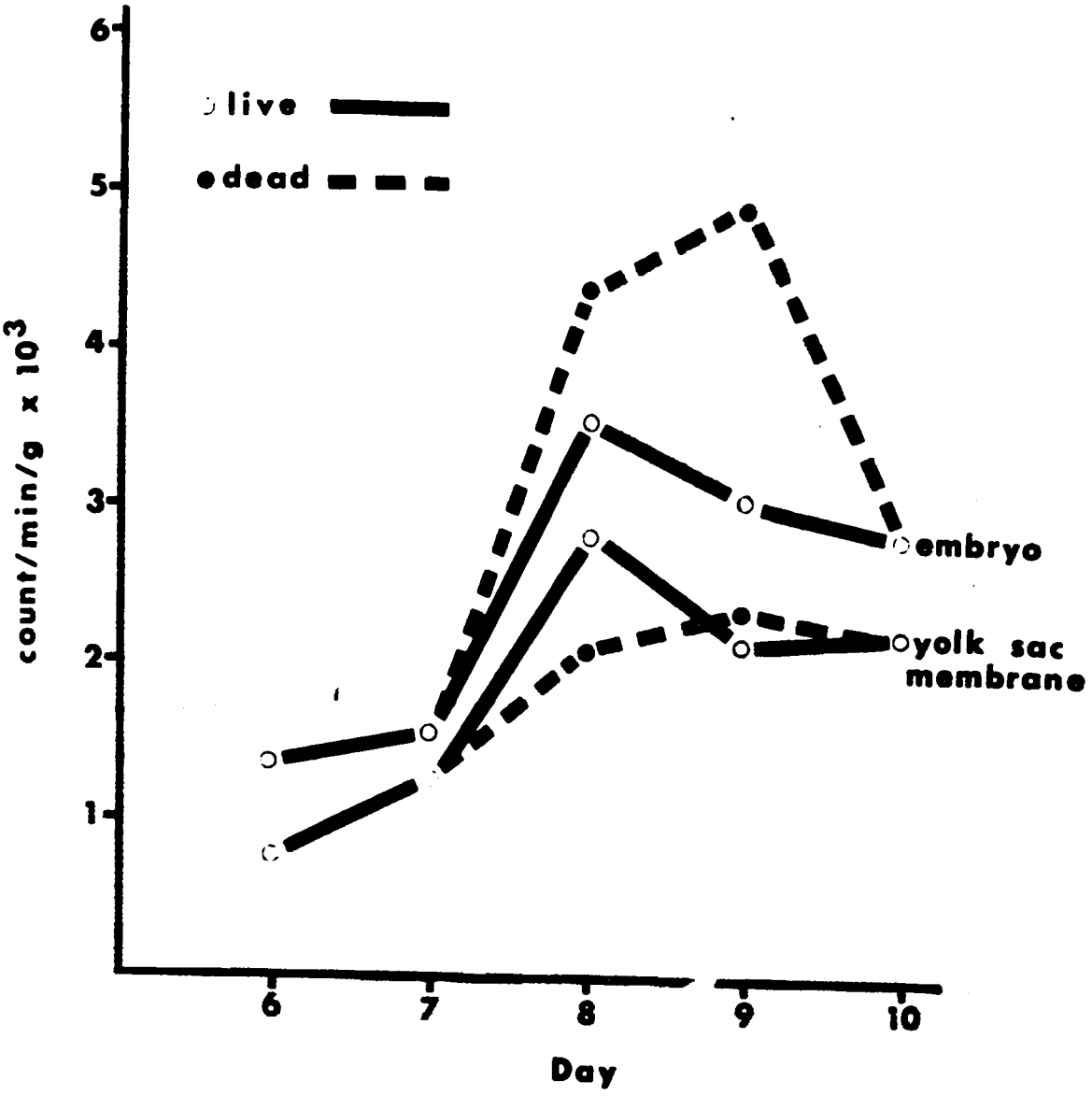


Fig. 9 Distribution of 1,2 propanediol-1-C^{14} in the supernatant fraction of the embryos and their yolk sac membranes on days 6, 7, 8, 9, and 10 of incubation.

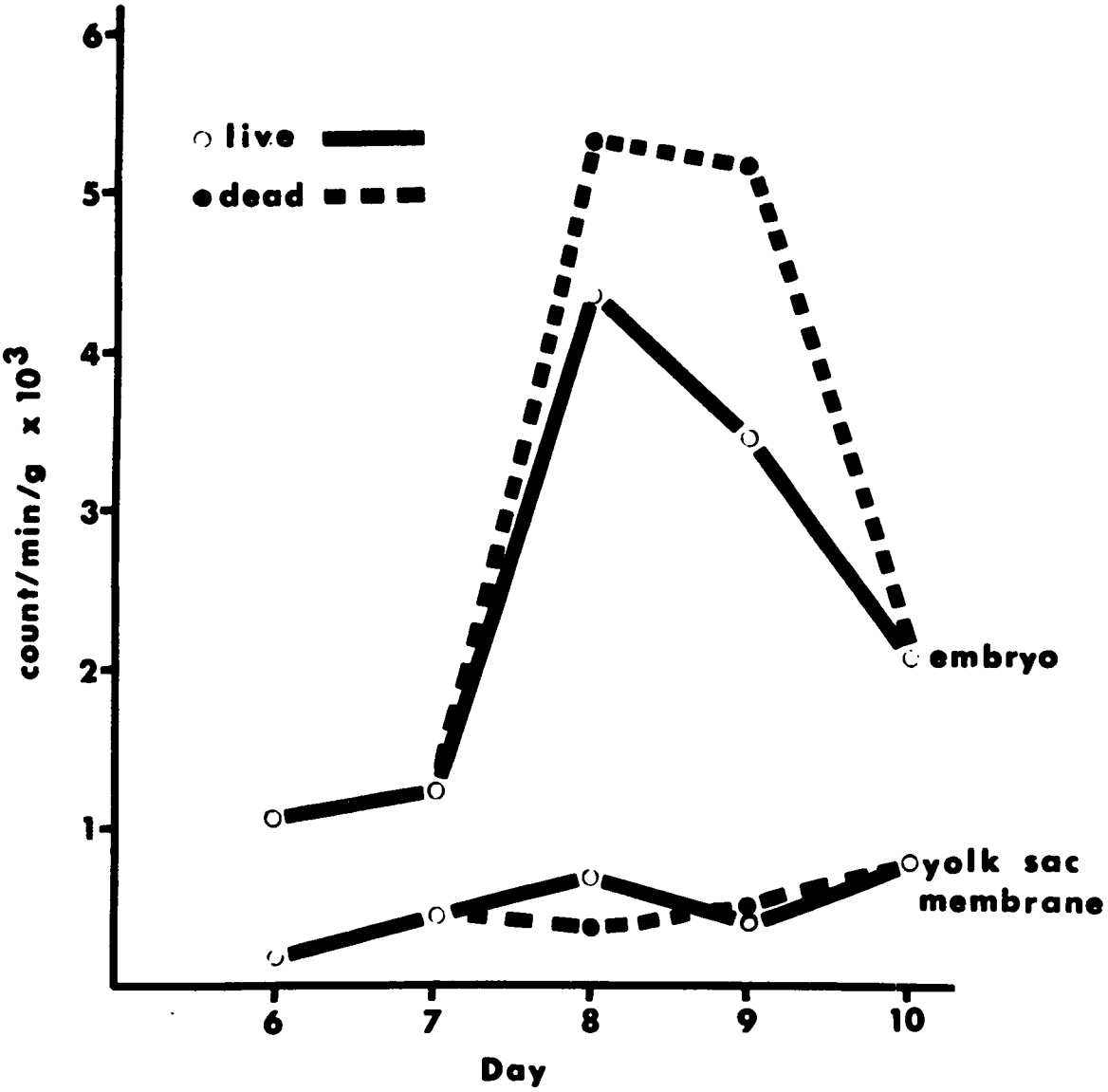


Fig. 10 Distribution of 1,2 propanediol-1-C^{14} in the precipitated fraction from embryos and their yolk sac membranes of day 6-10 of incubation

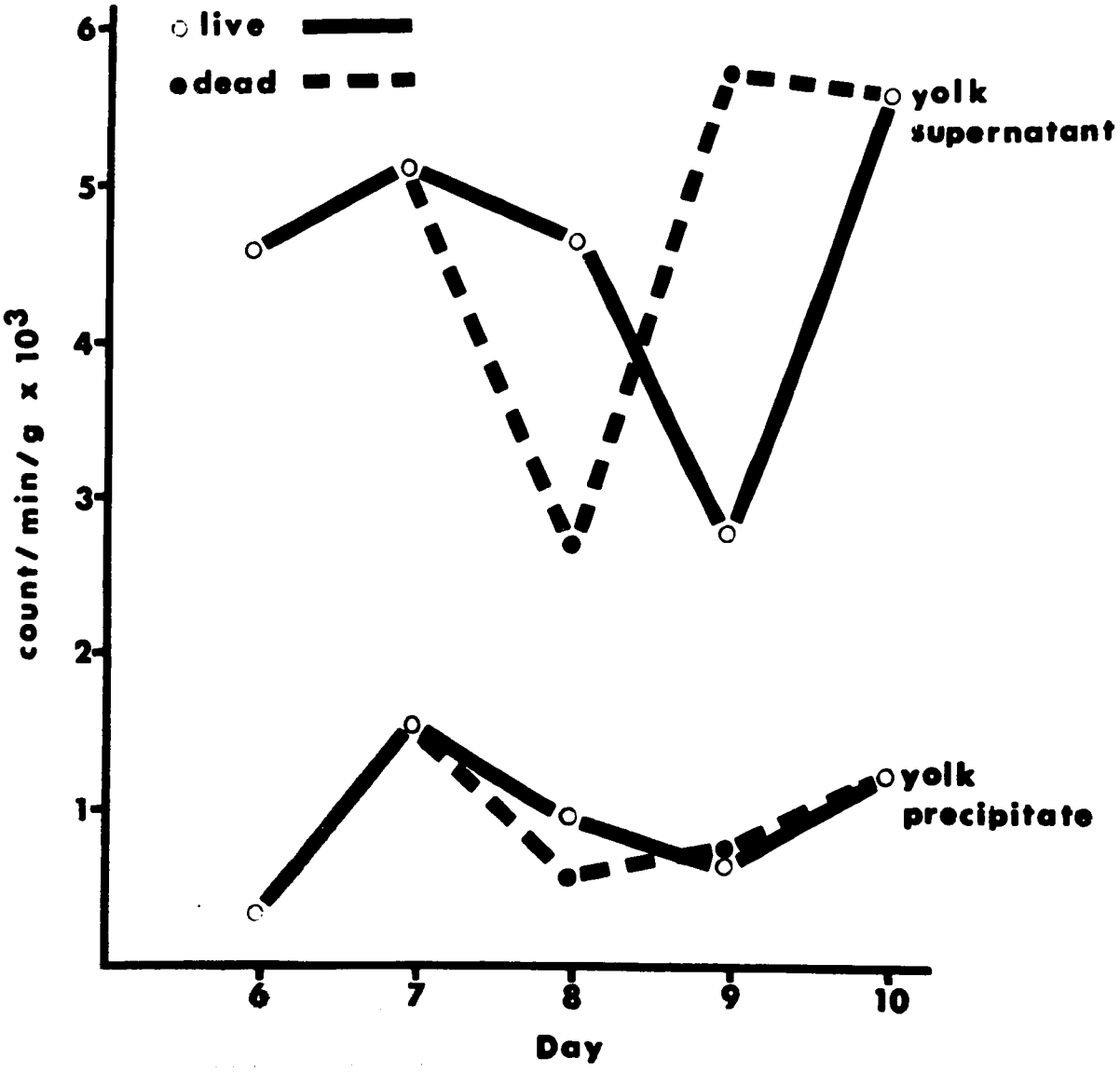


Fig. 11 Distribution of 1,2 propanediol-1-C^{14} in the supernatant and precipitated fraction of yolk of days 6-10 of incubation.

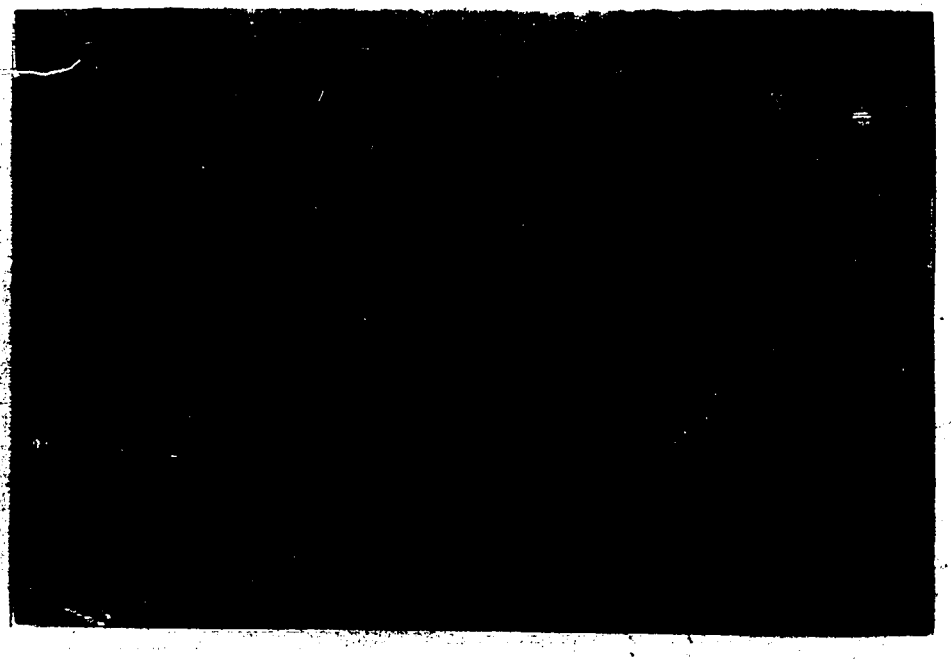


Plate I Technique and equipment for injection of eggs. At left, injection of eggs followed immediately, at right, with a drop of warm paraffin to seal the hole.

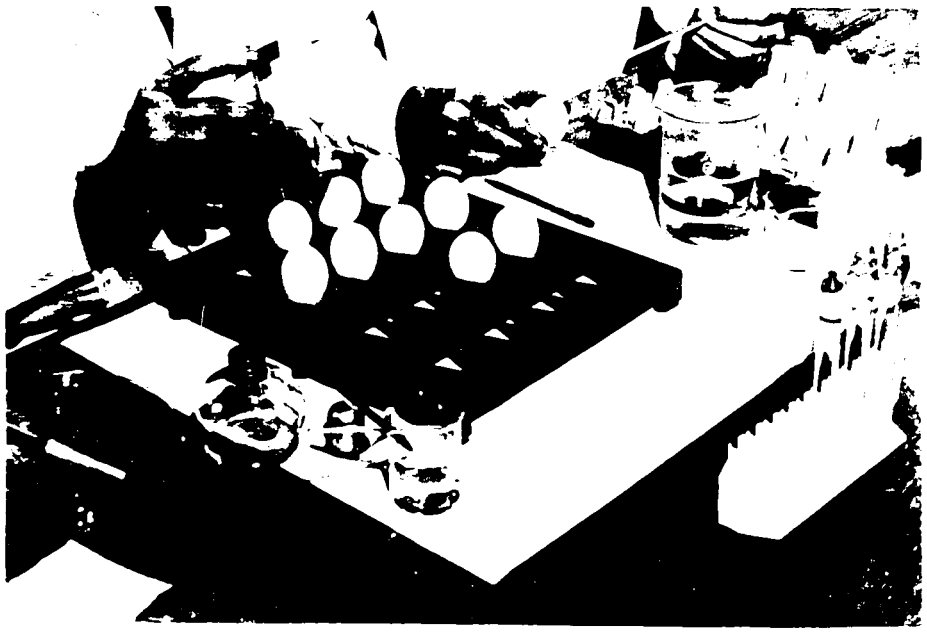


Plate II

Fig. 1 Neurotoxic chick after Phosphamidon treatment in comparison to a control

Fig. 2 Chicks with neurotoxicity of one or both hind limbs

Plate III

Fig. 1 Hydrocephaly and short mandible from injection on day 9 with 100 µg. of Phosphamidon

Fig. 2 Aeopthalmia and maxillia distortion from day 7 treatment of 100 µg. of Phosphamidon

Plate IV

Fig. 1 No left eye and crossed beak after treatment with 100 µg of Phosphamidon

Fig. 2 No right eye and crossed beak after treatment with 10 µg of Phosphamidon

Plate VI

Fig. 1 Acetylcholinesterase sites by Koelle's method in thigh muscle of 6 day old chick

Fig. 2 Acetylcholinesterase sites of control 6 day old chick

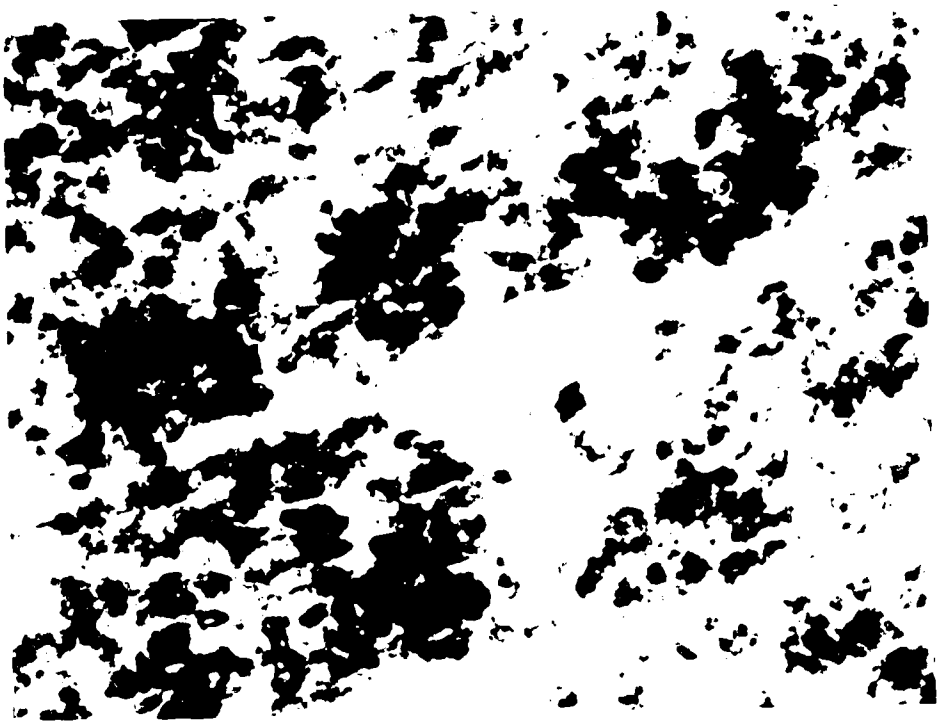
Fig. I

Fig. 2

Plate VII

Fig. 1 HPO stain of thigh muscle of neurotoxic chick

Fig. 2 HPO stain of thigh muscle of control chick



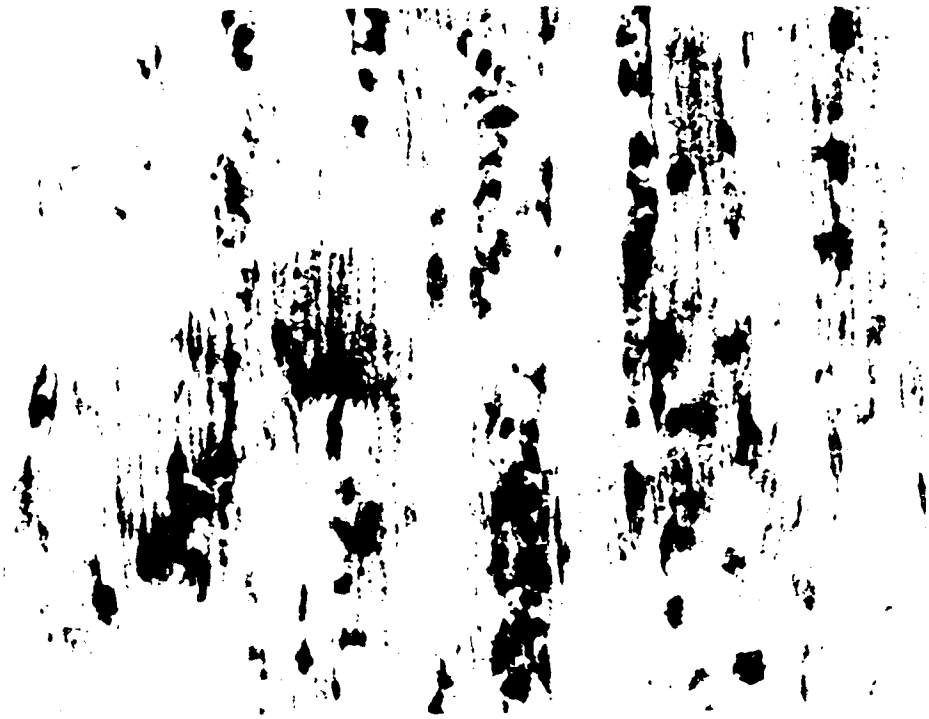
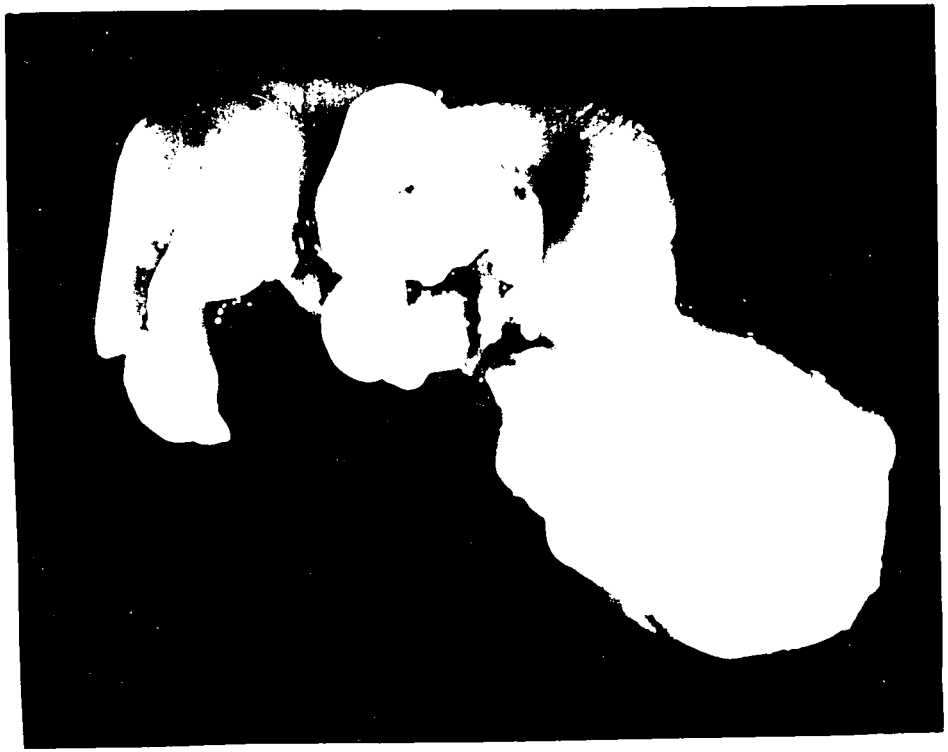


Plate VIII

Fig. 1 and Fig. 2 Embryos treated on day 2 with 10 µg of Sumithion showing different stages of development at death



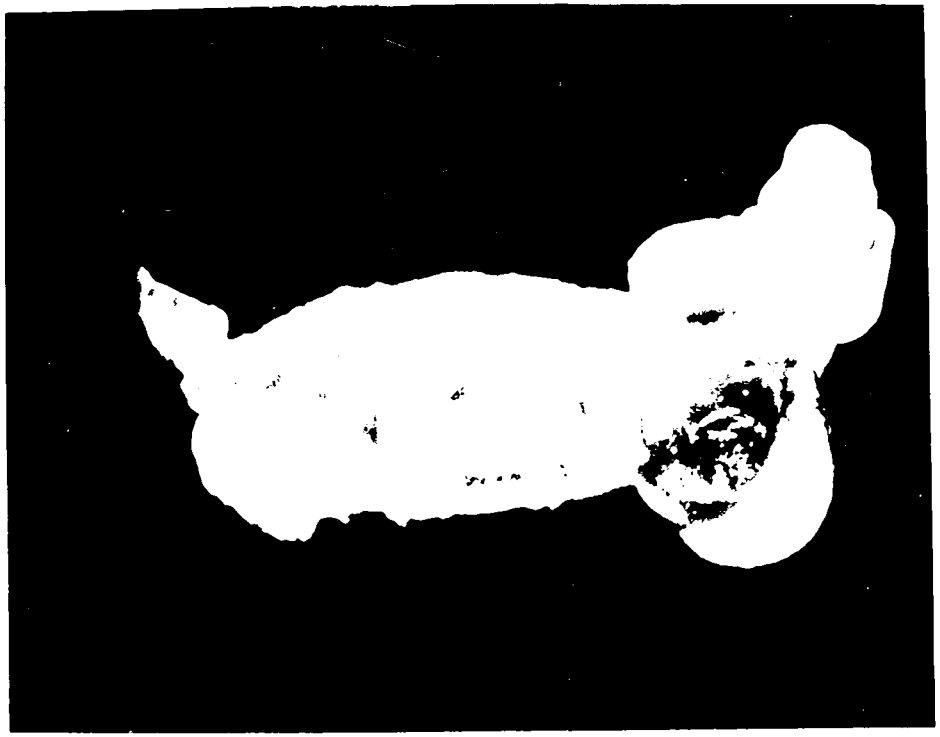


Plate IX

Fig. 1 Embryo treated on day 3 with 10 µg of Sumithion dying on day 5

Fig. 2 Embryo treated on day 3 with 100 µg of Sumithion

Plate X

Fig. 1 Embryo after treatment of 100 µg of Sumithion on day 4

Fig. 2 Hyperplasia of the mandible and abnormal development of the eye after treatment on day 1 with 10 µg of Baytex

Plate XI

Fig. 1 Embryo after 10 µg of Baytex on day 2

Fig. 2 Acrania, abnormal eye, and beak defect from 10 µg of Baytex on day 3

Plate XII

Fig. 1 Embryo treated with 100 µg of Baytex on day 1

Fig. 2 Embryo treated as above but on day 2

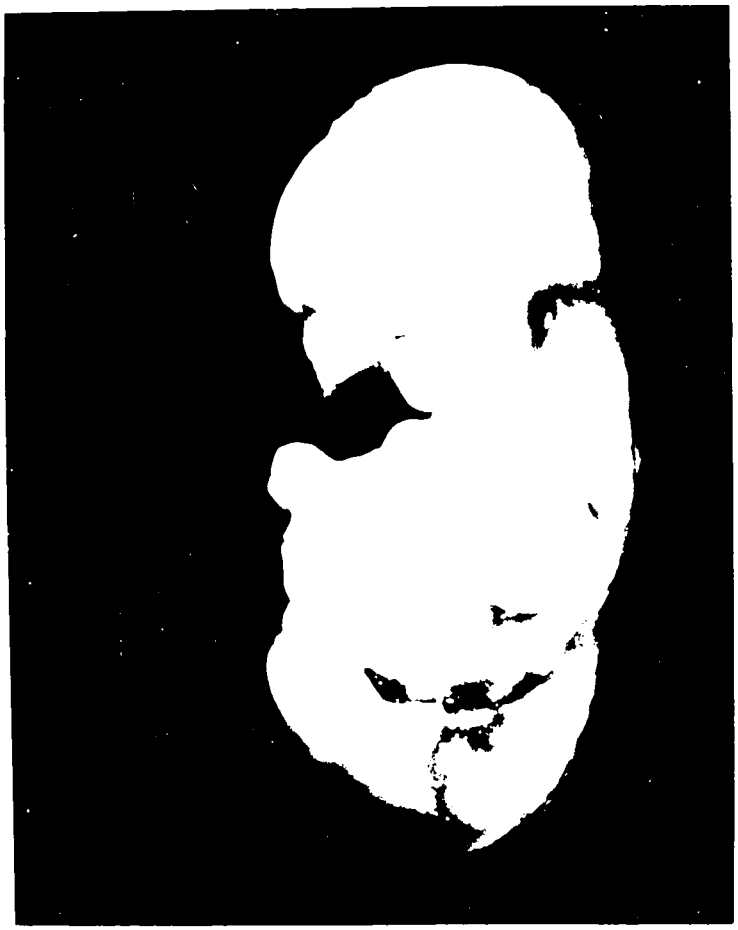


Plate XIII

Fig. 1 Embryo treated with 100 µg of Baytex on day 3

Fig. 2 Chick with hyperplasia of mandible, acrania and aeopthalmia after 10 µg of Dylox on day 7

Fig. 1

Plate XIV

Fig. 1 Eight day embryo 4 hours after 1,2 propanediol injection

Fig. 2 Control eight day embryo 4 hours after distilled water injection

Fig. 2

Plate XIV

Fig. 1 Eight day embryo 4 hours after 1,2 propanediol injection

Fig. 2 Control eight day embryo 4 hours after distilled water injection

Fig. 1

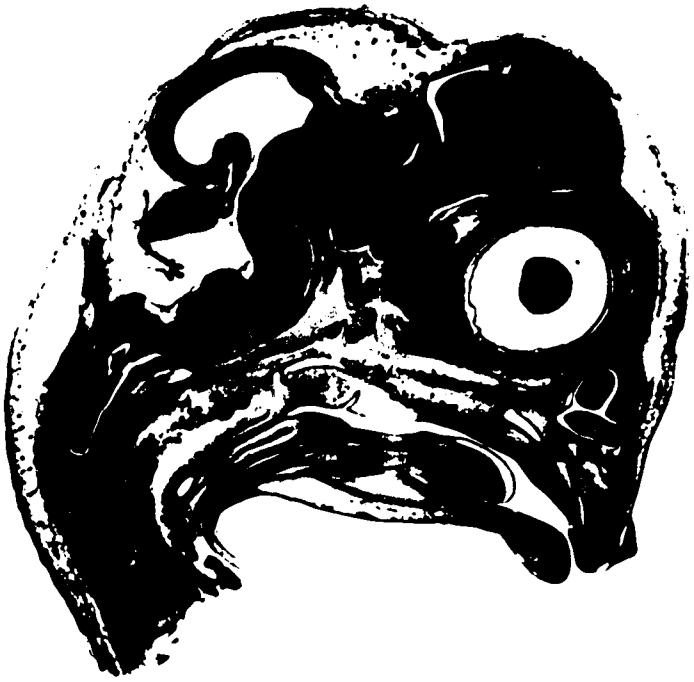


Fig. 2

Plate XVI

Fig. 1 Longitudinal section through eight-day experimental embryo showing hemorrhage in the body

Fig. 2 Control of above

Fig. 1

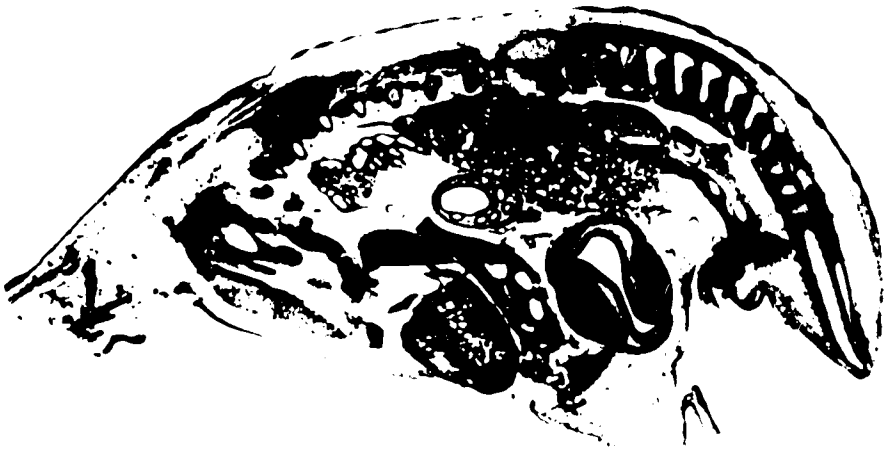


Fig. 2

Plate XVII

Fig. 1 Section through liver of experimental eight day embryo showing engorgement and cellular disruption

Fig. 2 Control section for above

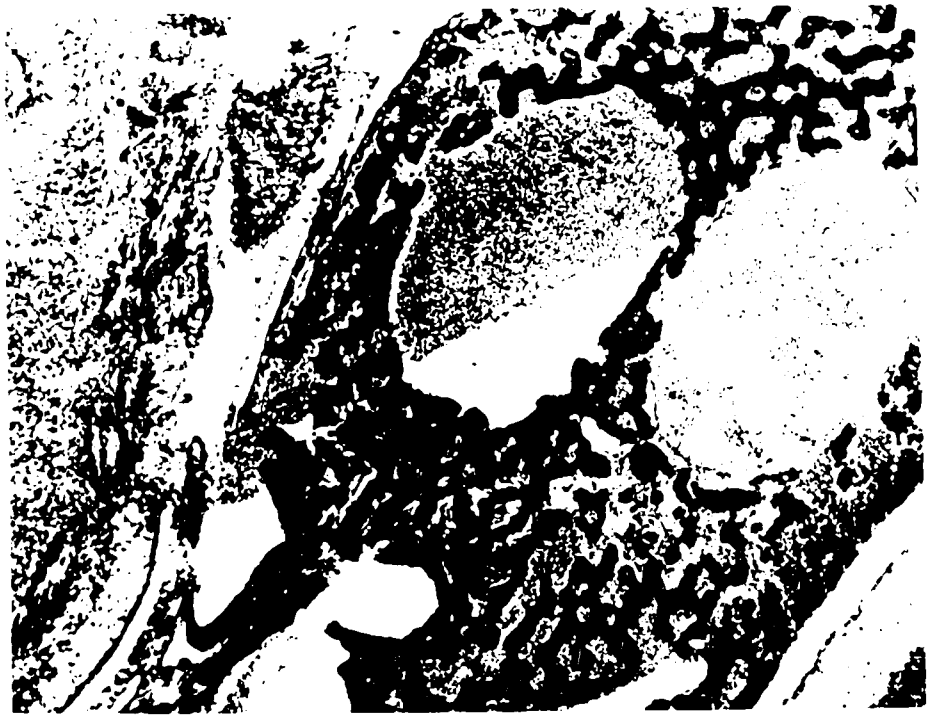


Fig. 1

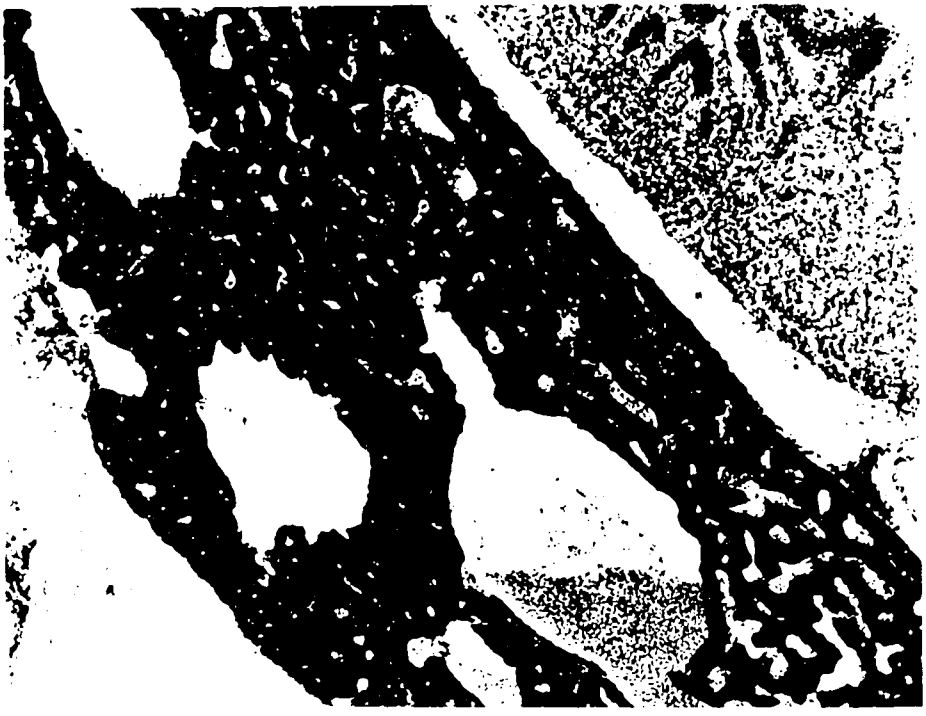


Fig. 2

Plate XVIII

Fig. 1 Kidney of eight day embryo treated with 1,2 propanediol

Fig. 2 Control for above

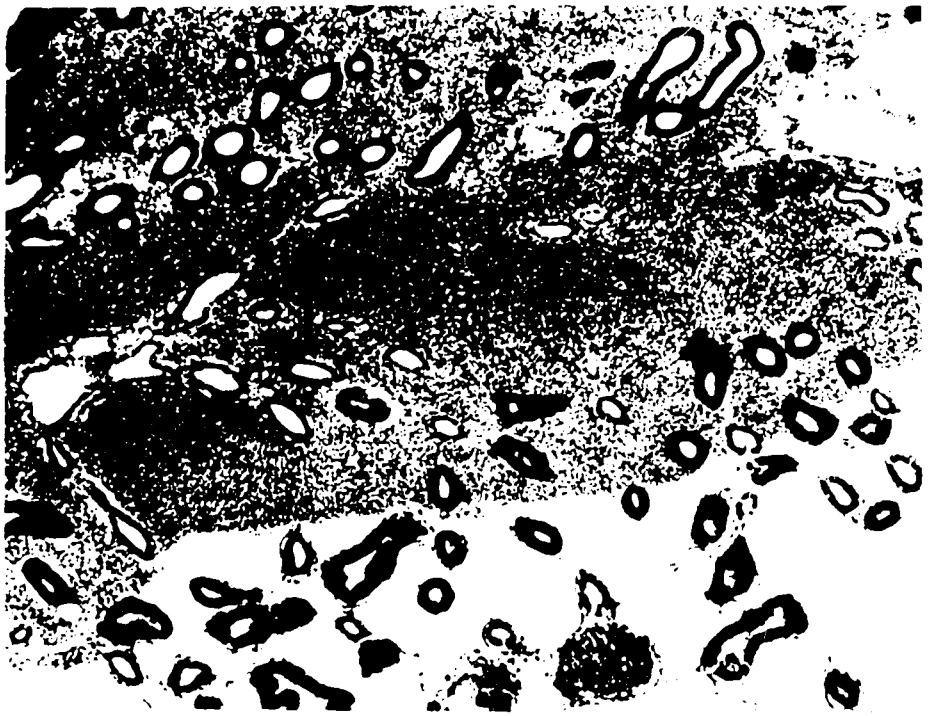


Fig. 1

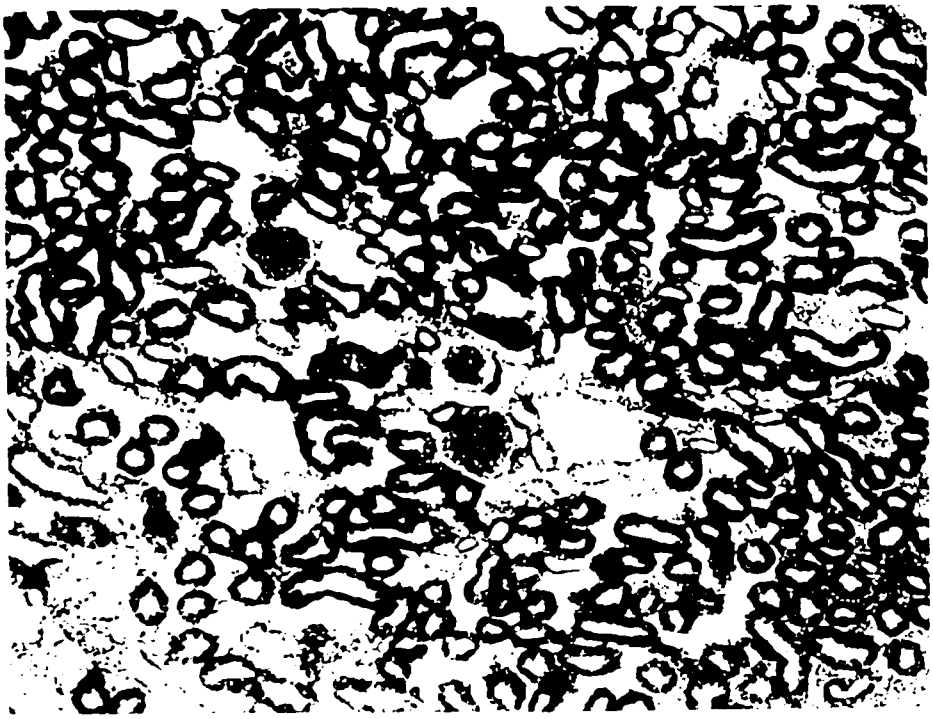


Fig. 2

Plate XIX

Fig. 1 Cross section of spinal cord of experimental eight day embryo

Fig. 2 Control for above

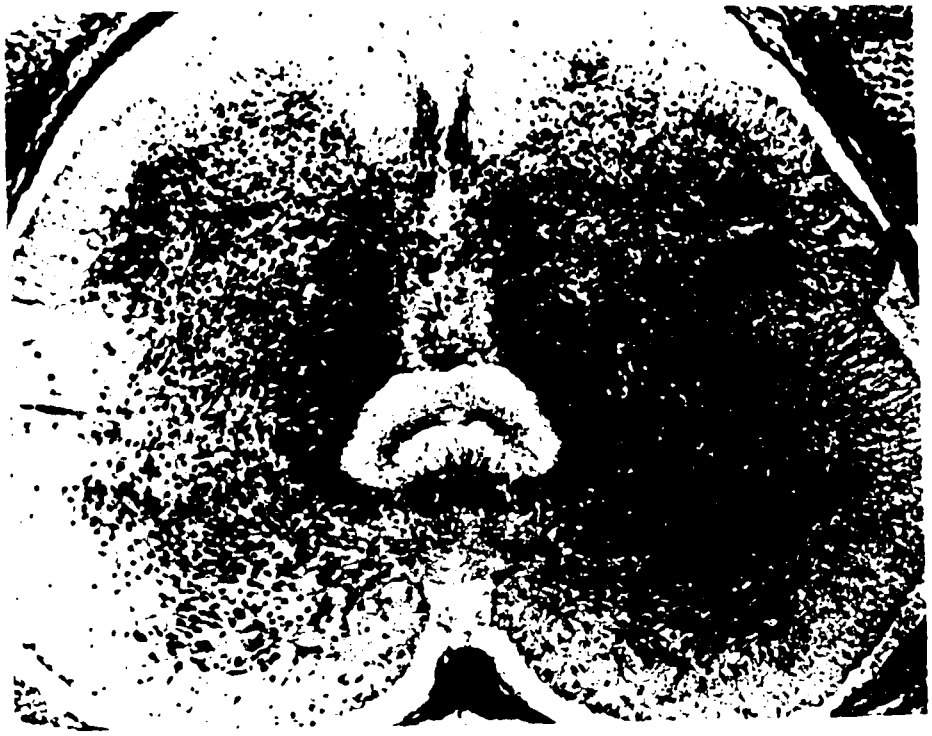


Fig. 1

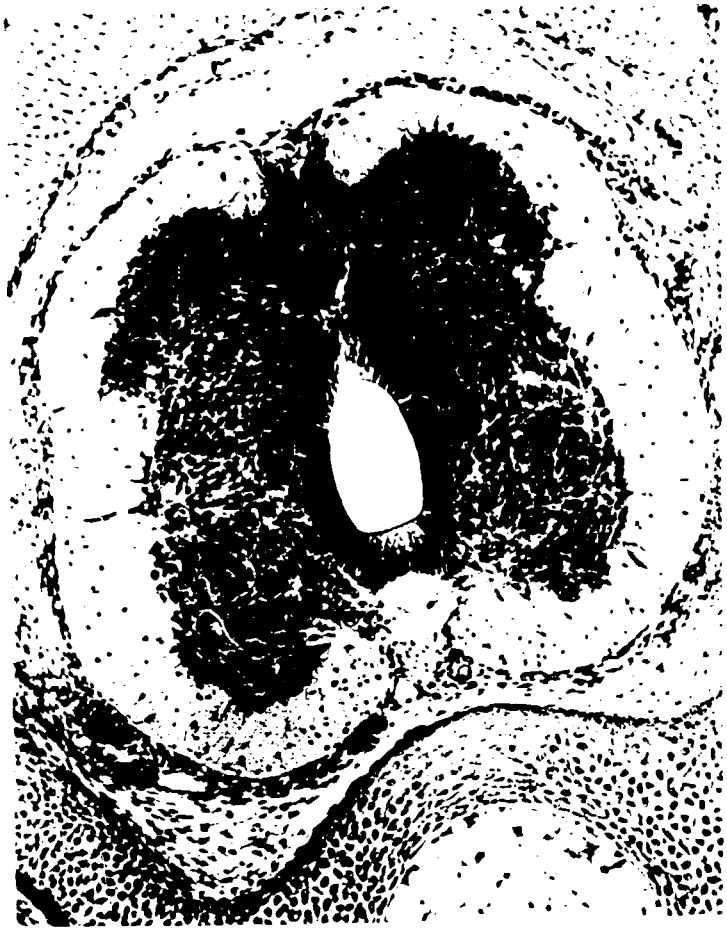


Fig. 2

Plate XX

Fig. 1 Cross section showing destruction of ependymal cells and neural debris of experimental eight day embryo

Fig. 2 Control for above

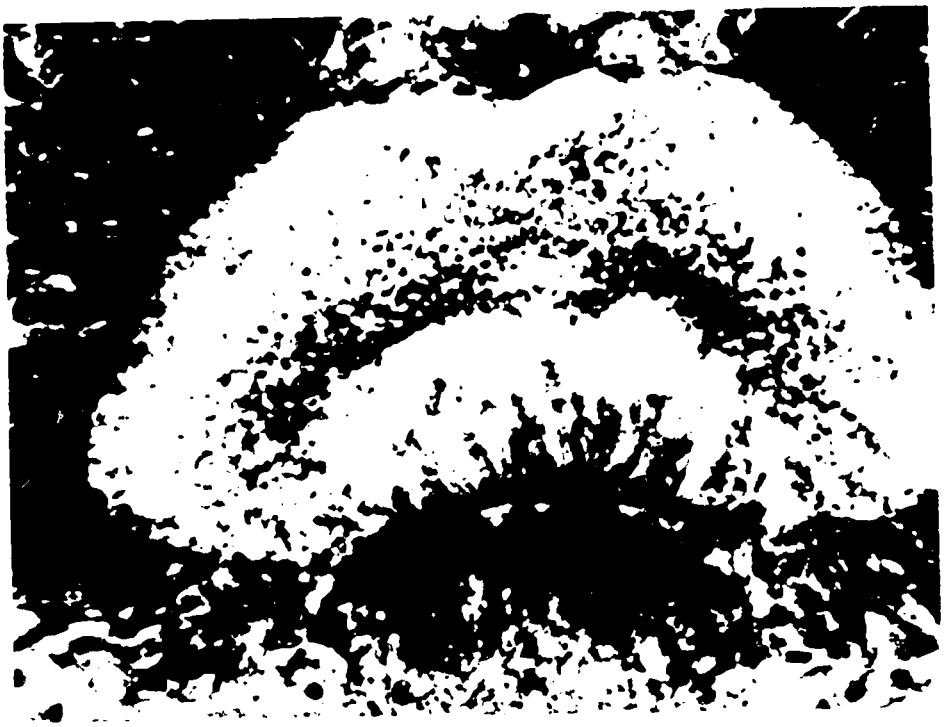


Fig. 1

Fig. 2

Plate XXI

Fig. 1 Sudan Black B stain of spinal cord of experimental eight day embryo

Fig. 2 Control for above

Fig. 1

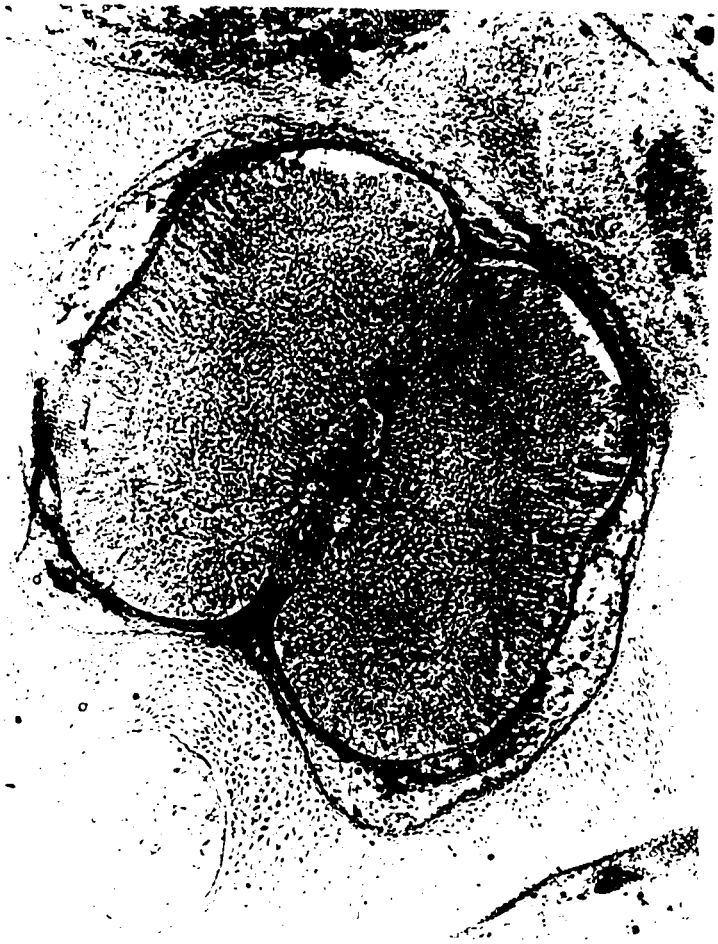


Fig. 2

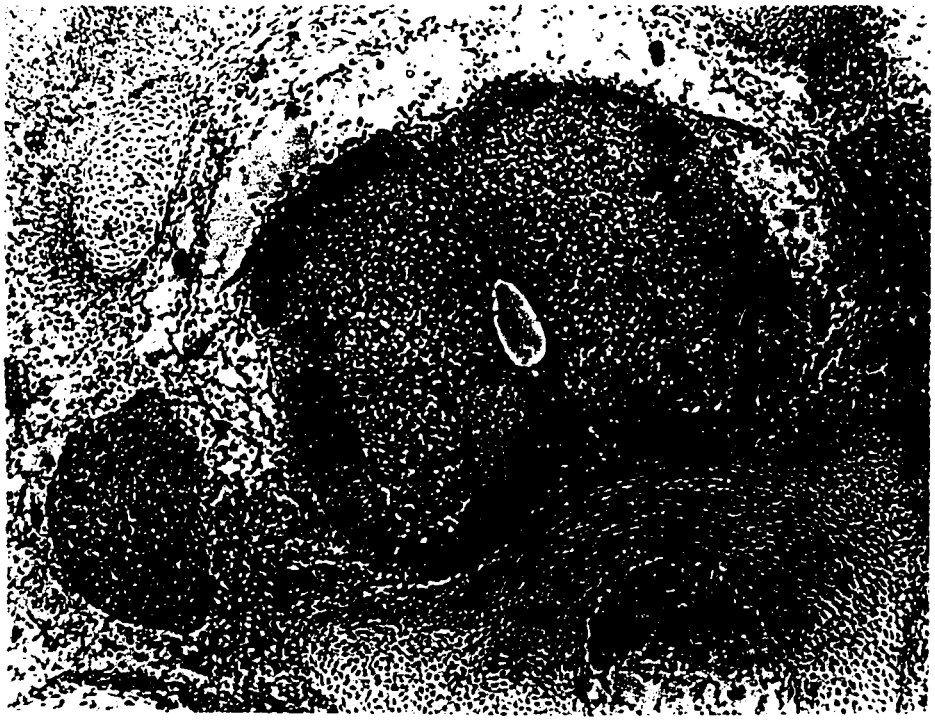


Plate XXII

Fig. 1 Sudan Black B stain of experimental eight day embryo showing lipid in neural canal

Fig. 2 Control section for above

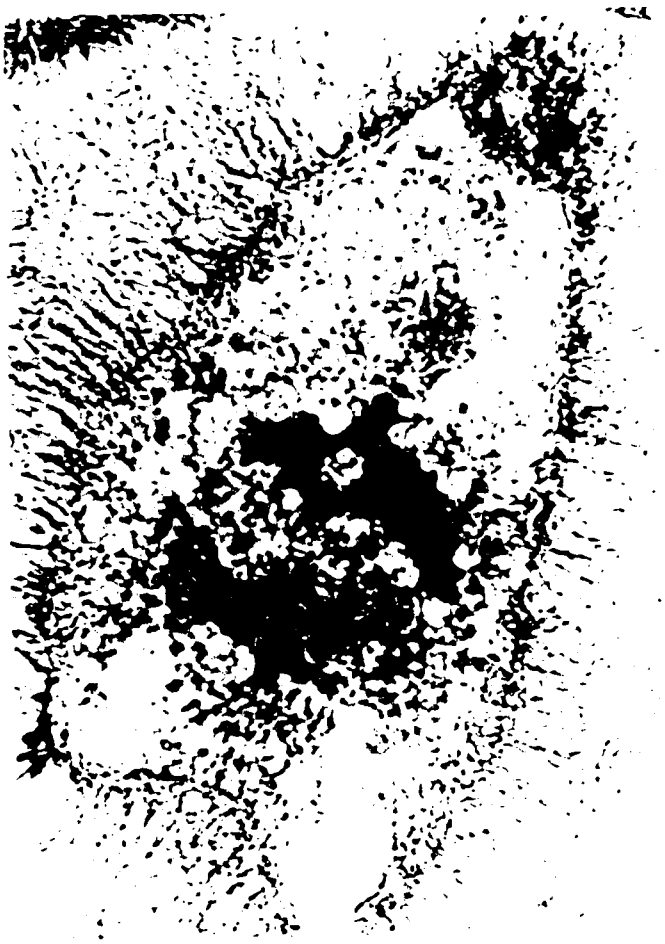


Fig. 1

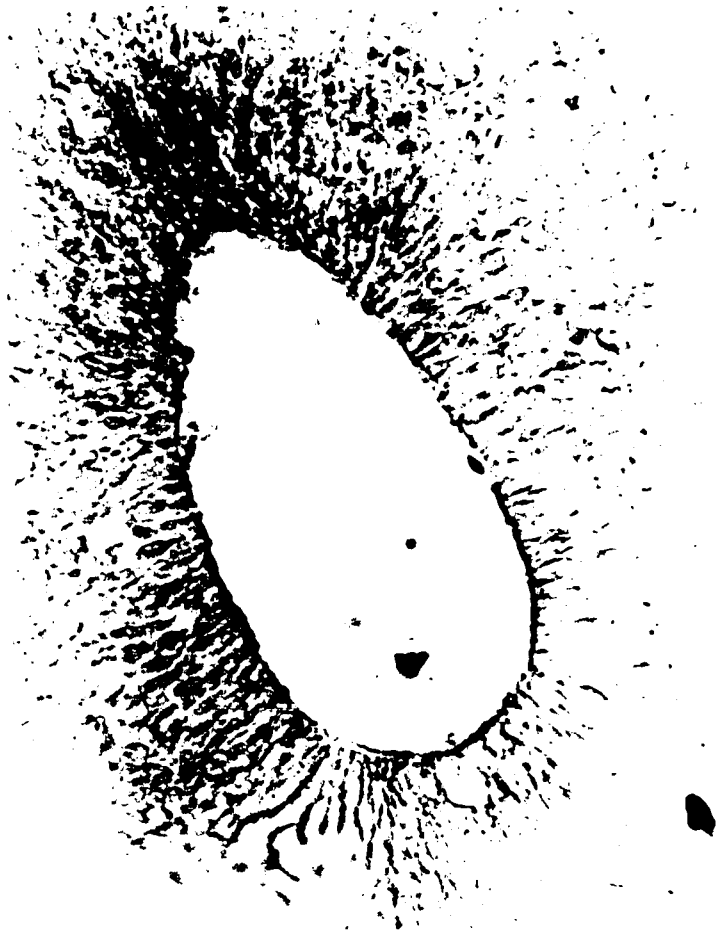


Fig. 2

Plate XXIII

Fig. 1 Mallory's connective tissue stain of the experimental embryonic kidney

Fig. 2 As above, control embryonic kidney

Plate XXIV

Fig. 1 Mallory's connective tissue stain of spinal cord of eight day experimental embryo

Fig. 2 Control for above

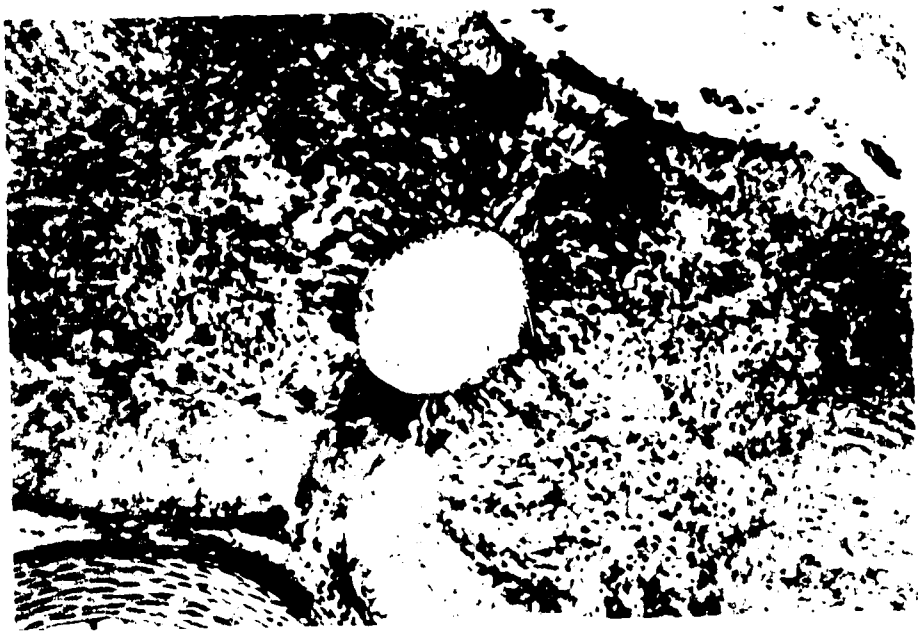


Fig. 1

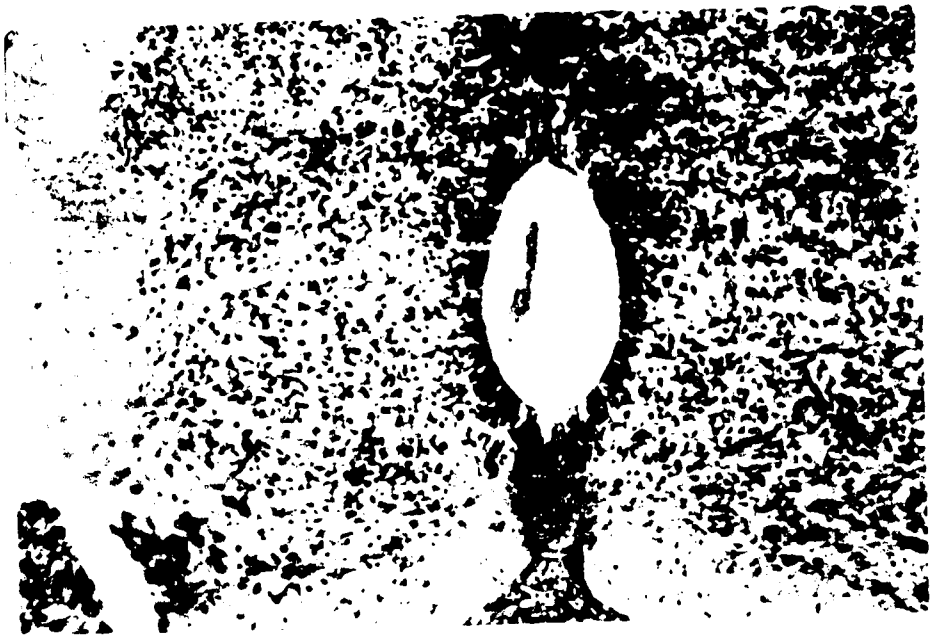


Fig. 2

Appendix

Properties and Metabolism of 1,2-Propanediol

Although introduced by Charles Wurtz in 1859 (Seidenfeld and Hanzlik 1932, and Heine et al 1950), 1,2-propanediol (propylene glycol, 1,2-dihydroxy-propane) was not considered for biological purposes until 1931. The first recorded use was as a replacement for the more toxic ethylene glycol (Hanzlik et al 1939, Mulinos et al 1943, Gessner et al 1961, Gershoff and Andrews 1962, and Coen and Weiss 1966) in the treatment of syphilis and neurosyphilis (Seidenfeld and Hanzlik 1932).

In versatility and utility, 1,2-propanediol is now, next to water, the most widely used solvent. Its characteristics of solvent, humectant, and preservative have resulted in its employment in food manufacturing and processing (Curme and Johnston 1953). It is used as a solvent for flavouring material in baking and candy production. This glycol prevents fermentation and mold growth in syrups (Heine et al 1950, Curme and Johnston 1953, Gray and Soa 1956, Selenka 1963, Rae 1948 and 1951). As a humectant 1,2-propanediol keeps packaged foods fresh and cereals crisp. Being odorless, innocuous, and unobjectionable, it is employed as a solvent for inks for printing food wrappers (Curme and Johnston 1953).

In the pharmaceutical industry an extensive contribution is made by 1,2-propanediol as a solvent for drugs, a

stabilizer for vitamins, and pastes for medicinal purposes (Heine et al 1950, and Curme and Johnston 1953). In cosmetics it acts as a preservative, humectant, spreader, and emollient. It has also been used as a therapeutic agent for the treatment of ketosis in cattle (Johnson 1954).

Toxicity of 1,2-Propanediol

Toxicity studies have been made with various types of administration, but no system or organ has been established as the cause of death. Histological investigations have indicated the 1,2-propanediol did not damage the kidneys, heart, spleen, and liver (Seidenfeld and Hanzlik 1932, Weatherby and Hoag 1938,and Newman et al 1940). But toxicology studies by Seidenfeld and Hanzlik (1932) demonstrated that intravenous injections to rats and rabbits resulted in an LD_{50} of 16.8 g/Kg and 5.25 g/Kg respectively. Intramuscular administration showed an LD_{50} of 14.7 g/Kg to rats and 7.5 g/Kg to rabbits.

In an extensive study (Weatherby and Hoag 1938), in which closer attention was directed to technique and route of administration, the above values were confirmed and it was established that the correct LD_{50} value for intravenous injections to rats is 6.8 g/Kg. It was also shown that the oral LD_{50} to rats was 33.5 g/Kg, while subcutaneously it was 22.5 g/Kg.

The study by Brown and Cartland (1936) has further confirmed these data. For mice (Davis and Jenner 1959) the

intraparitoneal LD_{50} was observed to be 11.5 g/Kg. The use of 1,2-propanediol as a carrier solvent in experiments with mice has been advised against (Lampe and Easterday 1953), since it was felt that the mouse is one species in which the glycol produces unfavourable results.

Selenka (1963) found that the antimicrobial property of 1,2-propanediol was due to inhibition of a specific enzyme, but the enzyme inhibited was not identified.

Sublethal doses of 1,2-propanediol administered to rats result in hematuria (Brown and Carland 1936). This prompted an investigation into the hemolytic effects of this compound. In vitro studies of aqueous solutions of glycol indicate that hemolysis occurs (Brown and Carland 1963, and Caldwallader 1963). The hemolysis may be prevented, up to a concentration of 30% propylene glycol, if the solutions are prepared with 0.9% sodium chloride (Caldwallader 1963).

Mallery and Randolph, and Randolph and Mallery (1944) have employed 1,2-propanediol in equal proportions with distilled water for leucocyte counts. The erythrocytes pass through three stages when suspended in this medium, first a turbid phase, then a transparent phase and finally a redevelopment of a turbidity due to precipitate formation. Microscopic observation reveals that the precipitate is caused by agglutination of red blood cells. The red blood cells eventually hemolyze. This occurs in concentrations of greater than 30%, 1,2-propanediol in water.

Cell-free plasma is also precipitated by 1,2 propanediol.

In an *in vivo* experiment (Brittain and D'Aray 1962) on rabbits, 12.5, 25.0, and 50.0% 1,2-propanediol in normal saline did not alter red blood cells, hemoglobin concentration, or total white blood cell counts.

Metabolism of 1,2-Propanediol

After oral and subcutaneous administration of 1,2-propanediol to a single rabbit, propylene glycol-monoglycuronic acid was detected in the urine, indicating that the glycol was excretable (Seidenfeld and Hanzlik 1932).

The oxidation products of the glycol, pyruvic and lactic acid, showed a possible glycogenic benefit (Seidenfeld and Hanzlik 1932, Lehman and Newman 1937, Hanzlik et al 1939, and Newman et al 1940), and inclusion of 1-10% aqueous propylene glycol in the food did not retard the growth or the body weight of rats (Lehman and Newman 1937). The compound was rapidly absorbed from the gastro-intestinal tract with a combustion rate directly proportional to 1,2-propanediol's concentration. When the concentration in the blood was high, 1,2-propanediol was found in the urine. Partial substitution of propylene glycol in a rat carbohydrate diet can result in a loss of body weight because of its unpalatability (Hanzlik et al 1939). Of the four glycols studied by Hanzlik et al 1939), only propylene glycol increased the glycogen content of the liver and of the whole body, and rats that ate a modified propylene glycol diet were more active.

Newman et al. (1940) further demonstrated the glycogenic capacity of 1,2-propanediol when they perfused isolated cat livers. The glycogen level in the perfused liver remained high, but there was reduced oxygen consumption and carbon dioxide production. An elevation in blood lactate followed perfusion, but there was no pathological damage to the liver. When insulin was added to the perfusion fluid it prevented an increase in the blood-lactate and stimulated utilization of the glycol. The glycogenic potential was also demonstrated in the blood and rumen of steers by Waldo and Schultz (1960). They observed that propylene glycol produced the greatest increase in the blood sugars of the six glycogenic compounds tested.

Recently, Bayley et al (1967) have shown that 9% propylene glycol could replace an equivalent amount of corn starch in a chicken diet.

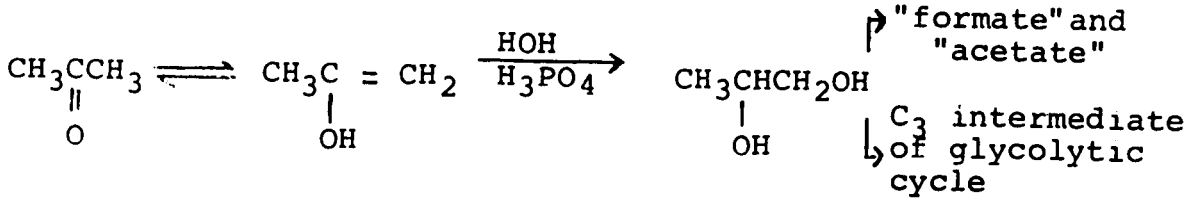
Biochemical Studies

The first biochemical study of 1,2-propanediol was carried out by Goepfert (1940), who demonstrated that the mold Fursarium lini Bolley dehydrogenated propylene glycol to acetol.

$$\underset{\underset{OH}{|}}{CH_3CHCH_2OH} \longrightarrow \underset{\underset{O}{\|}}{CH_3CCH_2OH} + H_2$$

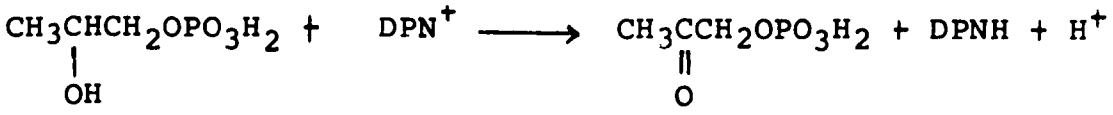
In the metabolism of acetone (Rudney 1950, Sakami 1950 a, b, Sakami and Welch 1950, and Sakami and Lafaye 1951) 1,2-propanediol or its phosphorylated form was sug-

gested as an intermediate with end products of "formate", "acetate", or a three carbon intermediate of the glycolytic cycle. A radioactive label on the methyl carbons of acetone is transferred to the β -carbon of serine and the methyl groups of choline and methionine. With the appearance of the label on the 3 and 4 carbons of glycogen, a portion of the labeled acetone may have been converted to lactate. The activity in carbons 1 and 6 of glycogen was much greater than in carbons 2 and 5, further suggesting a direct conversion of acetone to lactic acid.



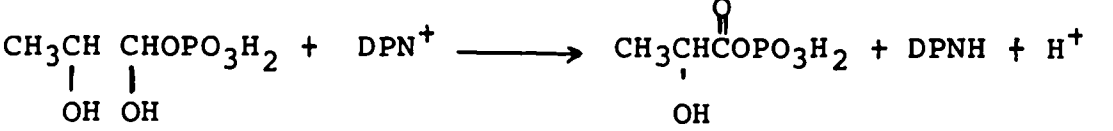
The possibility arose that acetone could be metabolized by carboxylation to acetoacetate or acetone cleaved to "acetate" and "formate" to give the observed labelling patterns. By labelling the carbonyl groups of acetone with C^{14}, Sakami and Lafaye (1951) confirmed that a direct oxidation to a 3-carbon intermediate was more likely since glucose possessed more activity in the 2 and 5 carbons than in the 1 and 6 carbons.

1,2-propanediol-1-phosphate stimulates keto acid production (presumely pyruvate) and oxygen consumption, whereas 1,2-propanediol will not (Miller et al 1953). In the presence of myogen A and DPN, the phosphorylated glycol is oxidized to acetol phosphate.



With the addition of an isomerase to the above reaction at equilibrium the reaction proceeds to lactaldehyde phosphate.

The lactaldehyde phosphate is further oxidized by D-glyceraldehyde-3-phosphate dehydrogenase to lactyl phosphate.



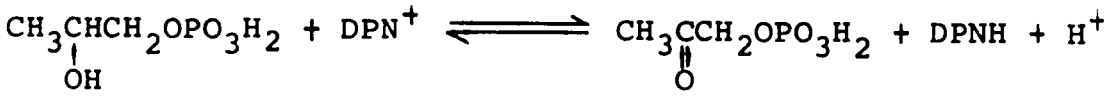
Sellinger and Miller (1959) corrected this last observation in which α-glycerol phosphate present in the rabbit muscle extract was attributed to the reduction of acetol phosphate to 1,2-propanediol-1-phosphate. The conversion was actually due to a specific enzyme 1,2-propanediol dehydrogenase, in the original mixture.

1,2-propanediol-1-phosphate is not foreign to biological systems. Rudney (1954 a,b) has isolated the phosphorylated form from rat liver, and Linberg (Miller and Bazzano 1965) has extracted it from sea urchin eggs and beef brain. Rudney also hypothesized that 1,2-propanediol may yield "acetate" and "formate", while 1,2-propanediol-1-phosphate may be oxidized to a glycolytic precursor.

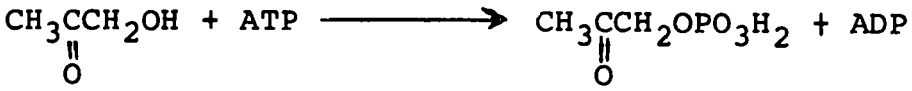
Groth and Lepage (1954), working with normal and neoplastic rat tissue under anaerobic conditions and using a fluoride block, demonstrated that a glycolytic precursor (pyruvate) can be converted to 1,2-propanediol-1-phosphate.

Baker's yeast metabolizes 1,2-propanediol, and 1,2-propanediol-1-phosphate either through acetol or acetol phosphate to lactic acid (Huggins and Miller 1956).

Huff and Rudney (1959) have supported the oxidation of 1,2-propanediol-1-phosphate to acetol phosphate by demonstrating the following reversible reaction:

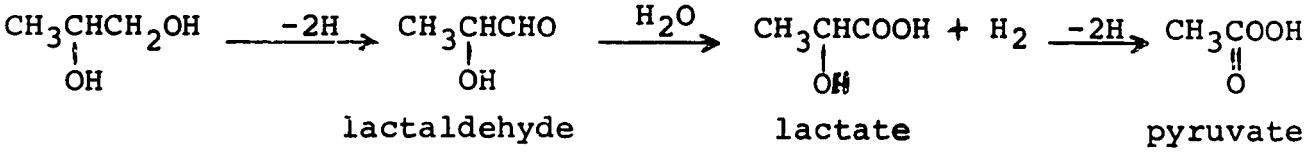


Rat liver and kidney homogenates convert acetol phosphate in the presence of ATP (Sellinger and Miller 1959).



Acetol and DL-lactaldehyde, two proposed intermediates of the propanediol-lactate pathway, are glycogenic in fasting mice (Skull and Miller 1960), and their product has been identified as 1,2-propanediol-1-phosphate (Sku-Mei et al 1964).

Two micro-organisms which use 1,2-propanediol as their carbon source have been isolated from soil (Taylor 1960). A pseudomonad oxidizes 1,2-propanediol to acetol then to a one carbon and a two carbon unit, while a diphtheroid attacks the primary alcohol in the following manner:



In a lactating cow (Emery et al 1967), 1,2-propanediol is converted in the liver to glucose by the pyruvate carboxylation pathway.

The metabolism of 1,2-propanediol does not always produce lactic acid. With a cell free extract of Aerobacter aerogenes and a cobamide coenzyme, Abeles and Lee (1961) and Lee and Abeles (1963) showed that isolated dioldehydrase oxidizes 1,2-propanediol to propionaldehyde.

$$CH_3\underset{\underset{OH}{|}}{C}HCH_2OH \xrightarrow[\text{cobamide}]{\text{dioldehydrase}} CH_3CH_2CHO + H_2O$$

In this reaction, the above hydrogen from the C-1 of 1,2-propanediol is transferred to the adenosyl moiety at the C-5' position of the cobamide, and from this position to the C-2 position of the 1,2-propanediol (Frey and Abeles 1966, and Frey et al 1967).

An enzymatic system from a different strain of Aerobacter aerogenes converts 1,2-diols into their corresponding deoxyaldehydes (Pawelkiewicz and Zagalak 1964).

Velle and Engel (1964) have isolated an enzyme from bovine placenta and seminal vesicles which is stereospecific for the D-form of 1,2 propanediol. The oxidation product is lactaldehyde.

The metabolism of 1,2 propanediol to lactic acid appears to go through two pathways (Rudney 1954b and Miller and Bazzano 1965). The free glycol takes the following route: lactaldehyde, methylglyoxal, and lactic acid, while the phos-

phorylated form passes through acetol phosphate, lactaldehyde phosphate and lactyl phosphate to lactic acid. 1,2-propanediol and its phosphate ester are intermediates in the metabolism of acetone. Micro-organisms can convert 1,2-propanediol into propionaldehyde (Abeles and Lee 1961, and Lee and Abeles 1963) or the deoxyaldehyde (Pawelkiewicz and Zagalak 1964).

The general metabolic features of 1,2-propanediol are shown in Chart 1.

Chart I Summary of literature review of 1,2-propanediol. C_1, C_2 refer to one carbon and two carbon units. Numbers in brackets correspond to references cited and are as follow:

1- Rudney 1950

2- Sakami 1950 a, b

3- Sakami and Welch 1950

4- Sakami and Lafaye 1951

5- Miller et al 1953

6- Sellinger and Miller 1959

7- Rudney 1954 a, b

8- Goepfert 1940

9- Miller and Bazzano 1965

10- Huggins and Miller 1956

11- Skull and Miller 1960

12- Sku-Mei et al 1964

13- Huff and Rudney 1959

14- Groth and Lepage 1954

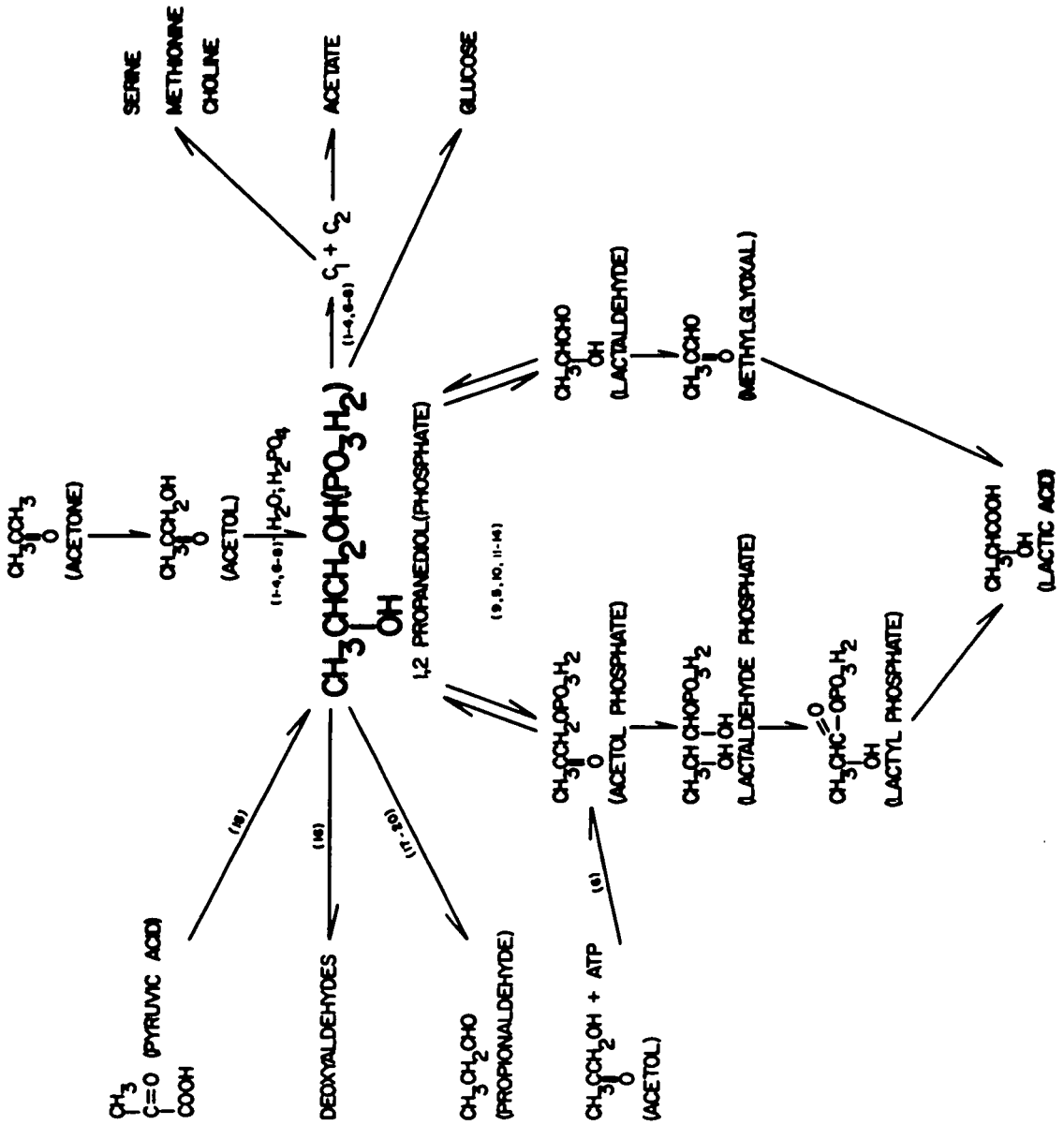
15- Pawelkiewicz and Zagalak 1964

16- Abeles and Lee 1961

17- Lee and Abeles 1963

18- Frey and Abeles 1966

19- Frey et al 1967


SERINE
METHIONINE
CHOLINE
ACETATE
GLUCOSE
(ACETONE)
(ACETOL)
(1-4, 6-9)
(1-4, 6-9) C1 + C2
1,2 PROPANEDIOL (PHOSPHATE)
(9, 8, 10, 11-14)
(LACTALDEHYDE)
METHYL GLYOXAL
(ACETOL PHOSPHATE)
(LACTALDEHYDE PHOSPHATE)
(LACTYL PHOSPHATE)
(LACTIC ACID)
(18)
(18)
(17-18)
(PYRUVIC ACID)
DEOXYALDEHYDES
(PROPIONALDEHYDE)
(ACETOL)
ATP
(9)

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