ACKNOWLEDGMENTS

This work was supported by a National Research Council of Canada Grant A-1019.

My thanks to all of the people who supported and encouraged me during my research. The number of people are too numerous to mention. I am sure you are all aware of who you are and will accept my sincere appreciation.

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ABSTRACT

Organophosphates because of their many advantages have in most cases replaced the chlorinated hydrocarbons such as D.D.T. Malathion (Cythion)^{R} is one of the organophosphate insecticides least toxic to mammals. The toxicity and the production of terata by Malathion has been recorded, but the causes of the malformations have not.

The organophosphate, Malathion^{R}, has been used in these studies to establish a reproducible system for investigating teratogenesis during development of the chick embryo. The teratogenic agent interfered with normal development of the limbs.

Staining with Toluidine Blue revealed a deficiency of cartilage characterized by a visible lack of ground substance surrounding the hypertrophied chondroblasts. Poor gamma metachromasia indicated a lack of anionic groups in the extracellular matrix. Specific staining for the chondroitin sulphates in the ground substance showed a deficiency of these sulphated mucopolysaccharides. This deficiency was borne out by radioautography with S^{35} which showed little incorporation into the chondroitin sulphates of the matrix in the cartilage of both forelimbs and hindlimbs of the embryo.

Preliminary studies have shown a lack of perichondrium surrounding the cartilage model. Thus it would appear that the synthesis of the protein collagen is being affected.

A histochemical study of the alkaline phosphatase involved in matrix formation and calcification of cartilage showed that the production of the enzyme was affected in the Malathion-treated limbs. Biochemical assay confirms the results of this histochemical study.

RESUME

Les avantages que présentent les organophosphates ont fait qu'ils ont remplacé très souvent les hydrates de carbone chlorés tels que le D.D.T.

L'organophosphate Malathion (Cythion)^{R} est un insecticide moins toxique pour les mammifères que le D.D.T. La toxicité du Malathion et la production de formes exceptionnelles (monstres) ont été étudiées et observées mais les causes des malformations sont inconnues.

Le Malathion^{R} a été utilisé dans le but d'établir un système permettant d'étudier la tératogénèse au cours de la formation des embryons de poulets. L'action tératogène se manifeste au niveau des membres. La coloration des tissus au bleu de toluïdine met en évidence des anomalies du cartilage caractérisées par l'hypertrophie des chondroblastes. La faible métachromasie γ suggère une déficience de groupes d'anions dans la matrice extracellulaire.

La coloration spécifique des sulfates de chondroïtine de la matrice indique une déficience de ces mucopolysaccharides. Cette déficience a été mise en évidence par la faible incorporation du S^{35} aux sulfates de chondroïtine de la matrice des cartilages des membres antérieurs et postérieurs des embryons.

Des études préliminaires ont révélé une absence de périchondre autour du cartilage embryonnaire ce qui porte à croire que la synthèse du collagène est touchée.

Une étude histochimique de la phosphatase alcaline utilisée dans la formation et la calcification du cartilage a démontré que la production de l'enzyme a été influencée dans les membres des embryons traités au Malathion. Des tests biochimiques confirment les résultats de cette étude histochimique.

INTRODUCTION

A- A History of the Development of Studies of Terata

Malformations occuring in embryos have been a subject of human concern since man first began to keep records of the history of mankind. One of the earliest recorded accounts was shown in Egyptian wall paintings made 5,000 years ago showing a club foot (Steindorf 1939). From many recorded observations the subject of malformations and their causes developed into the study of Experimental Teratology.

The first experimental teratologist was Etienne Geoffroy-de-Saint-Hilaire (1832). During the early 19th century he attempted to reproduce abnormalities in chick embryos by physical trauma and abnormal atmospheric conditions. Féré (1909) not only used physical measures but injected numerous chemicals into chicken eggs and described the abnormalities he induced.

Experimental mammalian teratology has progressed rapidly since 1940. The first experiments included the use of physical agents, temperature and humidity changes, hypoxia and irradiation. Various hormones such as cortisone, insulin and certain sex hormones have been employed. Certain nutritional deficiencies or excesses and some viral diseases have also produced malformations in embryos. Since the early experiments the study of teratology has extended to include the effects of chemicals and drugs during embryonic life and growth.

Stockard (1921) observed that the type of malformation produced depended on the stage during development when the treatment was administered (Ancel 1950 and Landauer 1954). It was shown that various agents administered at the same stage of chick embryo development tended to produce their own unique syndrome of malformations. Malathion administered at days $4\frac{1}{2}$ - 5 of development produces a type of syndrome between days 14 and 20 of embryogenesis in the chick embryo (Greenberg and LaHam 1969).

The drug Thalidomide^{R} is an example of a chemical that will produce terata. This tranquilizer, popular in the 1960's caused the reduction of one or all the bones of the upper and lower limbs. The malformations were usually symmetrical and bilateral with the upper limbs more often affected than the lower. Thalidomide caused these limb defects if the drug was taken early in pregnancy (McBride 1961).

B- The Role of Organophosphates and Their Toxicity

Many of the organophosphates used widely as insecticides appear to have a lower mammalian toxicity (O'Brien 1960) but are highly lethal to insects (Spiller 1961). Recent investigations have shown that certain organophosphates have produced gross teratisms in certain species of Teleost fishes (Matton 1970).

Organophosphates undergo self-degradation rapidly by isomerization or by hydrolysis with immediate loss of their actual potential biological activity (O'Brien 1960, 1967).

Another important factor in detoxification is the ability of the organism to degrade the organophosphate and to do so rapidly. The organophosphate can be degraded by various tissues including liver, kidney, lung, brain and muscle (Murphy and Dubois 1957; Seume and O'Brien 1960). Therefore, the mode of administration of a compound to an animal has a great influence; the toxicity will be largely decreased if the compound goes through the liver before reaching its target or organ systems.

In the chick embyro the cells of the liver are present at an early stage of development (day 5) but they do not begin to function until approximately day 10 of development (Romanoff 1960). Therefore, this makes the chick embryo an ideal experimental subject in which to locate and determine how the target of the teratogenic agent was affected. In this project Malathion was selected as an agent because it is a consistent producer of malformations and it is one of the most common pesticides in use.

C- Statement of Problem

It is known that various agents can induced congenital malformations or terata in developing avian and mammalian embryos (Landauer 1954; Landauer and Wakasugi 1968; Overman and Beaudoin 1971).

One of the less toxic organophosphates Malathion (Cythion)^{R} (0,0-dimethylS-(1,2-dicarbethoxyethyl) phosphorodithioate), has been used to achieve consistent reproducible malformations in chick embryos (Greenberg and Laham 1969).

The gross malformations seen in the hindlimbs at days 15 to 20 of embryogenesis consisted of micromelia, curled-toe paralysis and the absence of certain bones such as the tarso-metatarsus and phalanges. It was thought at this time that the forelimbs were unaffected.

A normal cartilage model must be produced in order that normal bone formation occurs in the long bones of the limbs. By studying the formation of cartilage or bone and its components in normal and malformed limbs it was hoped that we could demonstrate when and in what manner the target tissue was being affected.

A current theory states that teratogens are strong chelating agents and chelate metal ions such as iron, zinc, cobalt or copper. These metal ions are essential for the activity of enzymes (e.g. protocollagen proline hydroxylase) needed for collagen synthesis and maturation (Barr 1973). Inhibition of synthesis and maturation of collagen will result in malformations in developing structures of exposed embryos.

MATERIALS AND METHODS

Inoculations

A- Procedure

Fertile white leghorn eggs Strain F 934, purchased from a commercial hatchery (Neuhauser Poultry Co., Stratford, Ont.) were used during these studies. The eggs were incubated in a Jamesway single-stage incubator at dry and wet bulb readings of 38°C. and 30°C. respectively. On candling, eggs with improperly calcified shells, tremulous air-chambers or blood clots were discarded. The air-chamber, through which inoculations were to be made, was outlined and the position of the embryo within the egg was marked.

The blunt end of the egg was sterilized with 70% ethyl alcohol and a hole was punched in the mid-centre with an egg punch. Loose pieces of shell around the hole were removed and the incoulations were made. Injections were given into the yolk at an angle of 45° away from the embryo to avoid traumatizing it. The hole was sealed with a few drops of molten wax and the eggs were returned to the incubator when the wax was hardened. In all cases of inoculations a total of 0.1 ml solution was injected using a sterile tuberculin syringe and a 20-gauge, 1-inch needle.

The eggs were candled every 24 hours and samples of control and malformed embryos collected. Dead embryos were discarded.

B- Compounds injected -

1- Malathion - To achieve a consistent number of malformations an injection of 6% Malathion or 7.02 mg per egg at days 4½ - 5 of development (Hamburger and Hamilton 1951) was employed. This was shown by Greenberg and LaHam (1969) to produce malformations in 80% of surviving embryos.

Control samples were injected with 0.1 ml corn oil (Mazola)^{R} or 0.1 ml of physiological saline, also control stabs or untreated were run concurrently in each experiment.

Preparation of Malathion - 6% (7.02 mg)

9.4 mls of corn oil (Mazola) were mixed with 2.6 ml of 95% Technical Grade Malathion.

C- Preparation and fixation of materials

1- Fixative

In wax embedding the procedure of Williams and Jackson's Formula for cetyl pyridinium chloride was used (Clark and Grant 1961; Pearse 1969). Cetyl pyridinium chloride forms insoluble complexes with acid mucopolysaccharides (Conklin 1963).

2- Sample number selection

In histological and histochemical observations 100-150 slides were examined for each parameter of the individual experiments.

D- Measurement of limbs

Day 6-12 embryos were fixed in Williams and Jackson's fixative (Pearse 1968) for 48 hours after which all four

limbs were then amputated and measured using a dissecting microscope with an ocular micrometer. This was necessary to determine at what stage of development the limbs were being affected.

E- Initial Morphological and Histochemical Investigations

Group A - Controls - The controls were untreated, control stab and 0.1 ml inoculations of physiological saline and corn oil.

Group B - Treated - An 0.1 ml inoculation of 7.02 mg of 95% Technical Grade Malathion (Cyanimid of Canada) in corn oil was injected.

Whole limb samples were fixed in Williams and Jackson's Fixative for at least 96 hours. The limbs were dehydrated, embedded in paraffin and sectioned longitudinally at 6 micra and stained in 0.1% buffered Toluidine Blue (pH 4.4) for 2 minutes (Pearse 1968). The dye Toluidine Blue exhibits metachromasia in the presence of certain anionic groups e.g. the carboxyl and sulphate groups (Clark and Grant 1961; Pearse 1968). Toluidine Blue demonstrates metachromasia and is used to study cartilage.

F- Identification of Different Acid Groups of The Acid Mucopolysaccharides (Chondroitin Sulphates)

Histology - Sections of 6 μ from control stab and Malathion-treated samples were given identical treatment (Carlo 1963). The slides were stained for 30-60 minutes with Alcian Blue 8G X 300 0.5% (E. Gurr Ltd.) buffered at

pH 0.5; washed for 10 seconds in Walpole's sodium acetate-hydrochloric acid buffer pH 0.5 (Culling 1963); rinsed in water and stained for 30-60 minutes with Alcian Yellow GX (E. Gurr Ltd.) 0.5% pH 2.5.

The slides were examined at 10 minute intervals from 30-60 minutes for colour reaction, 100 slides were examined at each time period. Sulphated groups are coloured blue and carboxyl groups yellow. Acid mucopolysaccharides (chondroitin sulphates) which possess both carboxyl and sulphated groups are coloured blue-green.

G- Radioautography S^{35}

To determine the distribution of the predominant acid mucopolysaccharides (chondroitin sulphate) of the ground substance radioautography was done using S^{35} (aqueous solution carrier free, pH 6-8, Amersham and Searle, Toronto).

Controls of normal untreated, control stab and corn oil + isotope (S^{35}) were used. A dosage of 5-microcuries (µc) dissolved in 0.1 ml of physiological saline was used to make up the isotope solution. In the Malathion-treated embryos a dosage of 6% (7.02 mg) of Malathion was followed by an injection of 5 µc of S^{35} dissolved in 0.1 ml of physiological saline.

Histology - (Radioautography) - Sections of 6 micra were mounted on pretreated and subbed slices (Rogers 1967). They were dipped in a solution of celloidin and dried for 24 hours and then dipped in Kodak NTB_3 emulsion. The light

source was a Wratten series # 2 safelight. Temperature of the darkroom was 68°F and the relative humidity was 45-50%. The coated slides were dried and then exposed in sealed boxes containing drierite at 4°C. 10 test slides were developed every 24 hours from days 3 to 9 of exposure to determine the correct exposure time. Slides were developed in the darkroom for 10 minutes in Kodak D19 at 4°C and then washed for 30 seconds in distilled water at 4°C. The radioautographs were hardened in fixer (Kodak) at 4°C. and washed 1 hour in cold running water. If slides were not to be stained they were mounted in permount immediately.

Slides to be stained were next washed in cold absolute ethyl alcohol 5 minutes followed by 2 changes of 5 minutes each of absolute ethyl alcohol at room temperature. The slides were then stained with 0.1% buffered Toluidine Blue (pH 4.4) for 2 minutes, dehydrated, cleared and mounted in permount.

H- Connective Tissue Study of Cartilage

Cartilage contains collagen fibres which contribute to its tensile strength. The outermost layer of perichondium surrounding the cartilage model is predominantly of collagen fibres. A modified method of Weigert and Van-Gieson's Connective Tissue Stain was used in an attempt to stain the collagen fibres (Culling 1963).

The results show:

Nuclei - blue-black

Collagen - bright red

Cytoplasm and muscle - yellow-brown

I- Histochemical and Biochemical Study of Alkaline Phosphatase (E.C.3.1.3.1.)[a]

Alkaline phosphatases play a role in matrix formation and calcification of cartilage. Two methods were used to evaluate the presence of the alkaline phosphatases.

A- Histochemical Method - A modification of Gomori's Technique was used (Pearse 1968). This method depends on the deposition of calcium phosphate at sites of enzyme activity. Sections are incubated with an organic phosphate ester in the presence of Ca^{++} at pH of 9.

Treatment of specimens - Alkaline phosphatase was determined on treated and control embryonic limbs from days 6-12 of development.

The embryos were removed from the shell, rinsed in saline, blotted with filter paper and both forelimbs and hindlimbs were amputated. Each limb was placed in a scotch tape boat filled with Tissue-Tek^{R} compound (Ames Co.). Samples were quick frozen in liquid nitrogen and stored at -80^{o}C.

Longitudinal sections of 12 µ were cut and mounted on coverslips and Gomori's Modified Method for Alkaline Phosphatase used. Clean black deposits of cobalt sulphide indicate sites of enzyme activity.

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a- Nomenclature - Commission on Enzymes of the International Union of Biochemistry (1961).

B- Biochemical Method for Alkaline Phosphatase -

Forelimbs and hindlimbs were amputated from freshly killed day 6-11 embryos, washed in physiological saline, blotted dry and frozen in liquid nitrogen and stored at -60°C. The number of limbs in each sample was determined on a weight basis.

The sample (fore- or hindlimbs) was homogenized at 4°C. for 5 minutes in a glass tissue grinder at 4°C and centrifuged at 3,000 r.p.m. for 15 minutes at 4°C.

The alkaline phosphatase was determined on a sample of the supernatant using an enzyme kit (Boerhinger and Manheim Corp., New York). The test is based on the fact that Phosphatase splits p-nitrophenyl phosphate into organic phosphate and p-nitrophenyl which is yellow in an alkaline solution. Enzyme activity was measured by the yellow colour formed using a Gilford Spectrophotometer Model 2400. The wave length used was 460 nm.

RESULTS

D- Measurement of Limbs

Total sample numbers of limb measurements were taken for a period of 7 days (days 6-12 of embryogenesis). They consisted of:

Control stab	126 forelimbs	126 hindlimbs
Untreated	125 "	125 "
Corn oil injected	144 "	144 "
6% Malathion injected	420 "	420 "
TOTAL	815 forelimbs	815 hindlimbs

Using an analysis of variance (Scheffé test at 5%) Figs. 1-4 showed that the definite effect of Malathion on limb growth occurs between days 8-10 in development. Therefore all experiments perfomred subsequent to these measurements were carried out routinely between days 6-12. Unless otherwise indicated day 9 samples were used for illustration of results.

E- Initial Morphological and Histochemical Investigations

There was no observable differences in the gross morphological or histological appearance between the untreated controls corn oil injected and control stabs. Standard deviations of the measurement of limb growth were also insignificant. Therefore, the embryos from the control stab were chosen as representative control specimens.

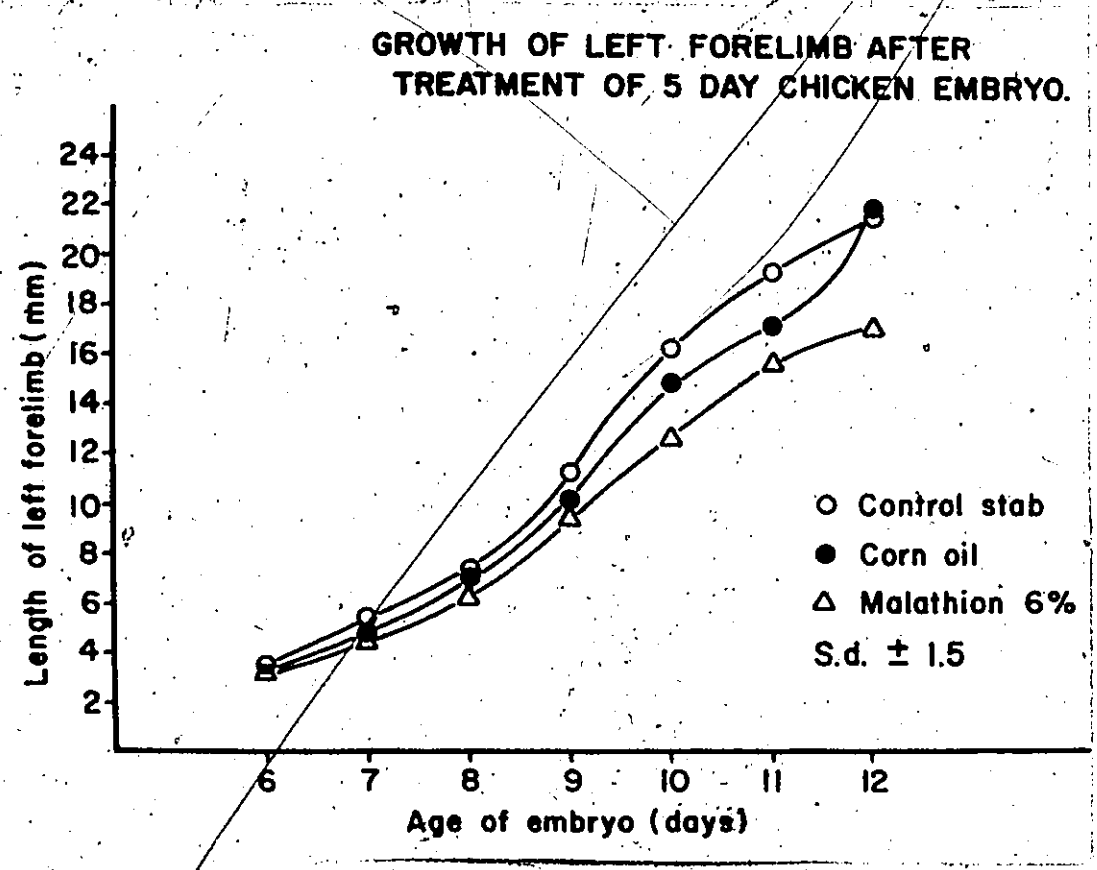

GROWTH OF LEFT FORELIMB AFTER
TREATMENT OF 5 DAY CHICKEN EMBRYO.
Length of left forelimb (mm)
24
22
20
18
16
14
12
10
8
6
4
2
6
7
8
9
10
11
12
Age of embryo (days)
Control stab
Corn oil
Malathion 6%
S.d. ± 1.5

Fig. 1

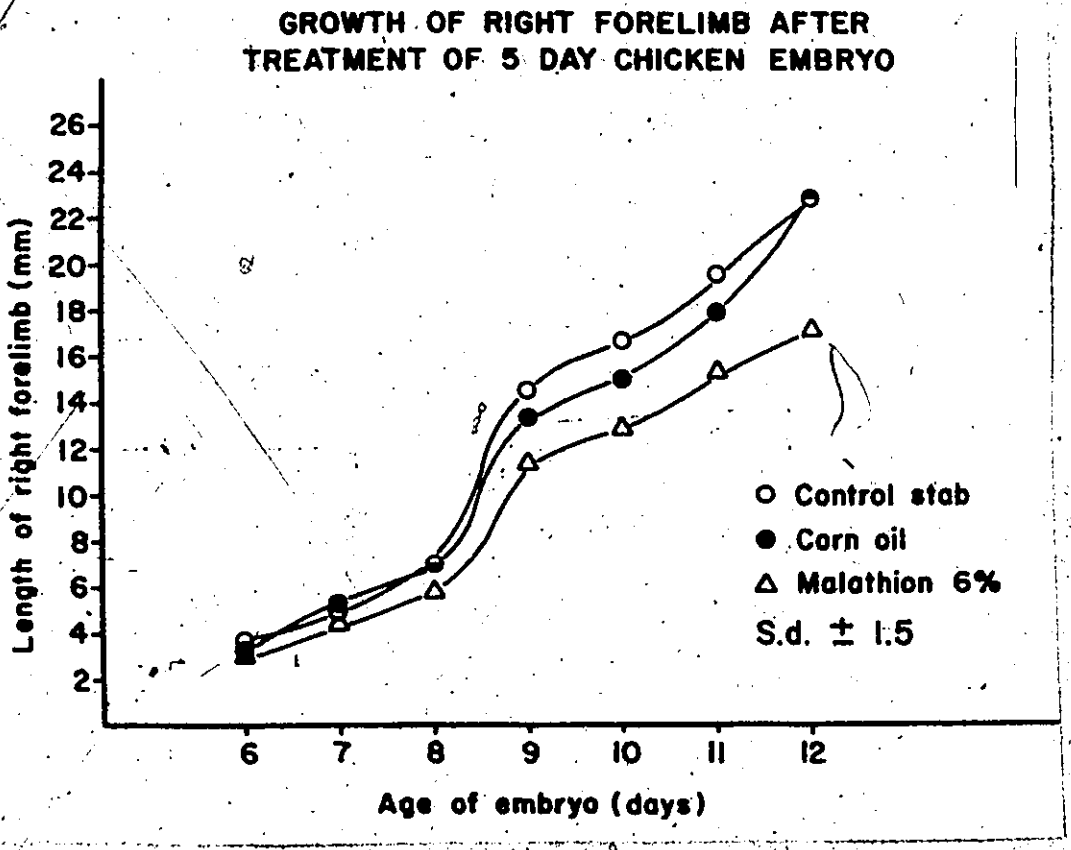

GROWTH OF RIGHT FORELIMB AFTER
TREATMENT OF 5 DAY CHICKEN EMBRYO
Length of right forelimb (mm)
26
24
22
20
18
16
14
12
10
8
6
4
2
6
7
8
9
10
11
12
Age of embryo (days)
Control stab
Corn oil
Malathion 6%
S.d. ± 1.5

Fig. 2

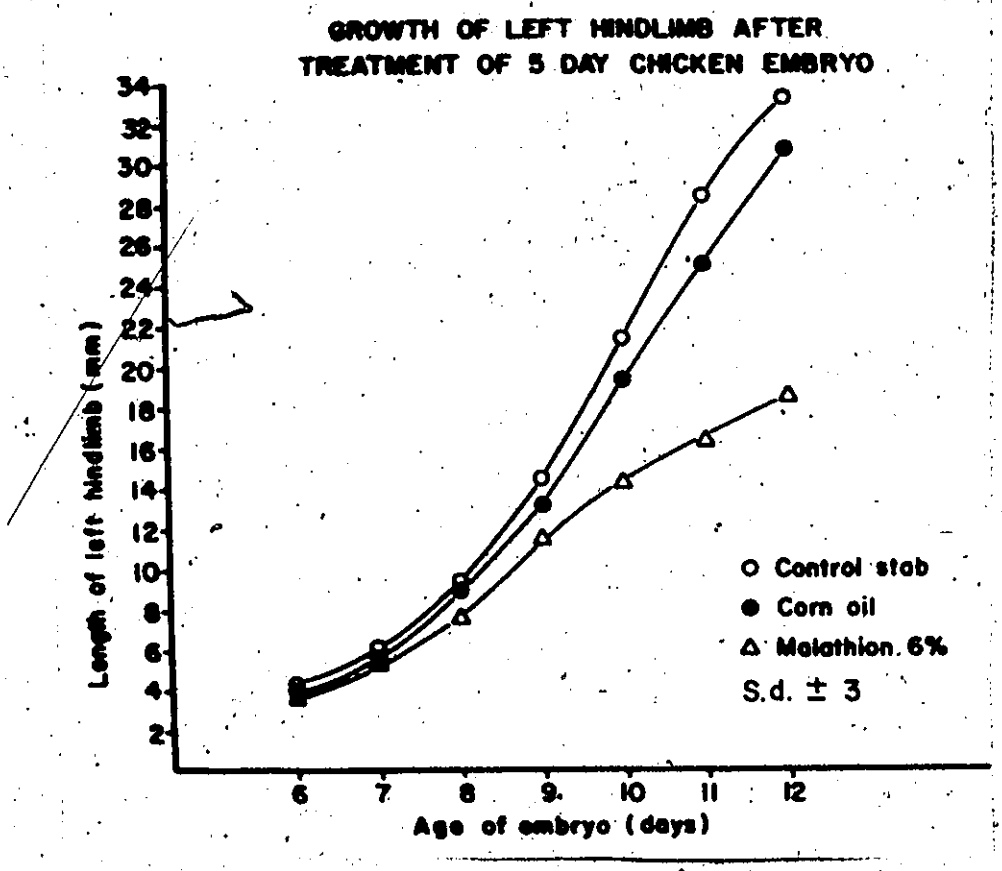
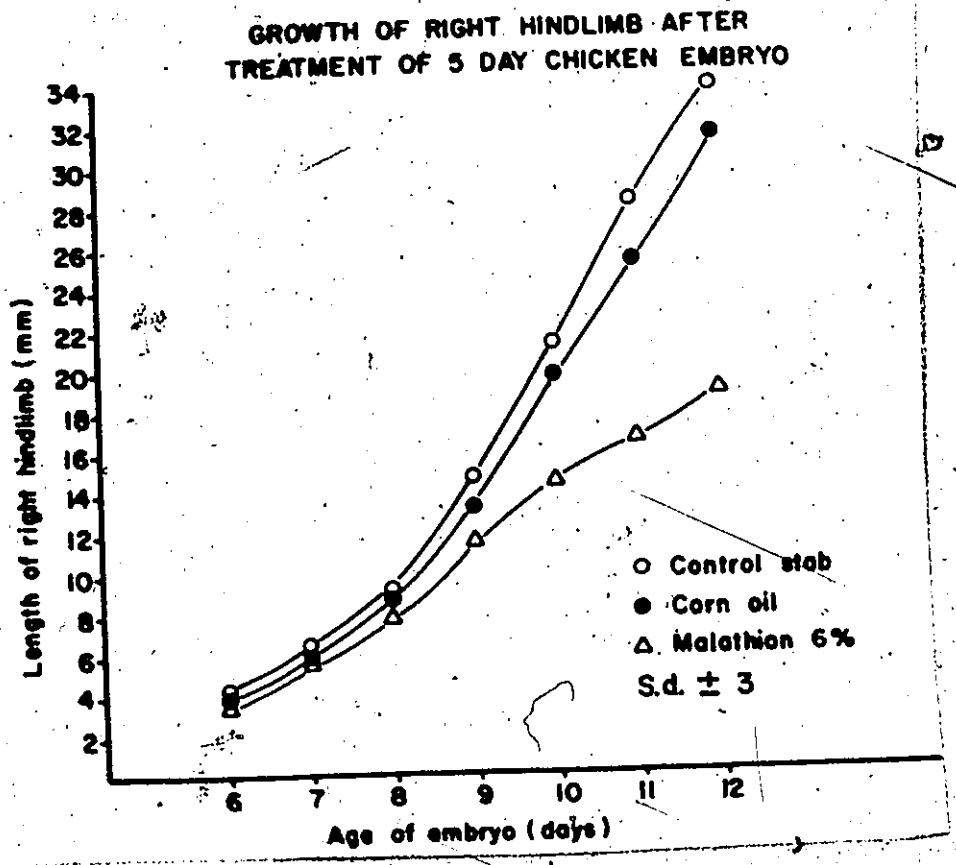

GROWTH OF LEFT HINDLIMB AFTER
TREATMENT OF 5 DAY CHICKEN EMBRYO
Length of left hindlimb (mm)
Age of embryo (days)
Control stab
Corn oil
Malathion 6%
S.d. ± 3

Fig. 3


GROWTH OF RIGHT HINDLIMB AFTER
TREATMENT OF 5 DAY CHICKEN EMBRYO
Length of right hindlimb (mm)
Age of embryo (days)
Control stab
Corn oil
Malathion 6%
S.d. ± 3

a- Forelimbs - The forelimbs of the control stab embryos had normal cartilage formation; the radio-ulnar development appeared normal and was well developed. Histochemical study showed γ metachromasia in the cartilage stained with the buffered Toluidine Blue especially over the matrix and around the lacunar margins (Fig. 5). The cartilage was normal in all respects.

The forelimbs of the Malathion-treated embryos showed abnormal development of the radii and a lack of chondrogenic tissue. The ulnae were absent (Fig. 6). The staining pattern was uneven with light pink areas interspaced by darker metachromatic areas resulting in a "banding effect" in the cartilage.

b- Hindlimbs - The hindlimbs of the control stab embryos had normal curvature at the tibio-tarsus joint (Fig. 7). All tibiae were well formed and the tibio-femur joints were normal in appearance. Gamma metachromasia was present in all cartilage elements.

Hindlimbs of Malathion-treated embryos exhibited oedema and lacked the normal tibia-tarsul curvature (Fig. 8). The tibia-femur joint was malformed and in some cases absent in the Malathion-treated embryos compared to the control (Figs. 9 and 10). In the affected hindlimbs certain chondroblasts were hypertrophied and even vacuolated in some cases along with an abnormal amount of extracellular matrix. In certain limbs the mesenchyme failed to differentiate into chondroblasts (Figs. 9 and 10).

Fig, 5. Section of a forelimb from a day 9 embryo- controls. The development of the cartilage is normal in the radius and ulna. Toluidine Blue. 45x.

Fig. 6. Forelimb section from a Malathion-treated embryo showing abnormal development of the radius and lack of chondrogenic tissue (arrow). Note the "banding effect" in the cartilage. Toluidine Blue. 45x.

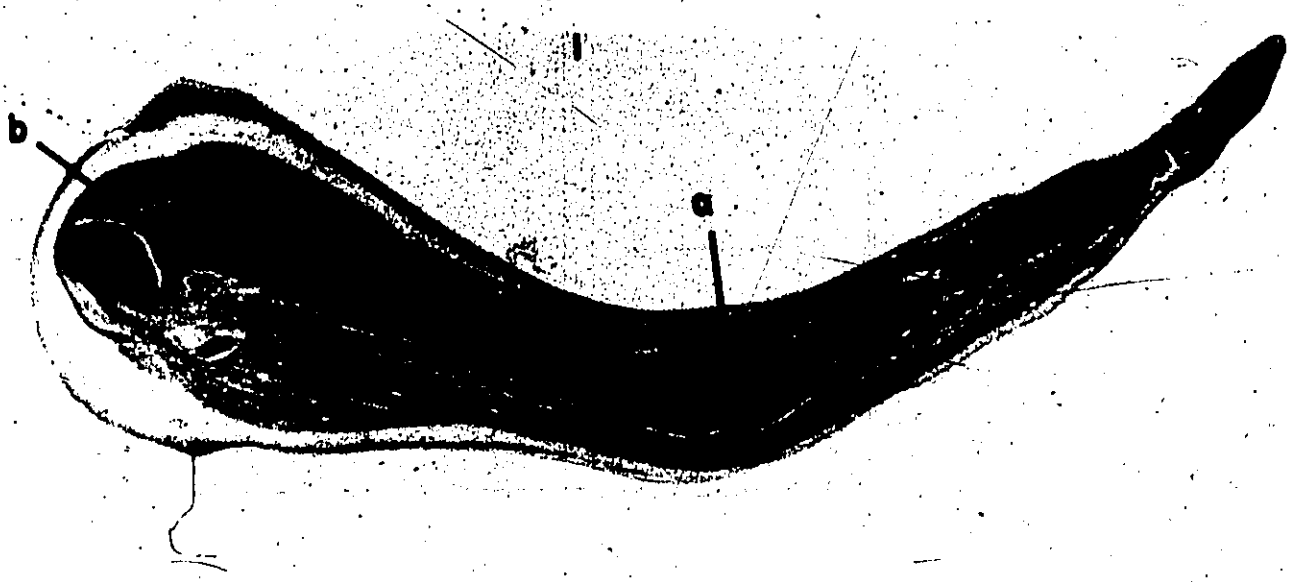


Fig. 7. Section of a hindlimb corresponding to the forelimb in Fig. 5. It has normal curvature at the tibia-tarsus joint (arrow a) and a well-developed tibia-femur joint (arrow b). There is an even pattern of γ- metachromasia. Toluidine Blue. 45x.

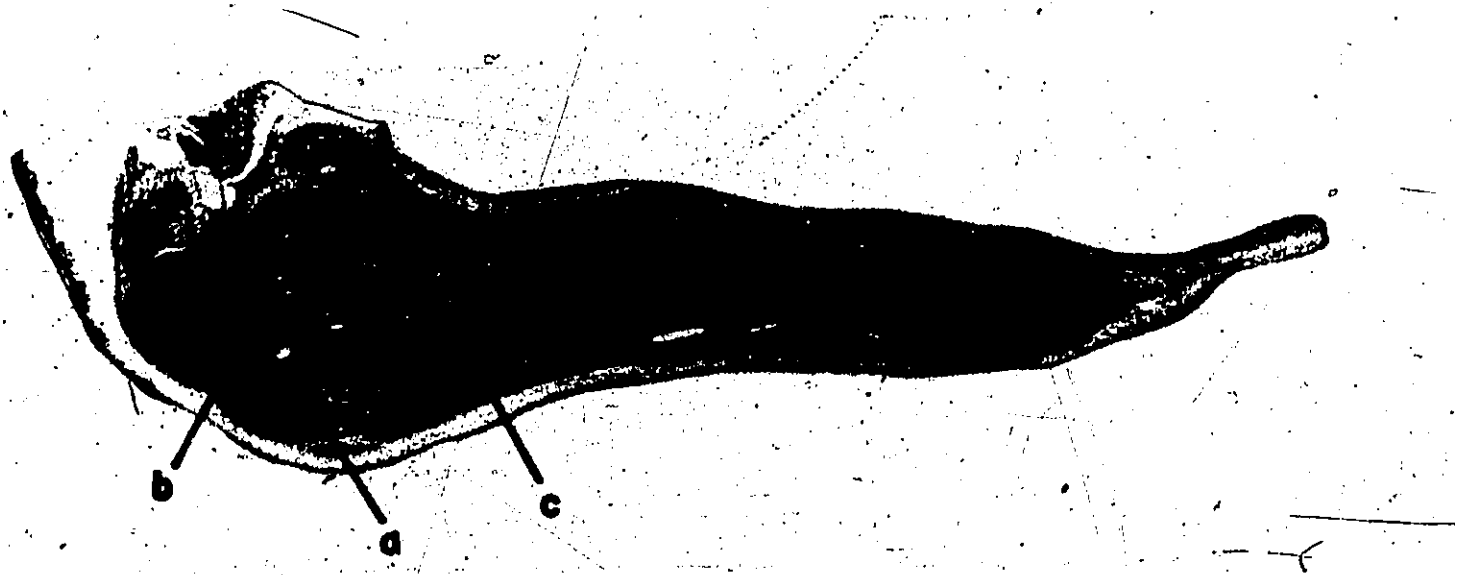


Fig. 8. Section of a hindlimb from a Malathion-treated embryo. It corresponds to the forelimb in Fig. 6. The tibia-tarsus joint is abnormal. Chondrogenesis is abnormal in the diaphysis of the tibia (arrow a) and in the proximal head of the tibia (arrow b). Some chondroblasts (arrow c) are hypertrophied with little matrix around them. There is no uniform γ-metachromatic pattern. Toluidine Blue. 45x.

Fig. 9. Hindlimb from a control 9-day embryo. It shows a normal tibia-femur joint and chondroblasts that are well-differentiated and have a normal amount of matrix. Toluidine Blue. 125x.

Fig. 10. A hindlimb from a 9-day embryo. The mesenchyme shows lack of differentiation. The chondroblasts that have differentiated are hypertrophied and have an abnormal amount of ground substance. Arrow A Toluidine Blue. 125 x.

F- Identification of Different Acid Groups of the Acid Mucopolysaccharides (Chondroitin Sulphates)

There was a maximum colour reaction seen in the specimens stained for 60 minutes in the Alcian Blue and Alcian Yellow.

In the control stab slides 3 colours were present. Yellow was predominant over the body of the cell and blue was seen in the extracellular matrix. The colour blue-green was especially predominant along the margins of the lactunae of the chondroblasts and the adjacent area of the ground substance (Figs. 11 and 13).

In the affected tissue 2 colours only were seen, yellow predominating over the cell body itself and green along the lacunar margins and over the extracellular matrix. The colour blue-green was not detected (Figs. 12 and 14).

G- Radioautography-S^{35}

Embryonic limb sections from the untreated and control stab groups were used to evaluate the degree of background (Rogers 1967). The limbs from the oil plus the isotope injected specimens served as control samples in comparing isotope uptake.

a- Forelimbs - The forelimbs of embryos injected with S^{35} in corn oil were normal in all respects. The even distribution of the silver grains attests to the uniform incorporation of the isotope (Fig. 15). In contrast, the forelimbs of the Malathion-treated chicks had an uneven uptake of

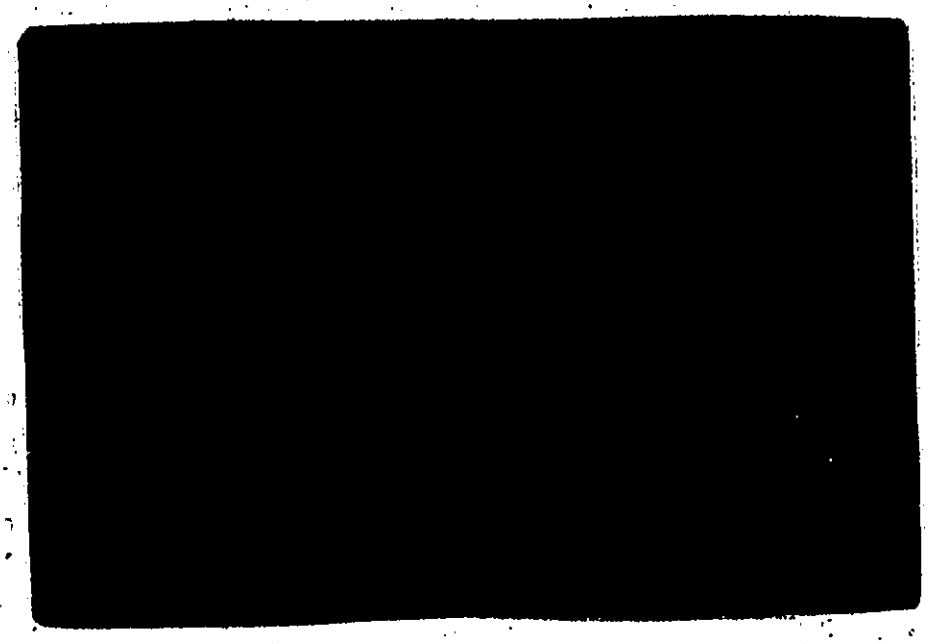


Fig. 11. Control tibial cartilage of a 9-day embryo. There are 3 colours present. Yellow is present over the cell body and blue is seen in the ground substance. The colour blue-green predominates along the lacunar margins of the chondroblasts and in the adjacent area of the matrix. Alcian Blue Alcian Yellow. 125x.

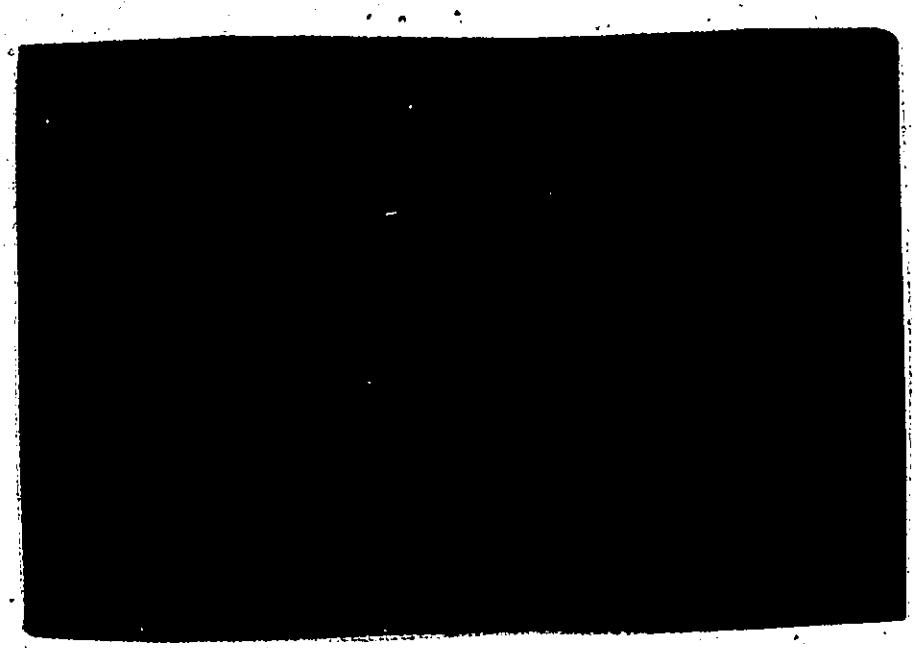


Fig. 12. Tibial cartilage of an embryo corresponding to that in Fig. 11 from a Malathion-treated embryo. Here 2 colours only are seen, yellow over the cell body and green along the lacunar margins and in the adjacent extracellular matrix. Alcian Blue Alcian Yellow. 125x.

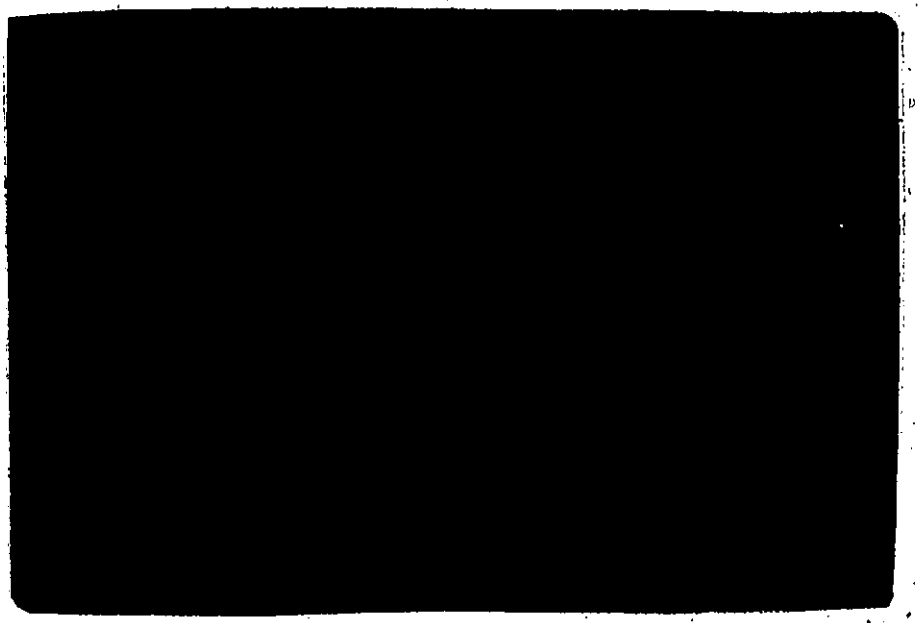


Fig. 13. This corresponds to the cartilage shown in Fig. 11. 500x.

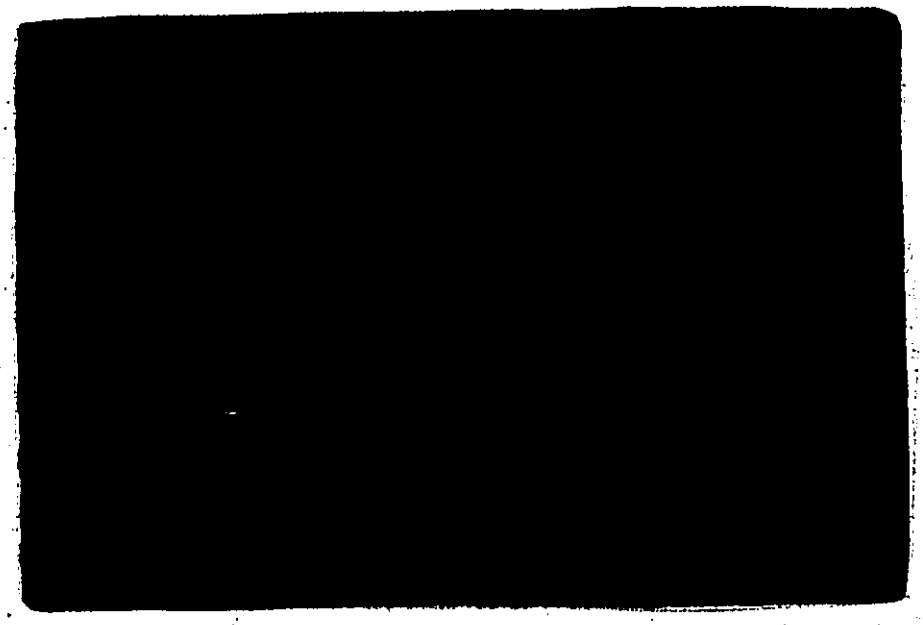


Fig. 14. This corresponds to the cartilage seen in Fig. 12. 500x.

Fig. 15. Photoradiograph of a forelimb from a 9-day control embryo. Both the uptake of S^{35} and the pattern of grain density is even. Unstained. 45x.

Fig. 16. Section of a forelimb from an embryo treated with Malathion plus S^{35}. There is an uneven uptake of S^{35} particularly in the radius. A "banding effect" is present. Unstained. 45x.

the isotope. This resulted in a "banding effect" in the pattern of grain development (Fig. 16), which corresponds exactly to that observed in the affected limbs stained with Toluidine Blue.

b- Hindlimbs - The radioautographs of the control hindlimbs also had a uniform S^{35} uptake and grain density. This was seen in the proximal and distal heads of the tibia and tarsus (Fig. 17). The tibia in Fig. 18 was shorter than the tibia in Fig. 17 and there was less isotope incorporation than in the control. There was also an uneven pattern of grain density in the tibia of the Malathion-treated embryos (Fig. 18).

H- Connective Tissue Study of Cartilage

In this study samples from control stab embryonic limbs and Malathion-treated limbs were used. In the control 9-day limbs the chondroblasts were normal in size and structure. The collagen fibers of the perichondrium surrounding the cartilage were well-developed as evidenced by the bright red colour. The mesoblasts appeared normal. The cartilage in the Malathion-treated limbs lacked perichondrium and the mesoblasts and chondroblasts were immature in appearance (Figs. 20 and 22).

I- Histochemical and Biochemical Studies of Alkaline Phosphatase (E.C. 3.1.3.1.)

A- A histochemical method using a modification of Gomori's Technique

The form of the control cartilage was regular and straight

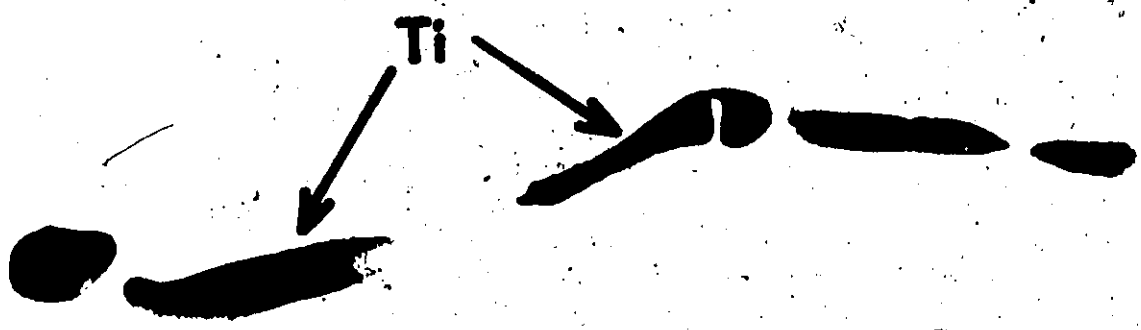


Fig. 17. Hindlimb, corresponding to the forelimb in Fig. 15. Development of the tibia (Ti.) is normal and the uptake of S^{35} is uniform. Unstained. 45x.

Fig. 18. Section of a hindlimb corresponding to the forelimb in Fig. 16. Isotope uptake is uneven. The tibia is short compared to the control specimen in Fig. 17. Unstained. 45x.

Fig. 19. Section from the tibial cartilage of a 9-day embryo. The perichondrium is well-defined. VanGieson's connective tissue stain. 125x.

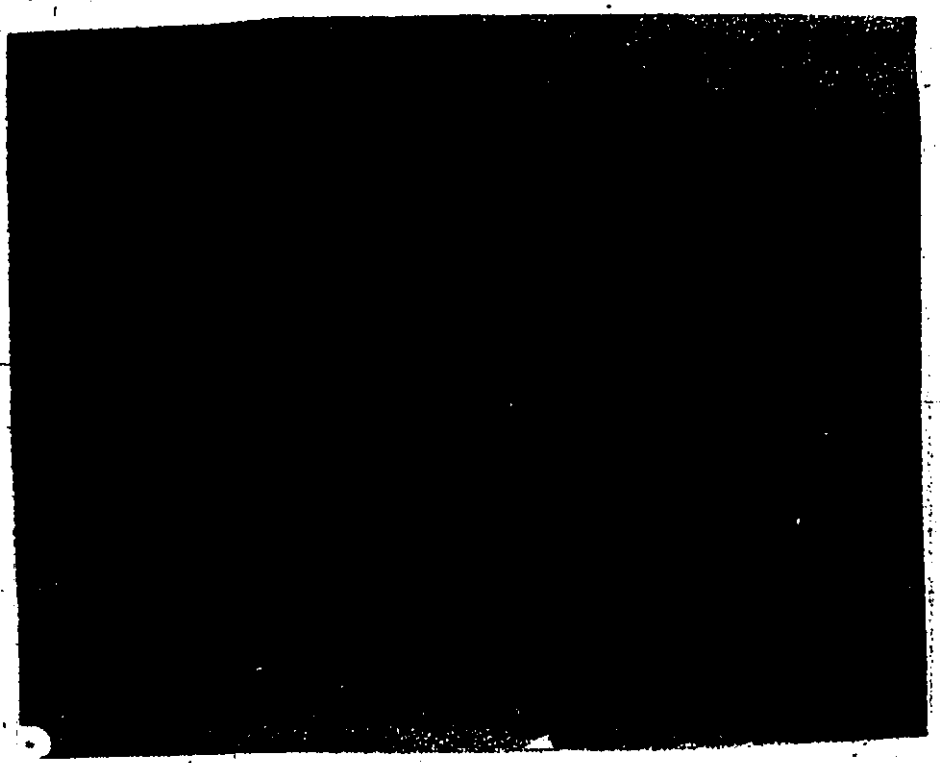


Fig. 20. Section from the tibial cartilage of Malathion-treated chick embryo of 9 days. There is no perichondrium present and the chondroblasts look immature in appearance. VanGieson's connective tissue stain. 125x.

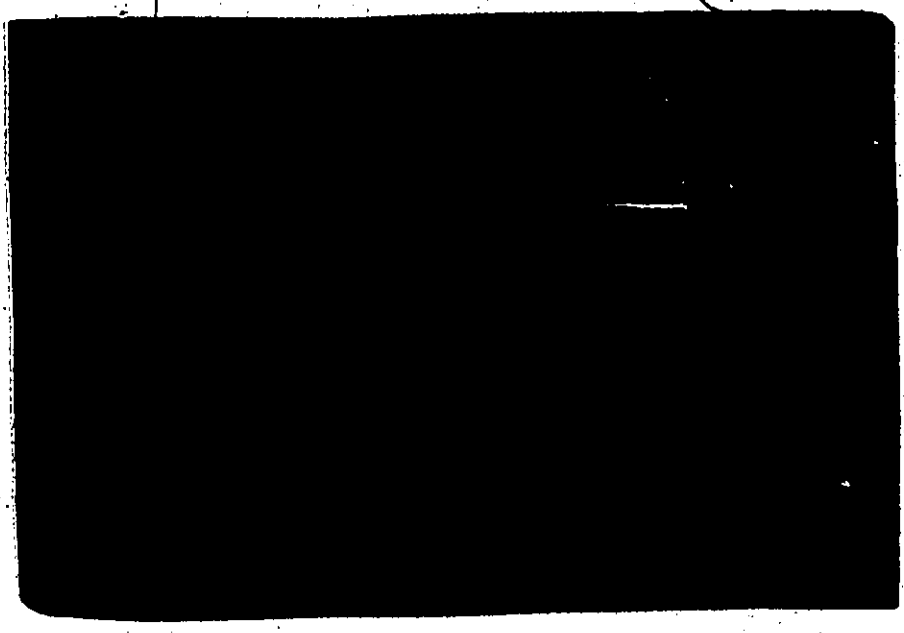


Fig. 21. This is a 500x magnification of the cartilage seen in Fig. 19. The collagen fibres in the perichondrium are normal and the cartilage chondroblasts are normal in appearance and size. VanGieson's connective tissue stain. 500x.

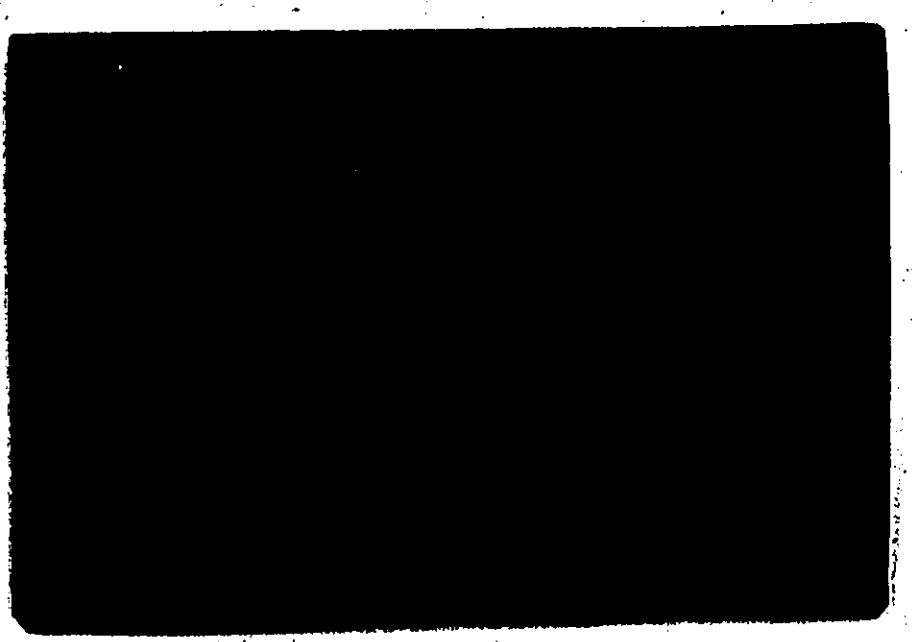


Fig. 22. This corresponds to the tibial cartilage as seen in Fig. 20. Note the complete absence of the collagen fibres of the perichondrium and the immature appearance of the associated chondroblasts and fibroblasts. VanGieson's connective tissue stain. 500x.

Fig. 23. The form of the tibial cartilage is regular and straight in outline. There is a heavy precipitation along the perichondrium and the adjacent margins of the cartilage. Precipitation is heaviest in the mid-diaphysis showing the most enzyme activity. Gomori's Modified Method. 125x.

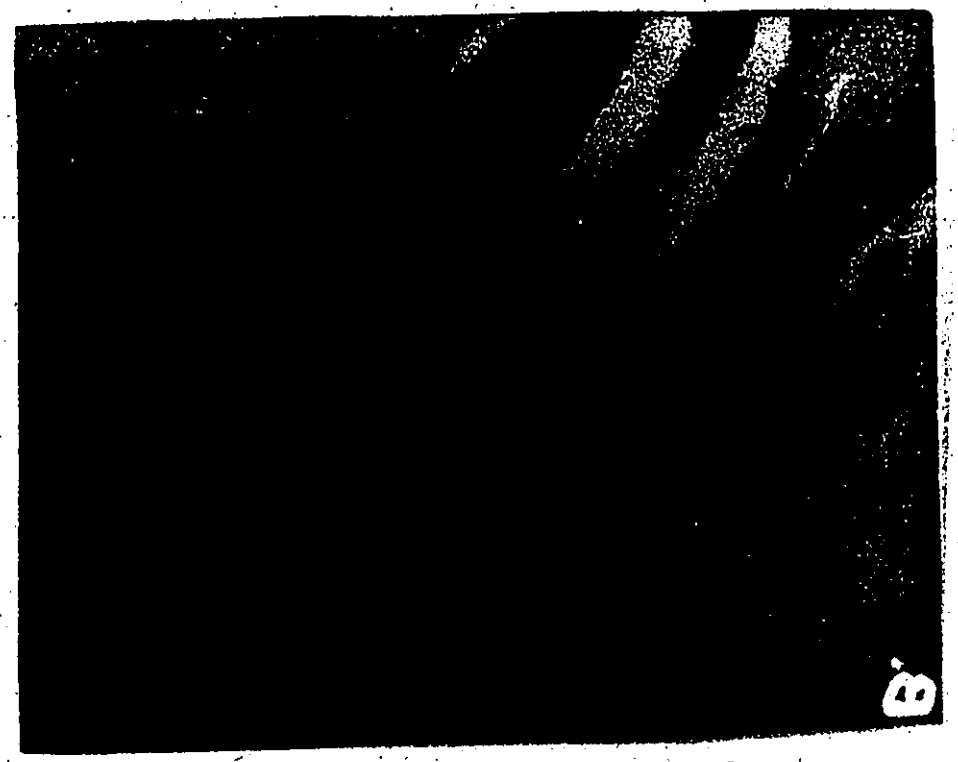


Fig. 24. This corresponds to the same area in the tibial cartilage as seen in Fig. 23. The Malathion-treated cartilage is bent in appearance and the chondroblasts of the fibula are poorly differentiated. There is less precipitation over the mid-diaphysis showing poor enzyme activity. Gomori's Modified Method. 125x.

in outline. There was heavy precipitation of black granules i.e. enzyme activity along the perichondrium and adjacent margins of the cartilage. Precipitation was heaviest over the mid-diaphysial region of the tibia (Fig. 23) showing the greatest amount of enzyme activity.

The Malathion-treated tibia was bent in appearance and the cells of the adjacent piece of cartilage (fibula) had chondroblasts that were poorly differentiated (Fig. 24). There was heavy precipitation over the tip of the malformed fibula. The tibial cartilage had poor precipitation over the mid-diaphysial region compared to that seen in the control. The enzyme activity along the perichondrium was not as extensive as in the normal case.

B- A biochemical method - an assay of alkaline phosphatase

Statistical analysis shows that the amount of alkaline phosphatase activity is depressed in the affected limb samples as compared with the controls (Student's t test). The effect is seen as early as day 8 in the analysis of both forelimbs and hindlimbs (Figs. 25-26).

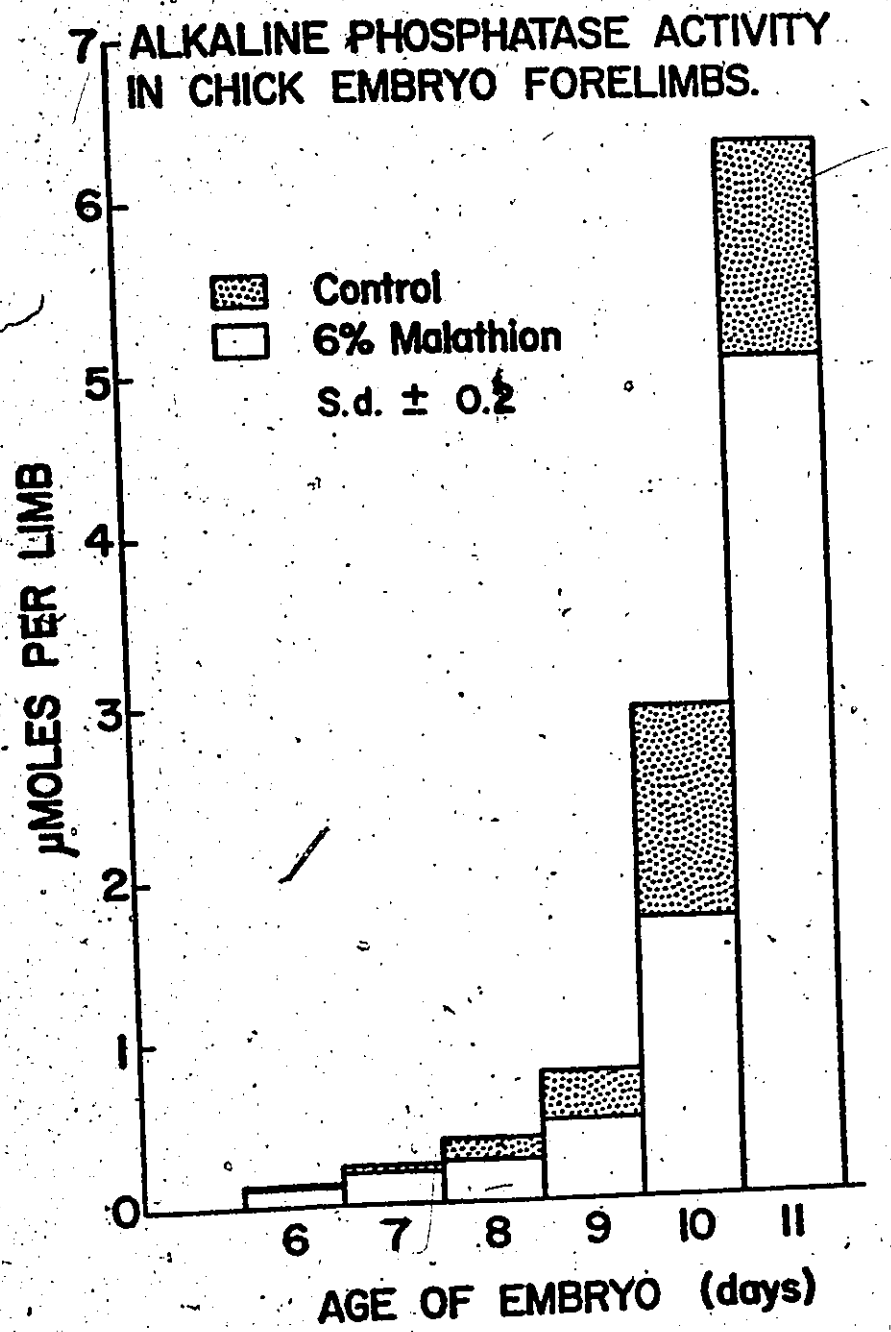


Figure 25. Alkaline Phosphatase Activity in Control and Malathion-treated chick forelimbs.

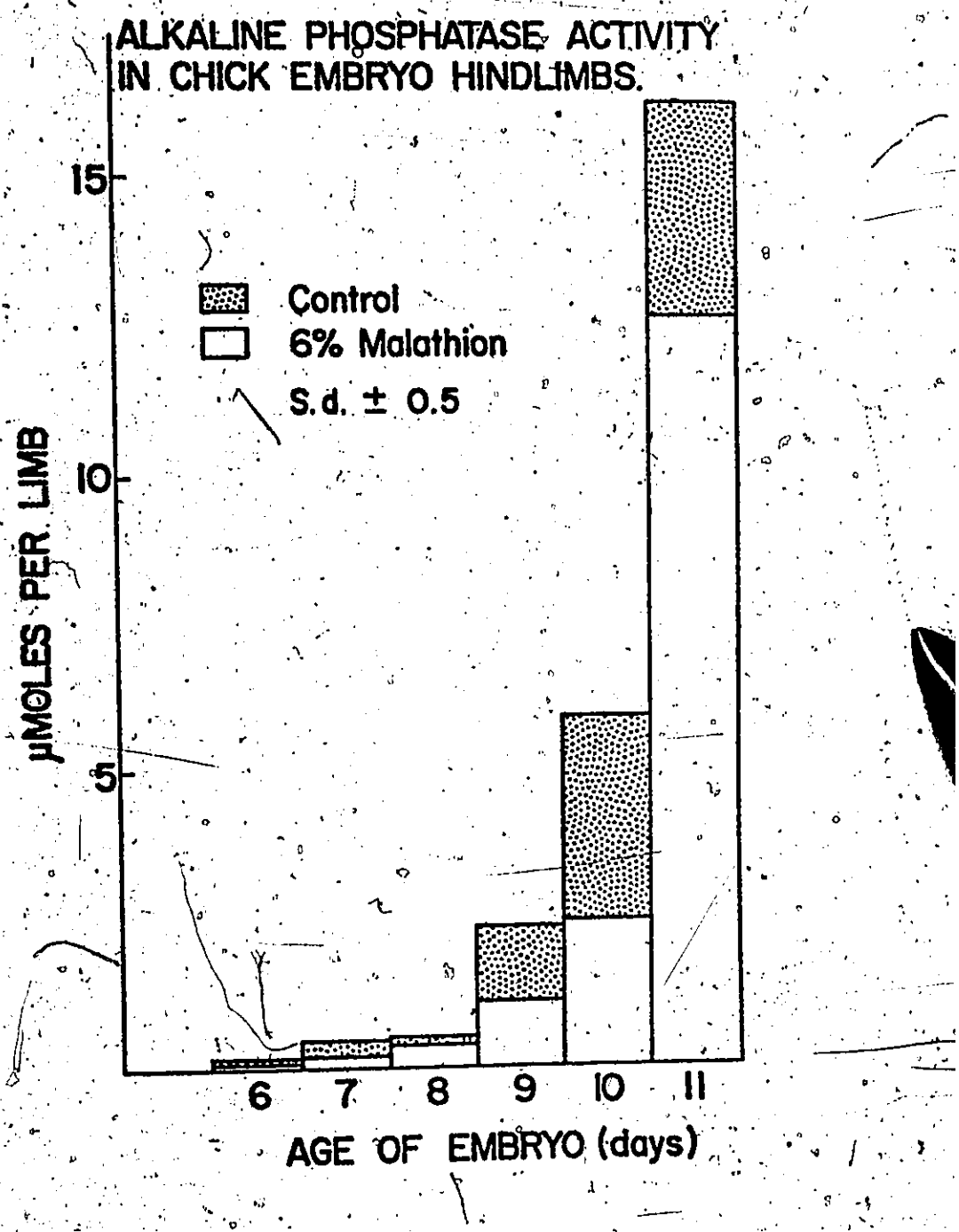


Figure 26. Alkaline Phosphatase Activity in Control and Malathion-treated chick hindlimbs.

DISCUSSION

Malathion injections on days 4½-5 of development (Hamburger and Hamilton 1951) produced a maximum effect between days 8-10 of embryogenesis. The general body size of the affected embryos was 24-36 hours behind the controls.

This "Malathion Syndrome" was described by Greenberg and LaHam in 1969 and consisted of sparse plumage, micromelia, overall growth retardation and beak defects. One of the most prominent results seen in the current experiments was marked oedema in the hindlimbs and to a lesser degree in the forelimbs. This phenomenum demonstrates the deleterious effect of the organophosphate on the cartilage cells. This could be due to the autolysis of organic substances within the cell or an impairement of the sodium pump at the cell membrane (de Robertis, Nowinski and Saez 1966).

D- Limb Measurements

By 3½-4 days (Stage 21) the leg buds of the chick embryo are distinct and larger than the wing buds (Romanoff 1960); by day 4 to 4½ (Stage 24) the limb buds are distinctly longer than wide and at 5 days (Stage 26) the first 3 toes are demarcated. Between 5½-6 days (Stage 28) the limbs have developed into well-defined structures.

The radius and ulna are in a prechondral stage after 4 days of incubation in the chick, with cartilage beginning to form in the diaphysis (Lillie 1952). In the chick embryo of 5 day's incubation time the wrist is composed of mesenchymal

condensations, which with the exception of Centrale 1, have not separated into anlagens of the carpals.

The skeleton of the hindlimbs is formed from a condensation which has the shape of a letter Y in the 5 day chick. The distal arms of the Y will give rise to the tibia and fibula. The elongation of the cellular rudiments is rapid, and the three major leg components are prechondral by the sixth day. Membranous precursors of the tarsus and metatarsals are present in the chick on the sixth day. The femur is separated from the pelvis by a dense cellular interzonal region at the presumptive acetabular articulation by day 5. Chondrification of the diaphysial region of the femur begins at 5.5 days and is in an advanced state in the thigh bone of the chick incubated at day 6. The femur is the stoutest element during the chondral stage but, its growth rate is second to that of the tibia. In the chick at 5 days the prechondral condensation of the tibia and the fibula are still connected to the femur and the metatarsal condensation.

Greenberg and LaHam (1969) demonstrated that 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. The developing limb buds during these early stages of embryogenesis would have a high metabolic rate due to their rapid growth and differentiation which could account for the rapid absorption of

Malathion into the embryo with the resulting inflammation, oedema and the subsequent teratogenic effects.

Two standard parameters used to determine growth in an embryo are weight and length measurements. Because of the marked oedema of the limbs the parameter of length measurement was used. By day 8 the embryo is doubling itself in weight, general body size and conformation. The increase in general metabolism could be affected by the organophosphate, Malathion. This could account for the maximum effect on the limbs between days 8 to 10. The effect is more obvious in the hindlimbs than in the forelimbs (Figs. 1-4).

E- Initial Morphological and Histochemical Investigations

It is agreed generally that metachromasia is due to the predominant anionic groups of the polysaccharide chains of the chondroitin sulphates in the ground substance (Quintarelli 1967 and Pearse 1968) Mucopolysaccharides from precartilaginous embryonic chick limb buds have been isolated (Searls 1964). The polysaccharide chain of the protein-polysaccharide complexes found in 14 day embryonic chick cartilage contained chondroitin 4- and chondroitin 6- sulphates (Shulman and Meyer 1968, 1970). The results using Toluidine Blue suggest that the ground substance in the various control groups is being produced normally since the cells were surrounded by a normal amount of matrix and demonstrated γ metachromasia.

The lack of uniform γ metachromasia suggested that many anionic groups of the polysaccharide chain were not avail-

at pH 0.5 it was possible to colour subsequent carboxyl groups using Alcian Yellow buffered at pH 2.5 (Carlo 1963).

Thus it was clearly demonstrated by using the Alcian Blue, Alcian Yellow dyes that the chondroitin sulphates were not being synthesized by the abnormal hind and forelimb chondroblasts. This was shown very markedly around the lacunar margins and the surrounding matrix of the chondroblasts where the chondroitin sulphates were found in the highest concentration.

The chondroitin sulphates are characteric of embryonic cartilage and add to its tensile strength and therefore, influence stability in structure. The chondroitin sulphates are also involved in certain disease processes, e.g. Osteoarthritis and Hurler's Syndrome (Pearse 1968) where abnormal amounts of these acid mucopolysaccharides are present.

Unless there is a normal cartilage model to serve as a base for calcification a normal bone will not form (Ham 1970). This is seen in the long bone formation in the limbs and is demonstrated in the Malathion-treated chick at hatch when the hindlimbs are malformed. This is also obvious in the "typical" posture (a keel resting position) in chicks with hindlimb malformation (Greenberg and LaHam 1969). The synthesis of abnormal amounts of the chondroitin sulphates of the matrix is one of the reasons for limb malformations.

It has been suggested that a certain amount of extracellular matrix is necessary for cells to differentiate (Shulman and Meyer 1970). The abnormal amounts of the ground

substance and its components, secreted by the affected chondroblasts, would seem to be preventing the mesenchyme cells from differentiating. Further cell development is related to the amount of ground substance (Florkin and Stotz 1968a).

The amount of extracellular substance serves a vital role in cell development in cartilage (Bloom and Fawcett 1970). As cartilage is avascular the ground substance and its contents are important for the diffusion of metabolites and ions during the initial stages of chondroblast development. A deficiency of vital ions and metabolites may affect the maintenance of the status quo of the chondroblasts and chondrocytes. This would lead to the cartilage development being abnormal.

G- Radioautography S^{35}

The sulphate ion must be metabolized by living cells which incorporate it in the early biosynthesis of the chondroitin sulphates (Schiller et al 1956). Radioactive sulphate has been detected in the embryonic chick limb buds as early as stage 23. An uptake of radioactive sulphate has been reported in the chick embryo as early as stage 4 (primitive streak) (Searls 1965).

The implied importance of mucopolysaccharides in cell aggregation and cell differentiation is well documented (Florkin and Stotz 1968a and 1968b). The predominant components of the ground substance mucopolysaccharides are chondroitin sulphate 4- and chondroitin sulphate-6. These chondroitin sulphates occur in large quantities in the young embryonic

cartilage and decreases in amount as the cartilage ages or becomes diseased or calcification starts. Thus, it would seem that chondroitin sulphates and the other contents of the matrix played a vital role in the normal development of the embr-onic chick limb cartilage.

In the autoradiographic studies of the malformed limbs there was little incorporation of the S^{35} into the chondroitin sulphates. This indicated that Malathion interfered with the normal synthesis of the chondroitin sulphates by the chondroblasts.

H- Connective Tissue Study of Cartilage

Collagen is the commonest supporting or connective tissue found in mammals, accounting for 25% of the total protein and is a major component in cartilage and bone (Florkin and Stotz 1968a).

The basis of organization of the whole animal body is therefore dependent on the synthesis and mode of deposition of the extracellular collagen fibres. This is seen in cartilage development where collagen is found in the extracellular matrix and the perichondrium. Collagen research is important in developmental biology and plays a significant role in wound healing and certain pathological processes. It has been implicated in a wide variety of diseases. There is also an important interrelationship between the acid mucopolysaccharides and collagen in connective tissue remodelling (Flint 1972). Hence the interrelationship between the acid

mucopolysaccharides and collagen could be an important factor in the development of embryonic cartilage.

In the affected limbs these two factors appear to be abnormal in amount, the acid mucopolysaccharides (chondroitin 4- and chondroitin 6- sulphates) and the collagen fibres in the perichondrium.

I- Histochemical and Biochemical Studies of Alkaline Phosphatases E.C.3.1.3.1.

Biochemical and histochemical parameters regulating cartilage and bone development have not been well understood in the past and have only recently been subject to intensive study (Makinen and Paunio 1970). Robison (1923) reported that actively calcifying and hypertrophic cartilages had high activity of the phosphatases; while resting cartilage had no demonstrable activity. The role of the phosphatases (Alkaline Phosphatase, E.C.3.1.3.1. and Acid Phosphatase E.C.3.1.3.2.) has been particularly controversial. The role of phosphatases in ossification has been confirmed recently (Vaes and Jacques 1968). It has been suggested that their role is in matrix formation and in energy release from ATP (Wergedal 1969).

Calcification in embryonic chick limbs begins at approximately 9-10 days of development. The Malathion-treated limbs had less enzyme activity than in the control at day 9. Inhibition of Alkaline Phosphatase would affect the secreting chondroblasts in the formation of the extracellular matrix

and during the calcification of the cartilage model.

These results are consistent with the dual role of the phosphatases i.e. (Alkaline Phosphatase) in matrix formation and in the calcification of cartilage prior to ossification.

CONCLUSIONS

1- An injection of Malathion (7.02 mg or 6%) not only produced malformations in the hindlimbs but also affected the forelimbs.

2- The maximum effect of Malathion on limb development occured between days 8-10 of embryogenesis.

3- Malathion affects the chondroblasts and the cell contents (cell hypertorphy and vacuolization).

4- The production of chondroitin 4- and 6- sulphates is impaired.

5- The synthesis of the protein collagen in the perichondrium is affected in the Malathion-treated embryonic limbs.

6- Histochemical and biochemical studies showed abnormal amounts of Alkaline phosphatase which were undoubtedly an influencing factor in the lack of matrix formation.

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