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MALATHION INDUCED TERATA AND THEIR BIOCHEMICAL IMPLICATIONS IN THE DEVELOPING CHICK EMBRYO

BY

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Supervisor

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ABSTRACT

Malathion is one of the organophosphorus insecticides least toxic to mammals. The toxicity of this compound in chick embryos is known; however, its teratogenic nature has not been recorded. Concentrations from 1.17 to 116.8 milligrams per egg were injected into the yolk sacs of 1 to 12 day incubated chicken eggs, and various types of abnormalities were produced. Injections of 3.99 and 6.42 milligrams malathion into the yolk sacs of 4 and 5 day incubated eggs produced deformed chicks with a combination of sparse plumage, micromelia, overall growth retardation and beak defects. All known breakdown and related products of malathion were investigated as to their teratogenicity; however, only the parent compound and malaoxon were capable of producing the aforementioned terata. Malathion causes a reduction in growth to embryos from eggs injected on days 4 and 5 of incubation. Various metabolites were injected along with malathion into fertile eggs in an attempt to determine the possible effect of the latter compound on metabolism. Of the compounds injected, tryptophan prevented both the malformations and the growth retardation while nicotinamide, nicotinic acid, and quinolinic acid prevented the malformations only. A tryptophan assay revealed a reduction of total tryptophan content in malathion treated embryos indicating a reduction in uptake of this amino acid from the yolk. Other compounds related to tryptophan by electronic reactivity enhanced the effect of malathion. Several NAD or NADP linked dehydrogen-

ases were investigated by histochemical and biochemical methods on both malathion treated and untreated embryos. A cholinesterase assay on embryo homogenates revealed no apparent relationship between cholinesterase levels and teratisms.

RESUME

Le malathion constitue l'un des organophosphates insecticides les moins toxiques pour les mammifères. Quoique la toxicité de ce produit sur les embryons de poulet soit connue, son action tératogène n'a pas été rapportée. Des concentrations de 1.17 à 116.8 milligrammes par oeuf furent injectées dans les sacs vitellins d'oeufs de poulet incubés (âges de 1 à 12 jours), et divers types d'anomalies furent ainsi produits. Des injections de 3.99 à 6.42 milligrammes de malathion dans les sacs vitellins d'oeufs incubés âges de 4 à 5 jours ont produit des poulets malformés présentant une combination de plumage réduit, micromélie, croissance retardée et déformation du bec. Tous les produits cataboliques connus du malathion et leurs dérivés ont été étudiés quant à leur pouvoir tératogène, cependant il s'est avéré que seuls le malathion et le malaoxon purent produire les tératismes mentionnés plus haut. Le malathion produit une réduction de la croissance dans les embryons de poulet quand il est injecté dans les oeufs incubés, âges de 4 à 5 jours. Divers métabolites ont été injectés en même temps que le malathion dans des oeufs fertiles afin de déterminer l'effet possible de ce dernier composé sur le métabolisme. Parmi ces composés, le tryptophane a empêché à la fois les malformations et le ralentissement de la croissance, tandis que la nicotinamide, les acides nicotinique et quinolinique n'ont empêché que les malformations. Un test avec le tryptophane a révélé une réduction de la quantité totale de ce produit dans les embryons traités au malathion, ce qui est symptomatique

d'une diminution de la consommation de cet acide aminé à partir du vitellus. D'autres produits s'apparentant au tryptophane par leur réactivité électronique ont accentué les effets du malathion. Diverses déshydrogénases à NAD et à NADP ont été étudiées chez des embryons traités au malathion et des témoins par des méthodes histochimiques et biochimiques. La mesure de la cholinestérase pratiquée sur des homogénats d'embryons n'a révélé aucun rapport entre les niveaux de cholinestérase et les tératismes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Various agents are capable of causing congenital malformations; they include viruses, cytotoxic compounds, antibiotics, alkaloids, azo dyes, hormones, dietary deficiencies, hypoxia, irradiation and others. Recently, in our laboratory, organophosphates have been implicated as possible teratogenic agents. This becomes important because these compounds are now used extensively as insecticides. As a result, we attempted to determine the effects of a relatively low toxic organophosphate on the developing chick embryo, but first a brief review of organophosphates.

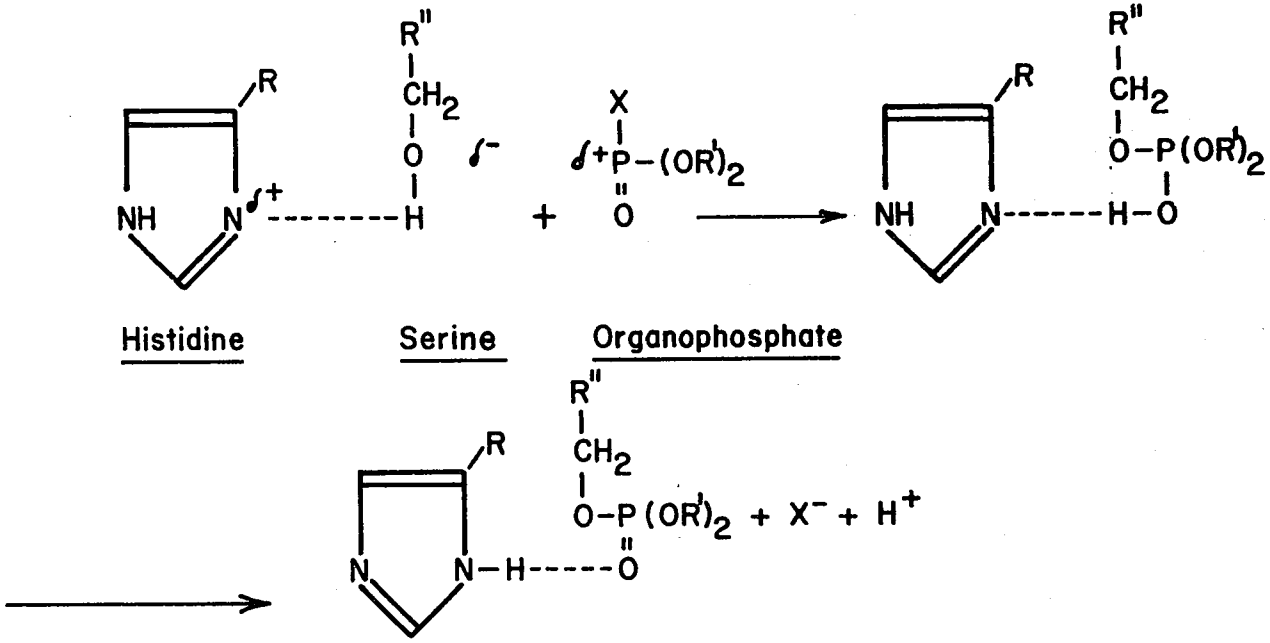
Organophosphates

In the 1930's, the organophosphates, tabun, sarin, and soman were prepared as nerve gases for the war effort. The insecticidal action of these compounds was realized by Gerhard Schrader and his associates of I. G. Farben Industries. They made dimefox in 1940, schradan in 1942, and parathion in 1944.

a. Enzyme inhibition

Organophosphates act by inhibiting cholinesterases. They do so by combining with serine at the esteratic site of the enzyme as shown in Fig. 1.

Fig. 1. Mechanism of cholinesterase inhibition by organophosphates



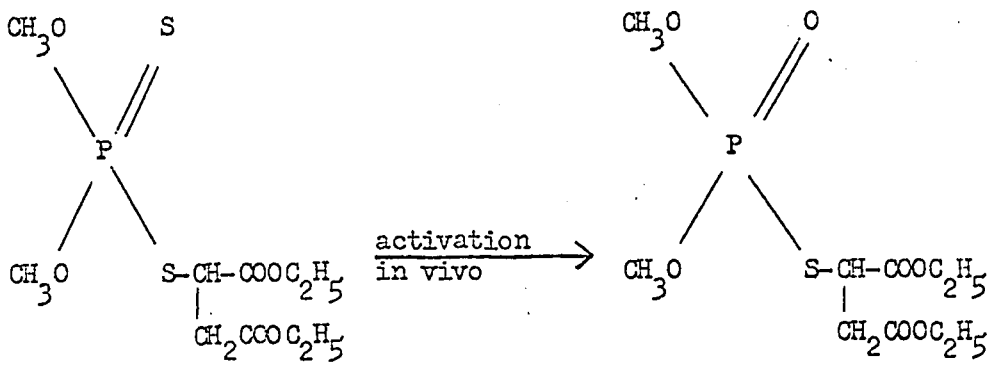
In simplified terms

$$(RO)_2P(O)X + EOH \longrightarrow (RO)_2P(O)OE + H^+ + X^-$$

Organophosphate — Cholinesterase — Phosphorylated enzyme

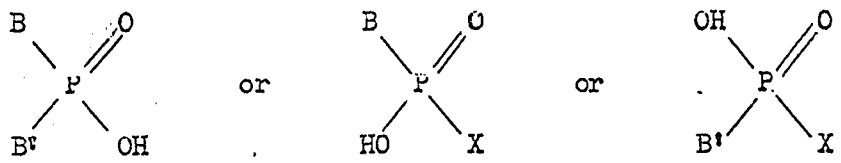
b. Activation and inactivation

Some organophosphates contain a thio group attached to the phosphorus atom. This thio group must first be oxidized for the compounds to be effective anticholinesterase agents; the P=S linkage is less electrophilic than the P=O linkage, thus the phosphorus in P=S compounds is inadequately positive to undergo rapid reactions with cholinesterases. For example, malathion is converted to malaoxon which is a much more potent cholinesterase inhibitor than malathion (O'Brien, 1960, 1967; Heath, 1961) as shown below:



malathion malaoxon

Inactivation resulting in the loss of all biochemical activity occurs upon hydrolysis of any of the bonds between the P atom and the acid or basic groups attached to it. The following compounds do not react with esterases:



c. Action of acetylcholine and related compounds

Acetylcholine is the chemical mediator which is responsible for the transmission of nerve impulses. It acts at the myoneural junction and at synapses. The enzyme acetylcholinesterase inactivates the compound soon after it is released. If acetylcholine is not destroyed, it would continue to stimulate and would disrupt the cholinergic system.

Compounds which mimic acetylcholine are nicotine and muscarine. Nicotine acts at the neuromuscular junction and ganglia. It stimulates voluntary muscles and indirectly activates smooth muscles or glands. Muscarine acts at the organ level and results in direct stimulation of the organ. For example, it may cause excess urination, lacrimation, decrease of heart rate, pupil constriction etc.

Compounds which antagonize acetylcholine are atropine and curare. Atropine antagonizes acetylcholine at the organ level by blocking the receptor site to which acetylcholine attaches. Curare

on the other hand, antagonizes acetylcholine at the neuromuscular junction.

d. Mode of action of organophosphates

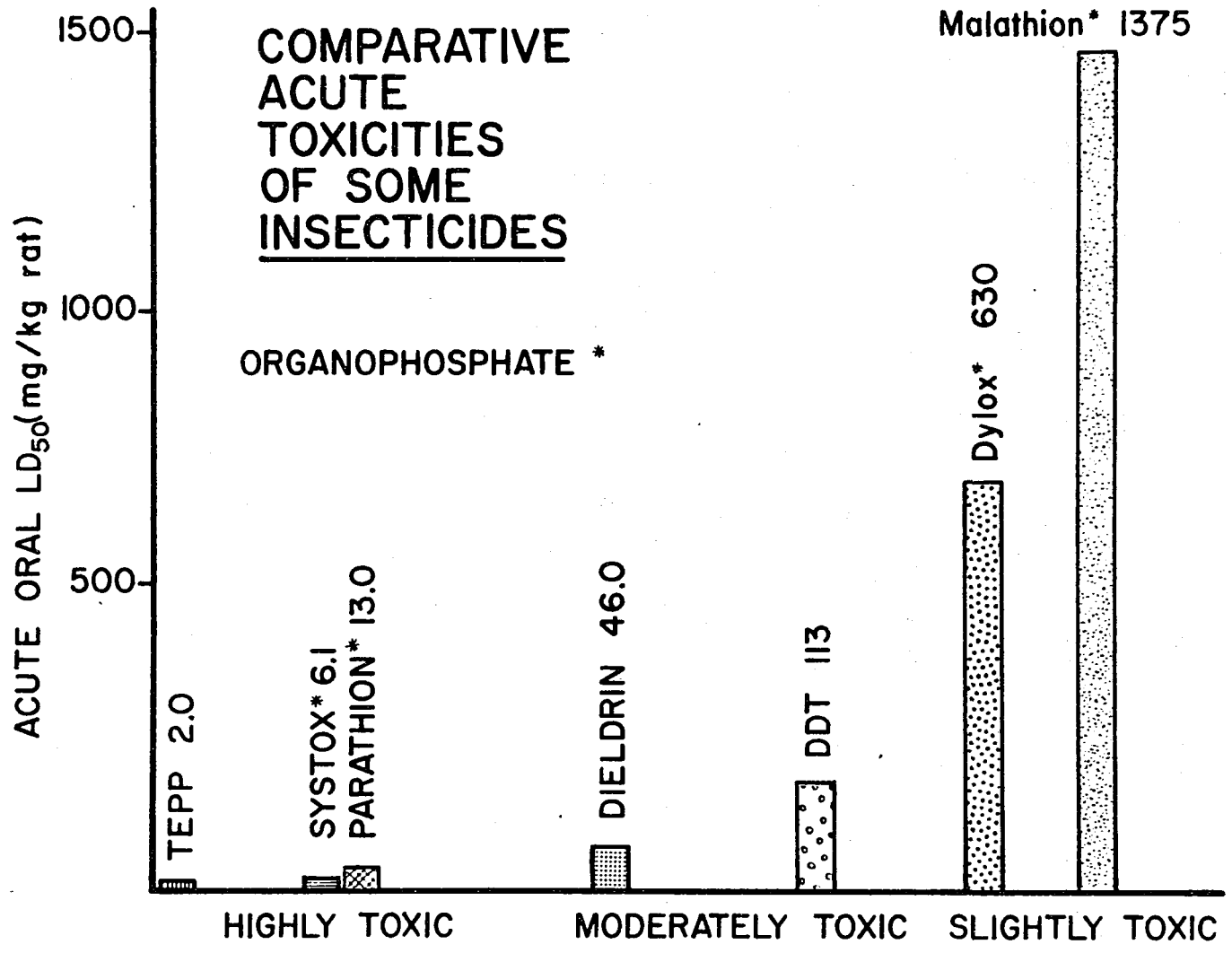
In vertebrates, the main effect of organophosphate poisoning is the inhibition of cholinesterases at cholinergic sites. The symptoms are excessive parasympathetic stimulation and may result in decreased heart rate, defecation, urination, and hypotension. Cholinesterase inhibition also affects the neuromuscular junction. This leads to twitching of voluntary muscles and paralysis. Death is ultimately due to asphyxiation either via paralysis of thoracic muscles, or by central action on the respiratory center. Atropine is an effective antidote at the organ level as well as centrally.

A few organophosphates cause nerve damage by destroying the myelin sheath (Koelle, 1963). Demyelinating compounds include diisopropyl phosphorofluoridate (DFP), diethyl phosphorofluoridate, and tri(ethyl phenyl) phosphate. Demyelination does not occur with diazinon, dichlorovos, demeton, chlorothion, sarin, soman, tabun, malathion, or schradan (O'Brien, 1960). In general, the organophosphate exhibiting the greatest cholinesterase inhibition is the most toxic. The relative toxicity of some organophosphates is shown in Fig. 2.

e. Malathion

Malathion was introduced as an insecticide in 1950 by the American Cyanamid Company. This compound has a low toxicity in mammals (Fig. 2), but is highly lethal to insects (Spiller, 1961). It is a liquid

Fig. 2. Comparative toxicity of some insecticides in the rat

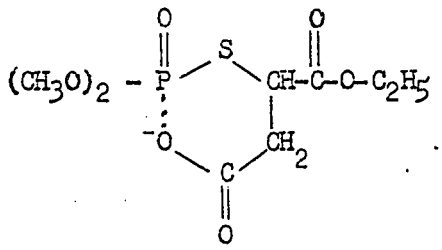

COMPARATIVE ACUTE TOXICITIES OF SOME INSECTICIDES
ORGANOPHOSPHATE *
ACUTE ORAL LD50(mg/kg rat)
1500
1000
500
TEPP 2.0
SYSTOX* 6.1
PARATHION* 13.0
DIELDRIN 46.0
DDT 113
Dylox* 630
Malathion* 1375
HIGHLY TOXIC
MODERATELY TOXIC
SLIGHTLY TOXIC

with a high boiling point, slightly soluble in water to the extent of 145 ppm, and soluble in many organic solvents and oils. Vertebrate toxicity is due to the anticholinesterase activity of malathion, however, the toxic signs last for only a short time and recovery occurs within a few hours. If death occurs, it is because of asphyxiation.

Walters (1957) reported the episode of a woman who drank 16 oz. of a malathion spray (equivalent to 130 mg/kg) to relieve a toothache. She was treated within 4 hours with atropine and she recovered the following day.

f. Degradation and metabolism of malathion

In mammals, malathion can be degraded by phosphatases and carboxyesterases; the latter enzyme can also cleave the second ester yielding the diacid. Malathion monoacid is a poor anticholinesterase agent probably because the carboxylate ion reduces the positivity of the phosphorus atom as shown below (O'Brien, 1967):



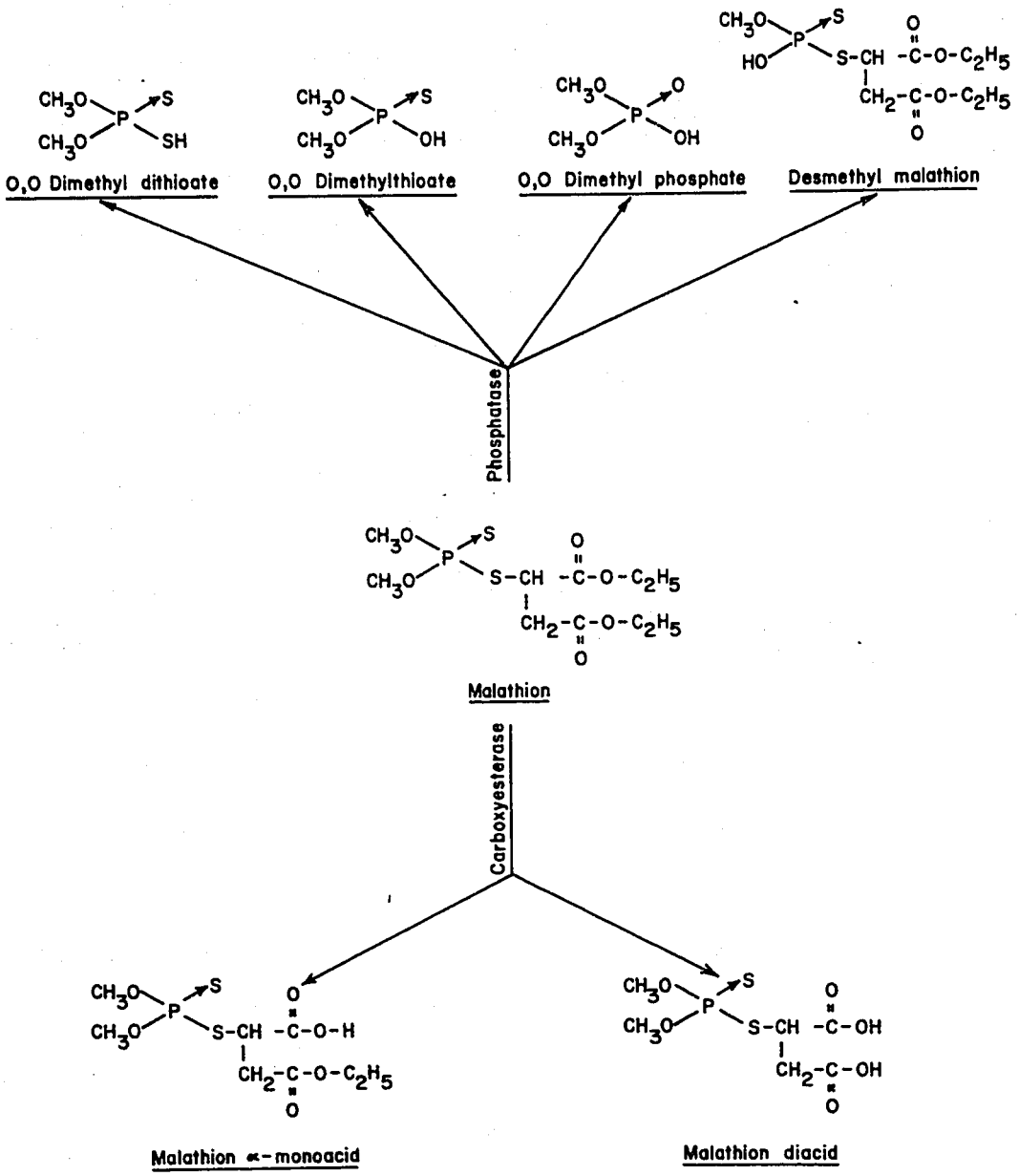
Malathion, and its oxygen analog malaoxon, can be degraded by various tissues including liver, kidney, lung, muscle and brain

(Seume and O'Brien, 1960; Murphy and DuBois, 1957). The percentage of urinary products, in the form of malathion α-monacid (Chen, Tucker, and Dauterman, 1969) and malathion diacid respectively, is 63 and 17 in the cow, 12 and 48 in the rat, and 40 and 21 in the dog. A scheme outlining the metabolism of malathion is shown in Fig. 3, (Knaak and O'Brien, 1960; O'Brien, Dauterman and Neidermeier, 1961; Krueger and O'Brien, 1959).

g. Organophosphates as teratogens

Khera et al. have shown that Systox^{R} (combination of O,O-diethyl-O-2(ethylthio) ethyl phosphorodithioate and O,O-diethyl-S-2 (ethylthio) ethyl phosphorodithioate) and EPN (O-ethyl-O-p-nitrophenyl phenyl phosphonothioate) cause permanent damage to the hind limb skeleton of ducks (Khera, LaHam and Grice, 1965; Khera et al., 1966). Ruddick (1968) injected various organophosphates into fertile chicken eggs; however, the results obtained were erratic and were not reproducible. The organophosphates that were injected with 1 to 500 µg per egg included water soluble phosphamidon (2-chloro-2-diethyl carbamoyl-1-methylvinyl 1-dimethyl phosphate) and water insoluble dylox (O,O dimethyl 222-trichloro-1-hydroxyethyl phosphonate), baytex (O,O dimethyl-O-(4-(methylthio)-M-toyl phosphorothioate), sumithion (O,O dimethyl (3-methyl-4-nitrophenyl) phosphorothioate), and parathion (O,O diethyl-p-nitrophenyl thiophosphate). These compounds were injected on days 1 to 10 of incubation. After phosphamidon treatment, the terata were inconsistent. The most frequent type of deformity was

Fig. 3. Metabolic scheme of malathion degradation in mammals


O,O Dimethyl dithioate
O,O Dimethylthioate
O,O Dimethyl phosphate
Desmethyl malathion
Phosphatase
Malathion
Carboxyesterase
Malathion α-monoacid
Malathion diacid

the "neurotoxic syndrome"; this consisted of chicks with outstretched hind limbs which caused the animals to rest on their sterna. Other terata included hydrocephaly, massive distortion of the maxilla, anophthalmia and cross beak. One phocomelic chick was produced with baytex after an injection of 500 µg per egg. Parathion was not teratogenic at the concentrations used. The "neurotoxic syndrome" was also common in control embryos if the eggs were not packed tightly in the hatching trays. The chicks use the eggs and shells for assistance during the early post-hatch period and if they are unable to support themselves in their first walking attempts, they will develop this syndrome. In Ruddick's work, the sample size used was small (10 to 30 eggs) and the amount of organophosphate administered was low. These factors probably account for some of the inconsistencies in his results.

Matton and LaHam (1969) treated trout larvae with dylox (0,0-Dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate) and produced fry with histological disturbances to the liver, kidney, heart and muscle. The authors concluded that these changes may be because of a decrease in heart rate and opercular muscle activity which leads to hypoxia and fatigue. These changes are due to the anticholinesterase action of dylox on the larvae. The teratogenic effects of malathion on chick embryos has not been previously recorded; however, its toxicity has been studied by various workers (Hadani and Egyed, 1966; Walker, 1966; McLaughlin et al. 1963).

The results of the current study will show that malathion produces teratisms in chick embryos consisting of feather, beak, and limb defects. These bear some similarities to the syndrome obtained by Landauer (1947, 1957) when insulin and 6-aminonicotinamide were employed.

STATEMENT OF PROBLEM

Long before the question of the danger to the environment of the slowly degraded persistent insecticides, such as the organochlorines, became a public issue, ultimately leading to their ban in many places, it was obvious to many biologists and chemists that the organophosphates would be the likely replacement. While more hazardous to handle and more costly, most of them are degraded rather rapidly in nature to harmless products. From the toxicity evidence available, it appeared that one of the most likely ones to be selected as a replacement was malathion because of its low toxicity in mammals. This has come to pass and today malathion is the most commonly used insecticide and is in constant use in crop protection and in the combat of disease-bearing insect vectors. In anticipation of its widespread usage, we were concerned that while it was apparently relatively non-toxic to adult mammals, no one had considered the possibility that ingestion of food-stuffs, possibly contaminated by malathion or its derivatives, by pregnant animals might prove toxic to the embryo and lead to congenital defects. Should this be the case and should the resulting teratisms be consistently reproducible and of a moderate nature, it would then be possible to carry out biochemical studies leading to the locus of the lesion. Malathion offered the added advantage of being relatively inexpensive, easy to obtain, available in the radioactive form, and information existed on its metabolism and breakdown products in mammals. For all these reasons, this study was begun.

MATERIALS AND METHODS

Injections

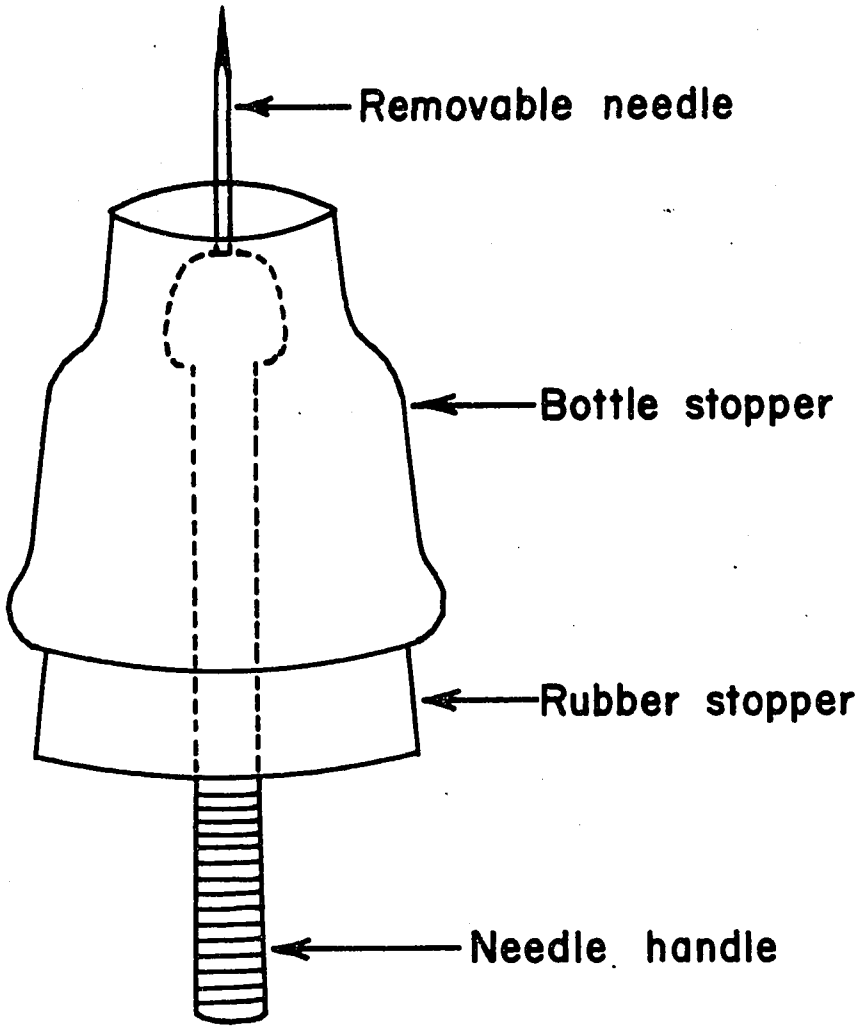
a. Method of injections

Fertile white leghorn eggs purchased from a commercial hatchery (Hy-Line Chicks, Chatham, Ontario) were used throughout this work. The eggs were set in a Jamesway single-stage incubator at dry and wet bulb readings of 38°C and 30°C respectively. Eggs with improperly calcified shells, displaced air cells, and blood clots were discarded. All injections were made through a hole drilled in the center of the blunt end of the shell with an egg punch (Fig. 4) which enabled us to treat large numbers of eggs in a short period of time. For all compounds used, 0.1 ml of a solution was injected into the yolk sac with a 20-gauge 1-inch needle.

When performing the injections, we were careful not to damage the vitelline membrane. If it is damaged, the yolk will spread out into the albumen. If the needle had yolk on it after it was removed from the egg, the egg was discarded.

Eggs were injected on days 1 to 3 of incubation (before the embryo is visible) placing the blunt ends horizontally facing the operator. Just prior to injection, the egg is shaken thereby ensuring that the embryo will come to lie at the top of the yolk thus precluding the possibility of damaging it upon injection. This is so because the blastoderm is less dense than the yolk.

Fig. 4c Diagram of egg punch


Removable needle
Bottle stopper
Rubber stopper
Needle handle

After day 3, the eggs are injected in a normal manner. The air cell is first outlined and the location of the embryo is marked on the shell. The needle is then inserted into the blunt end and is directed away from the embryo.

b. Compounds injected

1. Malathion

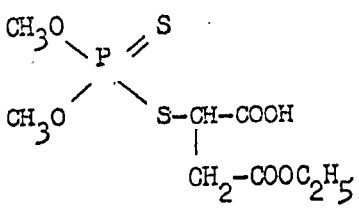
A solution of malathion in corn oil (0.1 ml) was injected into the yolk sac. Technical grade (95%) malathion (Cyanamid of Canada Ltd.) was used throughout. For each day from 0 to day 12 of incubation (Hamburger and Hamilton, 1951) each of 50 eggs was given a single injection. Because of the large number of eggs needed for each concentration, several runs were necessary to cover a range of concentrations from 1% to 100% including many intermediary values. Control series of uninoculated, distilled water inoculated, corn oil inoculated, and stab only inoculated eggs were run with each experimental series. In this way, the toxicity of the compound for each day of incubation could be determined.

A second series of experiments was conducted to determine and score the abnormalities obtained from malathion injected embryos.

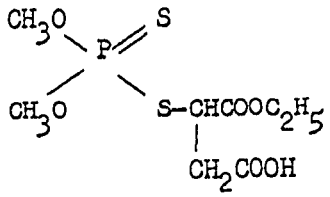
2. Malathion breakdown products and closely related compounds

Various malathion breakdown products (Cyanamid of Canada Ltd.) were injected into fertile eggs at doses of 5 and 10 mg. These compound and their structural formulae are listed as follows:

i. Malathion monocarboxylic acid



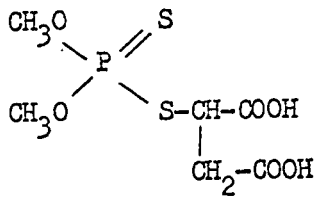
or



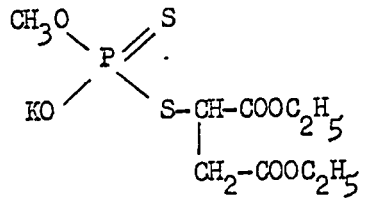
α-monoacid

β-monoacid

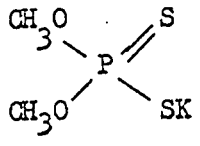
ii. Malathion dicarboxylic acid



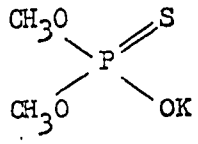
iii. O-Desmethyl malathion potassium sulfate



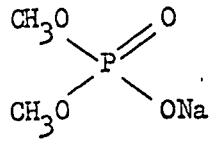
iv. Potassium dimethyl phosphorodithioate



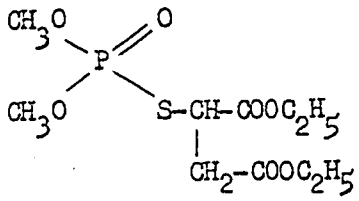
v. Potassium dimethyl phosphorothioate



vi. Sodium dimethyl phosphate

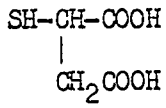


vii. Malaoxon

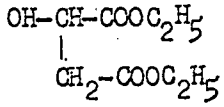


Five, 20 and 30 mg of each of the following carbon fragments of the malathion molecule were injected into fertile eggs:

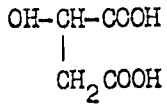
viii. Mercaptosuccinate (Nutritional Biochemicals-NBC)



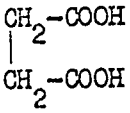
ix. Diethylmalate (NBC)



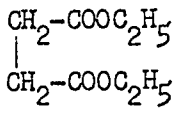
x. Malic acid (Eastman Kodak Chemicals)



xi. Succinic acid (Baker)



xii. Diethyl succinate (Baker)



3. Amino acids, vitamins, nicotinamide precursors and compounds related to tryptophan

A saline solution (0.1 ml) containing 0.025 mmole of one of the following compounds solubilized where required with a few drops of IN sodium hydroxide was injected together with the malathion in an attempt to counteract the effect of the insecticide on the embryo:

i. L-alanine (N. B. C.)

ii. L-glycine (Fisher)

iii. L-valine (Sigma)

iv. L-leucine (N. B. C.)

v. L-isoleucine (N. B. C.)

vi. L-serine (N. B. C.)

vii. L-cysteine (N. B. C.)

viii. L-methionine (N. B. C.)

ix. L-glutamic acid (British Drug House)

x. L-lysine (N. B. C.)

xi. L-arginine (N. B. C.)

xii. L-histidine (N. B. C.)

xiii. L-tryptophan (Baker)

xiv. L-phenylalanine (N. B. C.)

xv. L-tyrosine (N. B. C.)

xvi. L-proline (N. B. C.)

xvii. Thiamine (N. B. C.)

xviii. Vitamin A (N. B. C.)

xix. Riboflavin (N. B. C.)

xx. Indole (Eastman Kodak Chemicals)

xxi. Indoleacetic acid (Auxin) (Eastman Kodak Chemicals)

xxii. Serotonin Creatine Sulfate (N. B. C.)

xxiii. Adenine (N. B. C.)

xxiv. Hypoxanthine (N. B. C.)

xxv. Guanine (N. B. C.)

xxvi. Imidazole (Calbiochemicals)

xxvii. α-naphthol (Sigma)

xxviii. β-naphthol (Sigma)

xxix. Quinoline (British Drug House)

xxx. Anthranilic acid (British Drug House)

xxxi. Quinolinic acid (N. B. C.)

xxxii. Nicotinamide (5 mg/egg) (N. B. C.)

xxxiii. Nicotinic acid (5 mg/egg) (N. B. C.)

xxxiv. Atropine (5 mg/egg) (N. B. C.)

xxxv. Adenosine-5' Triphosphate Disodium Salt (ATP-5.0 and 2.5 mg/egg) (N. B. C.)

xxxvi. Adenosine-5' Diphosphate Trisodium Salt (ADP-5.0 and 2.5 mg/egg) (N. B. C.)

4. Insulin

Insulin was used as a teratogen (Landauer and Bliss, 1946; Landauer, 1947) to compare its effects on the embryo with those obtained with malathion. Various insulins were used including Toronto insulin (Connaught Laboratories), protamine zinc insulin (Connaught Laboratories), insulin crystals (Sigma), and Iletin^{R} (Eli Lilly Co.).

c. Duration of reversal effect of syndrome

Malathion (3.99 mg/egg) was injected into fertile eggs on day 4 of incubation. Five mg of either tryptophan, nicotinamide or quinolinic acid was injected into malathion treated eggs at 24 hour intervals from day 4 to day 12 of incubation. This was done to determine up to what day of incubation the alleviating compounds could still reverse the effect of malathion if injected at daily intervals into malathion treated eggs.

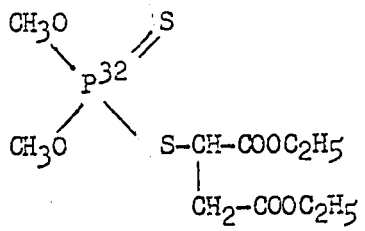
Skeletal Defects

Skeletal defects were determined by X-ray photographs and by alizarin red S staining (Gray, 1954). When staining with alizarin red S, the abdominal cavity was first opened and the embryo was placed in 95% ethanol for 3 to 5 days. The organs were removed and the animal was then placed in fresh ethanol for an additional week, and in acetone for 2 weeks. It was then immersed in ethanol for 2 days and in 1% potassium hydroxide for about 24 hours. The embryo was defeathered and re-immersed until it was cleared and was then dipped for 12 to 20 hours in 0.5% potassium hydroxide to which a few drops of a saturated aqueous solution of alizarin red S was added. It was then placed in increasing concentrations of glycerine (30, 60, 90%) each for 48 hours and stored in pure glycerine. Skeletal structures could now be observed.

Uptake of Malathion P^{32} in Embryo and yolk sac

The main biological effect of organophosphorus compounds is their inhibitory action of cholinesterase. We, therefore, chose malathion labelled in the P position for this experiment.

Malathion P^{32} obtained through Nuclear Chicago Corporation had the following structural formula:



The amount received weighed 0.1293 g and had 1 mc of activity.

a. Solutions

1. Stock solution

The shipping vial contained 1000 µc which was brought to a volume of 10 ml when mixed with corn oil.

2. 3.5% Malathion

Amount - 10 ml made up as follows:

1 ml stock solution (100 µc).

8.65 ml of oil.

0.35 ml of malathion.

Therefore, 0.1 ml of this solution contains 3.99 mg malathion and 1µc malathion P^{32}.

3. 5.5% Malathion

Amount - 10 ml made up as follows:

1 ml stock.

8.45 ml oil.

0.55 ml malathion.

Therefore, 0.1 ml of this solution contains 6.42 mg malathion and 1 µc malathion P^{32}.

4. 90% Malathion

Amount - 10 ml made up as follows:

1 ml stock solution.

9 ml malathion.

Therefore, 0.1 ml of this solution contains 105.12 mg malathion and 1 µc malathion P^{32}.

5. Control solution

Amount - 10 ml made up as follows:

1 ml stock.

9 ml oil.

Therefore, 0.1 ml contains 1 µc of P^{32} malathion and corn oil.

b. Schedule of injections

50 4-day incubated eggs were injected with 0.1 ml of solution 2.

50 4-day incubated eggs were injected with 0.1 ml of solution 4.

50 4-day incubated eggs were injected with 0.1 ml solution 5.

50 5-day incubated eggs were injected with 0.1 ml solution 3.

50 5-day incubated eggs were injected with 0.1 ml solution 4.

50 5-day incubated eggs were injected with 0.1 ml solution 5.

c. Procedure

1. The eggs were injected as per above schedule and 5 embryos of each group were removed 1, 2, 3, 4, 6 and 9 days after injection.

2. The embryos and yolk sacs were removed immediately and washed in saline solution and then dried.

3. The embryos or yolk sacs were then placed in glass homogenizing tubes and 1 ml of water was added to each.

4. The tissue was then ground for 5 minutes in a glass tissue grinder and the volume was noted.

5. A 0.2 ml aliquot of the homogenate was removed and placed on an aluminum planchet and spread thinly and evenly over the surface. The radioactivity was determined in a low background gas flow-counter (Nuclear Chicago Corporation).

6. An aliquot of yolk was also removed and counted on a planchet in a gas flow counter.

RNA, DNA, and Protein Synthesis

To determine if malathion had any effect on RNA, DNA or protein synthesis, uptake studies with labelled nucleotides and an amino acid were performed on malathion treated and untreated chick embryos in vivo.

Tritiated thymidine and uridine, and carbon-labelled valine were injected into the yolk sacs of fertile untreated and malathion treated chicken eggs. The uptake of the labelled compounds was to be followed at 2, 6, 12, and 24 hour intervals by liquid scintillation

methods on a scintillation counter (Nuclear Chicago Corporation), model number 8401, system 703, with a counting efficiency of 45%. The readings were made at a gate of 1500 and data of 1150 for tritiated compounds, and a gate of 1150 and data of 1050 for the carbon-14 labelled compound.

a. Solutions of radioactive compounds

1. L-valine-C^{14} (Amersham/Searle Ltd)

Specific activity 260 mc/mmole.

The shipping vial contained 50 µc in a volume of 0.8 ml or 62.5 µc/ml.

A 0.1 ml aliquot was diluted with 6.15 ml 0.9% saline for a concentration of 1 µc/ml.

In each egg, we injected 0.1 ml of a solution containing 0.1 µc.

2. Uridine-H^3 (Amersham/Searle Ltd)

Specific activity 4.25 c/mmole.

The shipping vial contained 250 µc in 0.5 ml or 500 µc/ml.

A 0.01 aliquot was diluted with 4.99 ml saline for a concentration of 1 µc/ml.

In each egg, we injected 0.1 ml of a solution containing 0.1 µc.

3. Thymidine-H^3 (Amersham/Searle Ltd)

Specific activity 11.60 c/mmole.

The shipping vial contained 250 µc in 0.5 ml or 500 µc/ml.

A 0.01 ml aliquot was diluted with 4.99 ml saline for a concentration of 1 µc/ml.

In each egg, we injected 0.01 ml of a solution containing 0.1 µc.

b. Scintillation solutions

1. Liquid scintillation mixture

1 part ethylene glycol monoethyl ether.

2 parts toluene.

1½ parts ethanol.

10 g PPO (2,5 diphenyloxazole)

0.2 g POPOP (1,4-bis-2(5-phenyloxazolyl) benzene)

2. Triton X-100 mixture

923 ml triton X-100

1077 ml toluene

4.3 g PPO

0.108 g POPOP

c. Schedule of injections and sacrifice of embryos

Embryos were injected with labelled uridine, thymidine and valine, with and without malathion on day 5 of incubation. The embryos were removed at 2 hour intervals up to 12 hours and then 24, 30, 36, 48, 72, and 96 hours after injection.

d. Procedure

1. Embryos were homogenized with a small amount of buffer in a glass mortar pestle type homogenizer for 5 minutes.

2. TCA (trichloroacetic acid) was added to the homogenate to a final concentration of 10%.

3. The solution was centrifuged for 5 minutes at 1800-3000 rpm and the supernatant discarded.

4. The precipitate was washed with 10% cold TCA containing 5% non-radioactive nucleotide or amino acid. It was washed thrice with acetone and thrice with ether, and then air dried for 2 hours.

5. A 5 mg sample of the dried TCA precipitate was solubilized in 3 ml hydroxide of hyamine and placed in a 55°C oven for 24 hours; or the dried TCA precipitate was solubilized with 2 ml of IN NaOH.

6. 15 ml of the liquid scintillation mixture was added to the hyamine solution; or the triton X-100 mixture was added to the NaOH along with water for a ratio of 13/10 producing a gel when cooled to 4°C. The radioactivity was determined on a liquid scintillation counter.

e. Control experiment

1. A control experiment was run using non-radioactive thymidine, uridine and valine to determine their effect on chick embryos when injected into the yolk sac of fertile chicken eggs. The eggs were candled each day after injection. The dead embryos were discarded and the viable ones removed on day 15 and examined for morphological defects.

2. To determine the scintillation system giving maximum results, we injected eggs on day 1 of incubation with radioactive compounds and removed the embryos on day 7. Each of the 2 scintillation systems was used and the results compared.

Tryptophan Assay

Total tryptophan content of 6 to 8 day old chick embryos injected with malathion on day 4 and 5 was determined by the method of Saifer and Gerstenfeld (1964).

1. The embryos were removed from the shell, freed of membranes and rinsed in 0.9% saline and patted dry with a paper towel.

2. The embryos were homogenized with 3.5% perchloric acid in a glass tissue grinder for 3 to 5 minutes.

3. The homogenates were hydrolyzed in hydrolysis tubes previously flushed with nitrogen, heat sealed and placed in an autoclave at 15 pounds pressure for a period of 24 hours, which kept the indole ring intact.

4. A photometric glyoxylic acid reaction for the indole nucleus of tryptophan produced a yellow colour which was read at 365 mμ in a Gilford Model 2400 Spectrophotometer.

5. Tryptophan values were determined from a standard curve prepared with L-tryptophan.

Cholinesterase Determination

Four day old chick embryos treated with malathion were removed 24, 48, 72 and 96 hours after injection and treated in the following way:

1. The embryos were removed from their shells, freed of membranes, and rinsed in saline and patted dry with a paper towel.

2. The sample was homogenized at 4°C for 5 minutes in a glass tissue grinder and centrifuged at 3000 rpm at 4°C for 15 minutes.

3. The acetylcholinesterase was determined on an aliquot of the supernatant with a cholinesterase kit (Boehringer Mannheim Corporation, N.Y.). The test is based on a method by Ellman et al., (1961) wherby cholinesterase in the homogenate splits acetylthiocholine into acetate and thiocholine; thiocholine combines with dithiobisnitrobenzoic acid to form a coloured product, thionitrobenzoic acid which is read at 405 mμ.

Lactate Dehydrogenase Assay

Incubated white leghorn eggs were injected with 3.50 mg malathion in corn oil on day 3, with 3.99 on day 4, with 6.42 mg on day 5 and with 8.12 mg on day 6. Ten embryos per group were removed for analysis 24 hours after injection and treated in the following way:

1. The embryos were extracted and homogenized in the same manner as for the cholinesterase determination.

2. Lactate dehydrogenase was determined on an aliquot of the supernatant with an LDH kit (Boehringer Mannheim Corporation, N. Y.). The test is based on the fact that LDH converts pyruvate and NADH into lactate and NAD. Enzyme activity is measured by the rate of decrease in absorbency of NADH.

Malate Dehydrogenase Assay

Two experimental runs were made. In the first run, the schedule of injections was the same as for lactate dehydrogenase. In the second run, the eggs were injected with 3.99 mg malathion on day 4 and 10 embryos of each group were extracted for analysis at 24 hour intervals.

1. The embryos were extracted and homogenized in the same manner as for the cholinesterase determination.

2. Malate dehydrogenase was determined on an aliquot of the supernatant with an MDH kit (Boehringer Mannheim Corporation, N. Y.). The test is based on the fact that MDH converts oxaloacetate and NADH into L-malate and NAD. Enzyme activity is measured by the rate of decrease in absorbancy of NADH.

Alkaline Phosphatase Assay

Incubated white leghorn eggs were injected according to the same schedule as for lactate dehydrogenase.

1. The embryos were homogenized in the same manner as for the cholinesterase determination.

2. Alkaline phosphatase was determined on an aliquot of the supernatant with an enzyme kit (Boehringer Mannheim Corporation, N. Y.). The test is based on the fact that phosphatase splits p-nitrophenyl-phosphate into organic phosphate and p-nitrophenol which is yellow in alkaline solution. Enzyme activity is measured by the intensity of the colour formed.

Histochemical Methods

Cholinesterase and some dehydrogenases were determined on treated embryos by histochemical methods. For all determinations, the embryos were first injected with 3.99 mg/egg malathion on day 5 and removed for analysis at 24 hour intervals up to day 8.

The embryos were extracted from the shell, freed of membranes, rinsed in saline, and patted dry.

1. The embryos were then either fixed in formalin containing 1% calcium chloride or unfixed.

2. They were then placed in cold gum sucrose (made by dissolving

1% gum arabic in 0.88M sucrose) until they sank. In this way, the tissue water was replaced by gum sucrose which enabled us to cut cryostat sections of good morphology (Pearse, LaHam, and Jannigan, 1963).

3. The embryos were then placed in scotch tape boats 2 cm x 1 cm x 1 cm made by placing scotch tape over a stove bolt head which was pre-moistened with glycerine.

4. The boat was filled with Tissue-tek^{R} OCT compound (Ames Co., Elkhart, Indiana) and then quick frozen in liquid nitrogen and stored at -80°C.

5. The blocks were then placed on a cork disc and then mounted on a microtome chuck with a few drops of water which sealed the cork to the cold (-25°C) chuck.

6. Frozen sections were cut and mounted on coverslips and the appropriate histochemical test was performed.

a. Dehydrogenases

Lactate, malate, succinate, DPN, TPN, glucose-6-phosphate and isocitrate dehydrogenase were determined on frozen sections (Pearse, 1960; Culling, 1963).

1. Solutions

MTT (3-4,5-dimethylthiazolyl-2) 5-diphenyl tetrazolium bromide) stock solution.

MTT (1 mg/ml).	2.5 ml
$CoCl_2$ (0.5 ml).	0.5 ml
Tris Buffer (0.2M).	2.5 ml

Distilled water. 3.5 ml

2. Substrates

i. 1.0M sodium DL-lactate.

ii. 1.0M sodium L-malate.

iii. 1.0M sodium succinate.

iv. 1.0M glucose-6-phosphate disodium salt.

v. 1.0M DL-isocitric acid.

vi. 6 mg DPNH.

vii. 6 mg TPNH.

3. Method

i. Add 0.9 ml MTT stock solution to 0.1 ml substrate (except NADH and NADPH).

ii. To this mixture, add a pinch of cofactor (NAD for lactate, malate, and isocitrate, none for succinate and NADP for glucose-6-phosphate).

iii. For NADH or NADPH, add 6 mg to 1.0 ml of stock solution.

iv. Adjust PH to 7 with tris buffer.

v. Add a few drops of the incubation mixture to the frozen section.

vi. Incubate at 37°C for 30 to 45 minutes.

vii. Postfix in 10% formalin containing 1% $CaCl_2$ for 5 minutes.

viii. Wash gently in distilled water.

ix. Mount in glycerogel.

The method is based on the fact that the tetrazolium salt accepts the electrons and hydrogens from the substrate liberated by the enzyme in question. The salt is reduced to formazan, an insoluble microcrystalline product which is deposited at the site of the enzyme.

b. Acetylcholinesterase

The method (Karnovsky and Roots, 1964) is based on the fact that acetylcholinesterase hydrolyses acetylthiocholine iodide liberating thicholine. The thicholine is believed to reduce ferricyanide to ferrocyanide preferentially. The latter combines with copper (Cu^{++}) ions to form the insoluble copper ferrocyanide. The Cu^{++} ions in the medium are complexed with citrate to prevent formation of copper ferricyanide.

1. Method

i. To 5 mg acetylthiocholine iodide dissolved in 6.5 ml 0.1M sodium hydrogen maleate buffer Ph 6.0 add the following in order:

0.5 ml 0.1M sodium citrate.

1.0 ml 30mM copper sulfate.

1 ml water.

1 ml 5mM potassium ferricyanide.

The final incubation mixture is green in colour and is stable for hours.

ii. Add a few drops of this medium to each

section and incubate at 37°C for 30 to 45 minutes. Reddish granules indicate the site of the enzyme.

APPENDIX I - METHODS AND MATERIALS

Autoradiography

To determine the distribution of malathion at a tissue level, radioautography was done using malathion P^{32}. Fertilized chicken eggs were each injected on day 4 and 5 with 1 µc malathion P^{32} containing 3.99 mg per egg of malathion on day 4 of incubation and 6.42 mg per egg of malathion on day 5 of incubation. Histological paraffin sections were made and mounted on pretreated glass slides (Rogers, 1968).

a. Pretreatment of slides

The glass slides were pre-cleaned by soaking overnight in sulfuric acid - potassium bichromate glass cleaner. They were next washed in cold running water followed by 2 changes of distilled water. They were then subbed in a solution of 5.0 g gelatin, 0.5 g chrome alum (potassium aluminum sulfate) and water to 1000 ml. The subbing mixture was filtered immediately before use and was made fresh before each subbing. The slides were drained and placed vertically in slotted wood blocks and dried in a dust free cabinet. Paraffin sections were cut and placed on one end of the pre-cleaned slides to which a few drops of albumen was first placed. The slides were then brought to the dark room fitted with a wratten #2 safelight.

b. Application of emulsion

The slides were deparaffinized by dipping them into 2 changes of xylol. They were next dehydrated in 2 changes of absolute alcohol for 5 minutes each and dipped in a celloidin solution made by dissolving 0.3 g parlodion (Mallinckrodt) in a 1 to 1 mixture of ethanol and ether. They were pre-warmed at 40°C on a slide warmer, and then dipped in NTB-3 emulsion in a plexiglass container made to receive a minimum number of slides (to conserve the emulsion). The slides were kept in a vertical position until the emulsion was drained off. The back of the slide was wiped with a kleenex tissue to remove any excess emulsion and they were then allowed to dry for 2 hours in the dark. The coated slides were stored in plastic slide boxes each containing a vial of drierite stopped with absorbent cotton to remove any condensation that might have formed. The boxes were sealed with black electrical tape and stored in a refrigerator at 4°C. Test boxes each containing 5 to 10 slides were developed at 1 week intervals to determine the proper exposure time.

c. Development and staining

The slides were developed for 10 minutes in D-19 developer (Kodak) at 4°C, washed for 30 seconds in cold water, fixed for 15 minutes in hardened fixer kept at 4°C, and then washed for 1 hour in cold running water. They were next placed in cold absolute alcohol

and then in absolute alcohol at room temperature, each for 5 minutes. They were stained for 10 minutes in 0.05% basic fuchsin at 4°C and then rinsed in absolute ethanol for 30 seconds at 4°C. The sections were dehydrated by placing them in absolute ethanol for 5 minutes at 4°C and then dipping them in 2 changes of absolute ethanol at room temperature each for 5 minutes. The sections were mounted with xylene-diluted permount and viewed microscopically.

RESULTS

Toxicity of Malathion

The toxicity of malathion in chick embryos was determined by injecting various amounts of the insecticide into fertile eggs at daily intervals. Mortality data which relates to the age of the chick is listed in Table 1 and Fig. 5.

As shown in Fig. 5, concentrations above 25% malathion were lethal throughout the injection schedule. When less than 25% was injected into 4 to 6 day incubated eggs, the mortality was dose dependent. Injections of 10% or less on days 7 to 12 or, injections of 1% or less throughout the injection schedule were innocuous since the per cent mortality was similar to the corn oil controls. Younger embryos (up to day 6) were more or less sensitve to concentrations greater than 1% malathion; however, when concentrations equal to or greater than 50% were injected on days 4 to 12 of incubation, none of the embryos survived.

To determine the length of time the embryos remained viable after each dose level, the eggs were candled at 24 hour intervals. After each day of injection, the deaths were scored from the time of injection to near the end of incubation (Tables 2 to 10; Fig. 6 to 11).

As shown in Fig. 6, when 3.99 mg per egg was injected on days 4 and 5, death occurred either soon after the treatment or at the end of the incubation period. From Fig. 7, when 6.42 mg per egg was injected on days 4, 5, and 6, death occurred soon after the treatment.

Table 1. Percentage mortality among developing chick embryos after the injection of malathion in corn oil[a]

Concentration of malathion injected[b]	Age of embryos at injection (days)								
	4	5	6	7	8	9	10	11	12
100% (116.80 mg/egg)	100	100	100	100	100	100	100	100	100
50% (58.40 mg/egg)	100	100	100	100	100	100	100	100	100
25% (29.20 mg/egg)	100	100	100	100	100	100	90	50	50
20% (23.36 mg/egg)	100	100	100	100	85	80	50	30	30
17% (19.86 mg/egg)	100	100	100	90	50	40	30	30	30
15% (17.52 mg/egg)	100	100	100	85	35	30	30	30	30
12% (14.02 mg/egg)	100	100	80	50	30	30	20	10	10
10% (11.68 mg/egg)	100	100	80	10	10	10	10	10	10
7% (8.12 mg/egg)	90	75	50	10	10	10	10	10	10
5.5% (6.42 mg/egg)	90	50	45	10	10	10	10	10	10
5% (5.84 mg/egg)	80	60	40	10	10	10	10	10	10
3.5% (3.99 mg/egg)	50	40	20	10	10	10	10	10	10
1% (1.17 mg/egg)	30	20	10	10	10	10	10	10	10
Control (corn oil)	30	20	10	10	10	10	10	10	10

[a] Embryos candled each day up to hatching.

[b] 0.1 ml per egg injected throughout.

Fig. 5. Toxicity of malathion in chick embryos after injection into fertile eggs on days 4 to 12 of incubation

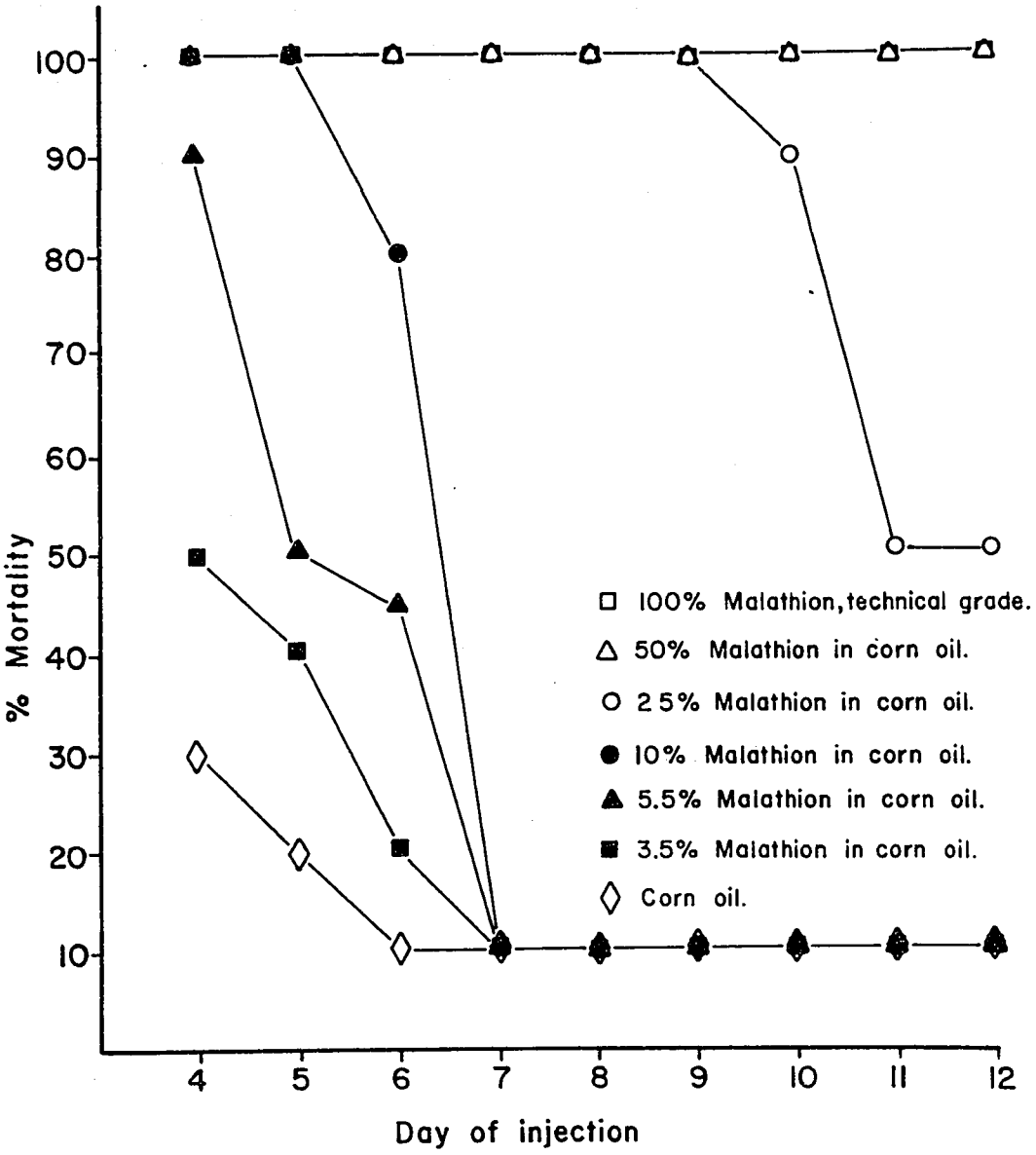

100
90
80
70
60
50
40
30
20
10
% Mortality
4
5
6
7
8
9
10
11
12
Day of injection
100% Malathion, technical grade.
50% Malathion in corn oil.
25% Malathion in corn oil.
10% Malathion in corn oil.
5.5% Malathion in corn oil.
3.5% Malathion in corn oil.
Corn oil.

Table 2. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 0.1 ml distilled water per egg[a]

Day of Incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	4								
5	0	8							
6	0	0	6						
7	4	2	0	0					
8	4	2	0	4	4				
9	0	6	0	4	0	0			
10	0	0	0	0	0	2	4		
11	0	0	0	0	0	0	0	0	
12	0	0	0	0	0	0	2	2	2
13	0	0	0	0	0	0	0	4	0
14	0	0	0	0	0	0	0	0	2
15	0	0	0	0	0	2	0	0	2
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	2	0	4	0
19	18	2	4	2	6	4	4	0	4

[a] Average values of 3 experiments after injecting 50 eggs each.

Table 3. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 0.1 ml corn oil per egg[a]

Day of Incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	14								
5	0	8							
6	0	0	8						
7	4	0	0	0					
8	2	0	0	2	8				
9	0	0	0	2	0	0			
10	0	0	0	0	0	0	2		
11	0	0	0	0	0	4	0	0	
12	0	4	0	0	0	0	0	2	2
13	0	0	0	0	0	0	4	0	4
14	2	0	0	0	0	0	0	4	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	2	0	0
17	0	0	0	0	0	2	0	2	0
18	2	0	0	0	0	0	2	0	0
19	6	8	2	6	2	4	0	2	4

[a] Average values of 3 experiments after injecting 50 eggs each.

Table 4. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 1.17 mg malathion per egg[ab]

Day of Incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	8								
5	0	8							
6	0	0	4						
7	2	0	0	0					
8	2	0	2	6	0				
9	0	0	2	2	2	4			
10	6	0	0	0	2	0	0		
11	0	0	0	0	0	2	4	0	
12	0	0	0	0	2	0	0	0	2
13	0	0	0	0	0	0	0	2	0
14	0	2	0	0	0	0	0	4	2
15	8	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	2
18	0	4	0	0	0	0	2	2	2
19	4	0	6	2	4	4	4	2	2

[a] Average values of 3 experiments after injecting 50 eggs each.

[b] 0.1 ml malathion in corn oil per egg.

Table 5. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 3.99 mg malathion per egg[ab]

Day of Incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	20								
5	0	10							
6	0	12	0						
7	0	0	8	2					
8	0	0	0	2	4				
9	0	0	0	0	0	6			
10	0	0	0	0	0	0	4		
11	0	0	0	0	0	0	2	4	
12	0	0	0	0	0	0	0	0	6
13	2	0	4	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	2	0	0	2	0
17	4	0	0	2	2	2	0	2	0
18	4	4	0	0	0	0	0	0	0
19	20	14	8	4	2	2	4	2	4

[a] Average values of 3 experiments after injecting 50 eggs each.

[b] 0.1 ml malathion in corn oil per egg.

Table 6. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 6.42 mg malathion per egg[ab]

Day of Incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	62								
5	24	38							
6	2	8	28						
7	2	0	10	4					
8	0	0	0	0	2				
9	0	0	0	0	4	6			
10	0	0	0	0	0	2	4		
11	0	0	0	0	0	0	0	2	
12	0	0	0	0	0	0	2	0	4
13	0	0	0	0	0	0	0	0	2
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	2
16	0	0	0	0	0	0	0	0	0
17	0	0	0	2	2	0	0	2	0
18	0	0	3	0	0	0	0	0	0
19	0	4	4	4	2	2	4	6	2

[a] Average values of 3 experiments after injecting 50 eggs in each

[b] 0.1 ml malathion in corn oil per egg.

Table 7. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 11.68 mg malathion per egg[ab]

Day of Incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	0								
5	100	0							
6	0	100	0						
7	0	0	76	8					
8	0	0	2	0	6				
9	0	0	0	0	0	0			
10	0	0	0	0	2	4	2		
11	0	0	0	0	0	2	0	0	
12	0	0	0	0	0	0	4	6	0
13	0	0	0	0	0	0	0	2	6
14	0	0	0	0	2	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	2	2	0	4	4	2	4

[a] Average values of 3 experiments after injecting 50 eggs each

[b] 0.1 ml malathion in corn oil per egg.

Table 8. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 29.2 mg malathion per egg[ab]

Day of Incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	0								
5	94	0							
6	6	82	0						
7	0	18	86	0					
8	0	0	14	86	94				
9	0	0	0	14	0	48			
10	0	0	0	0	0	34	22		
11	0	0	0	0	6	0	4	26	
12	0	0	0	0	0	0	4	8	32
13	0	0	0	0	0	0	0	0	10
14	0	0	0	0	0	0	44	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	10	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	8	16	16	8

[a] Average values of 3 experiments after injecting 50 eggs each

[b] 0.1 ml malathion in corn oil per egg.

Table 9. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 58.4 mg malathion per egg[ab]

Day of incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	16								
5	4	12							
6	4	10	26						
7	4	0	0	4					
8	24	2	2	10	62				
9	14	0	0	0	6	30			
10	6	2	2	18	6	0	52		
11	8	6	0	4	0	18	26	0	
12	10	0	6	6	6	2	2	98	0
13	0	4	0	6	0	6	16	2	92
14	0	2	0	0	0	4	2	0	6
15	2	20	10	16	8	0	0	0	2
16	0	0	0	6	0	6	0	0	0
17	0	10	2	0	2	12	0	0	0
18	2	8	0	4	0	0	0	0	0
19	6	24	52	26	10	22	2	0	0

[a] Average values of 3 experiments after injecting 50 eggs each

[b] 0.1 ml malathion in corn oil per egg.

Table 10. Percentage mortality among chick embryos examined up to day 19 of incubation after an injection of 116.8 mg malathion per egg[ab]

Day of Incubation	Age of Embryos at Injection (days)								
	4	5	6	7	8	9	10	11	12
4	15								
5	2	11							
6	9	4	4						
7	0	2	2	12					
8	5	0	2	5	47				
9	6	0	0	2	0	36			
10	2	2	2	2	7	0	28		
11	6	6	0	2	0	0	6	4	
12	5	2	4	4	6	8	0	46	6
13	0	2	2	5	0	2	26	10	84
14	0	2	2	5	13	8	18	26	6
15	4	2	4	2	0	0	6	0	0
16	4	0	0	10	0	0	6	2	0
17	2	6	10	4	10	10	4	0	0
18	4	9	12	4	0	2	0	2	4
19	36	52	56	43	17	34	6	10	0

[a] Average values of 3 experiments after injecting 50 eggs each.

[b] 0.1 ml malathion per egg.

Fig. 6. Toxicity of 0.1 ml of a solution of malathion in corn oil containing 3.99 mg malathion per egg injected into the yolk sac on various days of incubation. The eggs were candled each day and the per cent survivors was recorded up to hatching.

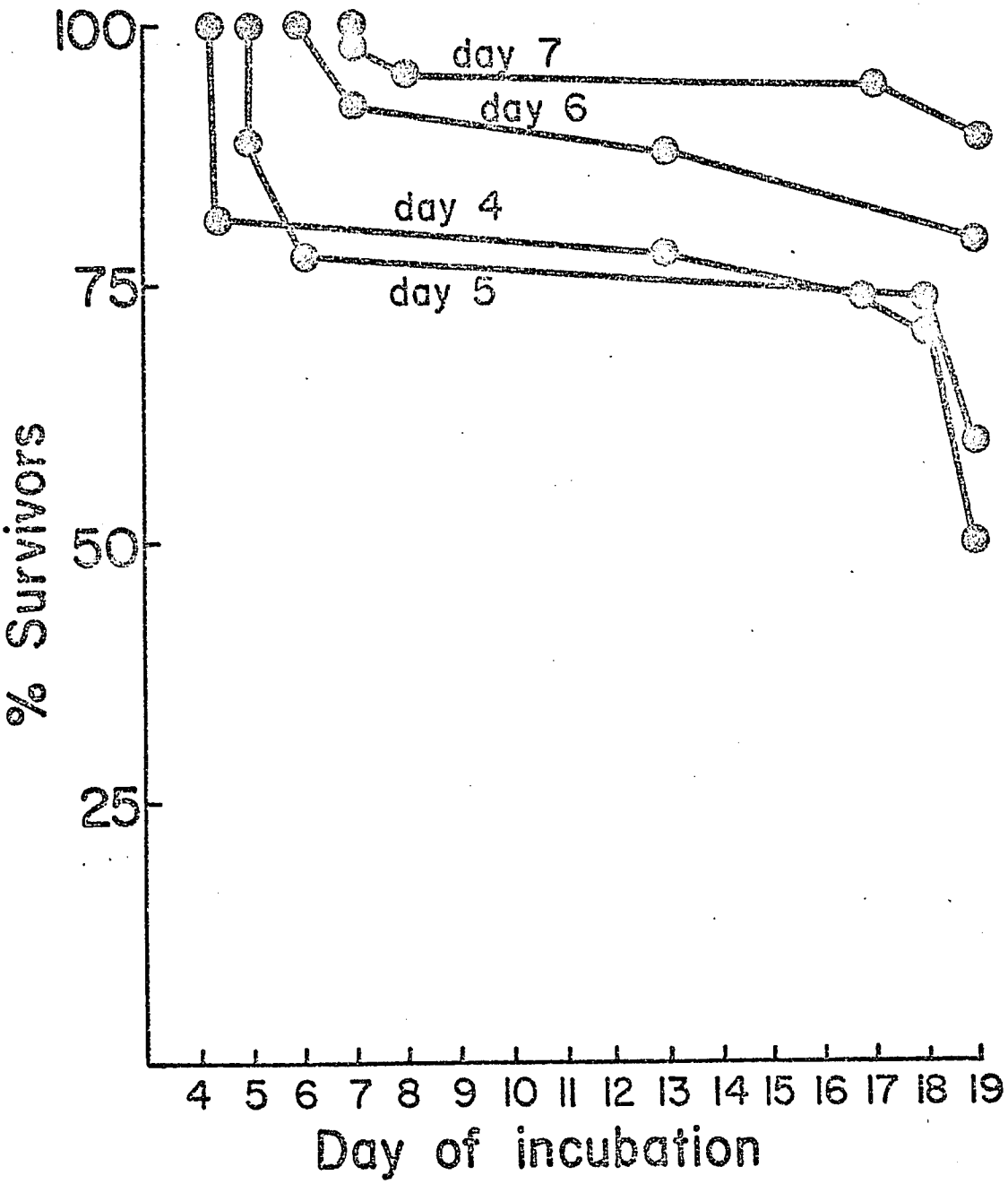

100
75
50
25
% Survivors
day 7
day 6
day 4
day 5
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Day of incubation

Fig. 7. Toxicity of 0.1 ml of a solution of malathion in corn oil containing 6.42 mg malathion per egg injected into the yolk sac on various days of incubation. The eggs were candled each day and the per cent survivors was recorded up to hatching.

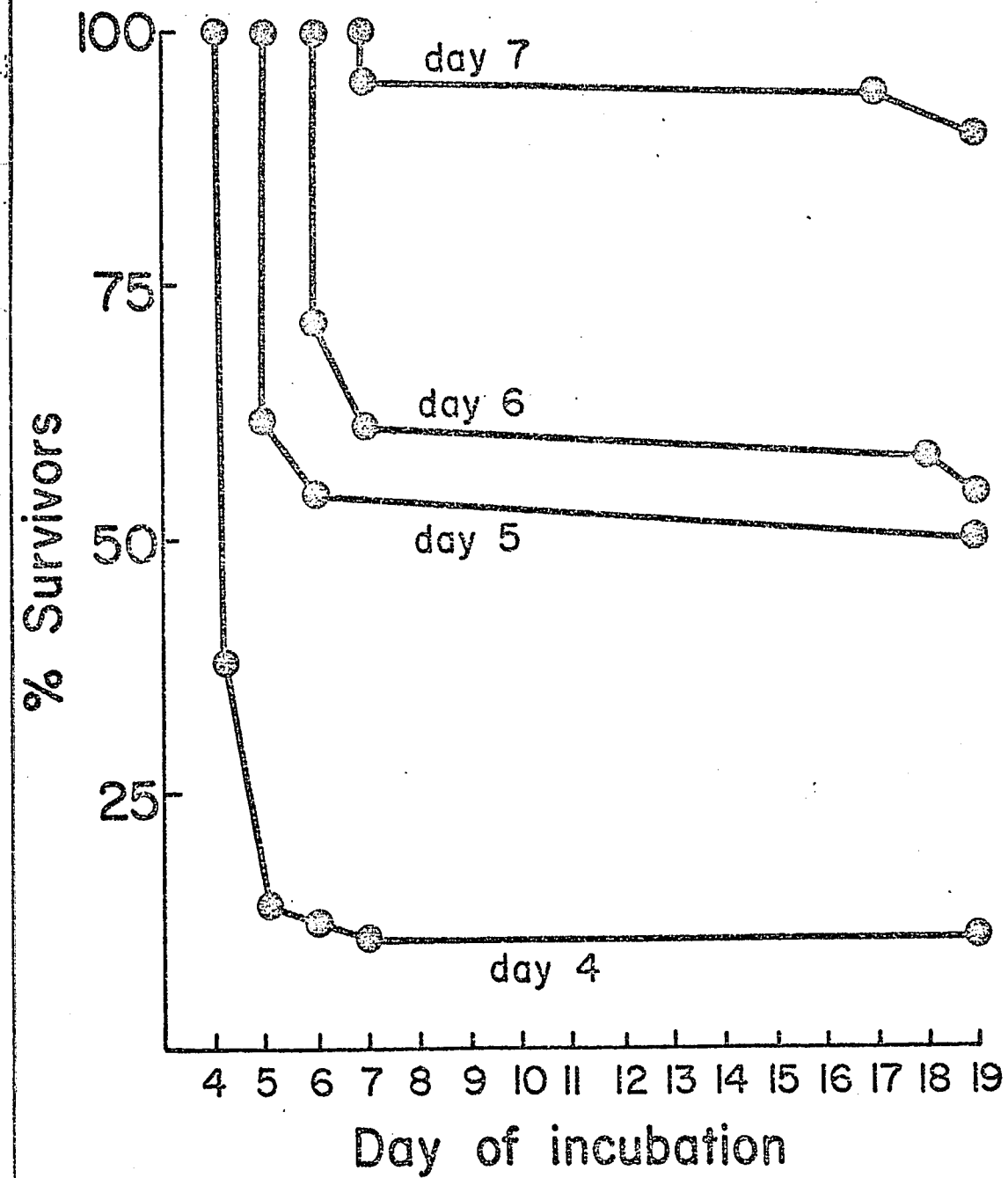

100
75
50
25
% Survivors
day 7
day 6
day 5
day 4
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Day of incubation

Fig. 8. Toxicity of 0.1 ml of a solution of malathion in corn oil containing 11.68 mg malathion per egg injected into the yolk sac on various days of incubation. The eggs were candled each day and the per cent survivors was recorded up to hatching.

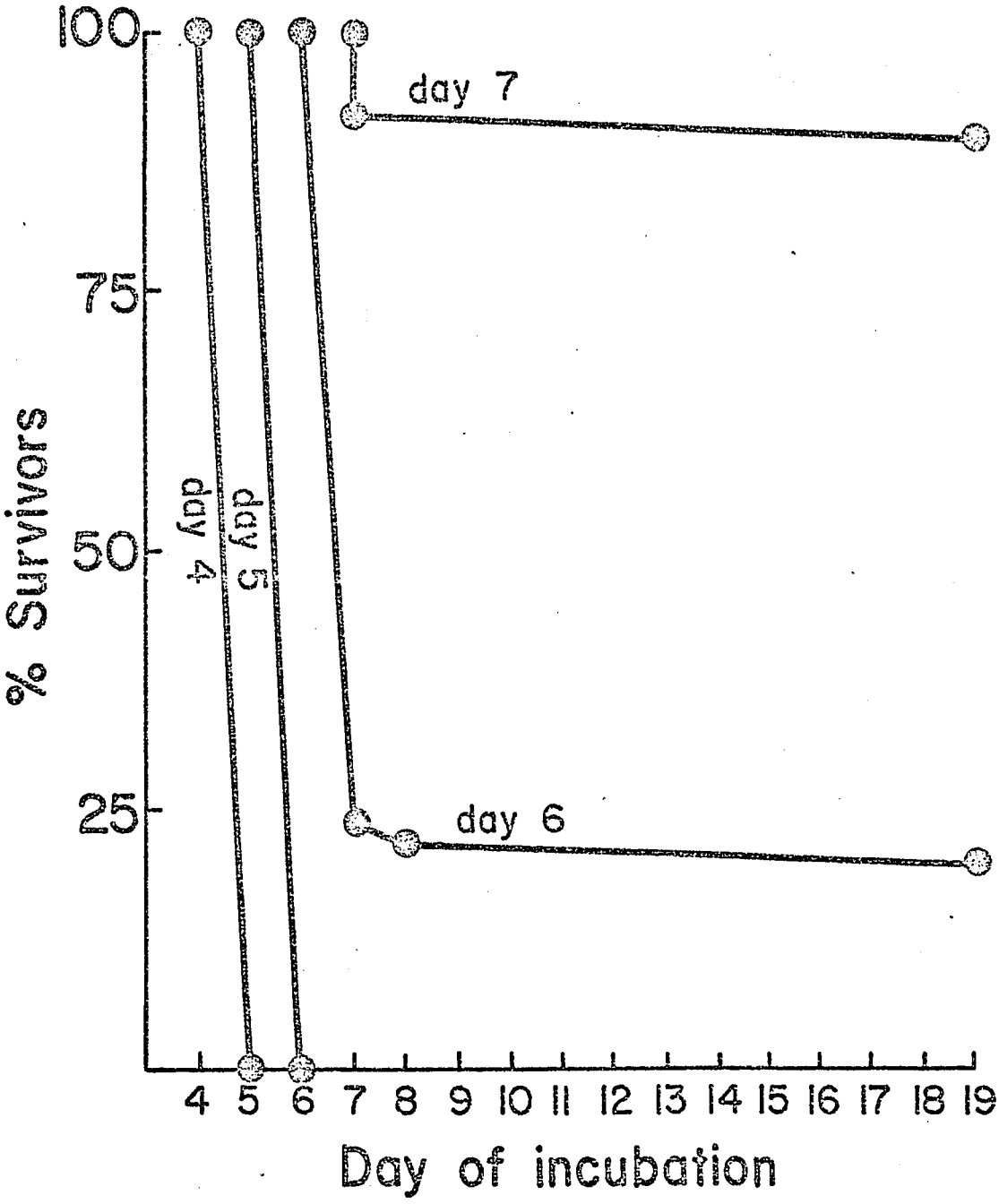

100
75
50
25
% Survivors
day 4
day 5
day 7
day 6
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Day of incubation

Fig. 9. Toxicity of 0.1 ml of a solution of malathion in corn oil containing 29.2 mg malathion per egg injected into the yolk sac on various days of incubation. The eggs were candled each day and the per cent survivors was recorded up to hatching.

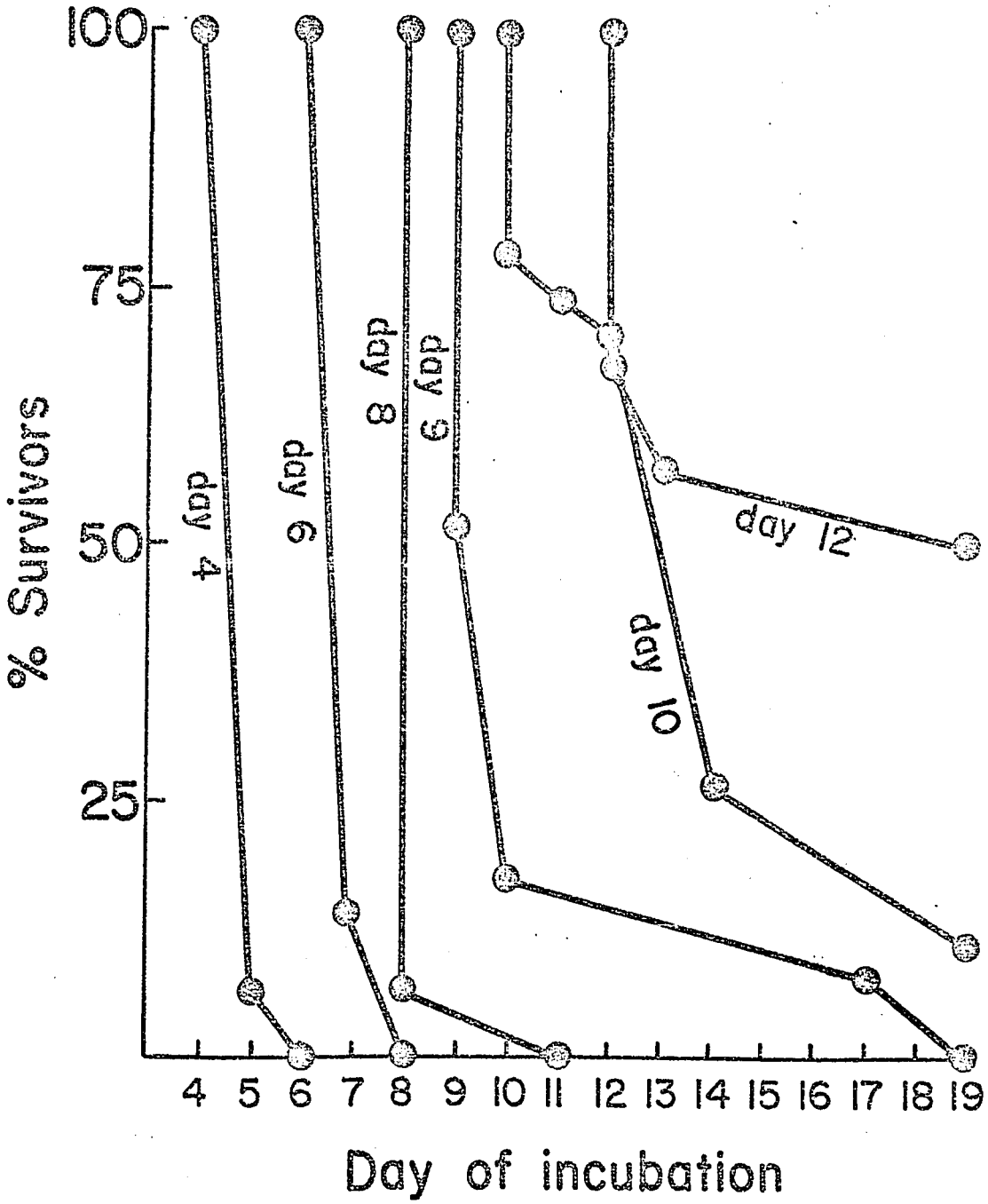

100
75
50
25
% Survivors
day 4
day 6
day 8
day 9
day 10
day 12
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Day of incubation

Fig. 10. Toxicity of 0.1 ml of a solution of malathion in corn oil containing 58.4 mg malathion per egg injected into the yolk sac on various days of incubation. The eggs were candled each day and the per cent survivors was recorded up to hatching.

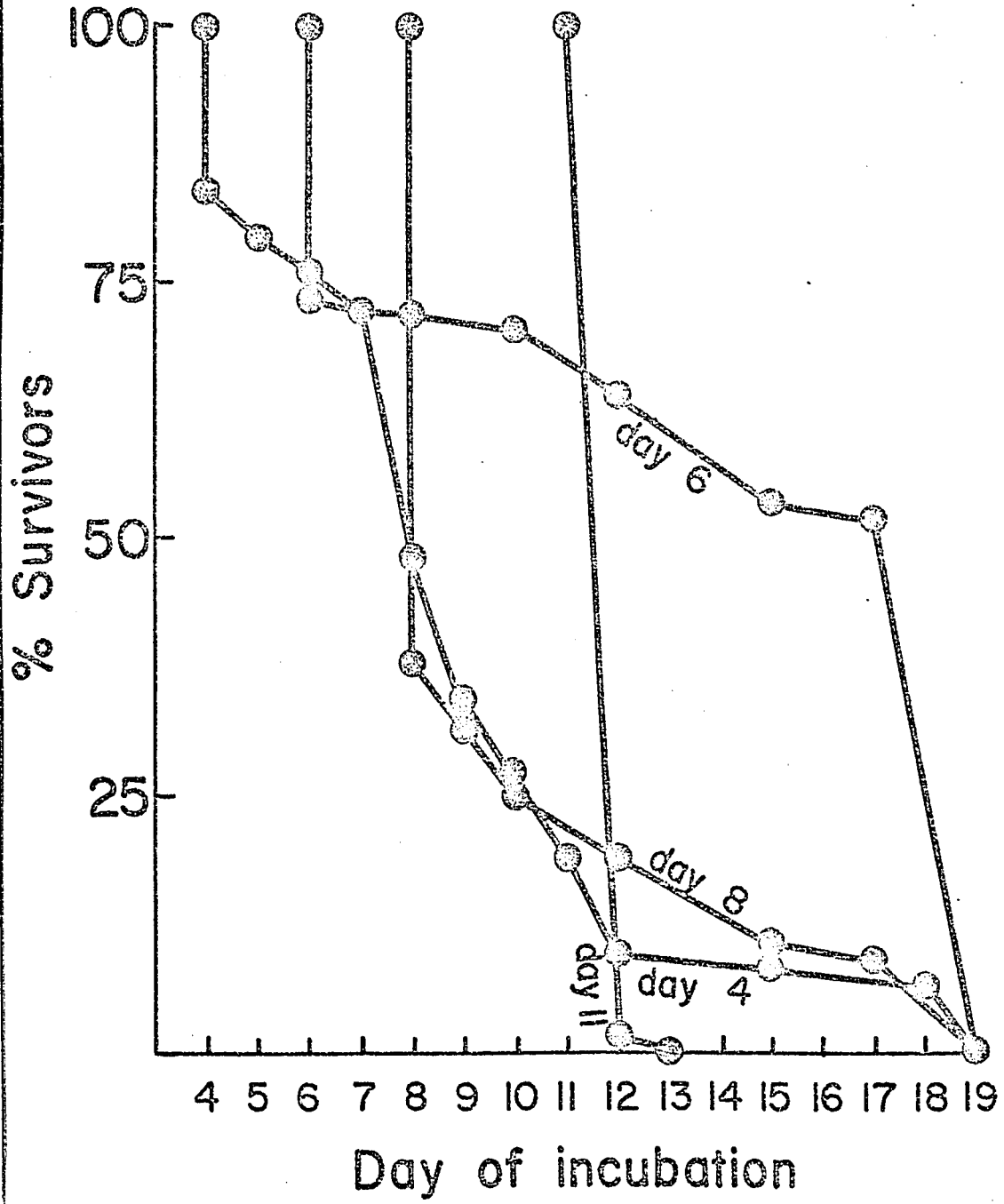
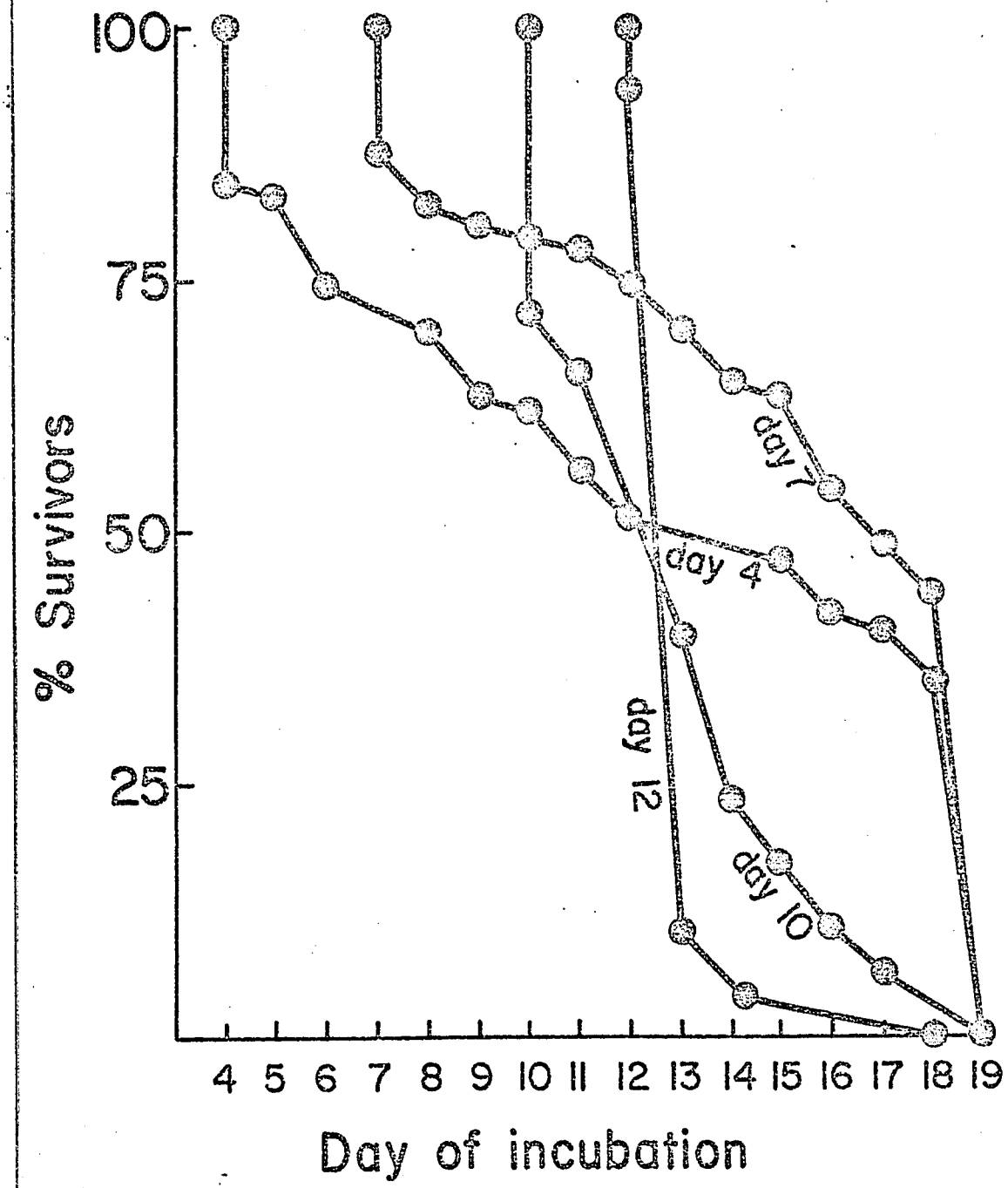

100
75
50
25
% Survivors
day 6
day 8
day 4
day 11
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Day of incubation

Fig. 11. Toxicity of 0.1 ml of a solution containing 116.8 mg malathion per egg injected into the yolk sac on various days of incubation. The eggs were candled each day and the per cent survivors was recorded up to hatching.


100
75
50
25
% Survivors
day 7
day 4
day 12
day 10
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Day of incubation

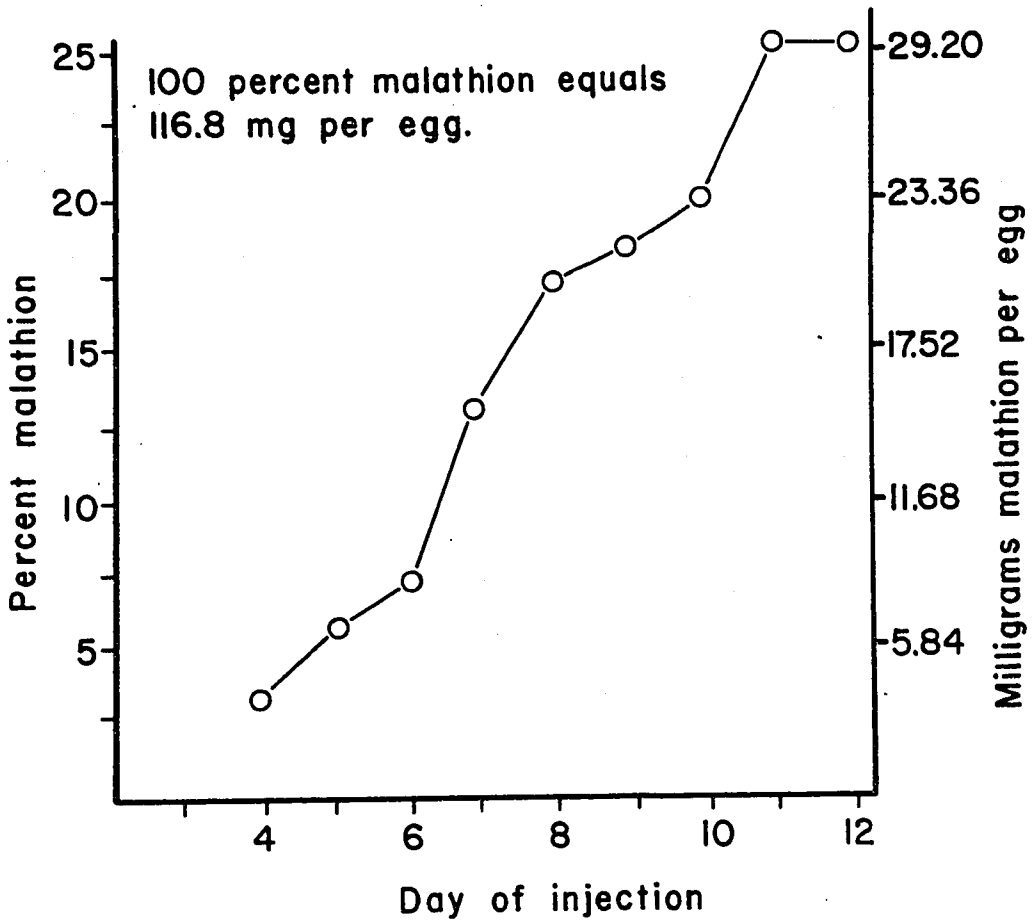
In general, 100% and 50% malathion injections on days 1 to 7 proved lethal in the latter stages of incubation, and injections on days 8 to 12 proved lethal within the first few days of the treatments. After injections of 25% malathion on days 1 to 9, most of the deaths occurred within the first 3 days of the treatments. Mortality was spread out to day 19 after injections on days 9 to 12. This same trend continued for the 10% injections. After injections of 5.5% and 3.5% malathion, mortality decreased and was spread out over the 19 day period except for the 5.5% injections on day 4 which yielded a high mortality within 2 days after treatments.

Abnormalities

The concentrations which killed 50% of the embryos during the incubation period (uncorrected LD_{50}), as shown in Fig. 12, yielded the maximum number of malformed chicks. One-half of the survivors of the 8 to 12 day injections were either featherless or had sparse feathers in the abdominal region. Ninety-five per cent of the survivors of those injected on days 6 and 7 were smaller in size than the controls, while 50% had fewer feathers throughout.

Ninety-eight per cent of the survivors of the 4 and 5 day injections displayed a combination of plumage, hind limb, beak and size defects. This congenital deformity will henceforth be referred to as the "malathion syndrome". The external characteristics of 15-day-old embryos (injected

Fig. 12. Concentrations of malathion which killed 50% of the embryos after injections on days 4 to 12 of incubation (LD_{50}-uncorrected)


100 percent malathion equals
116.8 mg per egg.
Percent malathion
25
20
15
10
5
29.20
23.36
17.52
11.68
5.84
Milligrams malathion per egg
4
6
8
10
12
Day of injection

with 3.99 and 6.42 mg of malathion per egg on day 4 and 5 respectively) are compared in Table 11. Photographs of 2-day-old (post-hatch) chicks (Figs. 13 to 19) illustrate some of the abnormalities of the legs, beak and overall size.

a. Legs

In all the affected chicks, the hind limbs were reduced to about one-half of the normal size and the phalanges were permanently flexed (curled toe paralysis). In 10%, the joint between the tarsometatarsus bone projected dorsally. One chick in 50 bilaterally lacked the tarsometatarsus bone and phalanges.

b. Beak

The length of the mandible was reduced. In 50%, the distal end of the maxilla was curved downwards over the mandible (parrot beak).

c. Plumage

Twenty-five percent of the chicks lacked feathers, especially in the abdominal region. Four percent were featherless. All hatched chicks had "clubbed down" (the down is coarse and hair-like).

d. Size

The overall size was two-thirds normal (proportionately distributed with the exception of the legs and beak). Six percent were dwarfs (about one-quarter of the normal size).

Table 11. The effect of malathion on the length of the hind limbs, body and mandible in chick embryos treated on incubation days 4 and 5, and examined on day 15

Treatment		Control (100% Corn Oil)	Malathion - 3.99 mg per egg on day 4	Malathion - 6.42 mg per egg on day 5
No. of eggs per group		25	25	25
Weight (g)		11.51 ± 0.14[a]	9.64 ± 0.19	8.84 ± 0.18
	Range	10.00-12.90	7.00-11.60	6.40-10.20
Body Length[b] (mm)		67.30 ± 0.37	62.00 ± 0.37	60.20 ± 0.45
	Range	64.00-71.00	51.00-67.00	56.00-65.00
Leg Length[c] (mm)		43.80 ± 0.25	28.00 ± 0.73	25.30 ± 1.06
	Range	41.00-45.00	20.00-34.00	9.00-35.00
Mandible[d] Length (mm)		10.90 ± 0.07	8.20 ± 0.18	8.00 ± 0.19
	Range	10.00-11.00	6.00-10.00	5.00-9.00

[a] Standard error.

[b] Crown to rump length.

[c] From acetabulum to phalanges.

[d] From gape to distal end of bone.

Fig. 13. Photograph of a group of 2 day old chicks from eggs injected on day 4 of incubation with 3.99 mg malathion per egg. The malformed chicks (in the foreground) are unable to walk because of limb deformities. Two control chicks from uninjected eggs are in the background

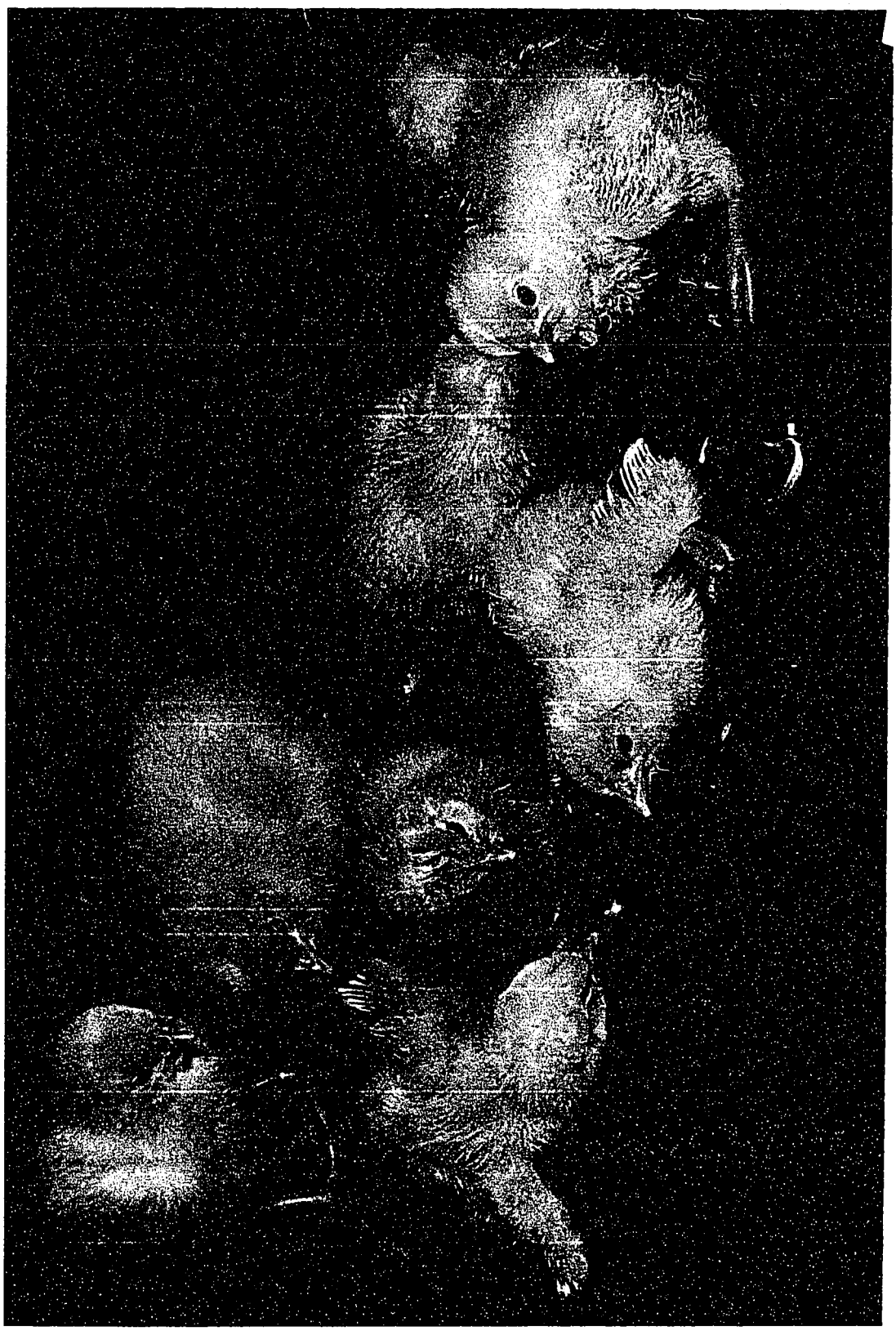
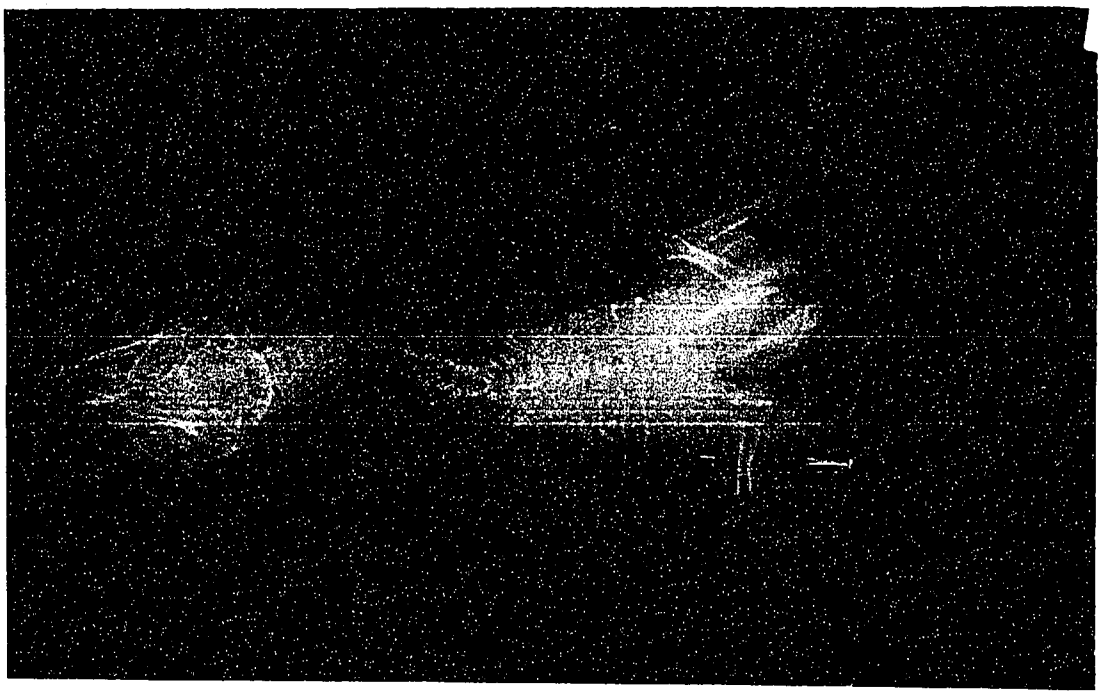


Fig. 14. Photograph of two day old post-hatched chicks. The left chick is from an uninjected egg and the right chick is from an egg previously injected with 3.99 mg malathion on day 4 of incubation. Note the malformed hind limbs and the down-curved maxilla. The chick is also unable to open its eyes

Fig. 15. Photograph of 2 day old chicks. The chick on the left is from an uninjected egg and the chick on the right is from an egg previously injected with 3.99 mg malathion on day 4 of incubation. Note the lack of digits on the right leg and the reduced overall size.

Fig. 16. X-ray photographs of 2 day old chicks. The left chick illustrates normal skeletal development. The right chick, previously treated with malathion on day 4 of incubation, illustrates micromelia and overall growth reduction



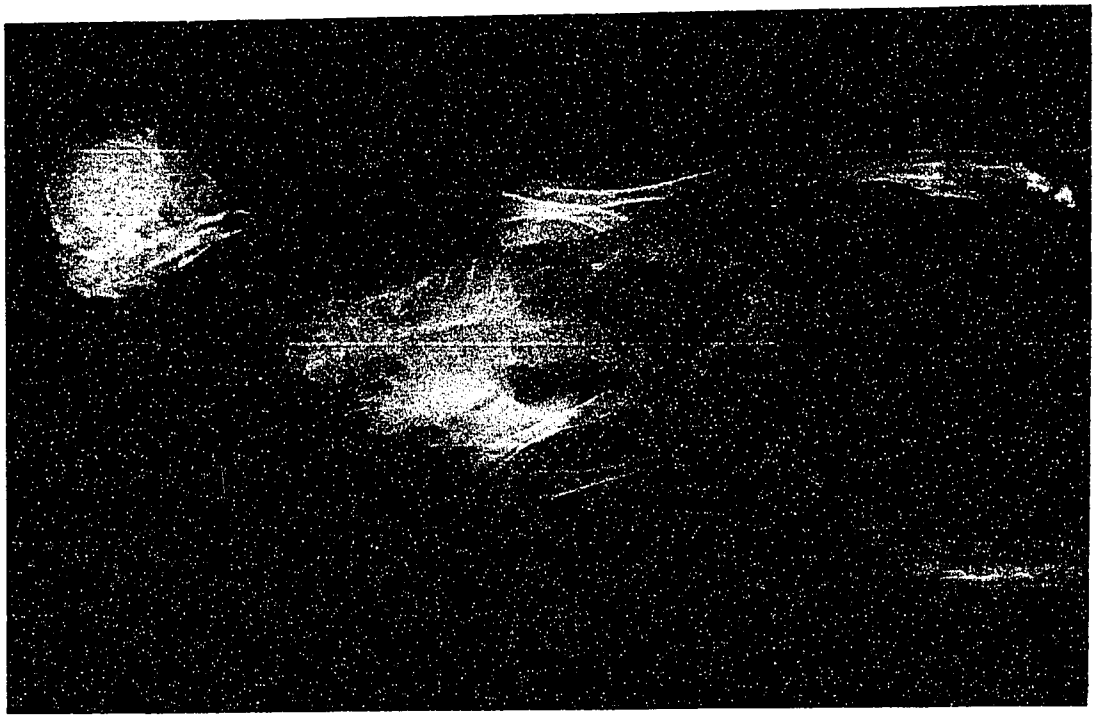


Fig. 17. Photograph of 19 day old chick embryos stained with alizarin red S and cleared by alkaline hydrolysis. The left chick is from an uninjected egg and the right chick is from an egg previously treated with 6.42 mg malathion on day 5 of incubation. Note the in-curved hind limbs, parrot beak, and reduced size in the malathion treated embryo

Fig. 18. The upper photograph illustrates the jaw skeleton from a control chick, while the lower one illustrates the deformed jaw skeleton of a malathion treated embryo. The mandible of the treated chick is reduced in size and the maxilla is curved over it in the form of a parrot's beak

Fig. 19. Photograph of dismembered hind limbs stained with alizarin red S. The left limb is from an untreated chick and the right limb is from a malathion treated chick. The length of the bones is markedly reduced. The tibiotarsus bone is bowed medially, while the tarsometatarsus bone is shortened and is projected medially

The malformations of embryos examined at 15 days of incubation produced by the various amounts of malathion tested are listed in Tables 12 to 20 and in Fig. 20 to 28. As shown in Fig. 20, 21, and 22, the controls and 1.17 mg malathion produced normal embryos except for a few which hemorrhaged.

As shown in Fig. 23, eggs injected with 3.99 mg on day 4 and 5 produced embryos with the "malathion syndrome". When 3.99 mg malathion was injected on days 6 to 12, the abnormalities included feather reduction, hemorrhages and neck blisters. Micromelia or beak defects were not evident.

As shown in Fig. 24, eggs injected with 6.42 mg on day 5 produced embryos with the "malathion syndrome". Those injected on days 6 to 12 had feather abnormalities, abdominal edema (Fig. 29), neck blisters, and hemorrhages. Micromelia or beak defects were not evident.

As shown in Fig. 25, eggs injected with 11.68 mg malathion on days 4 to 8 produced embryos with hemorrhages and feather reduction, whereas those injected on days 9 to 12 produced embryos with hydrocephaly, feather reduction, edema and neck blisters.

As shown in Fig. 26, eggs injected with 29.2 mg malathion on days 4 to 12 produced embryos with hemorrhages and feather reduction while those injected on days 9 to 12 produced embryos with edema and hydrocephaly.

As shown in Fig. 27, eggs injected with 58.4 mg on day 5 produced embryos with hemorrhages, feather reduction and the "malathion syndrome". Eggs injected on days 6 and 7 produced a few embryos with the "malathion syndrome" and a few with hydrocephaly. Eggs injected on days 8 to 12 produced embryos

Table 12. Autopsy results of chick embryos injected with 0.1ml distilled water per egg[a]

	Age of Embryo at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	4	4	2	0	4	0	0	0	0
Abdominal Edema	0	0	0	0	0	0	0	0	0
Hemorrhage + abdominal edema	0	0	0	0	0	0	0	0	0
Neck blister	0	4	0	0	0	0	0	0	0
Neck blister + feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction + hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
No apparent abnormalities	96	92	98	100	96	100	100	100	100

[a] Results of 3 experiments after injecting 50 eggs each.

Table 13. Autopsy results of chick embryos injected with 0.1 ml corn oil per egg[a]

	Age of Embryo at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	20	8	0	2	8	6	10	2	2
Abdominal Edema	0	0	0	0	0	0	0	0	0
Hemorrhage + abdominal edema	0	2	0	0	0	0	0	0	0
Neck blister	0	0	0	0	2	0	0	0	0
Neck blister + feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction + hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
No apparent abnormalities	80	90	100	98	90	94	90	98	98

[a] Results of 3 experiments after injecting 50 eggs each.

Table 14. Autopsy results of chick embryos injected with 1.17 mg malathion per egg[a]

	Age of Embryo at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	8	4	6	2	4	2	2	2	2
Abdominal Edema	0	0	0	0	0	0	0	0	0
Hemorrhage + abdominal Edema	0	0	0	0	0	0	0	0	0
Neck blister	4	0	2	2	0	0	0	2	0
Neck blister + feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction + hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
No apparent abnormalities	88	96	92	96	96	98	98	96	98

[a] 0.1 ml malathion in corn oil per egg - Results of 3 experiments after injecting 50 eggs each.

Table 15. Autopsy results of chick embryos injected with 3.99 mg malathion per egg[a]

	Age of Embryos at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	10	8	12	0	8	4	4	6	2
Abdominal Edema	8	10	0	4	2	6	4	2	0
Hemorrhage + abdominal edema	4	6	0	2	0	0	2	2	2
Neck blister	4	2	6	2	2	4	2	2	4
Neck blister + feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction	0	0	14	20	22	24	22	28	26
Feather reduction + hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	12	8	2	0	0	0	0	0	0
Hind limb micromelia + beak defects	38	18	6	0	0	0	0	0	0
No apparent abnormalities	24	48	60	72	66	62	66	60	66

[a] 0.1 ml malathion in corn oil per egg. - Results of 3 experiments after injecting 50 eggs each.

Table 16. Autopsy results of chick embryos injected with 6.42 mg malathion per egg[a]

	Age of Embryos at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	22	12	10	4	10	4	6	4	0
Abdominal Edema	0	2	4	2	2	2	2	2	2
Hemorrhage + abdominal edema	4	2	0	2	2	2	0	2	0
Neck blister	4	2	4	2	4	4	6	8	2
Neck blister + feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction	0	0	26	20	30	18	28	22	16
Feather reduction + hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	10	18	2	2	0	0	0	0	0
Hind limb micromelia + beak defects	40	32	4	2	0	0	0	0	0
No apparent abnormalities	20	32	50	66	52	70	58	62	80

[a] 0.1 ml malathion in corn oil per egg - Results of 3 experiments after injecting 50 eggs each.

Table 17. Autopsy results of chick embryos injected with 11.68 mg malathion per egg[a]

	Age of Embryos at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	58	52	46	35	48	6	10	10	14
Abdominal Edema	0	0	0	5	3	0	0	6	4
Hemorrhage + abdominal edema	0	0	6	0	0	2	0	0	2
Neck blister	0	0	0	0	0	0	0	0	0
Neck blister + feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction	18	22	16	18	20	22	28	20	24
Feather reduction + hydrocephalus	0	0	0	0	0	0	0	0	0
Hydrocephalus	0	0	0	0	0	24	37	28	18
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
No apparent abnormalities	24	26	32	42	29	46	25	36	38

[a] 0.1 ml malathion in corn oil per egg - Results of 3 experiments after injecting 50 eggs each.

Table 18. Autopsy results of chick embryos injected with 29.2 mg malathion per egg[a]

	Age of Embryos at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	62	50	43	48	82	34	20	28	26
Abdominal Edema	0	0	0	2	6	0	0	0	4
Hemorrhage + abdominal edema	0	0	0	4	0	18	10	12	4
Neck blister	0	0	0	0	0	0	0	0	0
Neck blister + feather reduction	0	0	0	0	0	0	0	0	0
Feather reduction	18	20	18	26	12	16	20	28	24
Feather reduction + hydrocephalus	0	0	0	0	0	0	15	0	0
Hydrocephalus	0	0	0	0	0	2	3	10	6
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + beak defects	0	0	0	0	0	0	0	0	0
No apparent abnormalities	20	30	39	20	6	30	32	22	42

[a] 0.1 ml malathion in corn oil per egg - Results of 3 experiments after injecting 50 eggs each.

Table 19. Autopsy results of chick embryos injected with 58.4 mg malathion per egg[a]

	Age of Embryos at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	26	10	10	28	64	24	8	80	80
Abdominal Edema	8	0	2	0	6	14	12	0	0
Hemorrhage + abdominal edema	6	2	4	0	4	26	74	18	8
Neck blister	0	4	10	0	2	0	0	0	0
Neck blister + feather reduction	0	0	6	0	0	0	0	0	0
Feather reduction	10	14	24	2	16	12	2	0	4
Feather reduction + hydrocephalus	0	0	0	12	0	0	0	0	0
Hydrocephalus	0	0	0	4	0	0	0	0	0
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	20	28	4	8	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + beak defects	10	14	4	6	0	0	0	0	0
No apparent abnormalities	20	28	36	40	8	24	4	2	8

[a] 0.1 ml malathion in corn oil per egg - Results of 3 experiments after injecting 50 eggs each.

Table 20. Autopsy results of chick embryos injected with 116.8 mg malathion per egg[a]

	Age of Embryos at Injection (Days)								
	4	5	6	7	8	9	10	11	12
Hemorrhage	28	4	10	16	4	38	16	34	70
Abdominal Edema	0	0	0	2	10	6	10	2	0
Hemorrhage + abdominal edema	2	4	4	0	10	10	54	22	12
Neck blister	0	0	6	6	8	0	4	0	0
Neck blister + feather reduction	0	0	16	0	0	0	0	0	0
Feather reduction	14	24	20	30	4	0	2	0	0
Feather reduction + hydrocephalus	0	0	0	4	0	0	0	0	0
Hydrocephalus	6	0	0	2	0	2	2	0	0
Hydrocephalus + feather reduction + hind limb micromelia + beak defects	28	26	4	6	0	0	0	0	0
Hind limb micromelia + feather reduction + beak defects	0	0	0	0	0	0	0	0	0
Hind limb micromelia + beak defects	10	8	2	4	0	0	0	0	0
No apparent abnormalities	12	34	38	30	64	44	12	42	18

[a] 0.1 ml malathion per egg - Results of 3 experiments after injecting 50 eggs each.

List of Abnormalities for Figures 20 to 28

A. Hemorrhage

B. Abdominal edema

C. Hemorrhage and abdominal edema

D. Neck blister

E. Neck blister and feather reduction

F. Feather reduction

G. Feather reduction and hydrocephaly

H. Hydrocephaly

I. Hydrocephaly, feather reduction, hind limb micromelia, and beak defects

J. Hind limb micromelia, feather reduction, and beak defects

K. Hind limb micromelia and beak defects

L. No apparent abnormalities

Fig. 20. Percentage of various types of congenital defects in chick embryos injected with distilled water on days 4 to 12 of incubation. The total height of each column represents 100 per cent

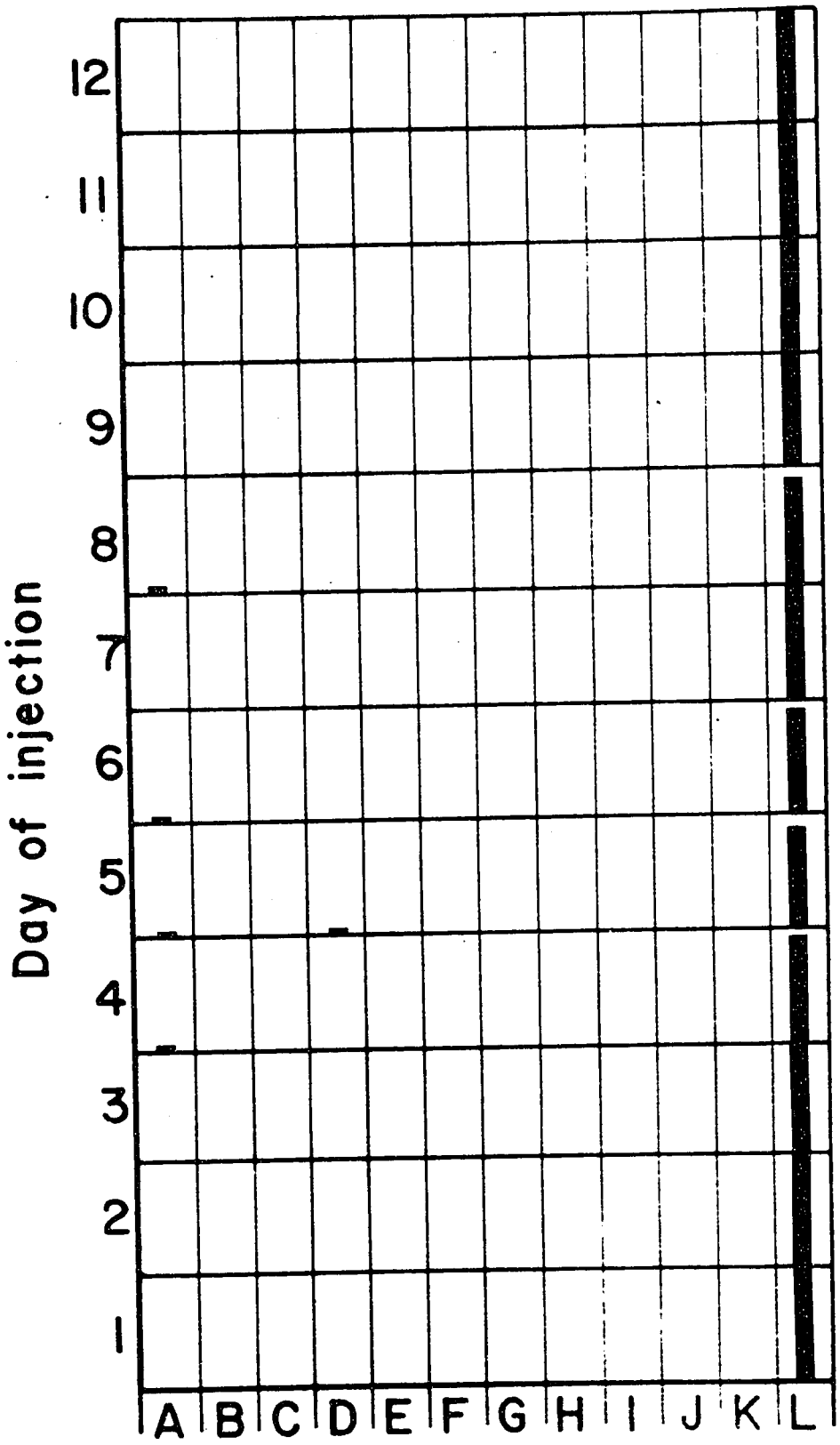

Day of injection
12
11
10
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Fig. 21. Percentage of various types of congenital defects in chick embryos injected with corn oil on days 4 to 12 of incubation

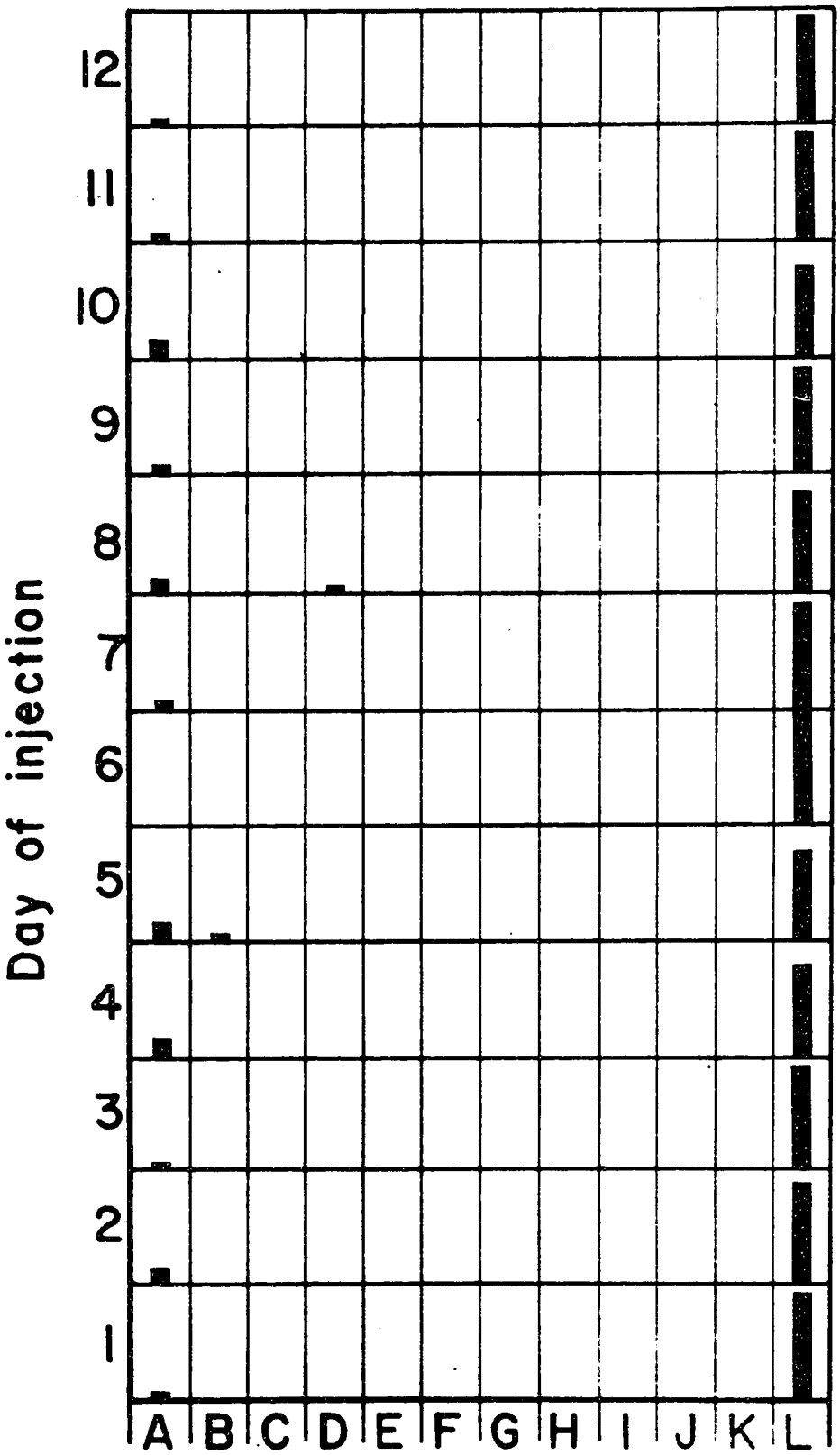
Day of injection
12
11
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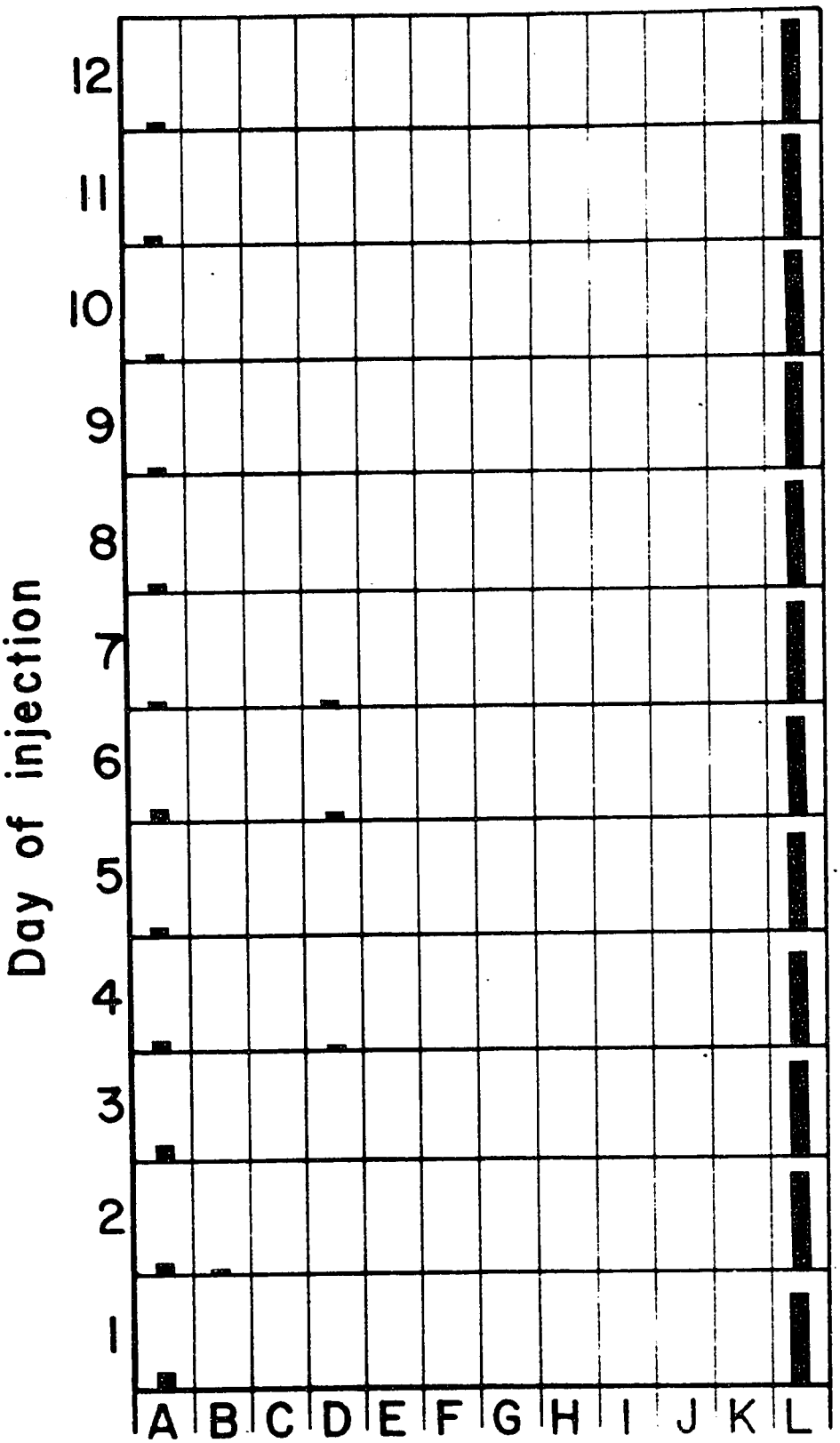
Fig. 22. Percentage of various types of abnormalities in chick embryos treated with 1.17 mg malathion per egg on days 4 to 12 of incubation


Day of injection
12
11
10
9
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7
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Fig. 23. Percentage of various types of congenital defects in chick embryos treated with 3.99 mg malathion per egg on days 4 to 12 of incubation

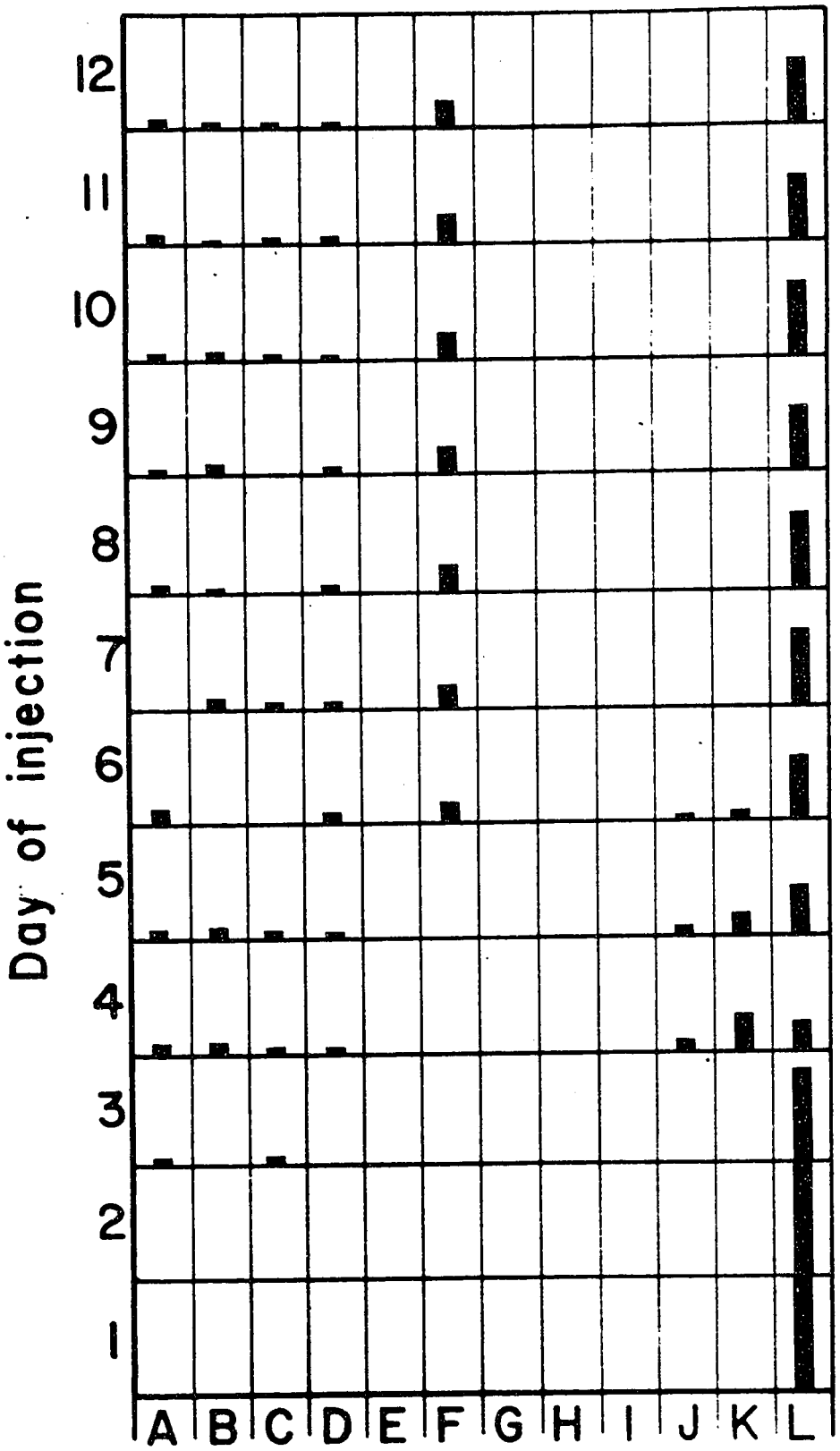
Day of injection
12
11
10
9
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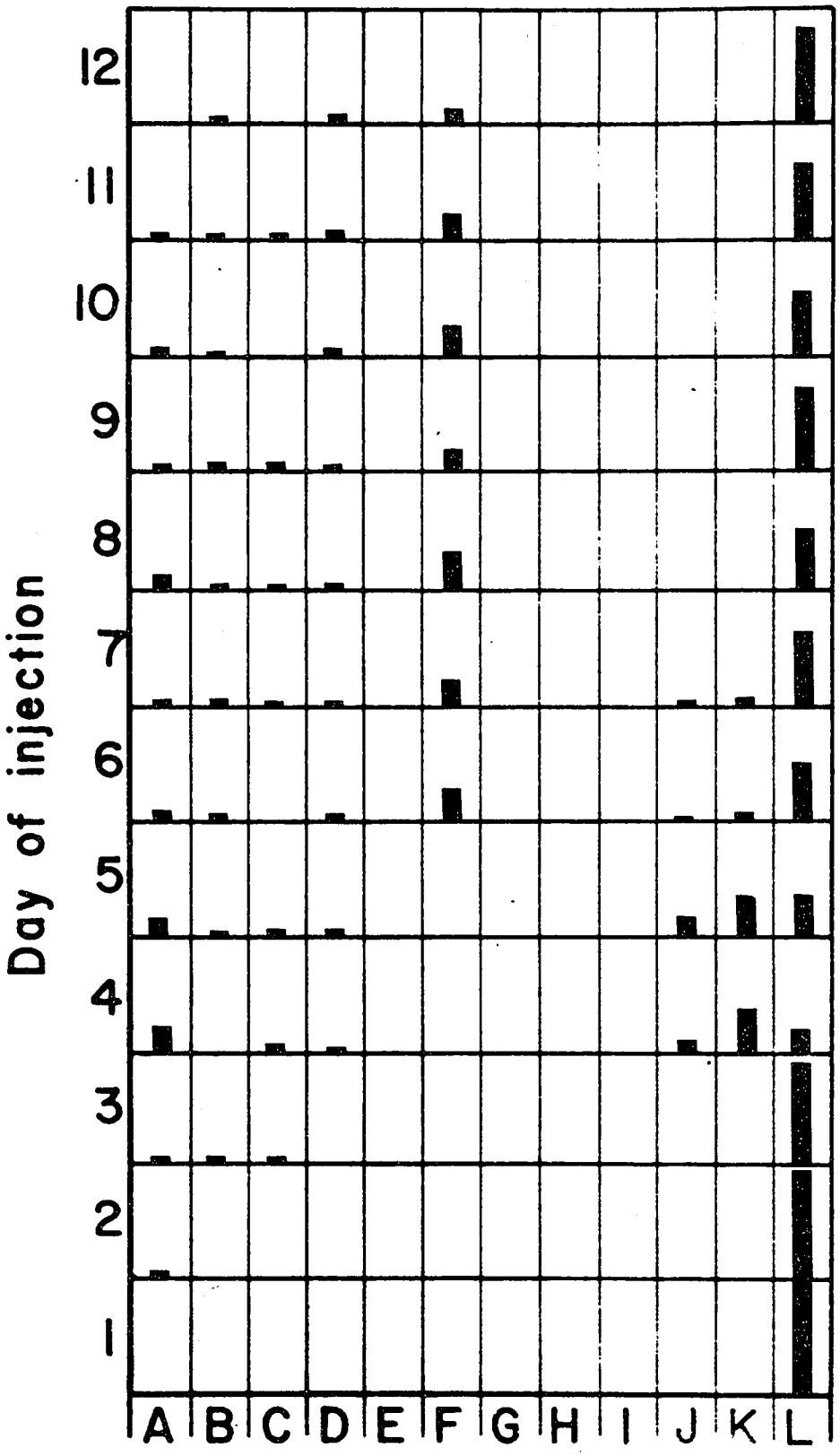
Fig. 24. Percentage of various types of congenital defects in chick embryos treated with 6.42 mg malathion per egg on days 4 to 12 of incubation


Day of injection
12
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10
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Fig. 25. Percentage of various types of congenital defects in chick embryos treated with 11.68 mg malathion per egg on days 4 to 12 of incubation

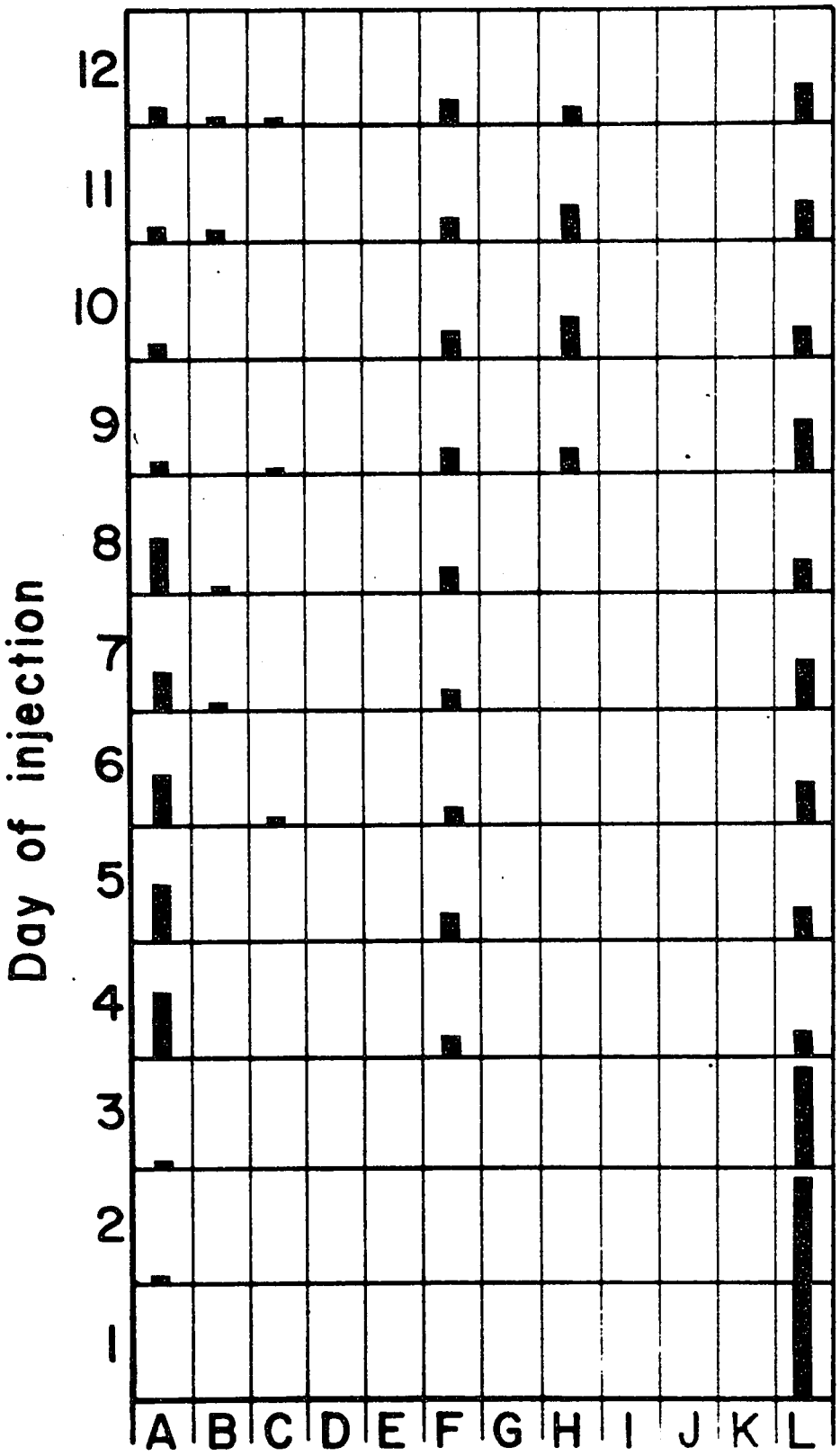
Day of injection
12
11
10
9
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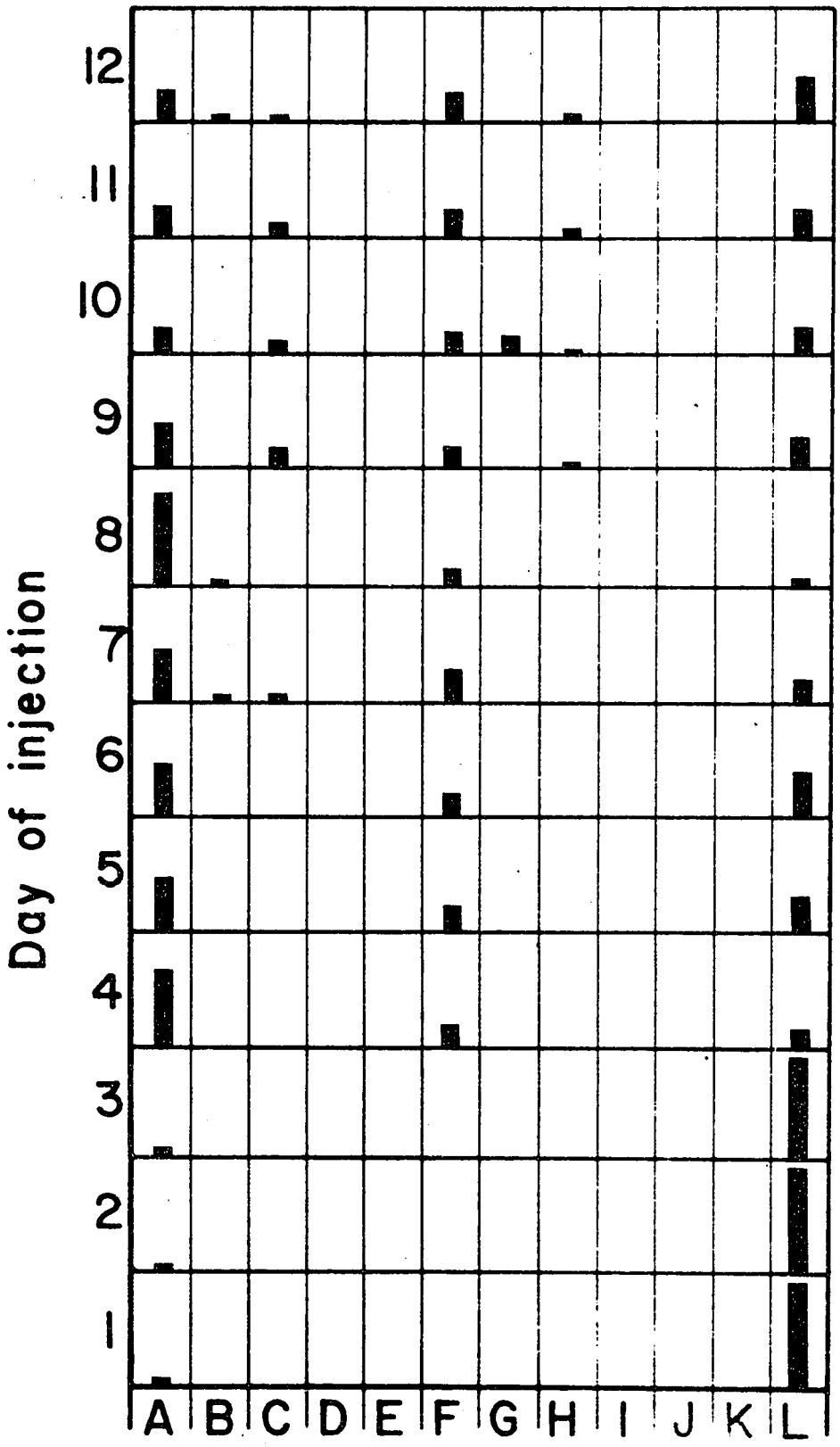
Fig. 26. Percentage of various types of congenital defects in chick embryos treated with 29.2 mg malathion per egg on days 4 to 12 of incubation


Day of injection
12
11
10
9
8
7
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4
3
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1
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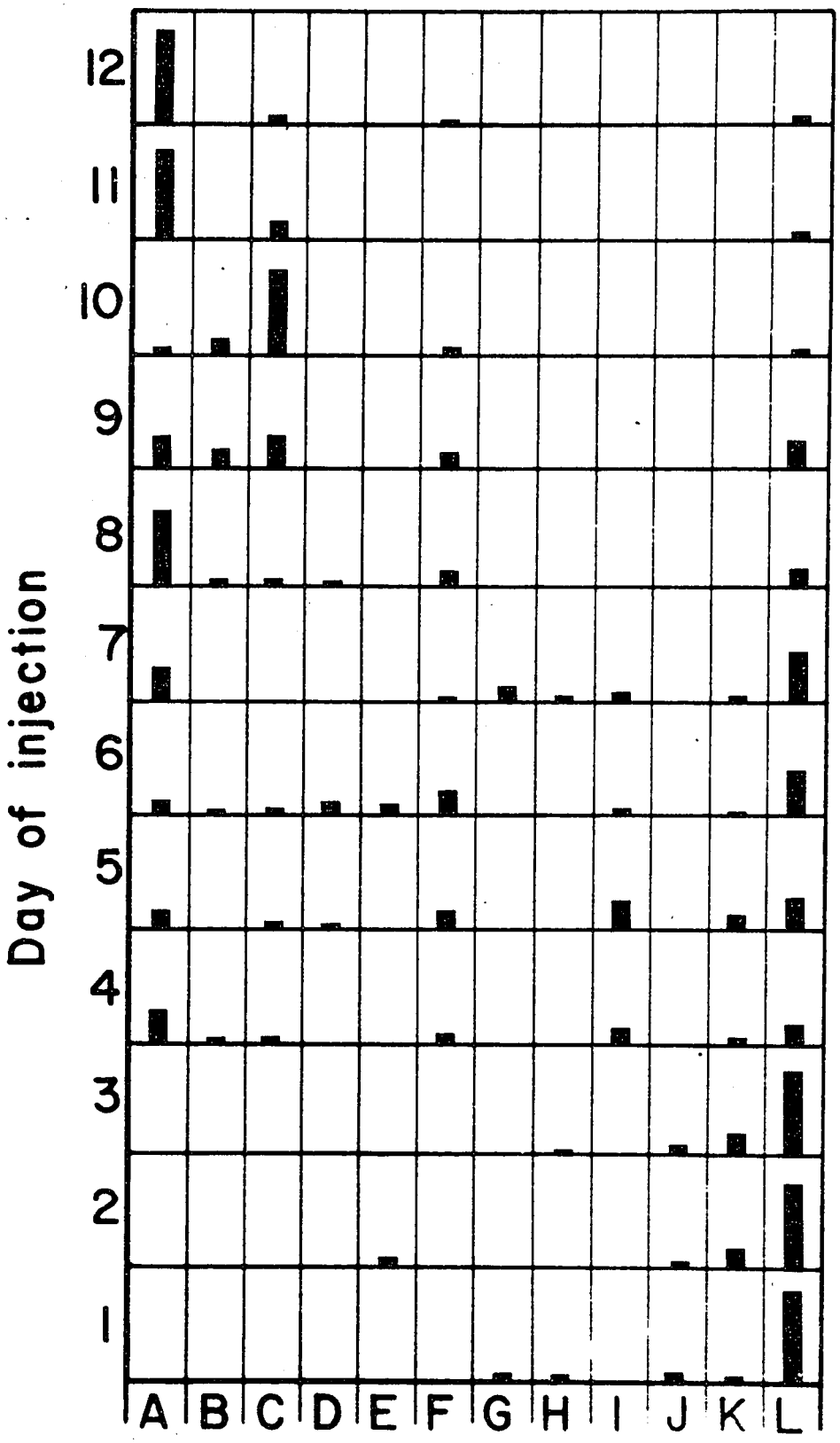
Fig. 27. Percentage of various types of congenital defects in chick embryos treated with 58.4 mg malathion per egg on days 4 to 12 of incubation


Day of injection
12
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10
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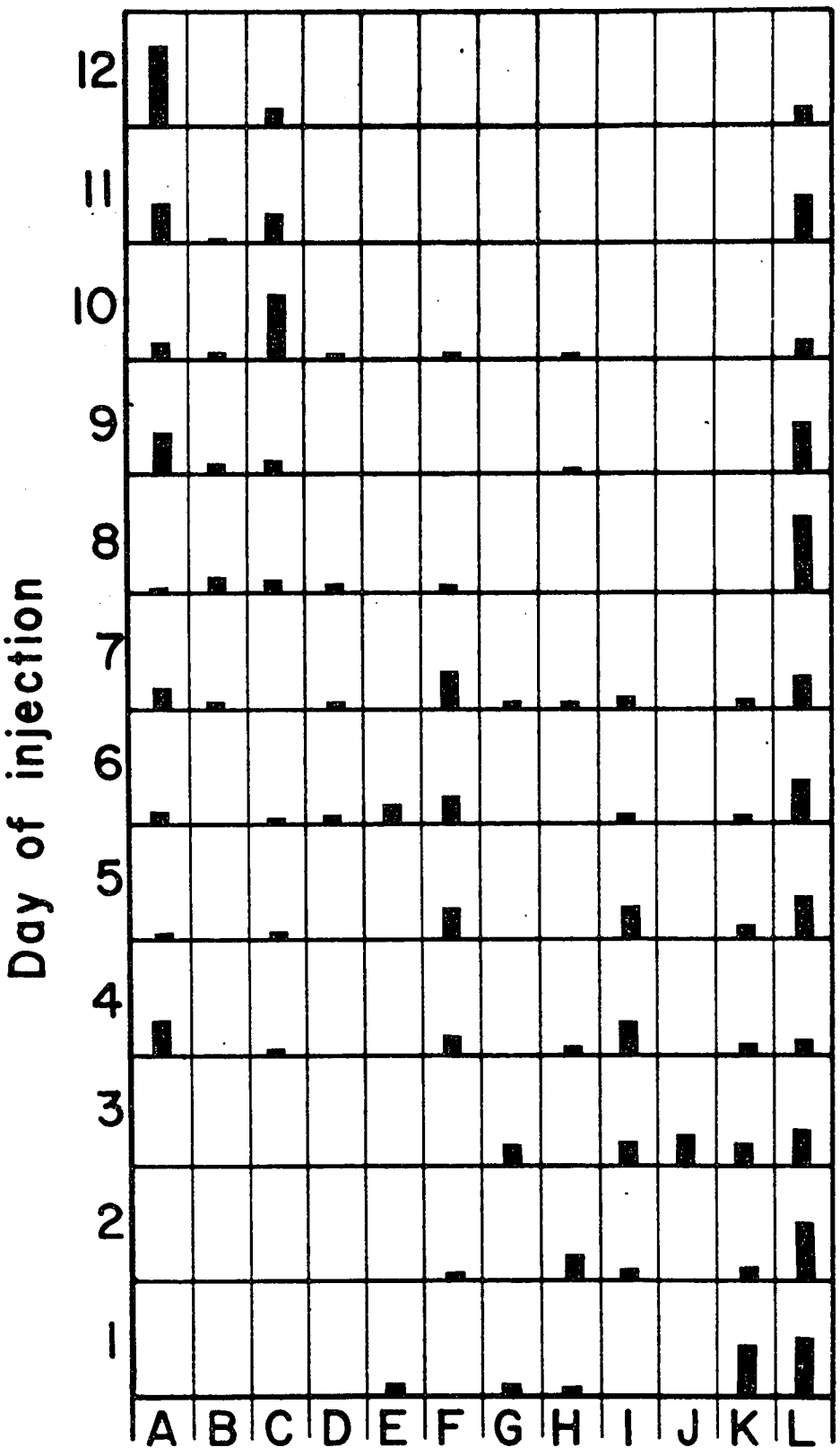
Fig. 28. Percentage of various types of congenital defects in chick embryos treated with 116.8 mg malathion per egg on days 4 to 12 of incubation


Day of injection
12
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10
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7
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Fig. 29. Dorsal and ventral views of a 13 day old chick embryo with abdominal edema after injections of 6.42 mg malathion per egg on day 7 of incubation

with abdominal edema, hemorrhages and feather reduction; however, the "malathion syndrome" was not evident.

As shown in Fig. 28, eggs injected with 116.8 mg malathion on days 4 to 7 produced embryos with hemorrhages, feather reduction, edema, neck blisters, and micromelia and beak defects. The micromelia and beak defects occurred after injections on days 4 and 5. Eggs injected on days 8 to 12 produced embryos with hydrocephaly, hemorrhages and edema.

The "malathion syndrome" was therefore evident after injections on days 4 and 5 with concentrations which killed 50%. Some embryos developed this syndrome after injections of either 58.4 mg or 116.8 mg on days 6 and 7. After day 7, the abnormalities were confined to hydrocephaly, hemorrhages and edema. The greatest number of malformations occurred at a dose level which produced 50% mortality. Below this level, the abnormalities decreased rapidly, whereas, above this level the embryos died and could therefore not be studied. The occurrence of the "malathion syndrome" was very predictable and was our main interest.

Distribution of Malathion-P^{32} in the Embryo and Yolk Sac

a. Gas flow counting

Having established the teratogenicity of malathion on the chick embryo, we next wished to determine at which level the malathion was acting. That is, does the label enter the yolk sac and/or the embryo, and if so, does it concentrate in any particular organ(s) or cell(s). We also wanted to confirm some of the peculiarities observed when undiluted malathion was injected into the egg. For example, when undiluted malathion was injected into the egg on days 4 to 7, there was

a delay in mortality. Was this delay due to the lack of entry of malathion into the embryo? We also wished to determine if there was a relationship between deaths and/or malformations, and the amount of label found in the yolk sac or embryo.

The distribution of malathion-P^{32} in fertilized eggs after injections of 3.99 mg per egg on day 4 of incubation and 6.42 mg per egg on day 5 of incubation is shown in Tables 21 and 22, and Figs. 30 to 33.

As shown in Fig. 30, after injections of 3.99 mg per egg malathion-P^{32} on day 4, the amount entering the embryo increased to day 13. There is a small increase between days 6 and 8 and a much larger increase on days 8 to 13. There is a larger amount of label in the yolk sac; however, the trend is similar to the uptake in the embryo. The amount of label in the yolk on the other hand, declines from day 6 to day 13. The albumen was not analyzed and therefore the total counts of the embryo + yolk sac + yolk will not be equal to each other on the days following injection.

As shown in Fig. 32, after injections of 6.42 mg per egg malathion-P^{32} on day 5 of incubation, the amount entering the embryo increased to day 13. The distribution pattern in the embryo and yolk sac is similar to the day 4 injections.

The uptake of malathion-P^{32} per gram embryo after day 4 injections, as shown in Fig. 31, increases to day 6. There is a sharp decline to day 8 and then a gradual decline to day 13. The amount of label per gram yolk sac reaches a peak on day 6, drops sharply to day 7 and reaches

Table 21. Distribution of P^{32} in fertilized chicken eggs after injection of 3.99 mg malathion containing 1 μc malathion P^{32} per egg on day 4 of incubation

	Day of incubation	Average wt (g)	Total vol homogenate (ml)	Average cpm per 0.2 ml[a]	Decay factor	Total cpm per homogenate	Cpm/g homogenate
Embryo	5	0.1949	1.20	332	0.7837	2,542	13,042
	6	0.3232	1.60	1,068	0.7837	10,902	33,731
	7	0.6322	1.70	1,015	0.7464	11,559	18,284
	8	1.0789	2.00	865	0.7464	11,588	10,741
	10	2.5914	3.20	985	0.7109	22,169	8,555
	13	5.5956	8.60	494	0.5849	36,317	6,490
Yolk-Sac	5	1.1975	1.30	3,051	0.7837	25,305	21,127
	6	0.7864	1.40	3,129	0.7837	27,948	35,539
	7	1.5939	1.60	2,637	0.7464	26,371	17,799
	8	1.4190	2.00	2,078	0.7464	27,840	19,619
	10	1.6277	2.20	2,634	0.7109	40,756	25,039
	13	3.8870	3.30	2,004	0.5849	56,532	14,544
Yolk	5	44.3911	42.90	2,241	0.7837	613,220	14,298
	6	40.8204	39.40	2,605	0.7837	655,488	16,619
	7	36.6907	35.40	2,626	0.7464	623,605	17,591
	8	34.7139	33.50	2,699	0.7464	606,406	18,080
	10	29.3422	28.30	2,547	0.7109	507,859	17,913
	13	24.4363	23.60	2,081	0.5849	420,003	17,789

[a] Average of 5 readings for each sample.

Table 22. Distribution of P^{32} in fertilized chicken eggs after injection of 6.42 mg malathion containing 1 μc of malathion P^{32} per egg on day 5 of incubation

	Day of incubation	Average wt (g)	Total vol homogenate (ml)	Average cpm per 0.2 ml[a]	Decay factor	Total cpm per homogenate	Cpm/g homogenate
Embryo	6	0.3246	1.60	553	0.7837	5,645	17,391
	7	0.7208	1.70	1,118	0.7208	12,731	17,662
	8	1.2027	2.00	875	0.6771	12,923	10,745
	10	2.4692	3.20	982	0.6771	23,205	9,398
	13	5.1492	8.60	681	0.5849	50,064	9,723
Yolk-Sac	6	1.4172	1.40	2,987	0.7837	26,679	18,825
	7	1.9806	1.60	2,638	0.7464	28,274	14,275
	8	1.8993	2.00	1,989	0.6771	29,375	15,466
	10	1.9032	2.20	2,536	0.6771	41,205	21,650
	13	3.2385	3.30	2,138	0.5849	60,312	18,623
Yolk	6	39.7233	38.40	2,506	0.7837	613,622	15,988
	7	32.7991	31.70	3,078	0.7464	653,416	20,619
	8	30.3462	29.30	2,519	0.6771	545,392	18,601
	10	28.5867	27.60	2,074	0.6771	423,000	15,315
	13	20.7000	20.00	2,123	0.5849	362,960	18,148

[a] Average of 5 readings for each sample.

Fig. 30. Distribution of malathion-P^{32} per embryo, yolk sac, and yolk after injections of 3.99 mg malathion containing 1 µc malathion-P^{32} per egg on day 4 of incubation (CPM/whole unit represents the counts recorded for the total structure; that is, the whole embryo, the whole yolk sac, or the whole yolk)

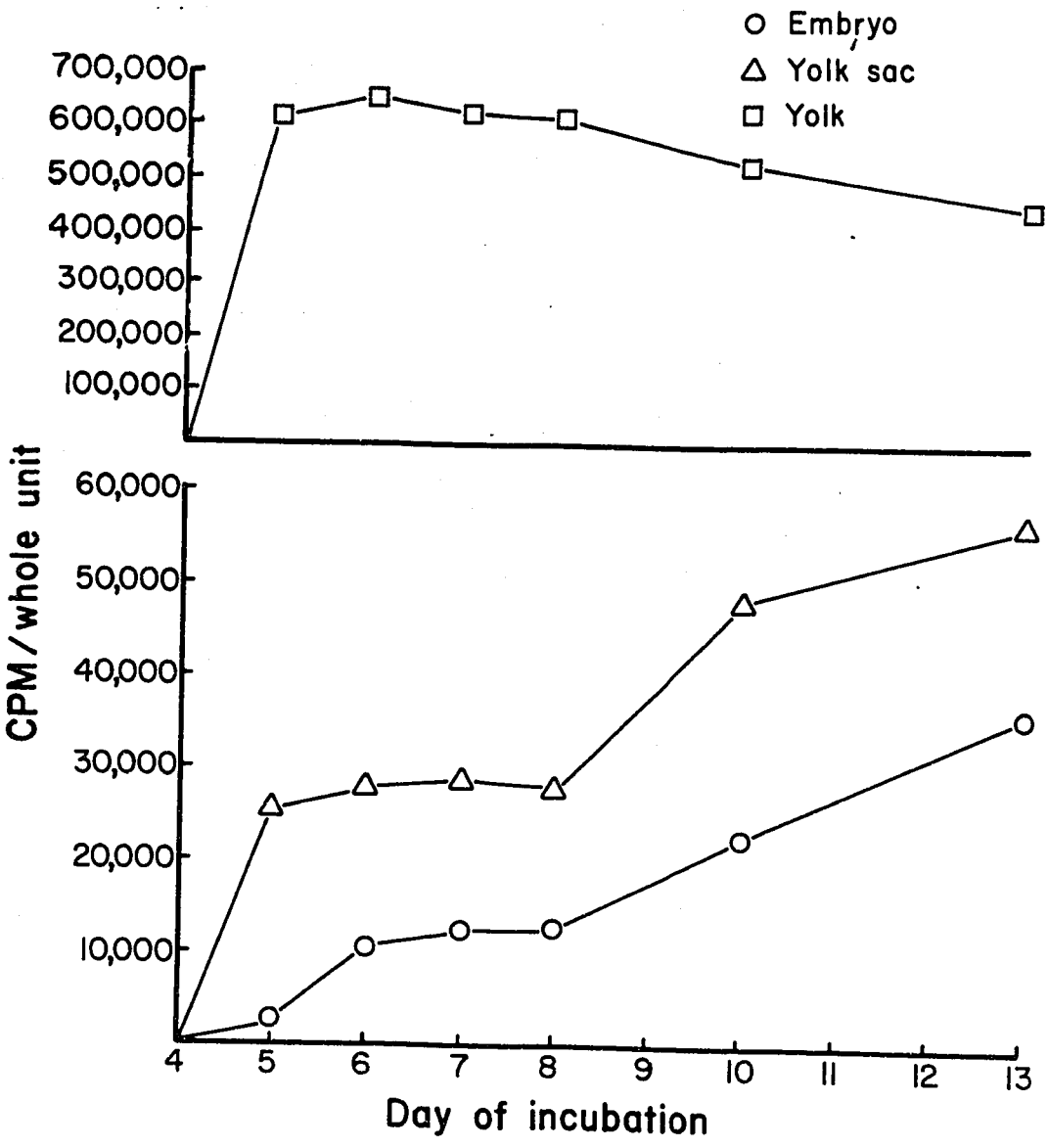

O Embryo
△ Yolk sac
□ Yolk
700,000
600,000
500,000
400,000
300,000
200,000
100,000
60,000
50,000
40,000
30,000
20,000
10,000
CPM/whole unit
4
5
6
7
8
9
10
11
12
13
Day of incubation

Fig. 31. Distribution of malathion-P^{32} per gram embryo, yolk sac, and yolk after injections of 3.99 mg malathion containing 1 µc malathion-P^{32} per egg on day 4 of incubation

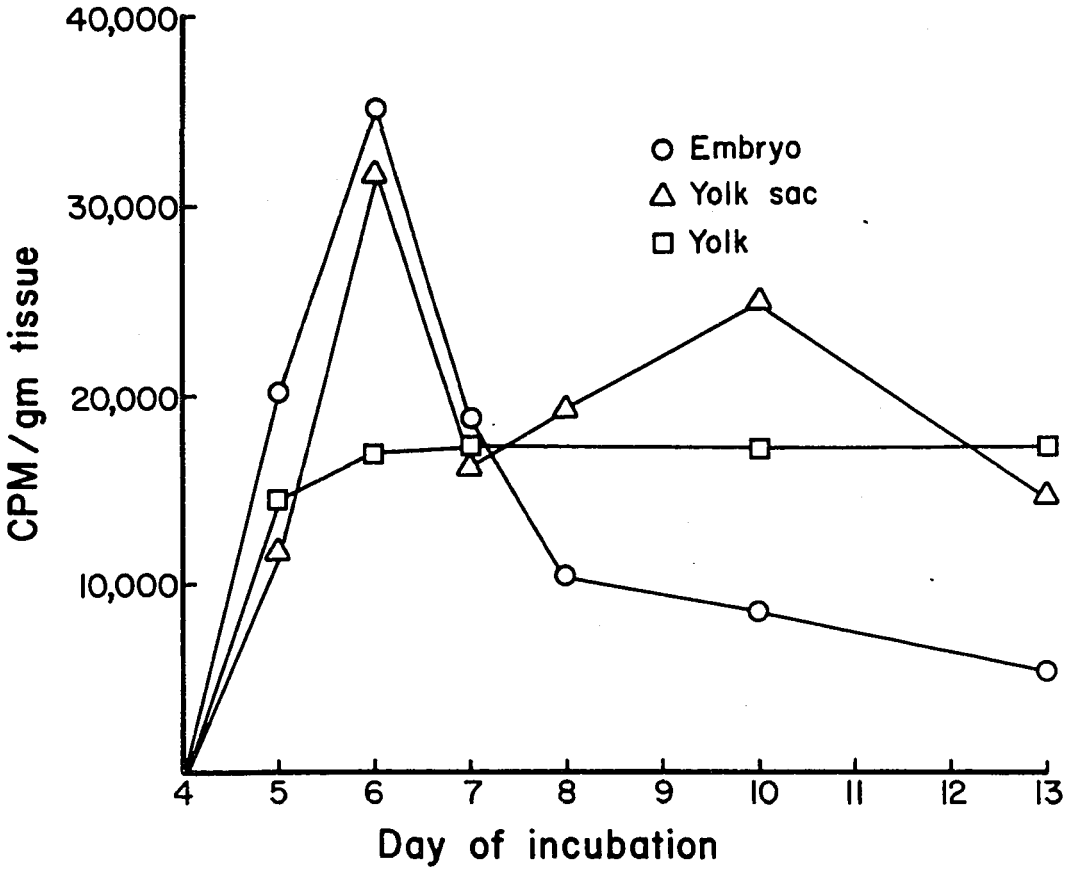

40,000
30,000
20,000
10,000
CPM/gm tissue
Embryo
Yolk sac
Yolk
4
5
6
7
8
9
10
11
12
13
Day of incubation

Fig. 32. Distribution of malathion-P^{32} per embryo, yolk sac, and yolk after injections of 6.42 mg malathion containing 1 μc malathion per egg on day 5 of incubation

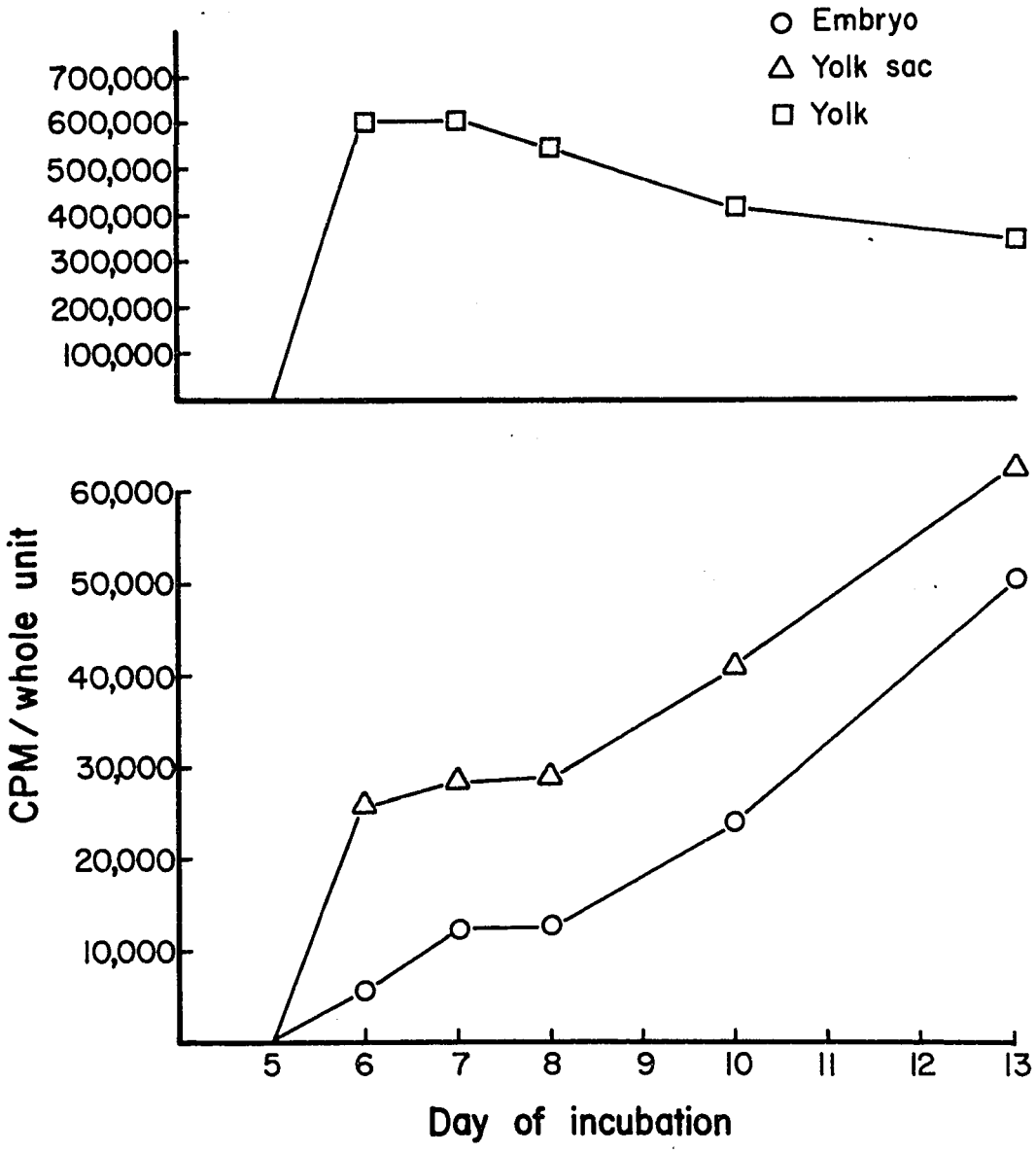
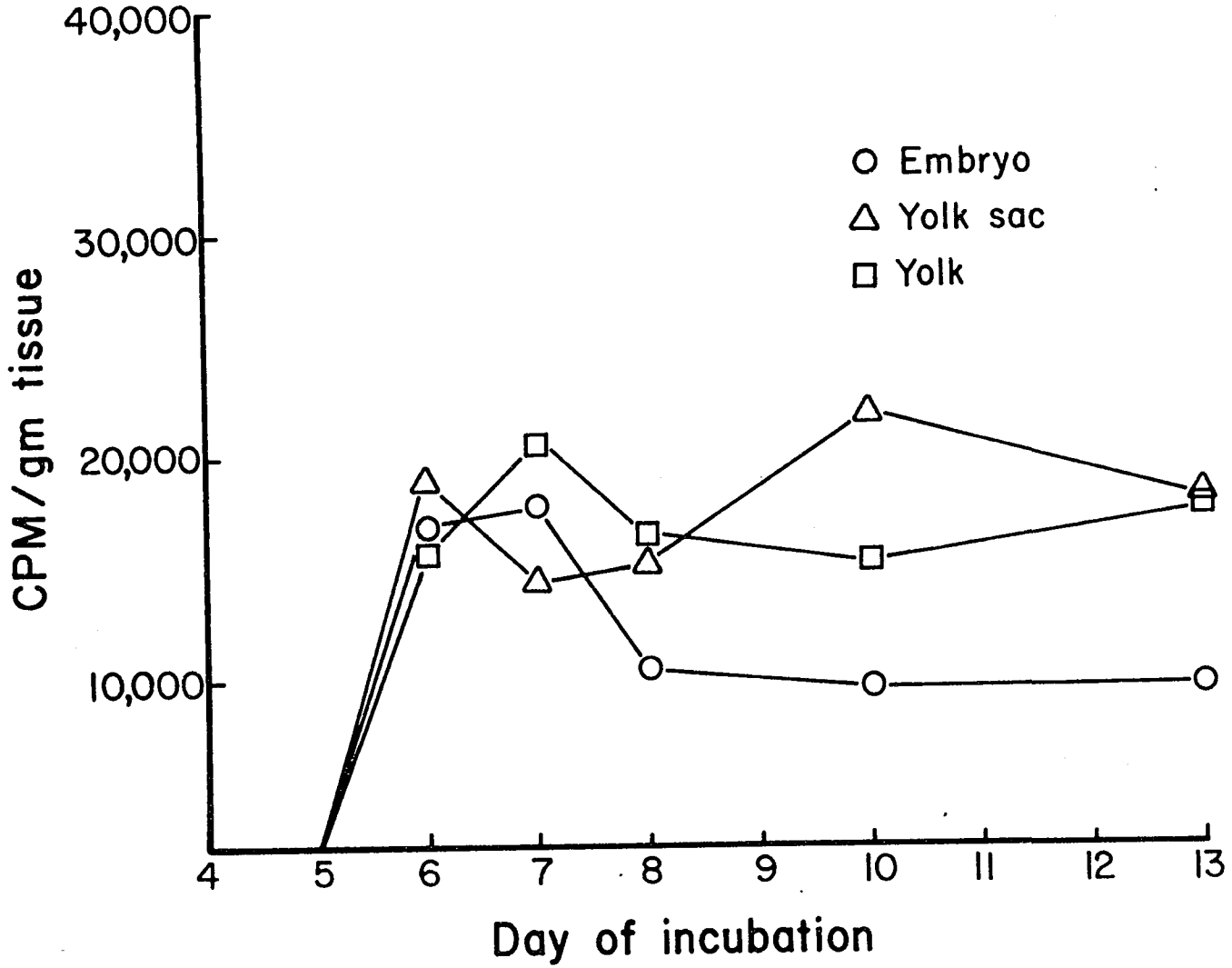

Embryo
Yolk sac
Yolk
700,000
600,000
500,000
400,000
300,000
200,000
100,000
60,000
50,000
40,000
30,000
20,000
10,000
CPM/whole unit
5
6
7
8
9
10
11
12
13
Day of incubation

Fig. 33. Distribution of malathion-P^{32} per gram embryo, yolk sac, and yolk after injections of 6.42 mg malathion containing 1 µc malathion-P^{32} per egg on day 5 of incubation


40,000
30,000
20,000
10,000
CPM/gm tissue
O Embryo
△ Yolk sac
□ Yolk
4
5
6
7
8
9
10
11
12
13
Day of incubation

another peak on day 10 and then declines to day 13.

The uptake of malathion-P^{32} per gram embryo after day 5 injections, as shown in Fig. 33, increases to day 7, declines on day 8 and levels off to day 13. As in the day 4 injections, the amount in the yolk sac membrane also has 2 peaks. The first on day 6, and the second on day 10.

The distribution of malathion-P^{32} in fertilized eggs after injections of 105.12 mg per egg (90%) malathion on days 4 and 5 of incubation is shown in Tables 23 and 24, and Figs. 34 to 37.

As shown in Fig. 34, after injections on day 4, the CPM (counts/min.) per embryo remains low to day 10 and then increases to day 13. The amount in the yolk sac parallels the embryo to day 10 and also increases to day 13. The amount of label in the yolk fluctuates on the various days tested. A similar pattern exists when the CPM per gram embryo is plotted against the day of incubation (Fig. 35).

As shown in Fig. 36, after injections on day 5, the CPM per embryo and yolk sac remains very low to day 8. The uptake by the embryo increases slightly to day 10 and levels off to day 13, whereas, the uptake in the yolk sac increases to day 13. The amount of label in the yolk fluctuates on the days tested. The CPM per gram embryo (Fig. 37) remains constant to day 13 while the CPM per gram yolk sac membrane remains low to day 8, increases to day 10 and levels off to day 13.

The CPM in embryos and yolk sac membranes after injections of 90% malathion-P^{32} on days 6 to 12 are listed in Table 25. After day 6 injections, the CPM per gram embryo on day 7 of incubation was 371.

Table 23. Distribution of P^{32} in fertilized chicken eggs after injection of 105.12 mg malathion containing 1 μc of malathion P^{32} per egg on day 4 of incubation

	Day of incubation	Average wt (g)	Total vol homogenate (ml)	Average cpm per 0.2 ml[a]	Decay factor	Total cpm per homogenate	Cpm/g homogenate
Embryo	5	0.2500	1.20	4	0.7837	31	124
	6	0.3652	1.60	3	0.7837	31	85
	7	0.6900	1.70	13	0.7464	148	214
	8	1.1556	2.00	10	0.7109	120	104
	10	2.7240	3.20	40	0.6771	945	347
	13	6.4133	8.60	347	0.5849	25,510	3,977
Yolk-Sac	5	1.2077	1.30	67	0.7837	556	460
	6	0.9330	1.40	45	0.7837	402	431
	7	1.6807	1.60	45	0.7464	482	287
	8	1.3375	2.00	17	0.7109	239	180
	10	2.0525	2.20	97	0.6771	1,603	781
	13	3.8551	3.30	296	0.5849	8,350	2,166
Yolk	5	43.6149	42.10	504	0.7837	135,500	3,107
	6	40.4167	39.00	235	0.7837	58,550	1,449
	7	38.2846	37.00	611	0.7464	151,395	3,954
	8	33.0165	31.90	1,330	0.7109	298,455	9,041
	10	28.0278	27.10	999	0.6771	199,770	7,130
	13	20.8552	20.10	1,056	0.5849	181,894	8,720

[a] Average of 5 readings for each sample.

Table 24. Distribution of P^{32} in fertilized chicken eggs after injection of 105.12 mg malathion containing 1 μc of malathion P^{32} per egg on day 5 of incubation

	Day of incubation	Average wt (g)	Total vol homogenate (ml)	Average cpm per 0.2 ml[a]	Decay factor	Total cpm per homogenate	Cpm/g homogenate
Embryo	6	0.4424	1.60	8	0.7837	82	185
	7	1.0570	1.70	9	0.7464	102	96
	8	1.2589	2.00	9	0.6771	133	106
	10	2.1850	3.20	26	0.6293	661	303
	13	6.7645	8.60	280	0.5849	20,585	3,043
Yolk-Sac	6	1.1695	1.40	42	0.7837	375	321
	7	1.7962	1.60	31	0.7464	332	185
	8	2.9703	2.00	26	0.6771	384	129
	10	1.7689	2.20	143	0.6293	2,499	1,413
	13	5.2828	2.30	155	0.5849	4,373	828
Yolk	6	42.0313	40.60	501	0.7837	129,800	3,088
	7	36.8563	35.60	560	0.7464	135,585	3,625
	8	28.8351	27.90	688	0.6771	141,541	4,909
	10	26.2683	25.40	55	0.6293	110,908	4,222
	13	26.4960	25.60	73	0.5849	159,753	6,029

[a] Average of 5 readings for each sample.

Fig. 34. Distribution of malathion-P^{32} per embryo, yolk sac, and yolk after injections of 105.12 mg malathion containing 1 µc of malathion-P^{32} per egg on day 4 of incubation

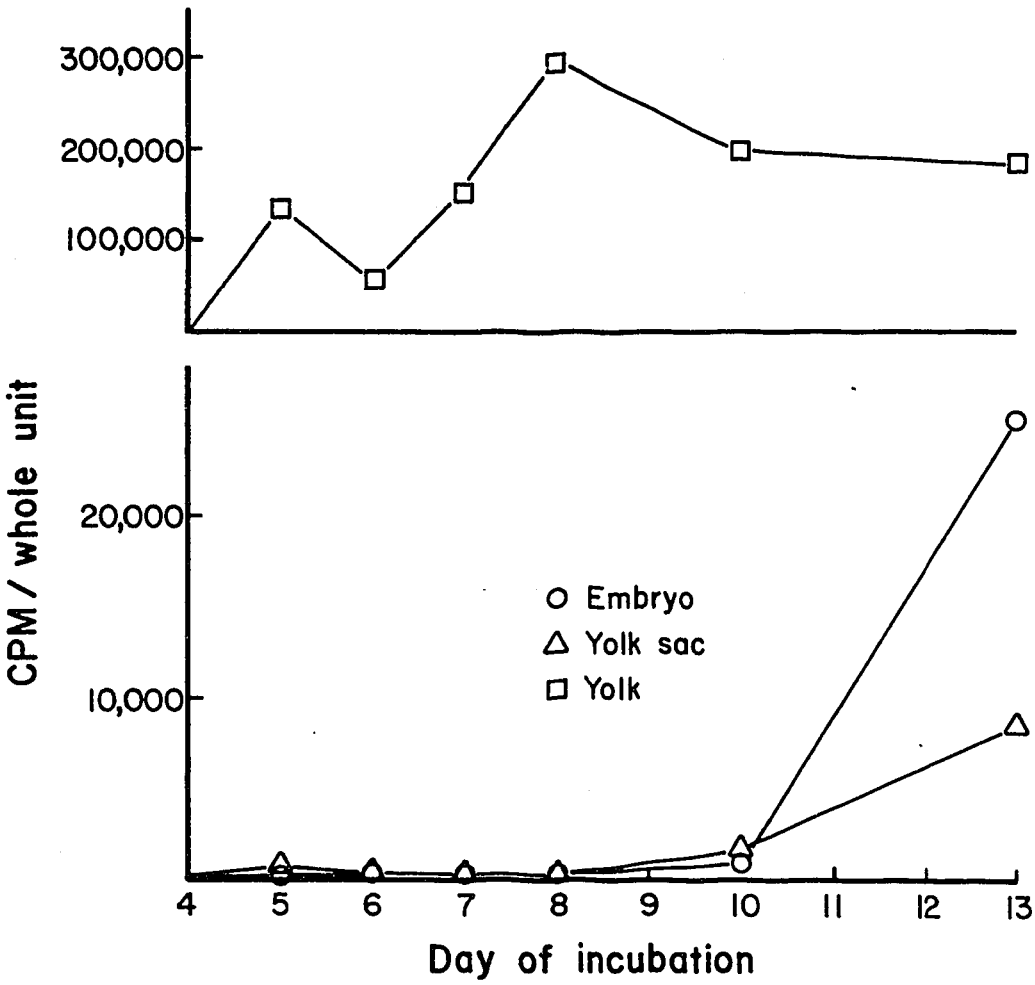

300,000
200,000
100,000
20,000
10,000
CPM / whole unit
O Embryo
Δ Yolk sac
□ Yolk
4
5
6
7
8
9
10
11
12
13
Day of incubation

Fig. 35. Distribution of malathion-P^{32} per gram embryo, yolk sac, and yolk after injections of 105.12 mg malathion containing 1 μc of malathion-P^{32} per egg on day 4 of incubation

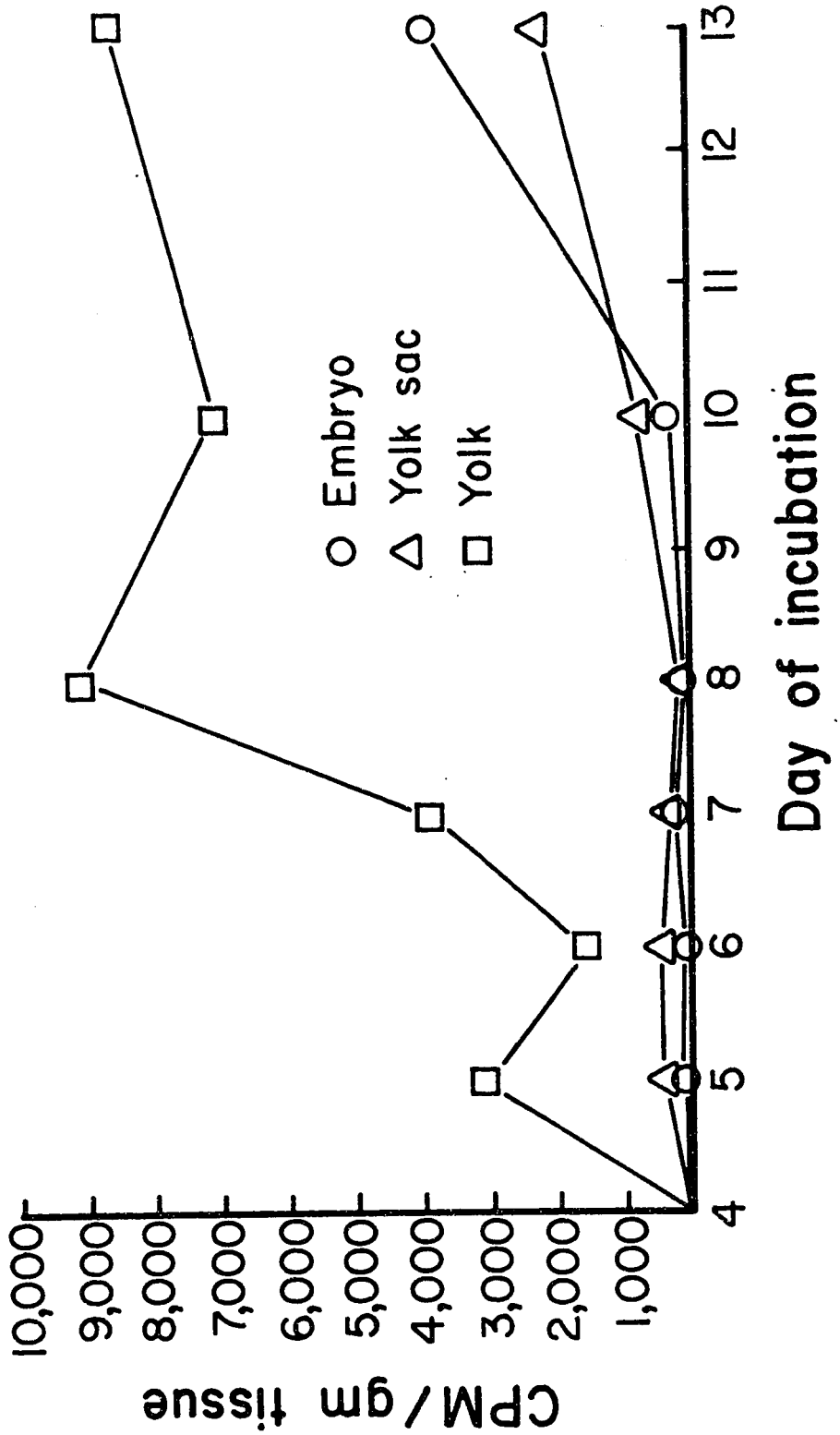

CPM / gm tissue
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
4
5
6
7
8
9
10
11
12
13
Day of incubation
O Embryo
Δ Yolk sac
□ Yolk

Fig. 36. Distribution of malathion-P^{32} per embryo, yolk sac, and yolk after injections of 105.12 mg malathion containing 1 μc of malathion-P^{32} per egg on day 5 of incubation

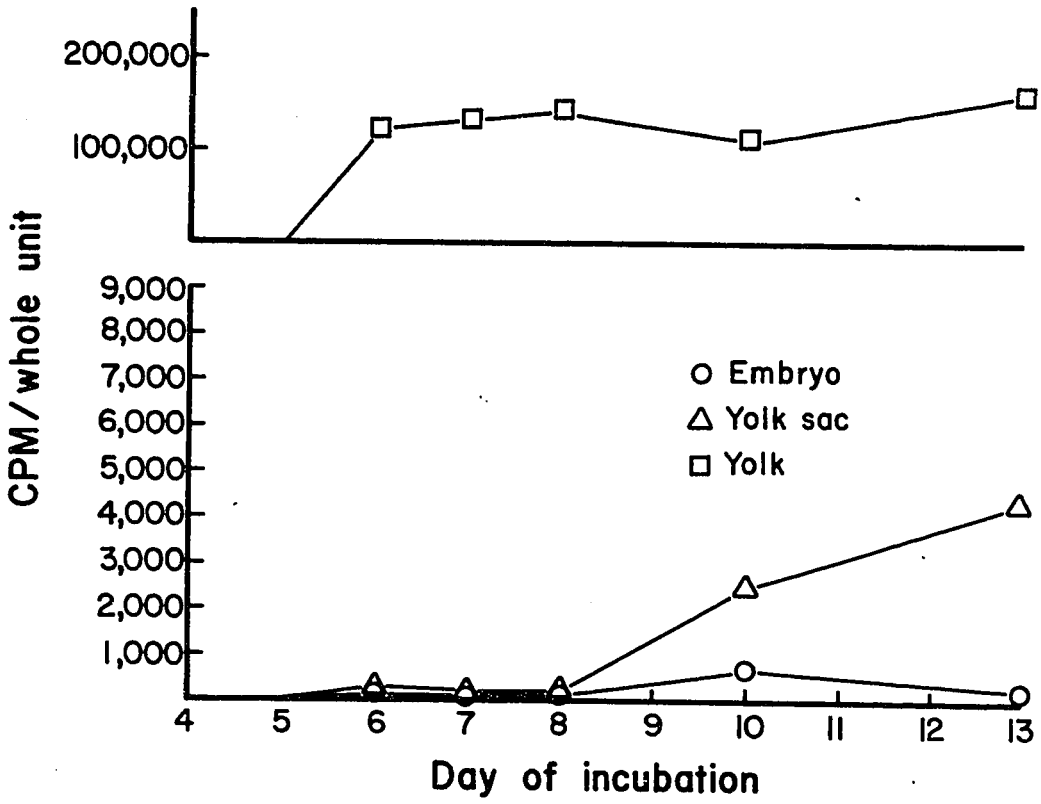

200,000
100,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
CPM/whole unit
O Embryo
△ Yolk sac
□ Yolk
4
5
6
7
8
9
10
11
12
13
Day of incubation

Fig. 37. Distribution of malathion-P^{32} per gram embryo, yolk sac, and yolk after injections of 105.12 mg malathion containing 1μc of malathion-P^{32} per egg on day 5 of incubation

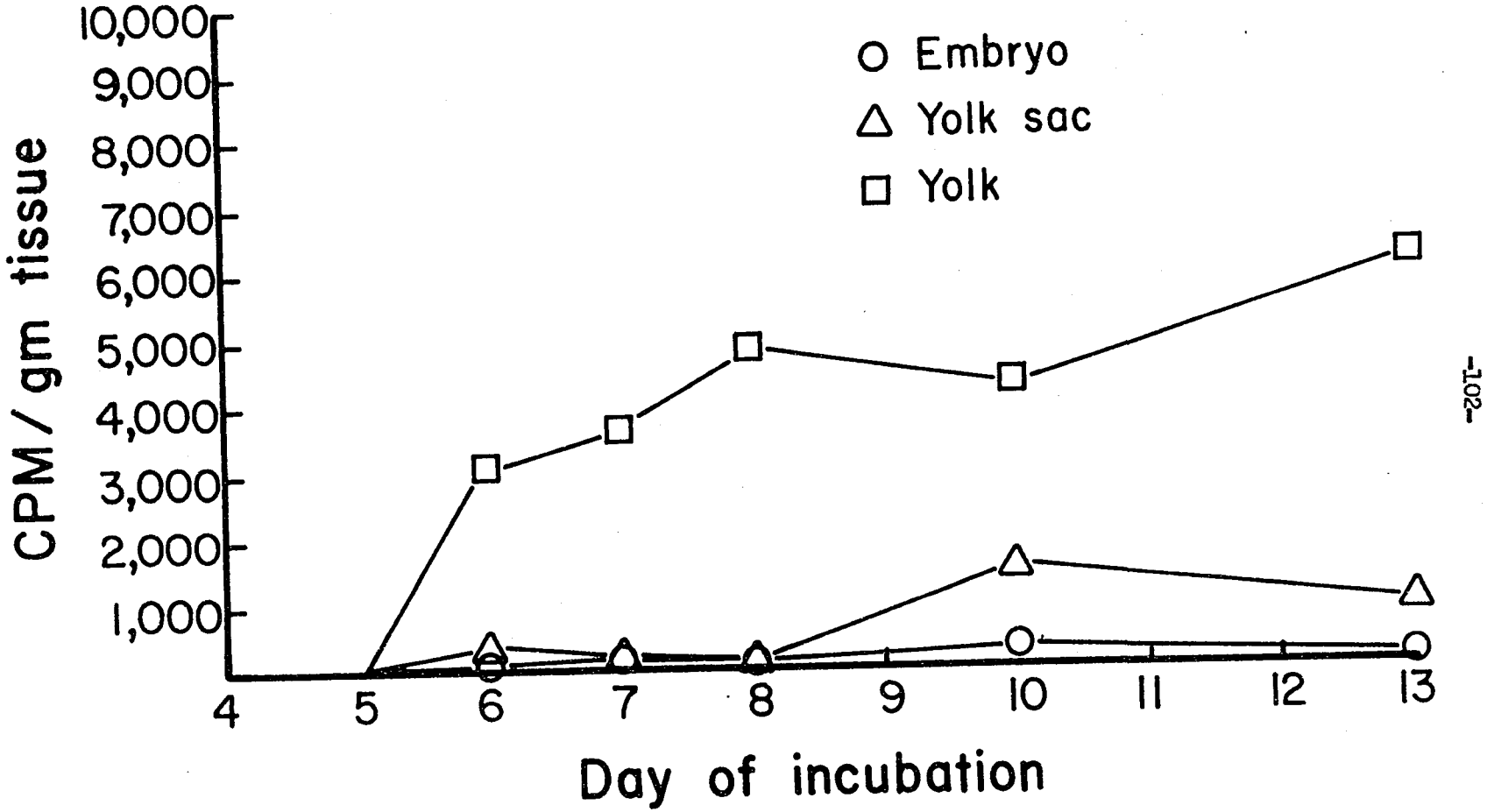

10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
CPM / gm tissue
Embryo
Yolk sac
Yolk
4
5
6
7
8
9
10
11
12
13
Day of incubation

Table 25. Distribution of P^{32} in fertilized chicken eggs after an injection of 105.12 mg malathion containing 1 μc per egg

	Day of incubation and extraction	Average wt (g)	Total vol homogenate (ml)	Average cpm per 0.2 ml[a]	Decay factor	Total cpm per homogenate	Cpm per gram homogenate
Embryo	6—7	0.7685	1.70	25	0.7464	285	371
	6—9	1.5589	2.20	33	0.6448	563	361
	6—13	6.6370	8.60	130	0.5849	9,557	1,500
	9—10	2.0223	3.20	61	0.5849	1,669	825
	10—11	2.9828	5.20	71	0.5571	3,314	1,111
	11—12	4.8588	6.50	160	0.5306	9,800	2,017
	12—13	5.6850	8.60	135	0.5054	11,486	2,020
Yolk-Sac	6—7	1.2887	1.60	303	0.7464	3,248	2,520
	6—9	1.5799	1.80	253	0.6448	3,531	2,235
	6—13	4.6390	3.30	91	0.5849	2,568	554
	9—10	1.9341	2.20	211	0.5849	3,968	2,052
	10—11	1.8164	2.50	249	0.5571	5,588	3,076
	11—12	2.2215	2.60	463	0.5306	11,344	5,106
	12—13	5.0127	3.30	775	0.5054	25,302	5,048
Yolk	6—7	32.0850	31.00	1,392	0.7464	289,060	9,009
	6—9	33.0444	31.90	490	0.6448	121,324	3,672
	6—13	23.4715	22.70	392	0.5849	76,001	3,238
	9—10	26.9488	26.00	211	0.5849	46,968	1,743
	10—11	29.4119	28.40	356	0.5571	90,805	3,087
	11—12	24.1753	23.40	624	0.5306	137,359	5,682
	12—13	24.5685	23.70	1,355	0.5054	318,240	12,953

[a] Average of 5 readings per sample.

This amount increased 4-fold to day 13. The amount of label in the yolk sac membrane decreased 4-fold from day 7 to day 13. The embryos died within 2 days of injections on days 9 to 12 and therefore the uptake could be followed for only 1 day after injection. The CPM per gram embryo injected with 90% malathion-P^{32} on days 9, 10, 11 and 12 and extracted 1 day later, increased from 825 to 2020 respectively. The CPM per gram yolk sac membrane extracted 1 day later were 2052, 3076, 5106 and 5048 respectively.

RNA, DNA and Protein Synthesis

To determine if malathion had any effect on RNA, DNA, or protein synthesis in vivo, we studied the uptake of labelled nucleotides and an amino acid in malathion-treated and untreated chick embryos.

The liquid scintillation system was checked for quenching by counting a known amount of the isotope and then adding a sample of the TCA extract which was solubilized with hydroxide of hyamine. The sample was recounted and the results are shown in Table 26. The CPM are reduced to 8% and 7% efficiency for embryo samples solubilized in hyamine and added to a known amount of tritiated thymidine and uridine respectively. For valine C^{14}, the counts are reduced to 18%. Because of the quenching problem, another system was used whereby the tissue is suspended in a gel made with Triton-X 100 (Patterson and Greene, 1965). A comparison of the 2 scintillation systems is shown in Table 27. The results indicate a large increase in the number of counts for the Triton-X 100 system over the hyamine system. The former system was therefore used to count the samples.

Table 26. The effect of quenching on embryonic tissue extracts solubilized in hydroxide of hyamine[a]

Compound	Counts per minute	Efficiency
Thymidine-H^3 (0.5 μc)	12,400	
Thymidine-H^3 (0.5 μc) + Hyamine sol. tissue	1,093	8%
Uridine-H^3 (0.5 μc)	11,800	
Uridine-H^3 (0.5 μc) + Hyamine sol. tissue	920	7%
Valine-C^{14} (0.1 μc)	57,200	
Valine-C^{14} (0.1 μc) + Hyamine sol. tissue	10,276	18%

[a] 3 samples of each.

Table 27. A comparison of 2 scintillation systems on the uptake of uridine-H^3, thymidine-H^3, or valine-C^{14} in TCA extracts of 7 day chick embryos injected 12 hours before extraction[a]

Sample	Hyamine-liquid system (cpm)	NAOH-Triton X-100 gel system (cpm)	Efficiency (%)
Blank	20	65	—
Uridine-H^3	47	215	18
Thymidine-H^3	40	204	15
Valine-C^{14}	119	561	20

[a] 5 samples of each.

Examination of 15 day embryos from eggs injected on days 4 and 5 with non-radioactive uridine, valine or thymidine revealed no abnormalities, or size decrease. The compounds were considered non toxic since the mortality was no greater than that observed when distilled water was injected (30%).

To determine if any radioactivity was in the TCA-non-precipitable fraction (supernatant), an aliquot of the supernatant of the TCA treated thymidine-H^3 sample was added to the Triton-X 100 mixture. The results are listed in Table 28. The counts were similar to the background. The supernatant fractions of the uridine-H^3 and valine-C^{14} samples were also counted; however, there was also no radioactivity in these fractions.

Table 29 lists the uptake of labelled uridine, thymidine, and valine in both untreated and malathion treated embryos. For the thymidine-H^3 treated embryos, the CPM increased to 4 hours, levelled off at 30 hours and then declined to 96 hours after injection. The uptake of thymidine-H^3 in the malathion treated embryos followed the same pattern. The Student's "t" test was used for the statistical analyses in comparing the effect of malathion. Seven of the 12 values were significantly lower than the untreated embryos. Three values showed no significant difference, and 2 were significantly higher.

Also from Table 29, the CPM for the uridine treated and uridine-malathion treated embryos increased to 6 hours, levelled off to 36 hours and declined to 96 hours after injection. Six of the 12 values for the malathion treated embryos were significantly lower than the untreated, 4 values showed no significant difference, and 2 were significantly higher (Student's "t" test).

Table 28. Determination of radioactivity in the supernatant fraction of TCA extracts after the injection of thymidine-H^3 in malathion treated and untreated 5 day chick embryos

Hours after Injection	Thymidine-H^3 (CPM)	Thymidine-H^3 + Malathion (CPM)
2	63[ab]	60
4	61	60
6	52	65
8	52	64
10	52	48
12	45	48
24	42	62
30	54	65
36	50	74
48	53	43
72	37	42
96	37	41

[a] CPM of blank is 55.

[b] average reading of 5 samples.

TABLE 29. UPTAKE OF URIDINE-H^3, THYMIDINE-H^3, AND VALINE-C^{14} IN MALATHION TREATED AND UNTREATED CHICK EMBRYOS INJECTED INTO THE YOLK SACS OF FERTILE CHICKEN EGGS ON DAY 5 OF INCUBATION

HOURS AFTER INJECTION	THYMIDINE (A)	MALATHION + THYMIDINE (B)	RATIO B/A	URIDINE (A^1)	MALATHION + URIDINE (B^1)	RATIO B^1/A^1	VALINE (A^{11})	MALATHION + VALINE (B^{11})	RATIO B^{11}/A^{11}
2	654[C]±2[D]	321±4*	0.49	762±12	788±29	1.03	136±3	136±5	1.00
4	913±25	800±38**	0.88	857±35	768±22**	0.90	172±2	126±13**	0.73
6	1011±28	820±60**	0.81	1107±42	822±13*	0.74	162±2	147±6*	0.91
8	973±62	1097±41**	1.13	1196±31	982±16*	0.82	150±3	209±6**	1.39
10	1170±78	947±71**	0.81	1189±19	1045±11**	0.88	245±1	282±9	1.15
12	1287±59	960±20*	0.75	1065±65	1026±16	0.96	284±4	257±6**	0.90
24	1180±34	1085±55	0.92	1045±43	1324±93*	1.27	338±4	314±3	0.93
30	1100±123	1011±37	0.92	1012±18	1090±20	1.07	443±2	355±18*	0.80
36	825±14	860±37	1.04	1010±48	909±13**	0.90	459±2	347±2*	0.76
48	977±20	705±12**	0.72	942±17	754±21*	0.80	500±3	535±3	1.07
72	568±20	485±43**	0.85	493±10	469±15	0.95	400±3	330±6*	0.82
96	329±22	424±13*	1.29 / 0.88[E]	294±9	358±18**	1.22 / 0.96	339±2	350±4	1.03 / 0.95

[C] AVERAGE READING OF 5 SAMPLES

[D] STANDARD ERROR

[E] MEAN VALUE

*DIFFERENCE BETWEEN MEANS SIGNIFICANT AT 1% LEVEL; **AT 5% LEVEL (STUDENT'S "T" TEST)

As shown in Table 29, the CPM for the valine treated groups increased to 48 hours, and then declined to 96 hours. Six values of the malathion treated series were significantly lower than the untreated, 1 was significantly higher, and 5 showed no significant differences between the 2 groups (Student's "t" test).

If we combine the values over the 4 day treatment period and take a mean of the ratios of the experimentals/controls (Table 29), we find that for thymidine there was a 12% drop (at 90% confidence limits, the resultant confidence interval ranges from a 4 to a 20% drop); for uridine, there was a 4% drop (at 90% confidence limits, the resultant confidence interval ranges from a 10% drop to no change); for valine, there was a 5% drop (at 90% confidence limits, the resultant confidence interval ranges from a 12% drop to no change). These results indicate a significant drop in DNA synthesis. Also, there is a slight drop in RNA and protein synthesis; however, the values are on the borderline of significance.

Tryptophan Assay

Since tryptophan alleviated the "malathion syndrome", we did a tryptophan determination on embryo and yolk sac homogenates treated with malathion in vivo to ascertain if there was a change in the total tryptophan in the treated tissue. As shown in Table 30, there is a marked decline in the total tryptophan of the embryo on day 6, 7, and 8, after an injection on day 4, and the values were significant at the 5, 1, and 10% levels respectively (Student's "t" test). The yolk sac values were significant at the 5 and 1% levels on days 6 and 7 respectively.

Table 30. Tryptophan assay of 5 to 8 day old chick embryo and yolk sac homogenates treated with malathion in vivo

	micrograms tryptophan per gram wet weight			
	Day 5	Day 6	Day 7	Day 8
Untreated embryos	591.80[a]±23.35[b]	611.66±31.64	626.61±18.96	776.14±33.31
Malathion treated embryos (3.99 mg-day 4)	589.65±22.98	491.87***±22.91	517.93***±19.29	690.24*±24.88
Malathion treated embryos (6.42 mg-day 5)			523.90***±15.83	685.98**±25.50
Untreated yolk sacs	1053.50±19.59	1213.78±42.68	1215.01±36.06	1267.05±38.64
Malathion treated yolk sacs (3.99 mg-day 4)	1026.21±64.95	1067.44**±33.77	1078.53*±59.66	1315.00±49.40
Malathion treated yolk sacs (6.42 mg-day 5)			1114.68*±44.96	1054.03***±41.28

*Difference between means significant at 10% level; **5% level; ***1% level (Student's "t" test).

[a]Mean value. [b]Standard error.

Embryos and yolk sacs of eggs injected on day 5 were assayed for tryptophan on days 7 and 8 of incubation. The day 7 and 8 embryos were significantly lower than the controls at the 1 and 10% levels respectively. The day 8 yolk sacs were significantly lower at the 1% level.

Acetylcholinesterase

The activity of acetylcholinesterase of untreated, malathion-treated, malathion-nicotinamide-treated and malathion-tryptophan-treated embryos is shown in Fig. 38. There is a marked reduction in the activity in all of the malathion treated groups. The number of milli-units per gram embryo drops from over 1700 in the 4 day control embryos to 130, 190 and 297 in day 5 embryos treated with malathion, malathion and nicotinamide, and malathion and tryptophan respectively. That is, the activity remains inhibited by malathion when in the presence of tryptophan or nicotinamide.

Lactate and Malate Dehydrogenases

Table 31 lists the results of the malate (MDH) and lactate (LDH) dehydrogenase assays on 4 to 7 day chick embryos homogenates. The embryos were injected with malathion on days 3, 4, 5, and 6 and removed for analysis 24 hours after injection. The activity of LDH on each of the days tested was similar in both the controls and treated embryos. The differences were not significant at the 5% level (Student's "t" test).

The activity of MDH was lower in the malathion treated embryos on days 4, 6, and 7. These values were significant at the 1% level.

To determine the duration of the effect of malathion on the activity of MDH, the embryos were treated with malathion on day 4 and removed for analysis on days 5, 6, 7, and 8. The results are shown in Table 32. The values were lower in the treated embryos on days 5, 6, and 8 and were

Fig. 38. Acetylcholinesterase assay of embryo homogenates from chicken eggs injected with 3.99 mg malathion on day 4 of incubation

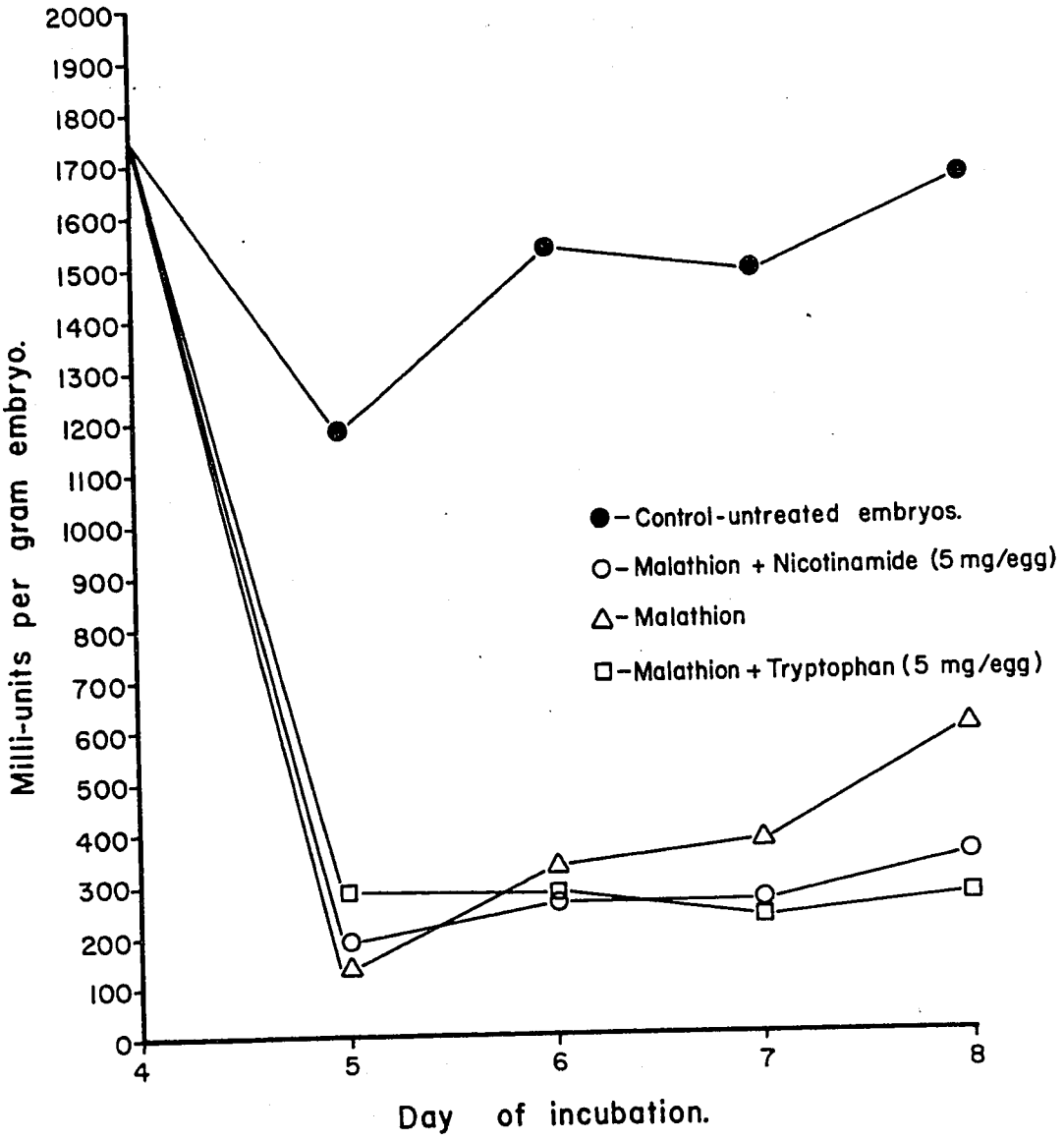

Milli-units per gram embryo.
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
4
5
6
7
8
Day of incubation.
● – Control-untreated embryos.
○ – Malathion + Nicotinamide (5 mg/egg)
△ – Malathion
□ – Malathion + Tryptophan (5 mg/egg)

Table 31. Assay of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) on 4 to 7 day old chick embryo homogenates treated with malathion in vivo[a]

		Units per gram of wet tissue			
		Day 4	Day 5	Day 6	Day 7
LDH	Untreated embryos	25.19[b] ± 0.80[c] 22.12-29.33[d]	27.11 ± 0.68 23.39-30.66	25.42 ± 0.52 20.25-27.13	33.30 ± 1.08 29.23-41.45
	Malathion treated embryos	26.18 ± 1.19 21.03-32.32	26.05 ± 0.49 23.67-29.04	21.92* ± 0.44 19.98-24.29	32.12 ± 1.12 27.33-37.55
MDH	Untreated embryos	55.69 ± 2.54 37.08-64.22	53.81 ± 2.24 44.63-65.56	49.14 ± 1.54 44.15-58.27	60.78 ± 2.38 43.20-68.95
	Malathion treated embryos	45.61 ± 2.14* 29.41-55.66	53.76 ± 1.97 44.99-65.56	35.73 ± 1.59* 29.14-42.12	38.85 ± 2.21* 28.41-50.77

[a] Incubated white leghorn eggs were injected with 3.50 mg malathion in corn oil on day 3, with 3.99 mg on day 4, with 6.42 mg on day 5, and with 8.12 mg on day 6. Ten embryos per group were removed for analysis 24 hours after injection.

[b] Mean value

[c] Standard error

[d] Range

* Significant at 1% level

Table 32. Malic dehydrogenase assay of chick embryo homogenates from fertile chicken eggs injected on day 4 with 3.99 mg malathion and removed for assay at 24 hour intervals

	Units per gram embryo			
	Day 5	Day 6	Day 7	Day 8
Untreated embryos	44.72[a]±1.41[b]	42.92±1.35	47.52±1.87	55.89±2.81
Malathion treated embryos	37.92*± 1.38	39.45**±0.83	48.80±1.75	52.75±2.57

[a] Mean value of 10 embryo homogenates

[b] Standard error

* Significant at 1% level

** Significant at 5% level

significantly lower on days 5 and 6 of incubation.

Alkaline Phosphatase

Table 33 lists the total alkaline phosphatase in embryos treated with malathion on days 3 to 7 of incubation and removed for analysis 24 hours after injection. The activity expressed in milli-units per gram wet weight, was lower in the malathion treated embryos on day 4, and higher on days 5, 6, and 7. The day 4 values were significantly lower at the 5% level and the day 5 values were significantly higher at the 5% level. The days 6 and 7 values were not significantly different than the controls.

Histochemical Determinations

The histochemical reactions revealed no observable differences between the malathion-treated and untreated groups except for acetylcholinesterase and MDH. The reactions for MDH, LDH, and DPN and TPN diaphorases were all strong especially in the kidney tubules, however, there was a slight reduction in the intensity of the MDH stain in the kidney tubules of malathion treated embryos (Fig. 39 and 40). The intensity of the staining for succinate and glucose-6-phosphate dehydrogenases were weaker in both the treated and untreated sections.

Acetylcholinesterase was markedly reduced in the malathion treated sections, especially, in the brain, ganglia and somites (Fig. 41 and 42). Recovery was not apparent up to day 8 since the enzyme was inhibited in all malathion treated sections that were examined (days 5 to 8).

Table 33. Assay of alkaline phosphatase on 4 to 7 day chick embryo homogenates treated with malathion in vivo[a]

	Units per gram embryo			
	Day of incubation			
	4	5	6	7
Untreated embryos	486.65[b] ±29.98[c]	487.89±32.07	332.42±25.84	311.94±25.76
Malathion treated embryos	351.98** ±46.81	620.33** ±47.48	358.96±10.83	353.60±23.06

[a]Incubated white leghorn eggs were injected with 3.50 mg malathion in corn oil on day 3, with 3.99 mg on day 4, with 6.42 mg on day 5, and with 8.12 mg on day 6. Ten embryos per group were removed for analysis 24 hours after injection.

[b]Mean value.

[c]Standard error.

**Significant at 5% level.

Fig. 39. Embryo sections demonstrating lactate dehydrogenase activity in kidney tubules. The lower 2 sections are from an uninjected control embryo and the upper section is from an embryo treated with 6.12 mg malathion on day 5 of incubation. The lower section is from an unstained control. The middle and upper sections have been stained to demonstrate lactate dehydrogenase activity. There is no detectable differences between the 2 upper sections

X 100

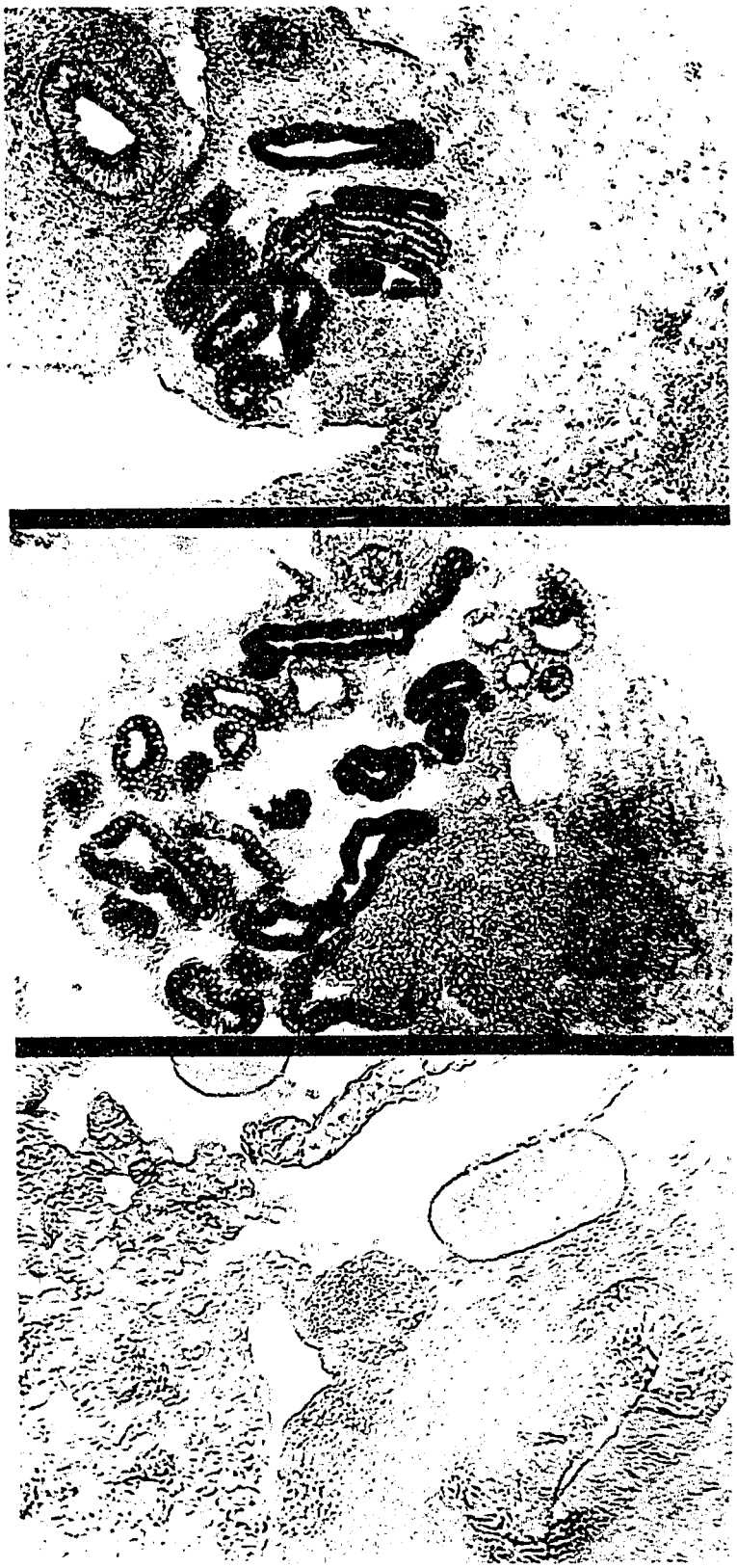


Fig. 40. Embryo sections demonstrating malate dehydrogenase activity in kidney tubules. The upper section is from an uninjected control embryo, and the lower section is from an embryo treated with 6.42 mg malathion on day 5 of incubation. There is a slight reduction in the intensity of the staining in the lower section X 100

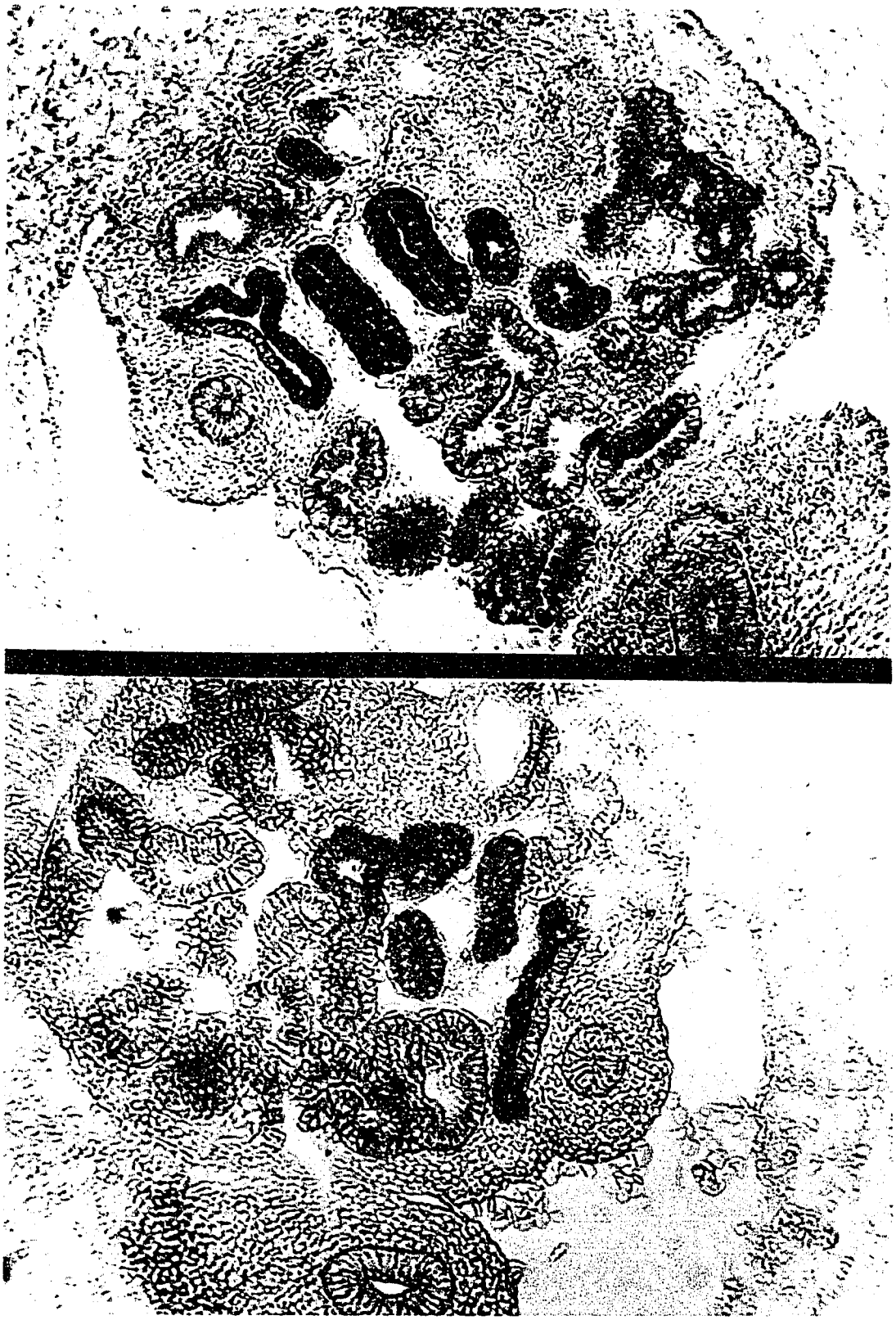


Fig. 41. Embryo sections from embryos demonstrating acetylcholinesterase activity. Note the absence of activity in the malathion treated upper section and the intense staining in the somite of the uninjected control X 400

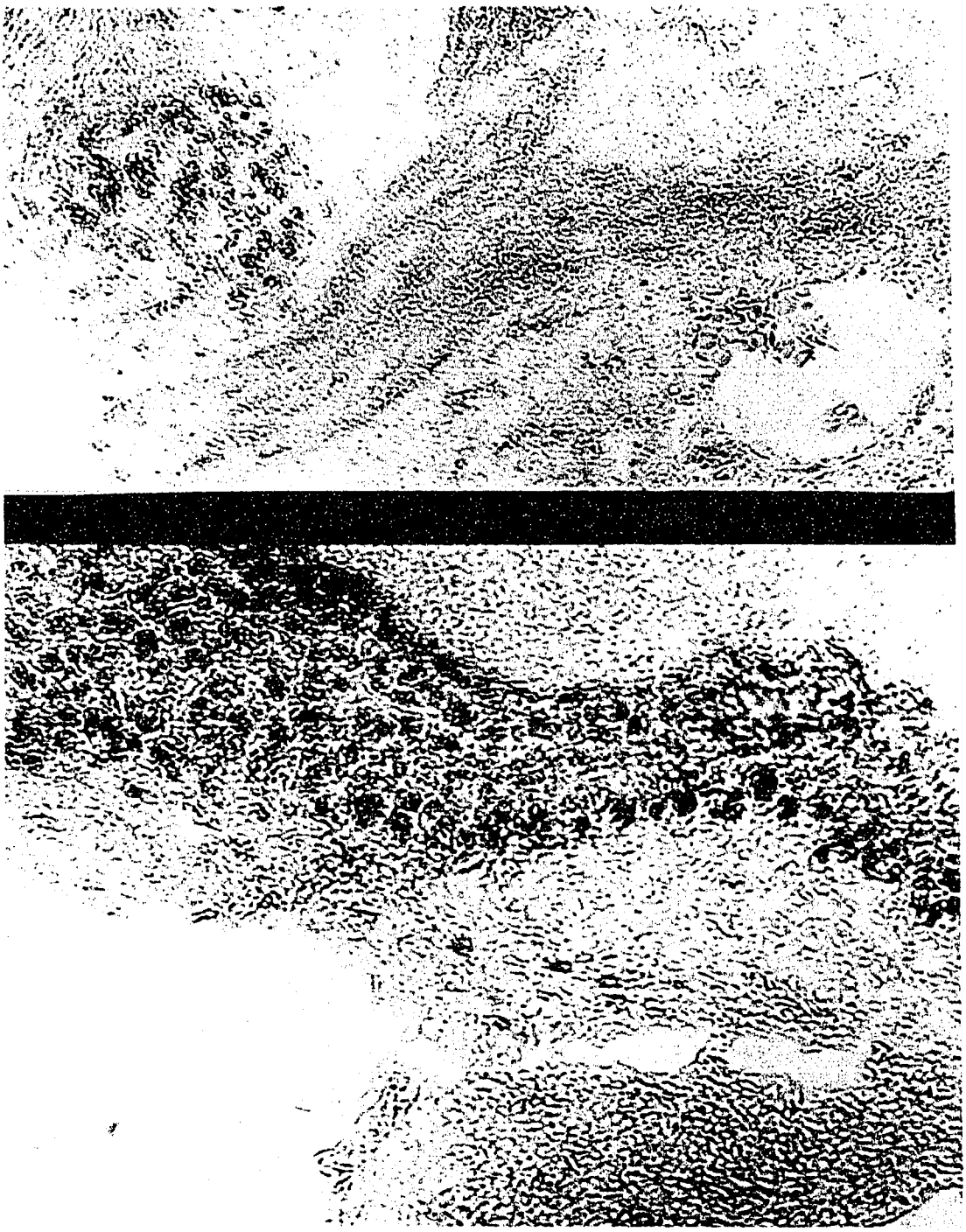
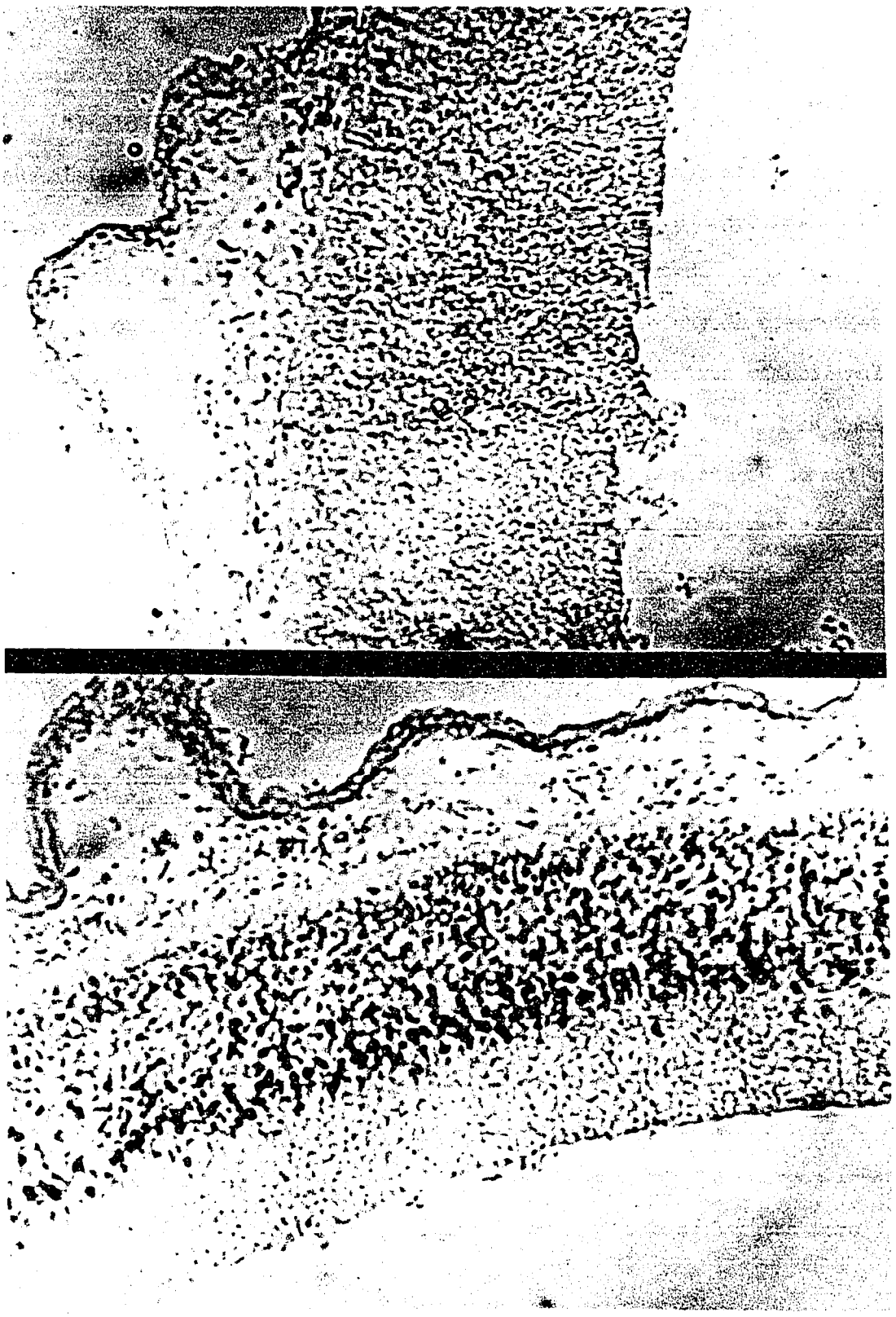


Fig. 42. Embryo sections of the optic lobe demonstrating acetylcholinesterase activity. Note the absence of activity in the malathion treated upper section and the intense staining in the uninjected control X 400



Malathion Breakdown Products and Related Compounds

Table 34 lists the mortality data after injections of mercaptosuccinate, diethylsuccinate, succinate, malonate, diethyl malate and malate on days 4 and 5 of incubation. All of the above compounds were less toxic than malathion at the concentrations injected.

Fifteen embryos of each group were extracted on day 14 of incubation, and examined for gross morphological defects. A comparison of external characteristics of mercaptosuccinate treated embryos are listed in Table 35 and 36. Treatment of 5 mg per egg on day 4 and 5 produced normal looking embryos after gross inspection. Treatment of 20 mg per egg on day 4 produced 2 embryos with skeletal defects (which included shortened maxillae and missing cranial bones) and 1 embryo with short legs. Treatment of 20 mg per egg on day 5 produced normal embryos. Treatment of 30 mg per egg on day 4 produced 5 embryos that were smaller than the controls, however, no other abnormalities were visible. Treatment of 30 mg per egg on day 5 produced 3 smaller embryos, however, no other malformations were apparent.

The weights of the day 4 treated embryos were significantly lower than the controls at the dose levels indicated (Table 35). The body lengths were significantly lower after 20 mg and 30 mg (10% level) treatments and the leg lengths were significantly lower after 5 mg (10% level), 20 mg (5% level) and 30 mg (1% level) treatments. After injections of 30 mg per egg on day 5, the weights and leg lengths of the embryos were significantly lower than the controls, however, the body lengths were not significantly different.

Table 34. Mortality of chick embryos after injections of compounds related to the side chain moiety of malathion[a]

Compound	Amount injected per egg (mg)	Day of injection	Percent mortality[b]
Malathion	3.99	4	54
	6.42	5	48
Mercaptosuccinate	5.00	4	28
		5	24
	20.00	4	34
		5	24
	30.00	4	34
		5	47
Malonic acid	5.00	4	24
		5	11
	30.00	4	23
		5	34
Malic acid	5.00	4	14
		5	14
	30.00	4	22
		5	20
Succinic acid	5.00	4	21
		5	11
	30.00	4	26
		5	24
Diethylsuccinate	5.00	4	27
		5	18
	30.00	4	28
		5	14
Diethylmalate	5.00	4	12
		5	16
	30.00	4	29
		5	22

[a] Each of 50 eggs injected.

[b] Eggs candled 10 days after injection.

Table 35. The effect of mercaptosuccinate on the length of the hind limbs, mandible, and body in chick embryos treated on day 4 of incubation and examined on day 14[a]

Parameter	Control (corn oil)	Malathion 3.99 mg/egg	mercaptosuccinate 5 mg/egg	mercaptosuccinate 20 mg/egg	mercaptosuccinate 30 mg/egg
Weight (g)	10.68±0.19[b]	8.89±0.36	9.66±0.42	8.98±1.06	9.26±0.29
Body length[c] (mm)	65.80±0.07	57.30±0.19	63.90±0.13	61.80±0.09	63.40±0.10
Leg length[d] (mm)	42.00±0.05	28.00±0.23	40.20±0.08	38.60±0.18	38.40±0.10
Mandible length[e] (mm)	10.00±0.01	8.00±0.04	9.70±0.02	9.70±0.03	9.50±0.02

[a] Fifteen embryos examined per group.

[b] Standard error.

[c] Crown to rump length.

[d] From acetabulum to phalanges.

[e] From gape to distal end of bone.

* Significant at 1% level.

** Significant at 5% level.

*** Significant at 10% level.

Table 36. The effect of mercaptosuccinate on the length of the hind limbs, mandible, and body in chick embryos treated on day 5 of incubation and examined on day 14[a]

Parameter	Control (corn oil)	Malathion 6.42 mg/egg	Mercaptosuccinate 5 mg/egg	Mercaptosuccinate 20 mg/egg	Mercaptosuccinate 30 mg/egg
Weight (g)	10.68±0.20[b]	8.36**±0.91	11.00±0.28	10.27±0.22	9.94**±0.23
Body length[c] (mm)	65.80±0.07	58.10*±0.12	66.30±0.08	64.30±0.06	64.40±0.06
Leg length[d] (mm)	42.00±0.05	28.40*±0.11	41.60±0.10	41.30±0.05	40.20***±0.09
Mandible length[e] (mm)	10.00±0.01	8.20***±0.09	10.00±0.01	10.10±0.01	9.80±0.02

[a] Fifteen embryos examined per group.

[b] Standard error.

[c] Crown to rump length.

[d] From acetabulum to phalanges.

[e] From gape to distal end of bone.

* Significant at 1% level.

** Significant at 5% level.

*** Significant at 10% level.

In a second series of experiments using the same injection schedule, 20 eggs of each group were allowed to hatch. Twelve chicks hatched after injections of 5 mg per egg of mercaptosuccinate on days 4 and 5 of incubation. One chick in each group was featherless in the abdominal region only, and 5 chicks treated with 5 mg on day 4 had bleached feathers. None of the embryos hatched after injections of 20 mg per egg on days 4 and 5. The embryos were extracted and examined, and none appeared malformed. After injections of 30 mg per egg on day 4, 1 chick hatched. The extracted embryos appeared smaller than the controls, and 4 had bleached feathers. None of the chicks hatched after injections of 30 mg per egg on day 5, however, the unhatched embryos appeared smaller than the controls and 3 had bleached feathers. All embryos examined on day 14 of incubation appeared normal, however, 3 were lighter in colour than the controls. Also, all embryos examined on day 14 appeared normal following injections of 5 and 30 mg per egg malonate, malate, diethylmalate, succinate, and diethylsuccinate on days 4 and 5 of incubation.

Table 37 lists the mortality data after several malathion breakdown products and standards were injected into fertile eggs on day 5 of incubation. One embryo in each group after injections of malathion monocarboxylic acid (5 mg) and potassium dimethyl phosphorodithioate (10 mg) was malformed. Both of them had 1 eye and a crossed beak. None of the other compounds which included malathion dicarboxylic acid, O-desmethyl malathion potassium salt, potassium dimethyl phosphorothioate, and sodium dimethyl phosphate were teratogenic at the dose levels administered.

Fifteen eggs of each group were allowed to hatch. The percent hatch

Table 37. Mortality of chick embryos after injections of malathion breakdown products and malathion standards into fertile eggs on day 5 of incubation[a]

Compound	Amount injected per egg (mg)	Percent hatch	Percent mortality[b]
Malathion monocarboxylic acid	5	86	14
	10	94	27
Malathion dicarboxylic acid	5	63	23
	10	77	12
O-desmethyl potassium salt	5	84	11
	10	63	20
Potassium dimethyl phosphorodithioate	5	70	8
	10	70	20
Potassium dimethyl phosphorothioate	5	77	18
	10	56	16
Sodium dimethyl phosphate	5	84	6
	10	77	16
Malaoxon	5	--	77
	1	--	64
	0.1	--	14
Malathion technical-95% (1968)	6.42	--	57
Malathion technical-95% Secondary standard	6.42	--	64
Malthion primary standard 99.3%	6.42	--	56

[a] Each of 30 eggs injected.

[b] Eggs candled 10 days after injection.

is listed in Table 37. Two embryos were malformed after injections of 5 mg per egg sodium dimethyl phosphate; 1 had an enlarged head and another lacked the left eye. Three embryos had sparse feathers after injections of 10 mg per egg malathion dicarboxylic acid and 3 embryos had bleached feathers after injections of 5 mg O-desmethyl malathion potassium salt.

After injection of malaoxon into fertile eggs on day 5 of incubation, the embryos exhibited the "malathion syndrome", however, the mortality was higher than with malathion. When malathion primary and secondary standards were injected into 5 day fertile eggs, the malformations were similar to those obtained with malathion technical grade (1968) used throughout this work.

Modifying Agents

Table 38 compares the effects of the injection of a second compound with that of malathion only. Of the amino acids, glycine prevented the malformations in 44%, but the embryos remained stunted. Tryptophan, on the other hand, not only prevented the malformations but also prevented the growth inhibition (Fig. 43), that is, the embryos appeared normal in all respects. Of the other compounds tested, quinolinic acid, nicotinic acid and nicotinamide prevented malformations in up to 92% of the embryos depending on the compounds used, but none were effective in preventing growth retardation. In 2 of 4 experiments, indole was effective in preventing malformations, however, in the other 2, it either had no effect or appeared to act synergistically with malathion.

Measurements of external characteristics of corn oil controls, malathion treated, malathion-nicotinamide treated, and malathion-tryptophan

Table 38. Percent malformations and growth retardation in 15 day old chick embryos injected on day 5 of incubation with malathion and various other compounds[a]

Treatment (0.025mmoles per egg except where indicated)	Percent malformations	Growth retardation
Malathion (Mal-6.42mg per egg)	80	Yes
Alanine	0	No
Ala + Mal	84	Yes
Glycine	0	No
Gly + Mal	44	Yes
Valine	0	No
Val + Mal	78	Yes
Leucine	0	No
Leu + Mal	80	Yes
Isoleucine	0	No
Iso + Mal	84	Yes
Sereine	0	No
Ser + Mal	80	Yes
Cysteine	0	No
Cys + Mal	90	Yes
Methionine	0	No
Meth + Mal	80	Yes
Glutamic Acid	0	No
Glu + Mal	78	Yes
Lysine	0	No
Lys + Mal	68	Yes
Arginine	0	No
Arg + Mal	76	Yes
Histidine	0	No
His + Mal	80	Yes

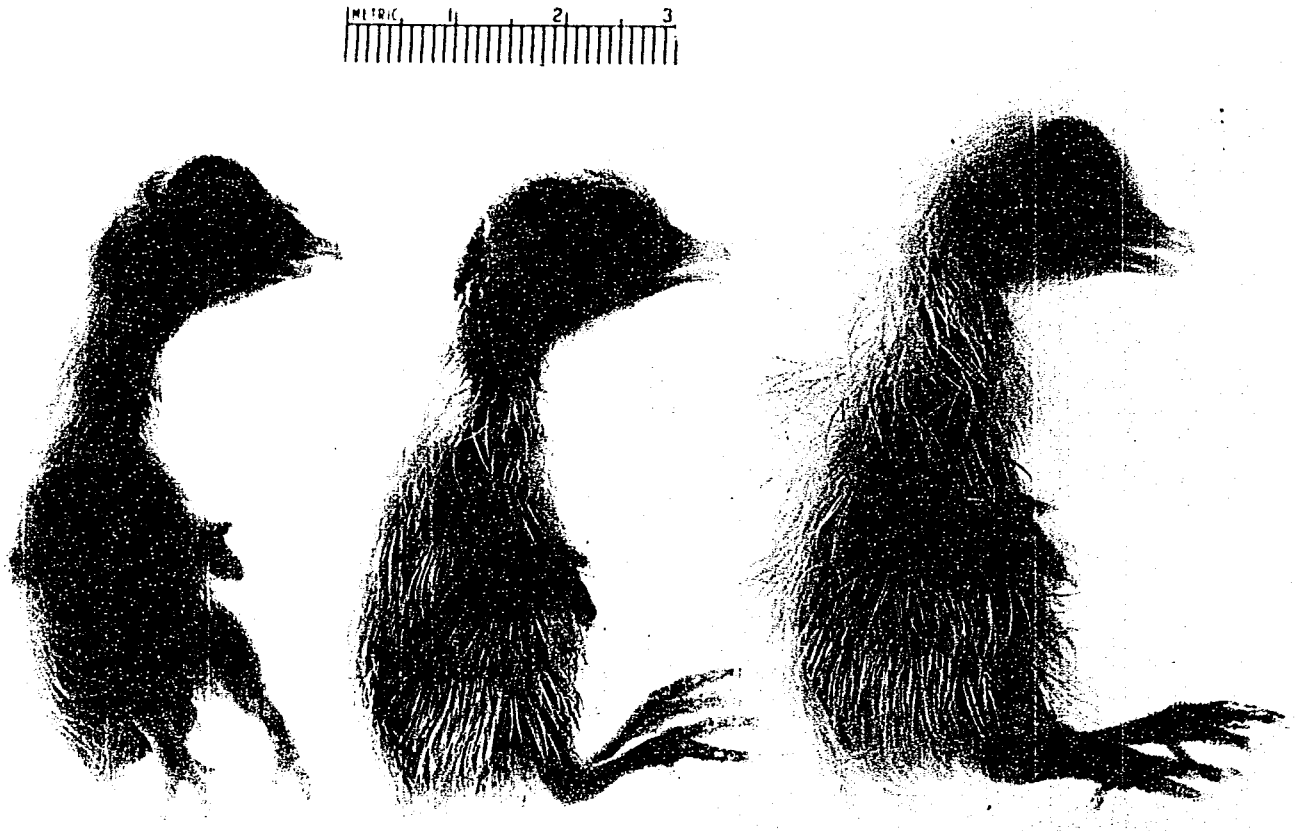
[a] 25 Embryos of each group were examined.

Table 38. Cont'd

Treatment (0.025mmoles per egg except where indicated)	Percent malformations	Growth retardation
Tryptophan	0	No
Try + Mal	0	No
Phenylalanine	0	No
Phe + Mal	84	Yes
Tyrosine	0	No
Tyr + Mal	80	Yes
Proline	0	No
Pro + Mal	68	Yes
Thiamine HCl	0	No
Thi + Mal	80	Yes
Anthranilic Acid	0	No
Anth. A. + Mal	76	Yes
Quinolinic Acid	0	No
Quin. A. + Mal	5	Yes
Serotonin Creatine Sulfate	0	No
Ser. Cr. Sul. + Mal	80	Yes
Indoleacetic Acid	0	No
Ind. Ac. A. + Mal	80	Yes
Vitamin A	0	No
Vitamin A + Mal	82	Yes
Riboflavin	0	No
Rib + Mal	80	Yes
Indole	0	No
Indole + Mal[b]	6(82)	Yes
Nicotinamide (5mg/egg)	0	No
Nic + Mal	10	Yes
Nicotinic Acid (5mg/egg)	0	No
Nic. A. + Mal	8	Yes

[b] In 2 of 4 experiments of 25 embryos each, indole counteracted the effect of malathion while in 2 other experiments there was no counteraction but rather a synergistic effect.

Fig. 43. Photograph of 15 day chick embryos. The left chick is from an egg treated with malathion, the middle chick is from an egg treated with malathion and nicotinamide, and the right chick is from an egg treated with malathion and tryptophan



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treated animals are listed in Table 39. In the malathion treated and malathion-nicotinamide treated groups, the body lengths and gross weights were similar but were less than the corn oil controls. The leg lengths were shorter and malformed in the malathion treated animals while the legs of those treated with malathion and nicotinamide appeared proportional to the size of the animal.

Table 40 lists the degree of malformations produced by compounds with k-values (measure of energy levels of molecules) which are in the same range as tryptophan. The embryos were not only malformed, but the degree of malformations were greater than the malathion controls (Fig. 44 and 45). The animals were smaller, their legs short and twisted, and in some instances the digits, tibiotarsus, and tarsometatarsus bones were absent, frequently on one side only. In others, a hematoma in the fore or hind limbs prevented the formation of digits. Exteriorization of the viscera and edema were also common features.

Insulin Injections

Table 41 lists the mortality data of chick embryos after insulin injections on days 4 and 5 of incubation. The mortality was in the same range as for malathion injections except for protamine zinc insulin which was higher on both days of injection.

Fifteen embryos of each group were examined for gross morphological defects on day 14 of incubation. After injections of Toronto insulin on day 4, 13 embryos had no apparent malformations; however, they were smaller than the controls. One embryo lacked the tarsometatarsus bone and digits from the right hind limb, and another had a shorter upper beak

Table 39. The effect of simultaneous injections of malathion and nicotinamide or tryptophan on the length of the hind limbs body and mandible in chick embryos treated on day 5 and examined on day 15 of incubation

Treatment		Control (corn oil)	Malathion 6.42 mg per egg on day 5	Malathion 6.42 mg and nicotinamide 5 mg per egg on day 5	Malathion 6.42 mg and tryptophan 5 mg per egg on day 5
No. of eggs per group		25	25	25	25
Weight (g)		11.51 ± 0.14[a]	8.84 ± 0.18	8.66 ± 0.14	12.08 ± 0.19
	Range	10.00-12.90	6.40-10.20	7.20-10.00	10.50-14.20
Body Length[b]		67.30 ± 0.37	60.20 ± 0.45	57.50 ± 0.61	64.68 ± 0.54
	Range	64.00-71.00	56.00-65.00	53.00-62.00	62.00-70.00
Leg Length[c] (mm)		43.80 ± 0.25	25.30 ± 1.06	38.64 ± 0.33	45.44 ± 0.55
	Range	41.00-45.00	9.00-35.00	37.00-42.00	40.00-50.00
Mandible[d] Length (mm)		10.90 ± 0.07	8.00 ± 0.19	9.28 ± 0.18	10.96 ± 0.15
	Range	10.00-11.00	5.00-9.00	8.00-11.00	10.00-12.00

[a] Standard error.

[b] Crown to rump length.

[c] From acetabulum to phalanges.

[d] From gape to distal end of bone.

Table 40. Degree of malformations in 15 day old chick embryos injected with malathion and other compounds with "k-values" in the same range as tryptophan [a]

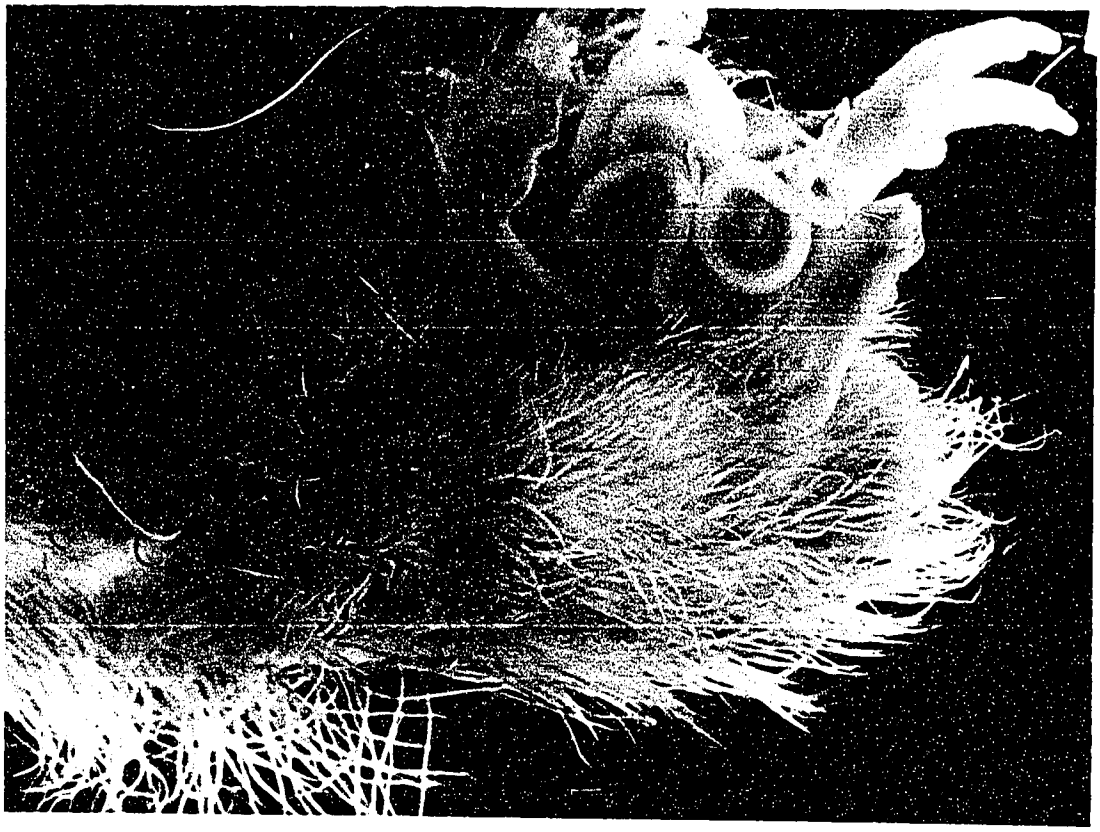
Treatment (mg per egg)	K-Values: Highest occupied molecular orbital	K-Values: Lowest empty molecular orbital	Degree of malformations [b]
Malathion (6.42)			+
Tryptophan (5)	0.534	-0.863	-
Try + Mal			-
Indole (2.5)	0.534	-0.863	-
Indole + Mal			+++; - [c]
Indoleacetic acid (5,2.5)	0.479	-0.863	-
Ind. Ac. A. + Mal			++++
Adenine (2.5, 1, 0.5)	0.486	-0.865	-
Adenine + Mal			+++
Guanine (1, 0.5)	0.307	-1.050	-
Guanine + Mal			+++
α-naphthol (2.5, 1, 0.5)	0.519	-0.671	-
α-naphthol + Mal			++++
β-naphthol (2.5, 1, 0.5)	0.569	-0.637	-
β-naphthol + Mal			+++
Quinoline (1, 0.5)	0.770	-0.440	-
Quinoline + Mal			++
Imidazole (2.5, 1)	0.660	-1.160	-
Imidazole + Mal			+

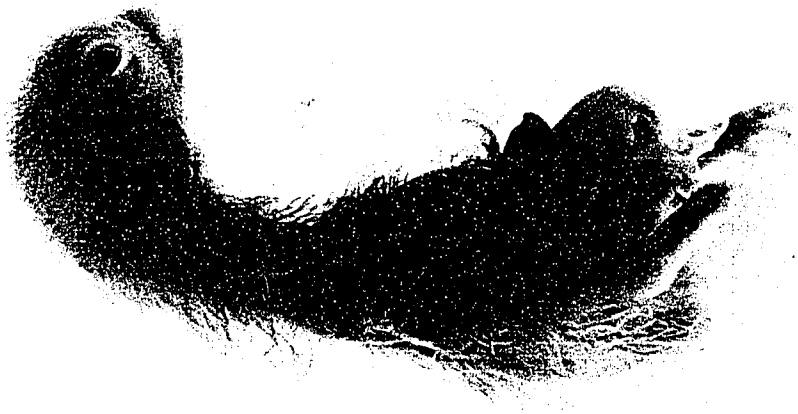
[a] 15 embryos of each group were examined for malformations on day 15 of incubation.

[b] + signifies the degree of malformations with malathion alone. Increasing plus signs signifies greater degrees of malformations - signifies no visible malformations.

[c] In 2 of 4 experiments embryos treated with indole and malathion were synergistically malformed, while in 2 other experiments indole prevented malformations.

Fig. 44. Photograph of a 15 day old chick embryo treated with malathion and indoleacetic acid on day 4 of incubation. The left view shows the entire embryo. Note the lack of digits on the right leg, the abdominal hernia, and the shortened beak. The right view illustrates a hematoma in the right wing in place of digits, as well as exteriorization of the viscera





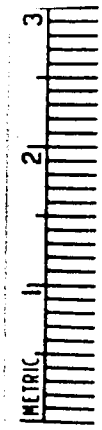

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Fig. 45. Photograph of 15 day old chick embryos. The left chick is from an uninjected egg. The second from left chick is from a malathion and guanine injected egg, the third from left chick is from an egg treated with malathion and α-naphthol, and the right chick is from an egg treated with malathion and adenine

Table 41. Mortality of chick embryos after injection of insulin into 4 and 5 day old incubated chicken eggs

Compound	Amount injected per egg	Day of injection	Percent mortality[b]
Insulin "Toronto"	4	4	53[a]
	4	5	60
Insulin "Lilly" (Illetin)	4	4	46
	4	5	57
Insulin crystals "Sigma"	5	4	47
	5	5	58
Protamine zinc insulin	4	4	70
	4	5	66

[a] Average value 3 experimental runs of 30 eggs each.

[b] Eggs candled 10 days after injection.

and was about half the normal size. All embryos appeared normal after Toronto insulin injections on day 5. After injections of Illetin on day 4, 13 embryos were free of visible malformations, and 2 had short hind limbs. After day 5 injections, 12 were free of visible malformations, and 3 had short hind limbs which were outstretched in a horizontal plane. None of the embryos developed malformations after injections of protamine zinc insulin or insulin crystals into day 4 or 5 incubated eggs.

Some eggs that were treated with each of the 4 insulins were allowed to hatch. More than 80% of the eggs hatched. None of the hatched chicks were abnormal; however, some of the Illetin treated embryos were smaller than the controls.

Duration of Reversal of Malathion Syndrome

To determine up to what day of incubation we could reverse the malathion-induced teratisms, we injected nicotinamide, quinolinic acid, or tryptophan at daily intervals from day 5 to day 9, into eggs pre-injected on day 5 with 6.42 mg malathion per egg. The results of these experiments are listed in Tables 42 to 44.

The reversal of the syndrome by quinolinic acid can occur after an injection of quinolinic acid up to day 8 of incubation. After this interval, reversal can still occur, however, 25% of the embryos were malformed on day 8 and the number malformed increased to day 10. An injection of quinolinic acid on day 11 or 12 cannot reverse the "malathion syndrome".

Nicotinamide can reverse the "malathion syndrome" up to day 9 of incubation. An injection of nicotinamide on day 12 has no alleviating

Table 42. Reversal of malathion-induced teratisms after injections of 5 mg nicotinamide at 24 hour intervals into fertile chicken eggs previously injected with 6% malathion on day 5 of incubation[a]

Compound(s) injected	Percent mortality	Malathion syndrome (percent viable embryos)	Other abnormalities including abdominal edema, neck blisters, and exteriorization of viscera
Malathion	42	88	10
Nicotinamide	28	0	0
Malathion+nicotinamide injected on day 5	64	0	9
Malathion+nicotinamide injected on day 6	60	0	18
Malathion+nicotinamide injected on day 7	27	0	22
Malathion+nicotinamide injected on day 8	47	4	16
Malathion+nicotinamide injected on day 9	62	10	6
Malathion+nicotinamide injected on day 10	60	38	0
Malathion+nicotinamide injected on day 11	34	77	0
Malathion+nicotinamide injected on day 12	22	90	4

[a] Each of 50 eggs injected.

Table 43. Reversal of malathion-induced teratisms after injections of 5 mg quinolinic acid at 24 hour intervals into fertile chicken eggs previously injected with 6% malathion on day 5 of incubation[a]

Compound(s) injected	Percent mortality	Malathion syndrome (percent viable embryos)	Other abnormalities including abdominal edema, neck blister, and exteriorization of viscera
Malathion	48	84	12
Quinolinic acid	28	0	0
Malathion+quinolinic acid injected on day 5	62	0	4
Malathion+quinolinic acid injected on day 6	60	4	4
Malathion+quinolinic acid injected on day 7	58	0	2
Malathion+quinolinic acid injected on day 8	52	25	6
Malathion+quinolinic acid injected on day 9	58	56	2
Malathion+quinolinic acid injected on day 10	69	65	0
Malathion+quinolinic acid injected on day 11	60	90	0
Malathion+quinolinic acid injected on day 12	42	92	0

[a] Each of 50 eggs injected.

Table 44. Reversal of malathion-induced teratisms after injections of 5 mg tryptophan per egg at 24 hour intervals into fertile chicken eggs previously injected with 6% malathion on day 5 of incubation[a]

Compound(s) injected	Percent mortality	Malathion syndrome (percent viable embryos)	Other abnormalities including abdominal edema, neck blisters and exteriorization of viscera
Malathion	58	86	8
Tryptophan	60	0	0
Malathion + tryptophan injected on day 5	74	0	8
Malathion + tryptophan injected on day 6	68	4	6
Malathion + tryptophan injected on day 7	52	6	2
Malathion + tryptophan injected on day 8	54	18	2
Malathion + tryptophan injected on day 9	40	35	4
Malthion + tryptophan injected on day 10	30	66	2
Malathion + tryptophan injected on day 11	32	78	6
Malathion + tryptophan injected on day 12	30	88	10

[a]Each of 50 eggs injected.

effect on the "malathion syndrome".

Tryptophan can reverse the syndrome up to day 8 of incubation. After this time, the syndrome can still be reversed but a greater number of malformed chicks are produced. An injection of tryptophan on day 12 has no alleviating effect on the "malathion syndrome".

APPENDIX II - RESULTS

Autoradiography

Paraffin sections from embryos treated on days 4 and 5 with 3.99 and 6.42 mg malathion in corn oil were coated with emulsion, stained, and examined for radioactivity. Test boxes of slides were developed at 1 week intervals, and the maximum activity was observed after 2 weeks of exposure.

Slides from embryos injected with malathion-P^{32} on day 4 and removed on day 5 were examined for the presence of black silver grains. Some activity was observed in the brain (cerebral hemispheres), retina, nerve cord, gut, and in the mesenchyme near the dorsal aorta. In all areas mentioned, the activity was extracellular, just a few areas in each tissue were observed, and in general, no particular pattern was evident.

Slides from embryos injected with malathion-P^{32} on day 4 and removed on day 8 had active areas in the brain tissue and around the eye. Some grains were present in the maxillary cartilage and in the connective tissue around the notochord; the liver, kidneys and heart tissues were devoid of activity.

Slides from embryos injected with malathion-P^{32} on day 4 and removed on day 10 had active areas in the brain, perichondrium, muscle tissue, and around the eye. In the mid-body regions, some active areas were observed in the nerve cord, and in the cartilages around it. Some tracks were seen emanating from 2 cartilage cells.

Slides from embryos injected with malathion-P^{32} on day 5 and removed on day 6, had active areas in the brain, mesenchyme, and in a few RBC's in the dorsal aorta. Some activity was observed in the neural tube, in the heart tissue and in the gut. Silver grains were observed emanating from cartilage cells in the limb buds.

Slides from embryos injected on day 5 and exposed to malathion-P^{32} for 3 days had active areas in the brain tissue, especially along the inner lining of the diencephalon and hemispheres. Some active areas were found in the nerve cord, in cartilages around it, and in the muscles of the limb buds.

Slides from embryos injected on day 5 and exposed to malathion-P^{32} for 5 days were examined for radioactivity. Silver grains were observed in extracellular regions of cartilages and muscles. Some activity was seen emanating from mesenchyme cells, and from ganglion cell bodies. Some activity was also found in the dermis of the skin at the site of the feather germs. Some activity was found in the liver, nerve cord and in kidney tubules.

In general, the radioactivity in both the days 4 and 5 injected embryos was extracellular, and only a few active areas in each organ or region was apparent. Also, there was no particular pattern of distribution.

DISCUSSION

The chicken egg has been used as a tool in testing the effects of chemicals since 1893 (Féré, 1893). Chemicals can be injected into the air cell, the chorioallantoic membrane, or the yolk. All the injections, in this study, were made directly into the yolk. To understand the connection between the yolk and the embryo, a brief discussion of the relationship of the various parts of the chicken egg follows.

The yolk is an emulsion consisting of a proteinaceous continuous phase and a dispersed phase made up of yolk spheres (4-50 μ diameter) and free lipid droplets (2 μ diameter) (Bellairs, 1961). The early embryo obtains nutrients by the phagocytosis of yolk particles by cells of the blastoderm. This process ceases after 2 days, and the embryo is then nourished by the yolk sac circulation. The epithelium of the yolk sac absorbs yolk and becomes the nutritive link between the embryo and its environment.

After 4 days of incubation, the yolk sac covers half the surface of the yolk and by 9 days of incubation, it covers almost the entire surface, except for the communication between it and the albumen sac (Lillie, 1952). Furthermore, the lining of the yolk sac is interrupted by septa projecting into the yolk; these folds have capillary networks in each part. At the end of incubation, the septa develop to the point whereby they divide the yolk sac into separate compartments each filled with yolk. Water soluble substances are moderately soluble in yolk whereas lipid soluble substances are not. The behaviour of oil in yolk occurs by gradual coalescence and growth of the droplets forming a system from which the oil does not separate (Romanoff and Romanoff, 1949). The behaviour of

malathion in yolk differs depending on the concentration used (Walker, 1967). Walker has shown that mixtures containing 80% or more vegetable oil rose rapidly and remained just under the yolk membranes while mixtures containing less than 80% vegetable oil sank to the bottom of the yolk sac.

In our work, after an injection of 25% or less malathion in corn oil, the mortality was dose and age dependent, and death occurred soon after injection. This is due (as was the case with Walker's results) to the fact that the malathion and corn oil suspensions rose to the top of the yolk to come in close proximity or in contact with the developing embryo. Thus, there is little in the way of a barrier to the compounds getting into the embryo. Both the 50% and undiluted malathion were lethal, but death occurred at a much later period of incubation than with lower concentrations. The immediate and latent death, after injections of various concentrations of malathion, was the result of the behaviour of malathion in the yolk. The density of malathion (1.23) is much greater than the yolk (1.035). Undiluted malathion therefore sank to the bottom of the yolk, not only away from the embryonal area, but removed from any vascular supply. This would account for the deaths at the end of the incubation period for injections on days 1 to 8. However, as the density of the solution approaches the density of the yolk ($\leq$25%), the malathion would be at or near the embryonal area soon after injection and would account for the early deaths. The toxicity of malathion, therefore, depends on the amount reaching the embryo which is dependent on

the density of the injected material, and the density of the injected compounds approaches the density of the yolk as more oil is mixed with the insecticide.

When 50% or undiluted malathion is injected on days 9 to 12, death occurs soon after the injection. This is due to the increased surface area of the yolk sac with its more abundantly developed vascular supply after day 8, and allows for easier access to the embryo so that when an injection is made on days 8 to 12, most of the embryos die within a few days after injection. Those injected prior to day 8 died at later stages of development indicating that in small amounts and at slow penetration, malathion is not immediately toxic to the embryo. Another explanation may be that malathion is degraded or detoxified in the yolk; however, in spite of the fact that the metabolism of malathion has never been elucidated biochemically in the chick embryo, none of the known breakdown products of malathion (as determined in mammals) were toxic or teratogenic to the chick embryo, thus, we are inclined to reject this explanation.

Dose levels which cause 50% mortality when injected on days 4 and 5 cause the "malathion syndrome" (Greenberg and LaHam, 1969). At a dose level which causes a greater than 50% mortality, the survivors are more severely malformed suggesting that more of the cells of the particular organ(s)

are affected. Many tiny embryos (one-quarter of the normal size) are produced at these higher dose levels indicating overall reduction in organ and tissue growth.

The only abnormalities that occurred after injections with corn oil, distilled water, or 1% malathion were hemorrhagic areas, abdominal edema, or neck blisters. The remaining deaths were of an undetermined nature. Injections of 3.99 mg per egg malathion on days 1 to 3 had no apparent effect; however, on day 4, the survivors had congenital defects to the hind limbs, beaks, and feathers, as well as overall size reduction. These abnormalities were also evident after an injection of 6.42 mg malathion on day 5. Micromelia of the hind limbs was the main lesion. The organs affected are related to structures of mesodermal origin.

In the developing embryo, at approximately 50 to 55 hours (Stage 15), the limb primordia are flat undifferentiated areas of mesoderm. By $3\frac{1}{2}$ to 4 days (Stage 21) the leg buds are distinct and are larger than the wing buds. By 4 to $4\frac{1}{2}$ days (Stage 24) the limb buds are distinctly longer than wide, and at 5 days (Stage 26) the first 3 toes are demarcated. By $5\frac{1}{2}$ to 6 days (Stage 28) the limbs have developed into well defined structures.

Cartilage formation begins at $5\frac{1}{2}$ days and is well advanced by 6 or 7 days of incubation. Osteogenesis of the long bones in the chick involves 4 stages. The first stage concerns perichondral bone formation whereby the perichondrium deposits a bony ring near the center of the cartilage; this extends towards the ends of the cartilage forming a hollow cylinder. By the time ossification commences the diaphysis (which is the middle of

the shaft of cartilage) has stopped growing; however, the ends of the bone continue to increase in diameter. The second stage involves absorption of cartilage. Erosion of cartilage begins about day 10 in the middle of the diaphysis and leads to the formation of the marrow cavity. By day 15, the cartilage of the middle third of the diaphysis is removed. The third stage involves calcification of cartilage. Calcium salts are deposited in the cartilage matrix immediately beneath the periosteium near the extremities of the diaphysis. The last stage involves endochondral bone formation which occurs only in the epiphysis and the distal 1/6 of the long bones in birds where formation begins later and less extensively than in mammals. The formation of endochondral bone begins late in the incubation period or after hatching. It involves the deposition of bone on the surface of rays of eroded cartilage within the marrow cavity. Osteoblasts then form layers of bone around the remaining cartilages. Injections on days 4 and 5, therefore, precede cartilage formation. Injections on days 4 and 5 cause feather reduction and limb defects while injections after day 5 result in feather reduction without limb defects. By day 6 and 7 (Stage 30) there are 2 dorsal rows of feather germs on either side of the spinal cord at the brachial level and 3 rows on the legs. By day 10, the flight feathers are evident and the coverts are visible in the wing web. As the feather primordia develop, they sink into the skin so that at the end of incubation, each primordium is located in a follicle. Each primordium undergoes 3 stages of development; the first is down feathers, the second is juvenile feathers and third is adult feathers. The down feathers originate from the dermis of

the skin. By day 8 or 9, this growth causes the dermis to grow outward forming a cap. The net result is an epidermal cylinder filled with a mesodermal core. Down feathers cease to grow after day 13, however, cornification continues. At day 15, the juvenile contour feathers start to develop at the base of the follicle under the down feathers.

Hydrocephaly occurred after injections on days 9 to 12 with 25% (or less) malathion and was not apparent after injections on days 1 to 9. Hydrocephaly was evident after injections of 50% and undiluted malathion on days 1 to 9 of incubation. This occurrence is probably because of slow penetration of the heavy material retarding its entry into the embryo to any great extent until day 9. It appears that the insult to the embryo by malathion on day 9 or later results in hydrocephaly. This condition was characterized by a gelatinous substance contained in embryos with enlarged heads.

The "malathion syndrome" was consistent and reproducible. Long bone-beak malformations occurred after the injection of fertile chicken eggs on days 4 to 7 with sulfanilamide (Ancel, 1950; Zwilling and DeBell, 1950), thallium (Karnofsky, Ridgway, and Patterson, 1950), eserine, insulin, boric acid and pilocarpine (Landauer, 1954). The effects of these chemicals on chick embryos differed from the "malathion syndrome" in the following ways:

1. In the malathion experiments, chicks with micromelia also had beak defects; however, with insulin about half of those with micromelia had beak defects.

2. After the malathion treatment on days 6 and 7, the overall size of the embryo was proportionately reduced; however, after treatment with

insulin etc., beak and long bone malformations occurred.

3. Insulin, pilocarpine and eserine caused syndactylism; however, malathion did not.

The "malathion syndrome" is therefore similar in kind but different in quality to the one described by Landauer. This implies that the chick embryo is sensitive to many teratogens injected on days 4 to 7 of incubation, but reacts somewhat differently to each, therefore, different metabolic pathways must be involved. The response of the embryo to these teratogens, including malathion, results in defects of the differentiating mesoderm; however, the teratogen inducing mechanism of the different compounds (or groups of compounds) is apparently not identical.

A clue to the teratogenic mechanism induced by malathion may be found in the work of Wilson and Walker (1966). In their experiments, malathion was added to cultured 14 day old chick fibroblasts. Concentrations above 1 µg/ml (3×10^{-6}M) inhibited the growth rate and peak cell populations. In all cultures containing 50 µg/ml (1.5×10^{-4}M) and 100 µg/ml (3.0×10^{-4}M) the cell populations rapidly decreased. In our experiments, 157 and 172 µg/ml (3.99 and 6.42 mg per egg respectively) were required to produce the "malathion syndrome"; these concentrations were in the same range as those used by Wilson and Walker. Gabliks and Friedman (1965 ab) found a 50% inhibition of protein synthesis when 13 and 15 µg/ml of malathion was added to human cell cultures. It is unlikely that the teratogenic action of malathion on the developing chick can be attributed solely to acetylcholinesterase inhibition; however, the abnormalities could be attributed to malathions' possible in vivo retard-

ation of cell growth and protein synthesis. Wilson and Walker also tested various fragments of the malathion molecule in cell cultures. Their results indicate that mercaptosuccinate was acutely toxic at 3.0×10^{-5}M, but not at 1.5×10^{-6}M. At a concentration of 1.5×10^{-4}M, mercaptosuccinate inhibited cell growth; however, the number of cells did not decrease at a rapid rate. That is, both mercaptosuccinate and malathion were toxic to chick embryo cells in vitro above 3.0×10^{-5}M, while succinate, malate, diethyl malate, and diethyl succinate were not toxic at levels as high as 1.5×10^{-4}M.

We performed experiments to determine if the carbon fragments of malathion or any of its other breakdown products were responsible for producing teratisms, and to determine if malathion affects the synthesis of proteins or nucleic acids in vivo.

The uptake of valine-C^{14} was checked in both the experimental and control embryos as an index of protein synthesis. The values after 4, 6, 12, 30, 36 and 72 hours of exposure to valine-C^{14} were significantly lower at the 5 and 1% level; however, exposure for 2, 10, 24, 48 and 96 hours showed no significant differences and exposure for 8 hours was significantly higher than the controls. Therefore, for an exposure period covering 96 hours, 6 values were lower, 1 was higher, and 5 were similar to the controls. When the changes were compared over 2 hour periods, there was no particular pattern for the differences. That is, they were randomized over 96 hours. When comparing the change in valine-C^{14} uptake over the total 96 hour period, there was a 5% decrease in the malathion treated group; with thymidine-H^3 and uridine-H^3 there was a

12 and 4% decrease in the treated groups, respectively. Thus, there was an indication of a reduction of protein and nucleic acid synthesis although these values were not statistically significant. However, since the treated embryos were smaller in size and had foreshortened hind limbs when measured on day 15, it was obvious that there had in fact been a reduction in protein synthesis. When comparing the weights of 4 to 8 day embryos, there was no significant differences between the treated and the controls indicating that the reduction of protein synthesis occurred after day 8.

None of the carbon fragments of the malathion molecule were teratogenic except for mercaptosuccinate (20, 30mg/egg) which produced smaller embryos, as well as 2 of 15 with cranial skeletal defects and 1 of 15 with micromelia; some of the external characters were smaller than the controls; however, no other defects occurred. In spite of this, we are reluctant to conclude that mercaptosuccinate is teratogenic because a 30 mg dose is physiologically so great that this is probably a non-specific effect. These in vivo results were similar to the in vitro results of Wilson and Walker (1966) in that none of the other carbon fragments of malathion such as malonic acid, malic acid, succinic acid, diethyl succinate and diethyl malate were teratogenic. A large dose (20, 30 mg/egg) of mercaptosuccinate was required to produce growth reduction. This dose was equal to about 10 times that used by Wilson and Walker to produce cell death in cultured fibroblasts. After an injection of malathion, the embryos are reduced in size; however, it

is unlikely that mercaptosuccinate is responsible for this reduction since the total administered dose of malathion was less than 7 mg per egg and of this, mercaptosuccinate would represent only a small fraction of the parent compound.

Mercaptosuccinate at a dose level of 30 mg per egg also prevented hatching. Some of the embryos were alive at the end of incubation; however, they were unable to get out of their shells on their own. Large doses of malathion also prevented hatching as the embryos were too weak to penetrate the shell. Again, it appears that the mercaptosuccinate does not cause this effect, after malathion injections, because of the small quantity (if any) of mercaptosuccinate generated.

None of the malathion breakdown products tested (Table 37) produced the "malathion syndrome". Anophthalmia and crossed beak were produced in 1 of 30 embryos after injections of malathion monocarboxylic acid (5 mg) and potassium dimethyl phosphorodithioate (10 mg); however, this defect was found in control embryos on many occasions and the small number affected discounts the teratogenicity of these compounds.

It would thus appear that the entire malathion molecule was necessary to produce the "malathion syndrome". Malathion (or malaoxon) is an anticholinesterase agent, and the entire molecule is necessary to produce this inhibition. It would, therefore, seem likely that cholinesterase inhibition would somehow be related to the production of teratisms; however, this is probably not so (the relationship between teratisms and cholinesterase inhibition will be discussed later).

Toronto insulin was injected into fertile eggs in an attempt to compare the teratogenic effects on the embryo with that of malathion. Three experiments of 30 eggs each were run. In general, the only apparent defect was a reduction in overall size. We repeated the experiment with Illetin^{R} (used by Landauer) which is a brand of insulin made by Eli Lilly and Company and sold in the United States under license from Connaught Laboratories. Forty-five chicks were examined and only 2 were malformed. The malformed chicks had short hind limbs which were outstretched in a horizontal plane and differed from the limbs of chicks with the "malathion syndrome". We were therefore unable to reproduce the teratisms obtained by Landauer with either Toronto insulin or Illetin^{R}. Protamine zinc insulin and insulin crystals had no effect on the chick embryo. The strain of eggs used may have been genetically different than those used by Landauer and may account for the observed differences in our results.

The uptake of malathion-P^{32} was followed to determine its distribution in the embryo. After injection of 3.5% malathion-P^{32}, the uptake in the embryo increased 4-fold at the end of the first day, remained relatively constant on days 7 and 8, and increased to day 13. The uptake in the yolk sac paralleled the uptake in the embryo, as expected, since this is the route by which materials in the yolk enter the embryo; however, the CPM were at a higher level. The amount of label in the yolk declined to day 13 as expected. If there is an increase in the amount of isotope in the yolk-sac and embryo,

the amount in the yolk should decline since we are dealing with a closed system. In view of the fact that the whole malathion molecule appears to be necessary to produce the teratisms, we believe that the counts obtained represent the phosphate of the intact malathion molecule.

When the results were expressed in CPM per gram embryo, the amount of isotope in the embryo increased sharply on day 6, and declined to day 13. There was also an increase in the yolk-sac values to day 6, a sharp decline on day 7 and a further increase on day 10 followed by a drop to day 13. We would expect these values to parallel the embryo values; however, when extracting the yolk-sac membrane, it is difficult to remove all of it. Also, after washing the membrane with 3 changes of saline, there was still some yolk adhering to it. These factors may result in a variation in the weights, and thus a discrepancy in the CPM. The amount of isotope per gram of yolk remains fairly constant as expected.

After injections of 6.42 mg malathion-P^{32}, the CPM in the yolk, yolk-sac membrane, and embryo followed the same pattern of uptake as the 3.99 mg doses. The amount per gram of embryo rose to day 7 and levelled off to day 13. These results indicate that, at least, the phosphate moiety entered both the yolk-sac and embryo in the 4 and 5 day injected groups, and both groups followed a similar distribution pattern.

The results of the 90% injections indicate that only a very small amount of the isotope entered the yolk-sac membrane and embryo; however, there was a wide variation between samples, because of the immiscibility of the malathion with the yolk. Therefore, only a small, variable amount would be in the vicinity of the embryo and would account for the discrep-

ancy in the amount entering the embryo and or yolk-sac. Another factor was the uneven distribution in the samples prior to removing a sample; that is, even though we thoroughly mixed the homogenates just prior to removing a sample, it either contained a small amount of the isotope or an unusually large amount. Furthermore, our technique of injecting a constant amount of radioactivity resulted in a decrease in the specific activities of malathion with increasing dose, since there was a proportionate increase in the amount of unlabelled malathion with increasing concentration. These factors may account for the large variation in the embryo, yolk-sac membrane and yolk values.

Malathion-P^{32} uptake was followed by autoradiographic methods in embryos injected on day 4 and 5 of incubation in an attempt to determine its tissue organismic, and cellular distribution. Embryos injected on day 4 were exposed to the isotope for 1, 4 and 6 days, and embryos injected on day 5 were exposed for 1, 3 and 5 days. In general, the radioactivity was dispersed in nervous, cartilage, and connective tissue. After 1 day's exposure, little or no activity was present in the kidney, liver or heart, but some activity was present in these tissues after a few days exposure. That is, exposure for longer periods did not cause any greater congregation in any particular tissue; however, more tissues became involved. Most of the activity observed was in the form of aggregates or tracks of the silver grains; the latter being very characteristic of P^{32}. The activity was mostly extracellular. No particular pattern was evident. In general, the low radioactivity may be due in part to the leaching of the malathion-P^{32} after exposure to tissue processing solvents.

Evidence from autoradiography and gas flow counting indicate that malathion does enter the embryo but does not concentrate to any extent in any one particular organ; however, it was observed in nervous tissue more than any other. Gerebtzoff (1959) reported that AchE can be detected in neuroblasts of the spinal cord especially in the anterior horns of 4 to 4½ day chick embryos. Also, at this time, acetylcholinesterase was present in the optic lobes and in the retina. It appears that there is some correlation between AchE activity and malathion-P^{32} distribution in young embryos, since the label was mainly present in those regions mentioned.

Reversal of the Malathion Syndrome

If a metabolic antagonist is injected into fertile eggs, teratisms may result which can be alleviated by supplying the agent which was blocked. For example, if ethionine (antagonist of methionine) was injected into fertile eggs, the embryos were abnormal with an enlarged liver and thin legs; this effect can be abolished by an injection of methionine (Karnofsky, Dagg and Lacon, 1955). Similarly, adenine will prevent the action of azaserine on the chick embryo (Dagg, Karnofsky, Lacon and Roddy, 1956), as previously mentioned.

Atropine which is an antidote for acetylcholinesterase inhibiting agents was injected into fertile eggs in an attempt to alleviate the teratisms produced by malathion; however, it had no therapeutic value. We are therefore not dealing with a simple cause and effect relationship.

Many unrelated compounds are capable of producing micromelia in

chick embryos which can be prevented by an injection of nicotinamide along with the teratogen (Landauer, 1948; Karnofsky, 1964). Only 6-aminonicotinamide, which is an analog of nicotinamide, appears to act in a cause-effect manner. The other compounds namely, pilocarpine, eserine, insulin, and sulfanilamide have various biological effects, however, all can produce micromelia when injected into chick embryos. Micromelia can be prevented by an injection of nicotinamide.

Pilocarpine and eserine are parasympathicomimetic agents. Pilocarpine acts by simulating acetylcholine at the receptor site and eserine acts by blocking acetylcholinesterase which increases the quantity of acetylcholine at the receptor site. The net result of both of these compounds is an increased acetylcholine effect on neuromuscular action. Nicotinamide has no known physiological effect on acetylcholine activity and yet it prevents the teratisms induced by pilocarpine or eserine.

In our work (Greenberg and LaHam, 1970) nicotinamide and nicotinic acid were found to counteract the teratisms but did not prevent the growth retardation. Tryptophan on the other hand, completely reversed the "malathion syndrome". Since tryptophan is an NAD precursor, we also tried another compound in the metabolic pathway of NAD synthesis from tryptophan, namely, quinolinic acid, in an attempt to mimic the effects of tryptophan. We found that quinolinic acid prevented the teratisms, but not the growth retardation. Anthranilic acid, however, which is a breakdown product and not a precursor had no therapeutic value.

All of the above data indicate that malathion has at least 2 adverse

effects in the chick embryo and that these do not appear to be related one to the other. To further elucidate this, we did a cholinesterase assay on chick embryos injected with malathion, malathion and nicotinamide, and malathion and tryptophan. If there was any correlation between teratisms and growth reduction, and cholinesterase inhibition, we would expect to find a difference in enzyme levels between the embryos free of malformations (malathion-nicotinamide treated), embryos free of both malformations and growth reduction (malathion-tryptophan treated), and embryos having both malformations and reduced size (malathion treated only). Since the enzyme levels were depressed in all of the treated groups, it does not appear that there is a relationship between malformations, malformations and growth retardation, and cholinesterase levels in chick embryos. This conclusion is reinforced by preliminary experiments in which we determined the effect of malathion, malathion and nicotinamide, and malathion and tryptophan on acetylcholinesterase in vivo, histochemically. These preliminary experiments strengthen our observations that enzyme levels were low in all the malathion treated embryos. Thus, the malathion syndrome can hardly be due to its effect on acetylcholinesterase. Also, as mentioned above, two other parasympathicoomimetic compounds are capable of causing a similar type syndrome; however, this parasympathomimetic effect is not directly responsible for producing terata at least as shown with malathion.

Nicotinamide and quinolinic acid are essential in preventing limb, beak and feather defects, while tryptophan is essential for preventing limb, beak and feather defects, as well as overall growth reduction. Malathion, therefore, interferes directly or indirectly with nicotinamide metabolism since two precursors or nicotinamide itself can prevent the

teratisms. Thus, the malformations could well be the result of interference with energy metabolism so that at essential periods of development there was an inadequate amount of energy available for normal growth of the limbs. Also, the complete reversal of the "malathion syndrome" was achieved by an injection of tryptophan together with the malathion. Since growth retardation and teratogenic effects in chick embryos are 2 separate entities (Zwilling and DeBell, 1950), a lack of the essential amino acid tryptophan, which is necessary for growth, may be responsible for the reduction in the size of the embryo. A tryptophan analysis revealed a significant decrease in day 6 and 7 embryos after malathion injections on days 4 and 5. This indicated a reduction of tryptophan uptake from the yolk.

Congenital malformations in chick embryos can be caused by a host of unrelated compounds. These malformations are similar in kind but different in many respects from one compound to another (as previously mentioned). It is difficult to believe that the lesions so induced are due to disturbances to 1 metabolic pathway or to 1 step in a particular pathway. An example is 3-acetylpyridine and 6-aminonicotinamide. Both of these compounds are capable of causing teratisms when injected into the 96 hour chick embryo (Landauer, 1957). The compound, 6-aminonicotinamide, caused 97% micromelia and 77% parrot or crossed beak. The compound, 3-acetylpyridine, on the other hand, did not cause micromelia, parrot or cross beak, but did produce a short upper beak in 24.2% and muscular hypotrophy in 97.1%. Both 3-acetylpyridine and 6-aminonicotinamide are capable of producing symptoms of niacin deficiency in mice which can be

alleviated by nicotinamide or nicotinic acid (Woolley, 1945; Johnson and McColl, 1955). It is thought that these analogues act by being synthesized into NAD or NADP and that the products are not physiologically active (West et al., 1966). Both of these compounds can be counteracted by exogenous nicotinamide yet, they produce totally different abnormalities.

In our work, we tested compounds which were structurally related but not necessarily metabolically related to tryptophan. One such group consists of compounds with an indole ring. Of those tested, indoleacetic acid, indole, and serotonin had no therapeutic value; however, in 2 of 4 experiments, indole had a more severe effect than malathion alone, whereas indoleacetic acid enhanced the effect of malathion in all instances. This synergistic behaviour suggests that we may not be dealing solely with a metabolic effect, but that there may be a sub-molecular or electronic effect. That is, there may be an electronic disturbance in the egg caused by the first agent (malathion), partially nullified by the second agent (nicotinamide, nicotinic acid or quinolinic acid), or completely nullified by the second agent (tryptophan); or the disturbance is enhanced by a synergistic agent, for example, indoleacetic acid.

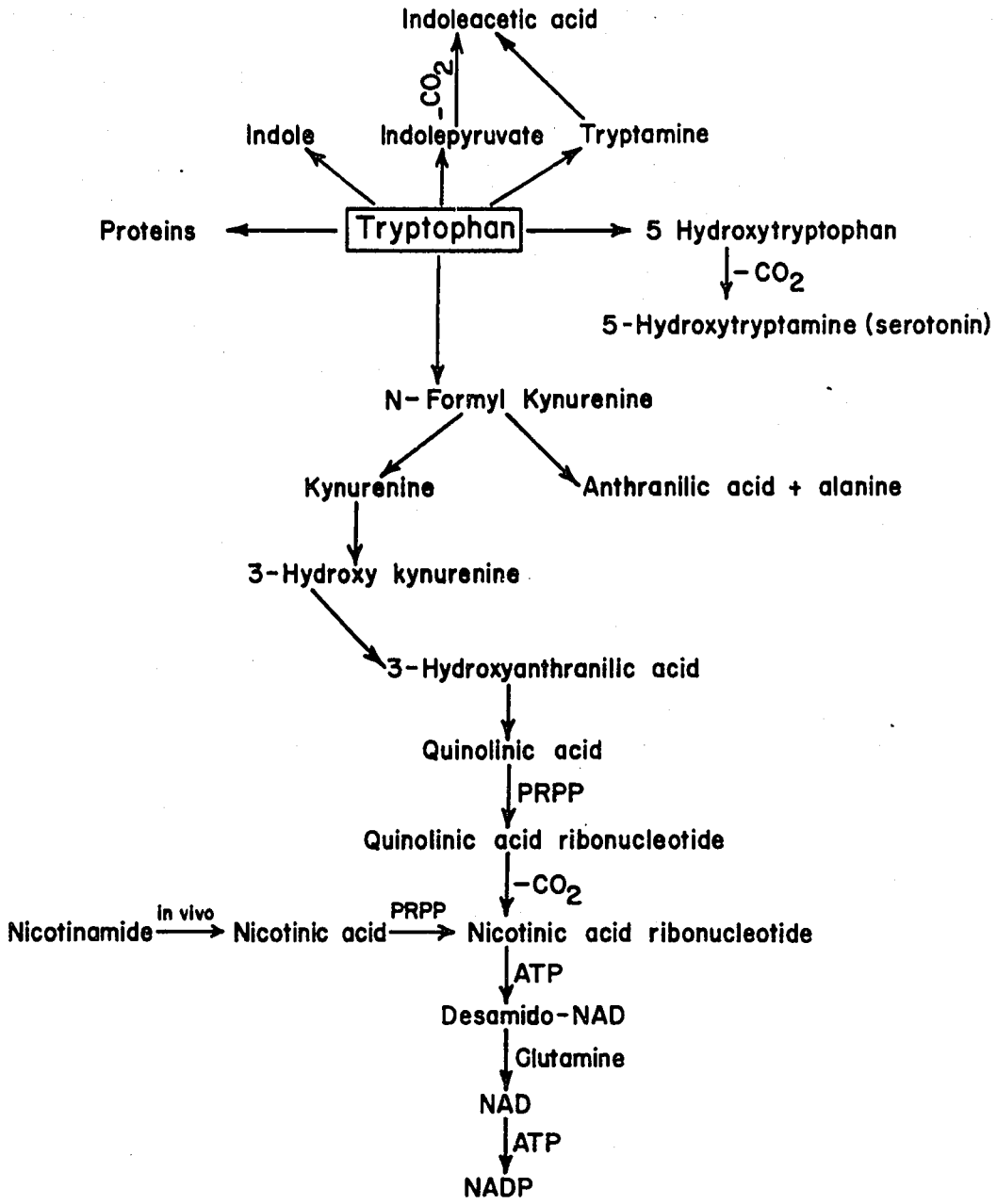
Compounds related to tryptophan electronically are so related because they possess similar k-values which are listed in a publication by Szent-Györgyi(1960). The smaller the k-value for the highest filled orbital (linear function of the ionization potential) the better is the electron donating ability of the compound, while the smaller the k-value for the lowest empty orbital the better is the electron accepting ability of the compound. Of those compounds injected, indoleacetic acid, adenine,

guanine, ∝-naphthol, β-naphthol, and quinoline had no effect on the embryos when injected alone; however, when they were injected with the malathion, they had a synergistic effect producing embryos which were more severely malformed. Imidazole which has k-values different from tryptophan was neither synergistic nor therapeutic. The above hypothesis was one which we felt we should test in our system, but at this time it would be premature for us to draw any definite conclusions.

The relationship of tryptophan and its metabolic products is shown in Fig. 46. Tryptophan is incorporated into proteins, it is the precursor of serotonin, (which is a potent vasoconstrictor) and can be converted to NAD or NADP.

Histochemically we tested several dehydrogenases to determine if malathion had any effect on NAD or NADP linked enzymes. We found no visible differences between control or experimental sections with lactate, succinate, DPN, TPN, glucose 6-phosphate, or isocitrate dehydrogenase. Slight differences were observed in the kidney tubules of experimental sections tested for malate dehydrogenase. Since histochemical methods were used for qualitative determinations, small quantitative differences would be difficult to detect. We, therefore, tested quantitatively for 2 NAD linked enzymes, namely, malate dehydrogenase and lactate dehydrogenase by biochemical assay. The former was tested to confirm our histochemical results, and the latter was tested because in early embryonic life, anaerobic glycolysis is prominent. LDH activity showed no differences between control and experimental embryos indicating that malathion has no effect on this enzyme. In the preliminary run with malate

Fig. 46. Metabolic scheme of the amino acid tryptophan


Indoleacetic acid
$-CO_2$
Indole
Indolepyruvate
Tryptamine
Proteins
Tryptophan
5 Hydroxytryptophan
$-CO_2$
5-Hydroxytryptamine (serotonin)
N-Formyl Kynurenine
Kynurenine
Anthranilic acid + alanine
3-Hydroxy kynurenine
3-Hydroxyanthranilic acid
Quinolinic acid
PRPP
Quinolinic acid ribonucleotide
$-CO_2$
Nicotinamide
In vivo
Nicotinic acid
PRPP
Nicotinic acid ribonucleotide
ATP
Desamido-NAD
Glutamine
NAD
ATP
NADP

dehydrogenase, there was a decrease in the enzyme 1 day after injection for injections of malathion on days 3 and 5, with a marked decrease on day 6. These results prompted us to run another experimental series with embryos injected on day 4 and exposed to malathion for a period of from 1 to 4 days. There was a decline in activity on day 5 and 6 of incubation which appeared to recover by day 7; this indicated that energy metabolism is affected with a reduction in ATP which could contribute to the production of teratisms. The quantitative differences were not large and therefore could not be detected histochemically except in some kidney tubule sections.

Nicotinamide was found to counteract the micromelia and facial skeletal defects produced by sulphonamides in chick embryos (Landauer and Wakasugi, 1968). They discovered that ADP could also successfully counteract the teratisms. In our work, ADP did not afford protection against malathion-induced terata. On the basis that the effects of many teratogens could be prevented by nicotinamide and or ADP, Landauer and Wakasugi concluded that "The morphogenetic disturbances produced by these compounds arose via diphosphopyridine-mediated pathways, interference with dehydrogenation, and with synthesis of ATP". They concluded that interference with dehydrogenation and ATP production were the main factors causing teratisms.

Since tryptophan, quinolinic acid, nicotinic acid and nicotinamide are all active alleviating agents against malathion-induced terata, malathion probably does not interfere with any of the steps in the path-

way leading to NAD, but rather, the block is probably at the level of tryptophan utilization. Roger, Upshall, and Casida (1969) found that the organophosphate bidrin is capable of producing teratisms in the chick embryo, and that the teratisms could be alleviated by nicotinamide. They also found that bidrin has no effect on the metabolism or quantity of nicotinamide or nicotinic acid produced in the embryo. They found that after an injection of an alleviating dose of nicotinic acid (1 mg per egg), NAD and NADP levels in the embryo were not greatly modified but the amount of nicotinic acid transferred to the embryo increased by a factor of 6. They also found that the metabolism of bidrin was unaltered by an alleviating dose of nicotinamide. Since studies with bidrin have shown that the quantity of NAD or like compounds is unaltered, it is possible that more is required to overcome the stress caused by malathion or other teratogens.

We contend that we are dealing with two problems, the first being growth retardation and the second being malformations. Tryptophan apparently satisfies the chick embryo in alleviating both conditions. An hypothesis consistent with our data is that malathion interferes with the uptake of tryptophan into the embryo during critical periods of development and that this leads to growth reduction. Tryptophan also supplies NAD or nicotinic acid or nicotinamide all of which are capable of preventing malathion-induced terata in the chick embryo.

This hypothesis has the advantage of being easily testable. First of all, a direct estimate of pool levels of tryptophan, as well as protein-bound tryptophan, should show that malathion causes a drop in the total tryptophan of the embryo and a proportionate rise in

that of the yolk. Secondly, injection of labelled tryptophan could be followed in both the controls and malathion treated embryos: in the latter, the rate of transfer of label to the embryo across the yolk-sac should be reduced.

Another interesting aspect is the fact that teratisms induced by an injection of malathion on day 4 can be alleviated by an injection of tryptophan, quinolinic acid, or nicotinamide up to day 10 of incubation. Zwilling (1959) found that cultured limbs from embryos injected with insulin on day 4 and extracted up to day 7 could be protected from malformations by nicotinamide, but after day 8 nicotinamide had no effect. This could be compared to our work, since reversal of the terata induced by malathion occurred up to day 8 after which reversal still occurred, but the number of malformed embryos increased to day 10. After day 10, the "malathion syndrome" could not be reversed.

In our laboratory, Gill and LaHam (unpublished results) are presently conducting a cytological and histochemical study of the limbs from chick embryos affected with the "malathion syndrome". Preliminary results have revealed morphological and staining differences in the cartilages. In many of the limbs, the processes at the ends of the bones were absent and the cells located in their place resembled undifferentiated mesenchyme suggesting that differentiation of these areas failed to occur. In the corn oil and stab-only controls, toluidine blue staining showed γ-metachromatic areas in cartilage tissue. The ground substance exhibited uniform shades of deep red, and the chondrocytes were surrounded by a normal amount of it. In the malathion treated embryos, toluidine blue staining showed γ-metachromasia; however, the shading was uneven

and the colours ranged from pink to deep red. Many of the cells were surrounded by a lesser amount of ground substance than the controls especially in the diaphysis of the femur. Also, the chondrocytes were hypertrophied. γ-metachromasia indicates staining of complex carbohydrates with negatively charged groups. Compounds such as mucopolysaccharides containing negatively charged carboxyl, phosphate, or sulfate groups are therefore somehow affected in the cartilages of malathion treated embryos.

SUMMARY AND CONCLUSIONS

1. Malathion, at dose levels which kill 50% of the embryos when injected into fertile chicken eggs on days 4 and 5 of incubation, is capable of producing consistent reproducible malformations to the embryos. The main defects are to the hind limbs, feathers, and beaks, as well as, overall growth reduction. The organs involved are related to structures of mesodermal origin. The limbs, feathers, and beaks are therefore vulnerable to a malathion insult on days 4 or 5 of incubation.

2. Malathion, at dose levels which kill 50% of the embryos when injected into fertile chicken eggs on days 6 to 12 of incubation, is capable of producing embryos with feather defects and stunted growth, but the hind limbs are rarely damaged. Therefore, only the feathers are vulnerable on days 6 to 12; however, edema and hydrocephaly may also occur.

3. The experimental evidence suggests that the entire malathion molecule is necessary to produce terata as well as cholinesterase inhibition, and furthermore, there is probably no relationship between cholinesterase inhibition and malformations.

4. Malathion causes a reduction in growth of chick embryos from eggs injected with an LD_{50} dose on days 4 or 5 of incubation.

5. Histochemical determinations of cholinesterase show that malathion treatment reduced the in vivo levels of this enzyme. Also, Histochemical determinations of lactate, succinate, DPN, TPN, glucose 6-phosphate, and isocitrate dehydrogenases revealed no detectable differences between malathion treated and untreated embryos. Slight differences were observed in kidney tubules of malathion-treated sections tested for malate dehydrogenase. A biochemical assay for malate dehydrogenase revealed a lesser amount in the malathion-treated embryos than in the untreated controls, indicating a general metabolic slowdown.

6. Nicotinamide, nicotinic acid, quinolinic acid or tryptophan can prevent the malathion-induced malformations, however, only tryptophan prevented the growth reduction as well.

7. Reversal of the "malathion syndrome" can occur after injections on days 5 to 10 of incubation with nicotinic acid, nicotinamide, quinolinic acid, or tryptophan into eggs pre-injected with malathion on days 4 or 5 of incubation.

8. Malathion causes both malformations and growth reduction in chick embryos, and tryptophan satisfies the embryo in relieving both the malformations, and the growth reduction. A tryptophan assay revealed a reduction of tryptophan in malathion treated embryos. This led to the hypothesis that malathion interfered with tryptophan uptake from the yolk. The malformation

alleviating chemical appears to be NAD or related compounds, and the growth retardation alleviating factor appears to the the essential amino acid, tryptophan.

APPENDIX III - HISTORICAL REVIEW OF TERATOGENESIS

Geoffroy de Saint Hilaire (1832) in the eighteenth century was the first to apply the term teratology to the study of monsters resulting from disorders in structural organization of animals. The prefix of the word is derived from the Greek meaning wonder. The science of Teratology deals with congenital malformations, which refer to the presence at birth of gross morphological changes from the norm, originating in fetal life. In general, teratogenic action causes inhibition or deviation of an organogenetic process and occurs during the period of organogenesis. Congenital malformations may occur spontaneously or be induced by external means; this review will deal mainly with induced malformations by physical or chemical means.

Various agents are capable of causing congenital malformations; they include viruses, cytotoxic compounds, antibiotics, alkaloids, azo dyes, hormones, dietary deficiencies, hypoxia, irradiation and others. These chemical agents may be dispersed into the aqueous habitat of fish, injected into the ova of birds or into pregnant mammals. In this outline, I will briefly discuss some of these agents and their relationships to congenital malformations, in mammals and birds with emphasis on the rat and chick embryos, after presenting a short history of teratology.

History

Isadore Geoffroy de Saint-Hilaire (1832) in his "Traité de Tératologie" described and classified congenital malformations in humans. He also attempted to reproduce congenital malformations in animals, but he had little success. He introduced the study of teratology; however,

the science of experimental teratology began in 1891 with the work of Dareste (1891) who obtained malformations in chick embryos by raising or lowering the incubation temperature, or by mechanical shaking. The embryos had poorly developed circulatory systems, and brain and eye abnormalities. From his experimental studies, Dareste concluded that both high and low temperatures produced the same type of defects; in general, similar types of malformations can occur regardless of the inducing agent.

Féré (1893-1901) was the first to propose the chick embryo as a tool to study the toxicity of various compounds. In his series of articles, he recorded the teratogenic action on the embryo of various chemical substances; embryos were either injected or were exposed to vapours. The injected substances included ethyl, methyl, and propyl alchohols; acetone, chloroform, sodium chloride, nicotine, morphine, hydrogen cyanide, atropine, peptone (hydrolysed mixture of meat protein), glucose, glycerine, potassium bromide and iodide, strychnine, creatine, antipyrine, cantharidin, and caffeine; tetanus, diphtheria, and tuberculosis toxins; blood of a dead rabbit containing pork cholera; syphilitic blood; and various venoms. The vapours included alcohol, chloroform, ether, mercury, phosphorus, ammonia, nicotine, muscarine, and various oils. Unincubated eggs were commonly used; however, in some experiments the eggs were pre-incubated for 24, 48, or 72 hours before injection. Féré studied the toxicity and the teratogenicity of the compounds by comparing the number of dead and malformed embryos with the controls. He described malformations which included omphalocephaly, cyclopia, spina bifida, and atrophy of the head, tail and optic vesicles. He observed these same malformations in the controls; however, they occurred less frequently. Féré also pointed out

that substances injected into unincubated eggs were more toxic to the embryo than if injected into pre-incubated eggs.

Stockard (1914) exposed fertilized, unincubated chicken eggs to the fumes of alcohol and ether for a period from 20 minutes to 30 hours. The shortest exposure time sufficient to produce malformations was 3 hours and 45 minutes, although in some instances exposure as long as 8 hours was ineffective; the best results were obtained after exposure of 14 to 20 hours. The treated embryos were smaller, had poorly developed brains, as well as various eye defects. He was very interested in the eye abnormalities since similar defects were produced in fish when developing fish eggs were exposed to ether or alcohol. In another series of experiments, Stockard exposed guinea pigs to alcohol and ether by intoxicating the parent. The offspring were eyeless and had nervous system defects. His experimental work led him to the conclusion that "The part or organ developing at the most rapid rate is inhibited more decidedly by treatment than are the less rapidly developing parts and is therefore most affected or modified in its development" (Stockard, 1921).

Alsop (1919) obtained abnormal chick embryos when incubation temperatures were altered. When she exposed fertilized eggs to low temperatures there was incomplete infolding of the neural plate into the neural tube, and a reduction in the number of somites. Increased temperatures produced brain abnormalities and extra somites lateral to the normal ones.

Child (1928) applied potassium cyanide, hydrochloric acid, copper sulfate, and ethanol to early chick embryos just after fertilization and produced embryos with microcephaly; however, if the exposure was at a later stage, after the head region was more developed, inhibition of the

posterior regions occurred.

Hammett and Wallace (1928) added lead nitrate to the yolk prior to incubation and examined the embryos after 18 to 72 hours. There was a marked reduction in the anterior regions, overall body size, and somite growth. The authors concluded that regions of rapid cell proliferation were selectively inhibited by lead.

Prior to 1935, the science of experimental teratology was well established; the chick embryo was the main organism used, and the outcome of these experiments laid the foundation for studies in higher vertebrates. The science of experimental mammalian teratology began in the 1930's with studies on the biological effects of X-rays (Job, Leibold, and Fitzmaurice, 1935) and dietary deficiencies (Hale, 1933) on mammalian development.

In this study, I will attempt to review the teratogenic agents and their effects on the rat and chick embryo. A comparison of the embryogenesis of these two animals reveals many similarities. In the chick embryo, the incubation period is 21 days; the primitive streak is apparent between 18 to 24 hours and the first heart beat is observed around the 9 somite stage (29 hours of incubation). The tail bud stage is reached between 3 and 4 days of incubation. Cartilage formation begins between day 5 and 6, while ossification occurs on day 11. The endocrine system starts to function in the second week of development between day 8 and 9. Organogenesis is complete by day 12.

In the rat embryo, the gestation period is 22 days; implantation occurs on day 7 and the primitive streak stage is evident on day $8\frac{1}{2}$ to 9. The first heart beat takes place on day 9 and the tail bud stage is established by $11\frac{1}{2}$ days of gestation. The endocrine system begins

to function on day 16 or 17.

The 4 day chick embryo is therefore equivalent to an 11 or 12 day rat embryo. Chick embryos are most susceptible to teratogens from days 4 to 7 of incubation, while rat embryos are most susceptible from days 10 to 12 of gestation.

Dietary Deficiencies

Dietary deficiencies have been known to be teratogenic since 1933 when Hale fed pregnant pigs vitamin A free diets during early pregnancy (Hale, 1933). In this study, all 11 of the offspring were born without eyehalls; 10 were alive at birth and 1 was stillborn. One piglet lived for 4 days, 1 lived for 3 hours and the remaining 8 died within 5 minutes of birth. Other malformations included facial clefts, accessory ears, misplaced kidneys and in rare instances, malformed hind limbs. To determine if the abnormalities were hereditary, he mated blind males to unrelated females as well as to blind sisters. The females were all fed normal diets during pregnancy yielding normal pigs.

Warkany and Nelson (1941) fed goitrogenic diets to pregnant rats on the assumption that the young would be directly affected since endemic goiter and cretinism existed in various countries where iodine deficiency occurred. One-third of the offspring developed congenital malformations including short mandibles, tibiae, fibulae, radii, and ulnae bones; syndactylism and fusion of the ribs. Iodized salt added to the mother's diet prevented the goiter in both the mother and young, but, did not prevent the congenital malformations; however, when the diet was supplemented with dried liver up to the 13th day of gestation, the malformations

were prevented. Warkany and Schraffenberger (1943, 1944) showed that the essential factor in the liver supplemented diets was riboflavin, and when pregnant females were fed diets lacking riboflavin, the young had malformations resembling those produced by the goitrogenic diets. They also showed that vitamins such as thiamine, niacin, pyridoxine, and calcium pantothenate did not prevent the teratisms. They mated the offspring in various combinations and found that the malformations were not transmissible.

In other experiments, Warkany (1943) fed pregnant rats diets lacking in vitamin D throughout pregnancy. The offspring had appendicular skeletal defects which were prevented by the addition of vitamin D to the maternal diets.

Diets containing folic acid antagonists were also teratogenic. Hogan, O'Dell and Whitley (1950) fed pregnant rats diets containing soybean oil meal as a protein source, and all vitamins except ascorbic acid, niacin, folic acid, and vitamin B_{12}. The young were normal except for hydrocephaly in less than 1%. When a folic acid antagonist, crude methyl folic acid, was added to the diets hydrocephaly increased to 20%. When the folic acid antagonists 2,4, diamino-5-(3'4'-dichlorophenyl)-6-methyl pyrimidine and 2,4, diamino-5-p-chlorophenyl-6-ethyl pyrimidine were given to pregnant rats after implantation, the young had cranial bone defects, hydrocephaly, and overall growth reduction (Thiersch, 1954).

Landauer (1936) described micromelic embryos from chicken eggs of hens fed deficient diets. The diets were lacking factors which were present in wheat germ, liver, and whey (Byerly et al., 1935). Couch (1948) experimented with chicken eggs from biotin deficient hens resulting in

embryos with parrot beaks, short and bent tibiotarsus bones, short tarso-metatarsus bones, and short wings.

Low Oxygen Tension

Ingalls, Curley and Prindle (1950, 1952) exposed pregnant mice to anoxia by placing them in a low pressure chamber at 260 to 280 mm Hg for 5 hours; when the mother was insulted on the 14th day of gestation, the offspring had fused ribs, cleft palate, cranioschisis, and anencephaly. Anencephaly was also the main abnormality following exposure to a simulated atmosphere of 25 thousand feet and was not found among offspring when the mother was exposed to 27 thousand feet, or maintained at sea level.

Grabowski (1961) exposed fertile chicken eggs to anoxia resulting in offspring which were edematous. Other malformations varied depending on the degree and duration of hypoxia. Eggs exposed to anoxia for 3 hours produced embryos with undersized heads containing multiple anomalies of the brain, eyes and beak. Exposure to moderate hypoxia for 3 hours produced embryos with hydrocephaly, microcephaly and folded midbrains. Cleft palate, crossed beak and short upper beak occurred after severe hypoxia for 3 hours or moderate hypoxia for 6 hours. Severe hypoxia for 6 hours produced microphthalmia and malformed or absent extremities. In short; brief acute exposures induced malformations of the head; moderate exposures affected individual structures of the head and trunk; prolonged exposures to mild hypoxia resulted in rumpless offspring. In all instances, edema was a common feature. According to Grabowski, most of the defects were caused by the edema syndrome. That is, hypoxia leads to fluid and electrolyte disturbances in the chick embryo which in turn

causes malformations due to the physical effect on nearby proliferating tissues (Grabowski, 1964). Analysis of the serum of hypoxia treated embryos showed an increase in lactic acid, free amino acids, potassium and carbon dioxide. Sodium, calcium, chloride, and glucose decreased while proteins and inorganic phosphates remained constant (Grabowski, 1966).

Irradiation

The main effect of irradiation on cells during early development is mitotic cell death, whereby cells are killed outright during metaphase or anaphase. Mitoses may be delayed or chromosome abnormalities and aberrations may occur because of chromosome damage prior to division. The effects of ionizing radiation on the fetus are due mainly to direct action on cellular mechanisms. These effects may cause sublethal biochemical injuries which affect cellular differentiation; or change the cell's proliferation rates; or kill certain groups of cells outright (Hicks and D'Amato, 1966).

The pioneer work on irradiation of mammals (Job et al., 1935) showed that irradiation produced consistent malformations in rats. After pregnant females were exposed to doses of 36 to 90r during day 9, 10, and 11 of gestation, more than one-third of the offspring had eye defects, jaw defects, and hydrocephaly. The dose level was important, since doses of 95 to 200r given on any of the first 18 days of pregnancy was lethal to the offspring, whereas doses of 100r during the latter days of pregnancy resulted in offspring with abnormal physiology. The timing was also important; when the animals were exposed on the 9th, 10th, and 11th days

of gestation, the brain primordia, the eyes, and the jaws were respectively malformed.

Warkany and Schraffenberger (1947) produced malformations in the offspring of Sprague-Dawley pregnant rats after exposure to single doses of between 190 to 950r of X-irradiation on the 10th to 16th days of gestation. Irradiation on day 10 produced offspring with cranial defects, cleft palate, fusion of the distal portion of the ribs, absence of ulnae, and shortening of the femurs. Irradiation on day 11 produced limb and tail defects, whereas irradiation on day 12 produced encephaloceles as well as short maxillae. Irradiation on day 13 and 14 resulted in malformed maxillae, syndactyly of the forelegs as well as short tails, tibiae, and fibulae. Irradiation on day 15 and 16 had no effect on the axial skeleton.

Russell (1950, 1956) experimented with irradiation on mouse embryos. He irradiated the embryos between day 1 and 14 of gestation with doses between 100 to 400r. Irradiation between day 1 and 5 produced a high mortality with no abnormalities, whereas irradiation between days 7 and 14 resulted in relatively low mortality with a great number of malformations. Irradiation between day 14 and birth resulted in a small number of deaths and malformations. He found that particular anomalies could be produced in the young when pregnant females were irradiated during certain periods between day 7 and 14 of gestation. For example, various ocular defects occurred when the mouse was irradiated between $7\frac{1}{2}$ and $9\frac{1}{2}$ days of gestation; malformations of the mouth and nose occurred when the pregnant mouse was irradiated between $6\frac{1}{2}$ and $8\frac{1}{2}$ days of gestation; skeletal defects of the limbs and cranium occurred after irradiation on

days 9½ and 10½, while tail abnormalities occurred after irradiation on days 11½ and 12½. At a dose level of 200r, no malformations occurred before day 6; however, exposure on days 6½ to 12½ produced a high incidence of thoracic and vertebral column defects.

Hicks (1950) exposed the whole bodies of pregnant rats and mice to doses between 200 and 600r; as a result, the fetuses had necrotic brains and spinal cords. This damage was attributed to the destruction of neuroblasts which was the direct effect of ionizing radiation on nervous tissue (Hicks, 1952).

Wilson, Jordon, and Brent (1953) produced malformations of the heart and related blood vessels in offspring from pregnant rats exposed to 200r on the 9th day of gestation; malformations of the spinal cord occurred after exposure to 200r on the 10th day of gestation. On the 9th day, 25r produced embryos with microphthalmia; 50r caused eye malformations and retarded brains; 100r caused overall growth retardation and eye abnormalities. Irradiation on the 10th day with more than 25r produced embryos with maldeveloped brains and spinal cord defects; the incidence for both types of malformations was directly related to the dosage given. In the aforementioned experiments, Wilson's group directly exposed the fetuses by opening the abdomen of the mother and irradiated the implantation sites (Wilson and Karr, 1951). Wilson observed that the effects of radiation was more severe on the 9th day than the 10th day and concluded that the 9th day embryos being less differentiated, were more vulnerable to mitoses delay and chromosome alterations.

In studies with radioactive isotopes, Sikov and Noonan (1958) injected from 0.3 to 2.0 μc of phosphorus^{32} (P^{32}) intraperitoneally

into pregnant rats on the 7th to 11th days of pregnancy; the abnormalities in the offspring were mainly restricted to central nervous system defects. Arnikar, Singh, and Udupa (1963) injected 50 μc of P^{32} into female rats on the 9th day of pregnancy; the offspring had limb defects. $Strontium^{90}$ administered to pregnant mice produced offspring with only skeletal defects (Hiraoka, 1961).

Irradiation exposure to man and its effects on the offspring was reported by Murphy and Goldstein (1929). They reported on the administration of X-rays to the pelvic region either before or after conception. Mothers irradiated before X-ray treatment had normal offspring, however, those irradiated after conception produced children with small heads and overall growth retardation. Japanese children who were in utero during the A-bomb explosion showed overall growth retardation, microcephaly, and mental retardation (Plummer, 1952; Miller, 1956). VanCleave (1963) reported 60 cases of microcephalic children exposed to irradiation in utero.

Irradiation of the Chick

It has been known since the turn of the century that exposure of fertile chicken eggs to irradiation caused congenital malformations. Gillman and Baetjer (1904) exposed chicken eggs to X-rays for 10 minutes each day during the first 4 days of incubation. The malformations included curved bodies and limbs, microphthalmia, and feather defects. Strangeway and Fell (1928) reported extensive vascular damage to 6 day embryos after exposure of 150 and 270r; cysts appeared on the body due to the distension of blood vessels. Various abnormalities were produced in the chick embryo

after X-ray exposure of up to 1800r (Karnofsky et al., 1950); the abnormalities included short mandibles, cross-beaks, corneal cysts, small heads, thin legs and deformed or absent toes.

An insight into the direct effects of irradiation on localized areas was made by Wolff (1936); he irradiated selected areas using a system of lead screens in a method perfected by Ancel and Wolff (1934). He obtained the following results:

1. Irradiation to the primitive streak, in front of Hensen's node produced omphalocephaly (body is twisted with heart on dorsal side and head is in heart region).

2. Irradiation at 20 hours in front of the cephalic fold produced diplocardia.

3. Irradiation of the medial region of the prosencephalon of an embryo of 8 to 15 somites produced cyclocephaly in which two eyes are found in the same socket.

4. Irradiation of the mesencephalon in an embryo of 8 to 15 somites produced octocephaly (derivatives of lower maxillary buds are completely or partially absent).

5. If the optic vesicles are irradiated at 12 to 40 hours of incubation, anophthalmia is produced.

6. If the lateral layers of an embryo of 15 to 23 somites are irradiated, the fetal viscera are exteriorized.

7. If the rhomboidal sinus is irradiated at the 12 to 18 somites stage, fused legs occurred.

Wolff was able to reproduce the above teratisms at will. Other workers

have used this technique and produced specific abnormalities. Stephan (1953) produced abnormalities of the aortic arches, pulmonary arteries and branchiocephalic trunks when the aortic arches were irradiated. Exposure of the limb buds to 4000r resulted in bone malformations (Wolff et al., 1958; Kirrman and Wolff, 1964); in the leg, the tibia developed at the expense of the fibula, whereas in the wing, the ulna developed at a more rapid rate than the radius. At 4500r, the radius and fibula ceased to develop, but if the ulna or tibia were irradiated, some growth did occur.

Hormones

Hormones are capable of causing teratisms in both mammals and birds. Fraser and Fainstat (1951) treated pregnant mice with cortisone resulting in offspring with cleft palate. They showed that the genetic makeup of the animal was responsible for a higher or lower incidence of the abnormality by using 5 different strains of mice. Treatment on the 10th or 11th days produced cleft palate in 79% of the offspring in the A and N strains, and 21% in the C-57, B, and H strains. Treatment on day 12 or 13, resulted in a lower incidence of cleft palate (29% in A and N and 8% in C-57, B, and H) for each strain of mice used. Cortisone caused overall growth reduction in offspring of treated pregnant mice (Kalter, 1957). When C^{14} cortisol was administered to Ajax and CBA mice, the uptake was similar in the 2 strains but the cleft palate sensitive strain (Ajax) retained a larger amount of the compound during 2 hours following injection suggesting that genetic differences in the metabolism or tissue binding may be responsible for the retention

differences and greater susceptibility (Levine, Yaffe and Back, 1968).

Insulin administered to pregnant rats was not teratogenic to the offspring (Kalter, 1968). However, insulin was teratogenic in the mouse producing exencephaly and other abnormalities (Smithberg, Sanchez and Runner, 1956); nicotinamide had no effect on the teratogenic action of insulin in mice. Insulin was also shown to be teratogenic in rabbits (Chomette, 1955); females were injected on 2 successive days of pregnancy; the offspring had microcephaly and ectopia cordis (displacement of the heart outside the thoracic cavity). When cortisone was injected onto the CAM (chorioallantoic membrane) of 8 day chicken eggs, skeletal defects were produced; when cortisone was injected into the yolk sac of 4 day embryos, bone deletions in the skull and abdominal hernia occurred (Karnofsky and Lacon, 1964). In the 8 day embryo, the bones did not stain well and were reduced in size. The authors suggest that in the 8 day embryo, the adrenal steroids seem to interfere with the process of ossification since the precursors of cartilage appear to be normal.

When insulin was injected into fertile chicken eggs, the embryos were completely or partially rumpless (Landauer and Bliss, 1946). Insulin was also found to cause abnormalities of the limbs, beaks, and eyes (Landauer, 1947). The timing and dosage determined whether rumplessness or leg-beak-eye-malformations would occur. Injections during the first 2 days of incubation produced a high incidence of rumplessness whereas injections on days 3 to 7 produced leg-beak-eye-malformations; the highest incidence occurred on day 5. Nicotinamide injections within 3

hours before or after the insulin treatment counteracted the micromelia and reduced the incidence of beak defects and rumplessness; an injection of α-ketoglutarate together with the insulin at 96 hours reduced the incidence of micromelia, but it offered no protection against rumplessness (Landauer, 1948). Zwilling (1951) found that insulin-treated abnormal embryos were hypoglycemic and the more pronounced the micromelia the more pronounced and prolonged was the hypoglycemia. According to Zwilling, the reduced blood sugar in the embryo resulted from inhibition of glycogen breakdown in the yolk sac membrane which serves as a storage for glycogen until day 8 when the liver begins to function. Zwilling also found a relationship between the length and duration of the hypoglycemia and the degree of leg shortening. The limbs were normal if normal blood sugar levels were reached by day 8, moderately micromelic if normal blood sugar levels were reached between day 8 and 10, and acutely micromelic if normal blood sugar levels were reached by day 14 or longer. Zwilling (1959) showed that the hypoglycemia was not directly responsible for the micromelia by producing the micromelia in cultured limbs grown in vitro in a medium containing insulin. The bones grown in the presence of insulin had short shafts and large ends. Bones grown in the presence of insulin and nicotinamide were normal. Histologically, there was more matrix between the cells in bones grown with insulin and nicotinamide than with insulin alone. In another experiment, Zwilling cultured bone rudiments excised on day 6 and 7 from embryos injected on day 4 with insulin. The limbs were abnormal with swollen ends and short long shafts. When day 6 bones were grown in the presence of nicotinamide, they were normal, however,

when day 7 bones were grown in the presence of nicotinamide, the shafts remained shortened but the ends were not swollen. Bones excised on day 8 and grown in the presence of nicotinamide remained distorted.

Azo Dyes

Gillman et al. (1948, 1951) discovered that abnormal offspring were produced when trypan blue was injected into the pregnant rat on day 8 or 9 of gestation. The dye was not present in the tissues of the affected offspring, and he concluded that the malformations must have resulted from a disturbance in the maternal metabolism. The malformations included hydrocephaly, spina bifida, as well as tail, eye, ear and skull defects. When the dye was injected on day 11 or later, the defects were not apparent.

Wilson (1955) studied the effects of 14 dyes by injecting rats with 10 mg of each on day 7, 8, or 9 of gestation. He found that trypan blue was highly teratogenic; Evans blue was less teratogenic and Niagara blue 4B and Niagara sky blue 6B were only slightly teratogenic. Wilson et al., (1963) using C^{14} labelled trypan blue failed to detect the dye in the embryonic tissue. A hypothesis to explain the mode of action of trypan blue has been postulated by Lloyd and Beck (1969). They presented evidence to show that the dye is taken up into the lysosome system of the yolk sac and that the dye inhibits 5 lysosomal enzymes in the liver and yolk sac. They further stated that trypan blue probably induces congenital defects by interrupting the flow of nutrients to the early post-implantation embryo by blocking the hydrolysis of nutritional macromolecules in the lysosomes of the yolk sac epithelium; this conclusion was based

on the fact that interruptions in the uterine blood flow could be teratogenic (Brent and Franklin, 1960).

Trypan blue was first reported to be teratogenic in the chick embryo by Ancel and Lallemand (1941). They exposed 48 hour incubated chicken eggs to the dye producing atrophy of the tail buds in 10% of the embryos. In other experiments, various other malformations occurred including gastroschisis, rumplessness, and hind limb defects; hemorrhages at the site of the defect appeared to be responsible for the malformations (Ancel, 1950). Other malformations attributed to trypan blue, after an injection into the yolk sac or subgerminal cavity at 24 or 36 hours of incubation, included microphthalmia, anophthalmia, cross beak and rudimentary limbs; injections later than 72 hours of incubation were not teratogenic (Beaudoin and Wilson, 1958). Kaplan and Grabowski (1967) reintroduced the theory (Ancel, 1950) that developmental defects caused by trypan blue were due to the presence of a hematoma at the site of the defect. They discovered that embryos which produced a caudal hematoma after trypan blue treatment at 48 hours of incubation produced rumpless chickens, while those that failed to develop a caudal hematoma had normal rumps. The hematoma occurred because of bleeding from a break in the dorsal aorta posterior to the hind limbs. Blood leakage in the tissue spaces resulted in cell death and resorptions which ultimately led to rumplessness.

Cytotoxic Drugs

Various cytotoxic drugs can be teratogenic when administered to embryos. They are capable of interfering with the multiplication of rapidly dividing undifferentiated cells by destroying enzyme function and interfering with

protein and nucleic acid synthesis. Cytotoxic drugs have been used in cancer therapy. Screening tests have revealed malformations in the offspring of test animals. The effect of these drugs have been reviewed by Cohen (1964) and Karnofsky (1963, 1965). The drugs concerned included alkylating agents, hormones, antimetabolites, alkaloids, antibiotics etc. Toxic levels of these compounds administered to intact animals caused inhibition of cell growth, retardation of oral or G. I. tract epithelium and cellular changes in the central nervous system of post-natal animals (Chaube and Murphy, 1968).

a. Alkylating agents

An example is nitrogen mustard which is teratogenic in various animals such as the mouse, rabbit, rat and chick. These compounds act by the substitution of an alkyl group in a molecule for an H-atom, or by substitution of a carbonyl or amino group in proteins, or a phosphate group in nucleic acids. They are capable of forming several types of cross linkages with the double stranded DNA causing its inactivation in vivo. Nitrogen mustard (2 mg) given intraperitoneally on the 10th to 12th day of gestation produced offspring with edema, exophthalmia, amelia and without rumps (Danforth and Center, 1954). Nitrogen mustard produced abnormal offspring in rats; they were reduced in size, and had limb defects, cleft palate, edema, and kidney malformations (Monie, 1961). Murphy et al., (1958) compared the effects of seven alkylating agents injected into rats on the 12th day of pregnancy. The young had defects of the tail, overall growth retardation, brain damage and cleft palate. Rats treated with myleran (1,4-Butanediol dimethanesulfonate) lacked brain damage while those treated with chlorambucil 4(p-(Bis-

(2-chloroethyl) amino) phenyl) butyric acid) had fetal edema.

In the chick, nitrogen mustard was highly toxic, but was only slightly teratogenic. When 0.01 mg was injected into the 4 day egg, death occurred within 1 to 5 days. The survivors were usually normal, however, the odd embryo had crossed beak, thin legs, and reduced weight (Murphy et al., 1957). Cyclophosphamide injected into fertile eggs produced embryos with micromelia and beak defects (Gerlinger, 1963).

b. Antimetabolites

Another group of cytotoxic agents are antimetabolites. They are structural analogues of naturally occurring compounds such as vitamins, purines, pyrmidines and amino acids. They interfere by causing a deficiency of the metabolite or by substituting for it in the corresponding metabolic pathway or system.

1. Purine analogues

The most active purine analogues in rats is thioguanine and thioguanosine; they produced offspring with cleft palate and retarded limbs. The least active is 9-ethyl mercaptopurine which produced only kinky tail (Chaube and Murphy, 1968). Another analogue, 8-azaguanine is metabolized in the same manner as guanine and is incorporated into DNA thereby inhibiting protein synthesis. When female mice were given a single intraperitoneal injection of 8-azaguanine, cleft palate and limb defects occurred (Nishimura and Numura, 1958). Deoxyguanosine is not teratogenic in the rat embryo, but it is teratogenic in the chick embryo; the malformations included bone deletions in the limbs, cleft

palate, beak defects, edema, and feather abnormalities (Karnofsky and Lacon, 1961). Deoxyguanosine interferes with the formation of deoxycytidine since an injection of the latter compound will prevent the teratogenic effects of the former compound (Karnofsky and Lacon, 1962).

2. Pyrimidine analogues

The pyrimidine analogues affect cell function by interfering with RNA and DNA synthesis. Single injections of the fluoropyrimidines in the rat from the 9th to 17th days of gestation produced embryos with cleft palate and deformed appendages, paws and tails (Murphy, Dagg, and Karnofsky, 1957).

In the chick, 5-fluorouracil injections into the 4 day egg produced embryos with skeletal defects including shortening of the lower beak, cleft palate, and malformed tibia and metatarsals; other halogenated pyrimidines are not teratogenic in the chick embryo (Karnofsky, Murphy, and Lacon, 1958).

3. Glutamine analogues

Glutamine analogues such as azaserine and diazo-norleucine inhibit purine synthesis by preventing the conversion of formylglycineamide ribonucleotide to formylglycineamidine ribonucleotide; glutamine transfers its amino group to the latter compound. These analogues may also prevent transamination reactions e. g., xanthylic to guanylic acid or uridylic to cytidylic acid, therefore blocking de novo purine synthesis. Azaserine is teratogenic in the rat; when pregnant females were given a single dose from the 7th to 14th day of gestation, the abnormalities included cleft palate, fused ribs and vertebral column defects. Those injected on day 7

or 14 had normal offspring. When azaserine was injected into 4 day incubated chicken eggs, the embryos had defects of the beak and appendicular skeleton, microthphalmia, edema, feather reduction and overall growth inhibition. When injected into 8 day incubated chicken eggs, the embryos had edema and feather reduction. Injections into 12 day incubated chicken eggs produced embryos with edema and feather inhibition, however, no skeletal defects occurred. Injections of nicotinamide, riboflavin, pyridoxine, ascorbic acid, and biotin failed to protect the embryo against azaserine (Dagg and Karnofsky, 1955).

4. Nicotinamide analogues

The nicotinamide analogue, 6-aminonicotinamide, was teratogenic in the rat (Chamberlain and Nelson, 1962; 1963); abnormalities included hydrocephaly, cleft palate, eye malformations, and urogenital and cardiovascular defects. In these experiments, rats were fed niacin-deficient diets during the second week of pregnancy for a period of from 48 to 72 hours; those fed the deficient diets on days 8 to 10 of pregnancy showed the highest incidence of abnormalities. The toxic effects and abnormalities caused by 6-aminonicotinamide can be prevented by an injection of 100 mg/kg of nicotinamide just before the injection of the analogue.

In the chick, 6-aminonicotinamide injected into the yolk sac of the 4 day incubated egg produced embryos with parrot beak and micromelia with bending of the tibiotarsus and tarsometatarsus bones. These effects could be prevented by a simultaneous administration of nicotinamide (Murphy, Dagg and Karnofsky, 1957). Landauer (1957)

obtained similar results with 6-aminonicotinamide, however, when he injected another analogue of nicotinamide, 3-acetylpyridine, into 96 hour fertile chicken eggs, most of the embryos did not hatch. Examination of the embryos revealed malformations, with the main defects being muscular hypoplasia, edema and a shortened upper beak; the body size was only slightly reduced and the long bones appeared normal in length but were thin. When both of these analogues were injected into 24 hour incubated chicken eggs, 51% were rumpless after 6-aminonicotinamide; whereas after 3-acetylpyridine, 25% had various abnormalities including eye defects, cerebral hernia, rumplessness and muscular hypoplasia. The teratogenic effects of 3-acetylpyridine could be reversed by an injection of nicotinamide (Ackerman and Taylor, 1948)

c. Antibiotics

Antibiotics have also been found to be teratogenic. These compounds inhibit nucleic acid and protein synthesis. An example is actinomycin D which is produced by soil bacteria. It blocks DNA-dependent RNA synthesis. When this drug was injected into pregnant rats on day 7 of gestation, the offspring had hydrocephaly and ectopia cordis; when injected on day 8, 16% were malformed with axial skeletal defects and hydrocephaly; when injected on day 9, 28% were malformed with similar malformations as day 8 injections; when injections were performed on day 10, the embryos had ocular and axial skeletal defects (Wilson, 1964). Another antibiotic, mitomycin C, blocks DNA synthesis in bacteria, viruses, and mammalian cells in culture. This compound does not produce malformations in rats; however, it is teratogenic in the mouse (Tanimura,

1967; Chaube and Murphy, 1968). Tetracycline, and penicillin and streptomycin have been shown to be teratogenic in rats producing cleft palate, hypoplasia of the mandible, micromelia and syndactyly for the former compound, and micromelia, syndactyly and ectrodactylia when the latter two compounds were injected together (Filipi, 1967).

Tetracycline was teratogenic when injected into the yolk sac of the chick, producing skeletal malformations and growth inhibition (Rolle and Bevelander, 1966). Tetracycline inhibited the rate of mineralization of the osteoid matrix which resulted in defects of the femur and mandible. The authors concluded that the concentration of tetracycline and not the time of administration was important in producing the observed terata.

Pierro (1961a) injected actinomycin D into 24, 48, and 96 hour incubated chicken eggs; 33%, 85%, and 4% of the embryos were rumpless, respectively. If DNA and actinomycin D were premixed and then injected together, the embryos were normal; however, the toxicity increased. Pierro (1961b) made a histological study and found that cell death and degeneration occurred in the trunk, tail, and unsegmented somatic mesoderm. The rumpless condition was attributed to cell death which completely blocked the development of the posterior trunk regions.

d. Alkaloids

Some alkaloids were found to be teratogenic in rats. Two of them, vinblastine and vincristine produced developmental defects in the mouse and rat (Cohlan et al., 1964; Cohlan and Kitay, 1965). When vinblastine was administered intraperitoneally from days 7 to 12 of

gestation, 60% of the embryos were resorbed and 8% had developmental malformations including stunted growth, hydrocephaly and hind limb defects. Vincaleucoblastine was also teratogenic; treated embryos showed a 6-fold increase in metaphase arrest which was responsible for the teratogenesis. Vincristine was not teratogenic in the rat.

Pilocarpine and eserine were teratogenic in the chick (Landauer, 1953 and 1954; Ancel, 1950). Eserine injections into the 4 day chicken egg produced embryos with parrot beaks, micromelia, and bent tibiotarsus bones, syndactylism, and feather defects. The limb defects could be reversed by injections of nicotinamide within 3 hours of the teratogen. Pilocarpine injections into the yolk sacs of 24 hour incubated chicken eggs produced rumpless embryos; when injected into 96 hour eggs, the embryos had limb defects. The dose level was important because an injection of 3 mg per egg at 96 hours produced embryos with syndactylism, whereas an injection of 6 or 12 mg per egg at 96 hours produced embryos with syndactylism and short, bent tibiotarsus bones. The terata produced by 3 mg pilocarpine could be reversed by nicotinamide, however, the terata produced by 6 or 12 mg per egg were irreversible. Colchicine was also found to be teratogenic in the chick producing micromelic embryos (Ancel, 1950).

e. Viruses

Gregg (1941) was the first to observe that a rubella virus infection in pregnant humans could cause malformations in offspring; they included congenital cataract, cardiac lesions, deafness, and mental retardation.

The number of malformed offspring increased if exposed during the first 4 weeks of pregnancy and declined up to the 13th week, after which none occurred. Forty-seven percent of the offspring were malformed if exposed during the first month of pregnancy; 22% were malformed if exposed during the second month of pregnancy, and 7% were malformed if exposed during the third month of pregnancy (Michaels and Mellin, 1960). The virus was found in cells of fetal organs as late as the fourth month of pregnancy (Monif, et al., 1965), and malformations were attributed to viral inhibition of cell multiplication.

Viruses were also teratogenic in other animals. Shimizu et al. (1954) found that Japanese B encephalitis virus injected into pregnant swine during early pregnancy caused stillbirths and hydrocephaly in the offspring. Young et al. (1955) gave live hog cholera virus to pregnant swine which produced malformations to 38% of the offspring. In the aforementioned articles, there was no illness to the adults and in the latter case, prior immunization of the sows did not prevent infection of the fetus.

In the chick, Hamburger and Habel (1947) injected influenza virus into the blastoderm of 48 hour chicken eggs; the defects included microcephaly, twisting of the axis, and impairment in growth of the amnion. The virus was not teratogenic when injected at later stages. Mumps virus, on the other hand was not teratogenic in the chick.

Thalidomide

Thalidomide is a tranquilizer which was prescribed to pregnant mothers in the early 1960's. Many cases of limb defects in newborn

children were reported by McBride (1961) and Lenz (1962) and were found to be caused by thalidomide taken in early pregnancy. The main defects (previously very rare) included the reduction of one or all bones of the upper or lower limbs. The malformations were mainly symmetrical and bilateral with the upper limbs being affected more often than the lower limbs. Other malformations attributed to thalidomide included hypoplasia of the pinnae, microphthalmia, congenital heart disease, and lesions of the intestine. The thalidomide incident led to many investigations in animals.

Giroud et al. (1962) gave thalidomide orally to pregnant rats, mice, and rabbits early in pregnancy and reported teratogenic effects in the mouse and rabbit but none in the rat. The malformations included cleft palate, anencephaly, and limb defects (the limbs were of the club foot type, however, no shortening occurred). Cook and Moore (1967) added thalidomide to the diet of female rats 3 days before mating and throughout pregnancy. The defects in the offspring included interference with ossification of vertebrae, ribs, and skull, as well as defects of the pelvic girdles and limbs. The susceptibility to thalidomide varies widely among species and strains. Human embryos were deformed when pregnant mothers were given daily treatments of from 0.5 to 1.0 mg/kg for several days (Lenz, 1964). Thalidomide was teratogenic in baboons after 5 mg per day was given from 12 to 22 days (Hendrick, Axelrod, and Claybcorn, 1966). Implantation was prevented in rhesus monkeys when 50 mg/kg was given daily for several days just after mating (Lucey and Behrman, 1963). Rabbits were very sensitive to thalidomide;

a daily dose of 30 mg/kg was teratogenic (Seller, 1962).

Thalidomide was also teratogenic in the chick. DeBock and Peters (1963) injected 1 to 5 mg per egg on day 1 of incubation; the embryos had short or twisted limbs, had only 1 lower limb, or had fused wings. Salzgeber and Salaun (1963) administered thalidomide to chicken eggs producing malformations in 30% of the embryos; limb malformations accounted for 15 to 22% of the malformed embryos.

The teratogenic mechanism of thalidomide-induced malformations is still unknown, however, the drug appears to enter the cells of the embryo and is then hydrolysed into polar compounds which are unable to cross membranes once formed (Williams et al., 1965). Fabro et al., (1964) have shown that the glutarimide ring is not essential for the production of terata.

Miscellaneous Compounds

Various sulphonamides were teratogenic in the chick (Ancel, 1950; Zwilling and DeBell, 1950; Landauer, 1968). Sulphanilamide produced parrot beak, micromelia with bent tibiotarsus bones, syndactylism and feather defects when injected into day 5 embryos; the micromelia was alleviated by an injection of nicotinamide (Zwilling and DeBell, 1950).

Various metals were teratogenic in the chick (Ridgway and Karnofsky, 1952). Of those tested, thallium produced micromelia and beak defects after injections into the 4 to 8 day yolk sacs. Arsenic produced stunted growth, micromelia, and edema after injections into 4 day yolk sacs, while rhodium produced stunted growth, feather inhibition, micromelia, and mild edema after injections into 8 day CAMS.

Lead nitrate produced brain hemorrhages and hydrocephaly after injections into the 4 day yolk sacs. Boron (boric acid) caused feather abnormalities, edema, and beak defects after injections into 4 day yolk sacs. Landauer (1954) found that boric acid caused short mandibles, micromelia, syndactylism, and curled toe paralysis after injections into 4 day yolk sacs; nicotinamide reduced the incidence of facial abnormalities, but offered no protection against micromelia.

Triazene (3,3 dimethyl-1-phenyltriazene) is an anticancer agent which caused developmental defects in chickens and rats (Murphy et al., 1957). Females treated on day 11 of gestation produced offspring with limb defects, edema, and growth inhibition; treatment on day 12 produced embryos with cleft palate, fused ribs and malformed paws. Injections of triazene in 4 day chicken eggs produced embryos with weight inhbition, edema, feather defects, micromelia with bent tibiotarsus bones, and beak abnormalities. Nicotinamide prevented the skeletal defects but did not prevent the stunting or reduce the mortality.

Thiadiazole (2-ethylamino-1,3,4-thiadiazole), an anticancer agent, which increased the uric acid content in chickens and man because of an increase in de novo purine synthesis, was teratogenic in rats. Rats treated on day 9 had normal offspring, however, those treated on day 11 had offspring with short or absent tails, and syndactylism. Rats treated on the 12th day had cleft palate, hare lip, and skeletal defects of the tail and limbs (Murphy et al., 1957). Thiadiazole was not teratogenic after injections into 4 day chicken eggs.

Although not all teratogenic agents were covered in this review, it is evident that the embryo is susceptible to congenital defects by a variety of unrelated compounds, especially at the time of organogenesis during the period of differentiation.

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