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Malathion and Insulin : An Investigation into a possible cause-effect relationship in chick teratogenesis.

by

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A thesis
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ABSTRACT

The injection of 2 IU of turkey insulin into the yolk sac of chicken embryos on the 5th day of incubation resulted in abnormalities of the limbs and beak similar to those observed with mammalian insulins. These findings suggest that endogenous insulin levels may function in limb and beak development. It is, however, difficult to determine if the insulin levels used were of physiological significance. In an attempt to investigate this question, malathion, an organophosphate insecticide suspected of exerting its teratogenic potential by increasing endogenous insulin levels (Arsenault and Gibson, 1974; Arsenault et al., 1975; Laley and Gibson, 1977) was injected into the embryos. The analysis of immunoreactive plasma insulin levels in these embryos, however, did not show a significant difference when compared to those of the controls but this observation is only suggestive because of the large variation measured for insulin levels in different chicks. In addition, although limb to body length ratios were decreased from the 9th to the 17th day, and plasma glucose levels were increased on day 7 and decreased on days 9 and 11, no correlations between insulin and either of these two parameters were observed until day 17. On this day a positive correlation was seen between the plasma insu-

lin and glucose levels. On the other hand positive correlations were seen between the plasma glucose levels and limb to body length ratios on the 9th, 11th and 13th days of incubation. Hematocrit levels were decreased on the 7th day after malathion treatment but were normal on the 9th and 11th days.

Therefore, it was concluded that homologous (turkey) insulin can induce abnormalities in chickens. Malathion exerted similar teratogenic effects but these were not associated with large alterations in endogenous plasma insulin levels.

RÉSUMÉ

L'injection de 2 IU d'insuline de dinde dans le sac vitellin d'embryons de poulet le cinquième jour de leur incubation a produit des difformités aux pattes et au bec semblables à celles qu'on observe avec les insulines des mammifères. Ces résultats suggèrent que l'insuline endogène peut affecter le développement des pattes et du bec. Il est cependant difficile de déterminer si les niveaux d'insuline utilisés ont une signification physiologique. En vue d'élucider cette question, le malathion, un insecticide organophosphoré soupçonné d'exercer son action tératogène en augmentant les taux d'insuline endogène (Arsenault et Gibson, 1974; Arsenault et al., 1975 et Laley et Gibson, 1977) a été injecté dans les embryons. L'analyse des niveaux de plasma insuline immunoréactifs dans ces embryons n'a cependant pas démontré des différences marquées par rapport aux témoins mais cette observation est seulement suggestive à cause de la variation marquée mesurée pour les niveaux d'insuline dans différents poulets. De plus, bien que le rapport membre/longueur du corps ait décru du neuvième au dix-septième jour et que les niveaux de plasma glucose aient augmenté au septième jour et diminué aux neufième et onzième jours, on n'a observé aucune corrélation entre ces paramètres et l'in-

suline avant le dix-septième jour. Ce jour-là il y avait une corrélation positive entre la plasma insuline et les niveaux de glucose. D'autre part on a obtenu des corrélations positives entre les niveaux de plasma glucose et les rapports membre/longueur du corps les neuvième, onzième et treizième jours d'incubation. Les niveaux d'hématocrite ont diminué le septième jour du traitement au malathion mais sont remontés aux neuvième et onzième jours.

On a par conséquent conclu que l'insuline homologue (c'est a dire de la dinde) peut induire des difformités chez les embryons de poulet mais que les effets du malathion ne dépendent pas de modifications significatives des niveaux d'insuline endogène.

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Chapter I

INTRODUCTION

The injection of 2 IU of insulin into the yolk sac of 96 or 120 hour incubated chick embryos resulted in an overall decrease in embryo size and a variety of symptoms including micromelia and beak anomalies, primarily short upper beak and parrot beak. Low frequencies of microphthalmia, anophthalmia, buphthalmia, syndactyly (in association with micromelia) and polydactyly also occurred (Landauer, 1947, 1951; Landauer and Rhodes, 1952). Each of these abnormalities mimic naturally occurring mutations in fowl. For example, abnormalities of the beak and extremities strongly resemble hereditary "short upper beak" (Landauer, 1947), and the insulin induced syndactyly primarily effects the 3rd and 4th toes as does hereditary syndactyly (Landauer and Rhodes, 1952). On the basis of these similarities, Landauer (1947) proposed that insulin's teratogenic effects were due to derangements in the same metabolic pathways as those effected by "genetic modifiers". This proposal led to an increased interest in elucidating insulin's mechanism of action. Zwilling (1948) studied the possible role of insulin on carbonydrate metabolism and found decreases in blood sugar levels. In these studies, embryos were injected with 2 IU of

beef insulin on day 5 of incubation and blood was sampled on incubation days 6, 8, 10 and 12. The data showed a positive correlation between the extent of hypoglycemia and the severity of the micromelia. Although Zwilling realized that the hypoglycemia and micromelia could be parallel effects of insulin he suggested the possibility of a cause-effect relationship between the two.

An alternative theory was proposed when nicotinamide-supplemented, insulin-injected embryos showed a decrease in the incidence of micromelia, beak defects and eye anomalies (Landauer, 1948; Landauer and Rhodes, 1952). The authors (1952) suggested that insulin might act by interfering with normal codehydrogenase activity. Further studies with nicotinamide showed that it also prevented hypoglycemia, thus supporting the earlier theory of indirect action (Zwilling, 1951).

Landauer's theory of a direct action by insulin was, however, supported by two other observations. The first of these was that limb and beak abnormalities and hypoglycemia are separable events (Zwilling and DeBell, 1950). That is, when sulfanilamide was injected into the yolk sac of 30, 48 or 120 hr incubated embryos they developed micromelia and parrot beak similar to those produced by insulin, but normoglycemia was maintained. As with insulin injections, supplementation with nicotinamide decreased the frequency and extent of the micromelia and parrot beak. These studies in-

dicate that hypoglycemia is not an absolute requirement for the production of the observed anomalies.

The second line of evidence derives from *in vitro* studies in which glucose concentrations were held constant and the effects of insulin on cultured limbs studied. The *in vitro* effects were similar to those found *in vivo*. That is, the treated bones were characterized by enlargement of the periosteal collar, shortening and bending of the bone shaft, abnormally small cells in the epiphysis, a decreased amount or total absence of the epiphyseal zone of flattened cells and an overall reduction in matrix. The only difference observed was that the characteristic necrotic areas seen in the epiphysis of *in vivo* treated femurs and tibias were not observed *in vitro*. Indeed, *in vitro* the epiphyseal cartilages were greatly enlarged (Chen, 1954, Hay, 1958 and Zwilling, 1959). The reason for this discrepancy is unclear but may be a general property associated with the culture technique as the normal necrosis associated with the formation of the joints does not occur *in vitro* (Zwilling, 1959).

Another similarity between the *in vivo* and *in vitro* systems is that nicotinamide protected the long bones from the effects of insulin. When limbs from 5 day insulin-injected (5 IU/egg) embryos were excised on day 6 and cultured on nicotinamide enriched medium, the limbs did not differ from the limbs of uninjected embryos excised on day 6 and grown on non-enriched medium. Conversely, the insulin treated

limbs grown on non-enriched medium showed abnormal development typical of cultured limbs excised from embryos on day 6 or day 7 of incubation and grown on normal medium enhanced with insulin (Zwilling, 1959). As no differences in the glucose concentrations were present in the treated and control cultures (Chen, 1954; Hay, 1956; Zwilling, 1959) these studies demonstrated that hypoglycemia and micromelia were separable events and that insulin might have direct effects on cultured limbs similar to those seen in vivo. Therefore, it seems unlikely that the in vivo action of insulin on limb development is mediated directly by hypoglycemia.

At the time of the above studies (1940's and 50's) histological examinations of the developing chick pancreas showed that differentiation began on the 7th or 8th day (Potvin and Aaron, 1927; Villamil, 1942; Lièvre, 1957). There was however, much controversy over the day on which beta granule formation and thus insulin potential secretory activity occurred. Indeed, the initial appearance of secretory granules in beta cells was reported on the 12th (Potvin and Aaron, 1927; Sandstrom, 1934; Villamil, 1942), 13th (Ghiani, 1956) or 17th (Lièvre, 1957) day of incubation, times well past when insulin injections exert their initial effects on limb and beak development. More recently, however, electron microscopic studies of the developing chicken pancreas have shown the presence of both alpha (Dieterlen-Lièvre, 1963; Prybrylski, 1967) and beta cells (Prybrylski, 1967) on the

third day of incubation. In addition, both insulin (Benzo and Green, 1974) and glucagon (Benzo and Stearns, 1976) have been measured in the pancreas and plasma of chicken embryos as early as the fifth day of incubation. In other words endogenous insulin is present at the time when spontaneous developmental abnormalities occur.

Not only is insulin present, but some evidence indicates that it may have functional activity during these early stages. For example, Benzo and DeLaBaba (1972) proposed that the normal development of the smooth endoplasmic reticulum (SER) and glycogen deposition in the embryonic liver may be zinc insulin dependent. The authors cultured livers from 5 day incubated chick embryos on media with or without zinc insulin for 6 days and then examined the cellular morphology. Livers grown on insulin-free medium showed no development of SER or glycogen. However, normal development of glycogen "rosettes" and a tubular, lattice-like network of SER were present in the livers grown on the zinc insulin rich medium. This suggests that zinc insulin is an absolute requirement for the normal development of SER and for glycogen deposition in the cultured embryonic chick liver.

In addition, roles for both insulin and glucagon have been proposed in the regulation of carbohydrate metabolism in the developing embryo. Changes in circulating levels of both hormones from day 8 to hatching are known to correlate with changes one might expect in liver enzymes and glycogen

content. That is, increases in glycogen phosphorylase a and decreases in glycogen are observed in the plasma when glucagon levels increase (Benzo and Stearns, 1976) and increases in glycogen synthetase and glycogen are observed at times of high plasma insulin levels (Benzo and Green, 1974).

The available evidence further indicates that the role of insulin may not be limited to the liver, as insulin specific receptors have been found in chondrocyte cell membranes isolated from chicken embryo pelvic cartilage on days 11 and 12 of incubation (the only days studied). These receptors had a frequency of distribution per mg membrane protein less than one half that of embryonic liver cells but showed binding characteristics typical of insulin specific receptors in the embryonic chicken liver and mammalian tissues. As in these tissues, binding to the receptors is temperature dependent, has a pH optimum of 8.0 and kinetics indicative of negative cooperativity (Stuart et al., 1979).

Therefore, insulin is present early in development and is likely to have some effect on liver development, carbohydrate metabolism and chondrocyte function. Thus, the naturally occurring anomalies which are mimicked by insulin injection may be due to precocious and/or enhanced secretion of insulin from the developing pancreas.

One problem common to all of the above studies is that mammalian insulins were used and the amino acid composition of the mammalian insulin is different from that of chicken

insulin (Smith, 1966). Consequently, it is impossible to determine if the observed anomalies are due to insulin "per se" or to steric or kinetic changes resulting from the differences in the insulin molecules. Therefore, the first objective of the present study was to minimize the steric and kinetic differences by examining the effects of a turkey insulin, which has the same amino acid sequence as chick insulin (Markussen and Sundby, 1973), on the embryonic development of the chicken.

Even if turkey insulin is found to produce anomalies similar to those found in embryos injected with mammalian insulins it would be difficult to determine if the injected dose was pharmacological or physiological. Evidence indicating that embryos may be physiologically capable of producing sufficient amounts of insulin to result in congenital abnormalities has been reported (Arsenault and Gibson, 1974; Arsenault et al., 1975; Laley and Gibson, 1977). These studies involved the use of an organophosphate insecticide, malathion, [O, O - diethyl S - (1,2 dicarboxyethyl) phosphorodithioate] (fig 1). This compound has a low mammalian high insect toxicity ratio and is therefore commonly used in crop protection and in the prevention of insect transmitted diseases such as malaria and yellow fever. As with other organophosphates, the toxicity of malathion is usually attributed to its anticholinesterase activity. In the specific case of malathion, the active inhibitor is one of its meta-

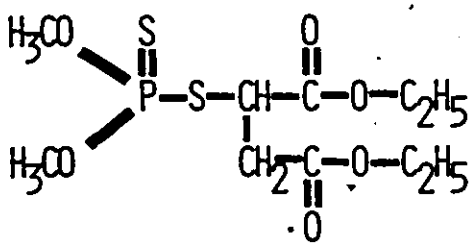
bolic breakdown products, malaoxon (March et al., 1956; fig 2). Malaoxon is formed when malathion undergoes oxidative desulphuration, a reaction catalzed by the mixed function oxidase system located in the gut and fat body of insects and the liver of mammals and birds (Cremlyn, 1978). Both malathion and malaoxon are detoxified by carboxyethylases which produce water soluble compounds that are easily excretable. This mechanism of detoxification is particulary important since it is believed to confer 'selectivity' to malathion's toxicity (Cremlyn, 1978). Both, insects and vertebrates rapidly metabolise malathion to malaoxon but insects, in general, have low carboxyesterase activities allowing malaoxon to accumulate at the nerve endings. High activities of these enzymes nerve endings in vertebrates prevents appreciable malaoxon accumulation in vertebrate nerve endings at the dose levels required for insect toxicity. Thus low vertebrate high insect toxicity toxicity is achieved.

Studies have shown that malathion has low toxicity in chickens but at high oral doses (392 mg/kg) malathion can lead to increases in mortality, depression, loss of appetite, laboured respiration, discharge from the eyes and nose, salivation, a preference to rest on knees, paralytic convulsions and death. The analysis of radioactive phosphorous excretion in surviving hens, however, showed that greater than 50% of the malathion is excreted within 8 hours

of administration and only traces remain after 48 hours (Gupta and Paul, 1977). These results indicate that accumulation is unlikely. Therefore, it is unlikely that a significant amount of malathion would accumulate in the egg prior to laying (Gupta and Paul, 1977 and March et al., 1956).

A study designed to simulate field spraying of mallard duck eggs at levels of 1/2 and 5 times the levels recommend by the U.S. Environmental Protection Agency (EPA, 25 lb/acre in aqueous emulsion and 2.8 lb/acre in oil) indicated that malathion was non-toxic and non-teratogenic in aqueous emulsion or oil at 1/2 the recommended concentration. Toxicity and teratogenesity were, however, observed when eggs were coated with an aqueous emulsion (but not in an oil vehicle) roughly equivalent to 5 times the recommended EPA level (Hoffman and Eastin, 1981). This study indicates that malathion spraying constitutes a potential hazard to avian embryos if concentrations of malathion exceed those recommended by the EPA.

Figure 1: Structural formula of malathion



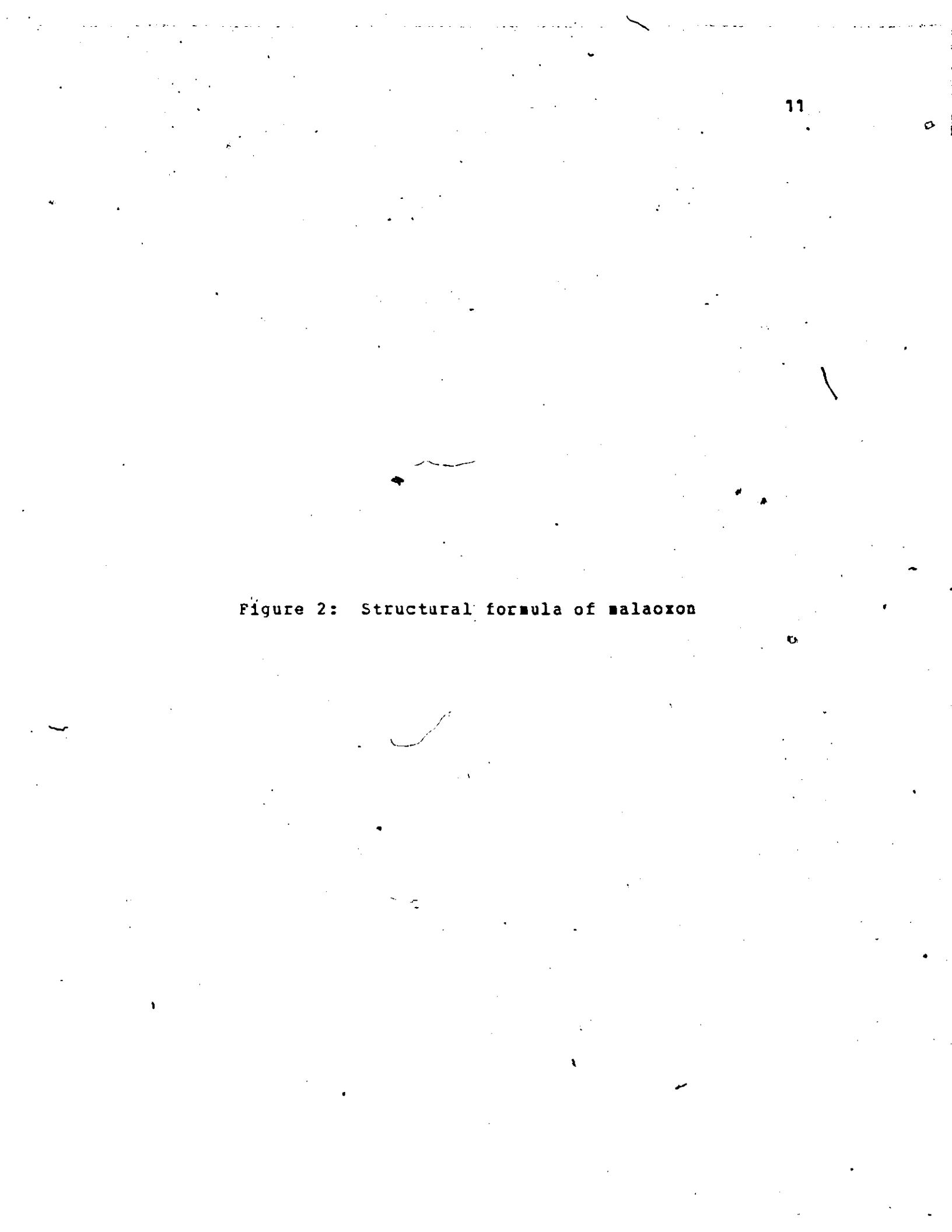
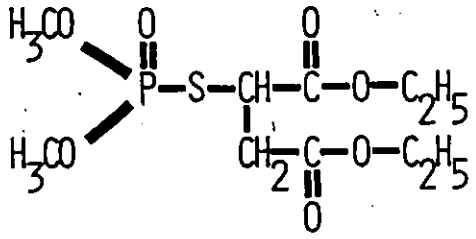


Figure 2: Structural formula of malaoxon



One theory of the mechanism of action of malathion in the production of anomalies in the developing embryos is that it stimulates insulin production and release by the embryo (Arsenault and Gibson, 1974; Arsensult et al., 1975; Laley and Gibson, 1977). The initial studies with malathion showed that the gross abnormalities produced in malathion treatment were similar to those produced by insulin except that syndactyly and polydactyly did not occur, whereas low frequencies of missing tarsometatarsus and phalanges did occur (Greenberg and LaHam, 1969).

Similarities between the effects of malathion and beef insulin were not limited to gross observations since hypoglycemia (Arsenault et al., 1975 and Laley and Gibson, 1977) and reversal with nicotinamide (Greenberg and LaHam, 1970, Wenger and Wenger, 1973) were also demonstrated. In addition, histological studies of the developing tibias from embryos injected with either insulin or malathion showed necrosis in the central portion of the epiphysis on the 8th day of incubation (Ho and Gibson, 1972a; Rabinovitch and Gibson, 1972a). This necrotic zone showed decreases in glycogen and matrix, and the adjacent cells showed an increase in glycogen storage, the latter being a consistant finding in malathion treated embryos but an inconsistant symptom of insulin treatment. Further, both treatments were observed to produce patchy decreases in the deposition of sulfated mucopolysaccharides (Duraiswami, 1950; Gill and LaHam, 1972;

Hinchliffe, 1974; Jackson and Gibson, 1977). Additionally, bone spicule deposition was irregular and asymmetrical resulting in a thickened layer along the concave side of the diaphysis (Zwilling, 1959; Rabinovitch and Gibson, 1972a, 1972b; Ho and Gibson, 1972a, 1972b). Throughout most of the incubation period calcification proceeded more rapidly and the intensity and extent of alkaline phosphatase staining was greater than controls. On day 20 of incubation the epiphyseal cartilage of treated embryos contained areas of hypertrophy which stained more intensely for glycogen, calcium and alkaline phosphatase, indicating that a secondary zone of ossification was forming. In control embryos this did not occur until the post-hatching stages (Ho and Gibson, 1972b; Rabinovitch and Gibson, 1972b).

Two studies have been reported which attempt to demonstrate a direct relationship between the injection of malathion and increases in insulin levels. In these studies malathion was injected into the yolk sac on the 5th day of incubation and the development of the pancreas was followed histologically (Arsenault and Gibson, 1974; Laley and Gibson, 1977). The results of these studies showed increases in both alpha and beta tissue in those embryos displaying extreme micromelia, defined as embryos with a limb to body length ratio less than 80 % of the control mean, on day 11. All treated embryos on day 15 had increased alpha and beta tissue regardless of whether or not micromelia was present.

Moderate and extreme micromelia on day 17 and extreme micromelia on day 19 were associated with increased beta tissue. Further, the extent of the increase in the alpha and beta tissue was positively correlated with both the extent of hypoglycemia and the degree of micromelia (Laley and Gibson, 1977).

In summary then, evidence from studies of gross anomalies, nicotinamide reversal, bone histology and histochemistry, blood sugar measurements and pancreatic histology suggest a correlation between increases in insulin levels and malathion treatment. It should be noted, however, that not all evidence points to this conclusion as there are some differences in the effects of the two compounds. Principally, syndactyly and polydactyly are seen in insulin treated but not in malathion treated embryos. Further, nicotinamide reversal is effective only up to 6 hours after insulin injection (Landauer and Rhodes, 1952) but up to 96 or 120 hours after malathion injection (Greenberg, 1971). Also, in malathion-treated eggs, tryptophan was a more effective reversing agent than nicotinamide as it reversed the decreases in embryo size, the micromelia and the incidence of beak defects. Conversely, Landauer and Rhodes (1952), using a ten fold higher dose of trypophan, found that it "probably produced no significant change in embryo mortality, nor in the teratogenic effects of insulin". Some differences in their effects on bone development were also seen (Ho and Gibson,

1972b; Rabinovitch and Gibson, 1972). That is, malathion affected glycogen levels in the chondrocytes on days 8, 10, 12, 14, 18 and 20 whereas the glycogen content of condrocytes in insulin treated limbs appeared similar to controls (except as mentioned above) in the region immediately adjacent to the necrotic regions of the epiphysis. Also, in insulin treated embryos, high calcium or alkaline phosphatase activities were seen in the area of reduced matrix near the end of incubaton or in areas of hypertrophied cartilage preceeding the arrival of vascular tissue to the area. This was not observed in malathion treated embryos.

There is no reason to presume that malathion acts specifically to increase insulin levels. In fact evidence points to the converse, as the amount of alpha tissue also increases. Additionally, there is no solid reason to predict that increases in endogenous insulin will have effects identical to those of injected mammalian insulins. Thus, the relatively minor differences outlined above do not necessarily refute the hypothesis that malathion acts primarily via an increase in embryonic insulin levels.

The strongest reason to question this hypothesis is that the changes in the limbs of malathion injected embryos occurred as early as day 8 (Ho and Gibson, 1972a, 1972b; Jackson and Gibson, 1977) with reversal of the syndrome up to day 10 or 11 of incubation (Greenberg, 1971), whereas changes in the pancreas have only been demonstrated on days 11

through 19 (Arsenalult and Gibson 1974; Laley and Gibson, 1977). Clearly evidence of increases in insulin levels in the latter half of incubation is not necessarily indicative of earlier increases when the initial changes in the limbs and beak occur.

The second part of this study was designed to test the hypothesis that malathion exerts its teratogenic potential by increasing endogenous insulin levels in the developing chick embryo. To test the efficacy of this hypothesis malathion was injected and plasma insulin levels were measured on alternate days from the 7th to 17th day of incubation. This time frame made it possible to compare the results of this study with those demonstrating changes in the pancreatic histology (Arsenault and Gibson, 1974; Laley and Gibson, 1977) and the time of initial changes in bone development (Ho and Gibson, 1972a, 1972b; Jackson and Gibson, 1977). In addition, plasma glucose levels were measured and an attempt was made to correlate the insulin levels with the extent of micromelia and hypoglycemia.

Chapter II

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Fertile eggs from White Leghorn chickens were obtained from Semetin Hatcheries, St. Canute, Québec. Prior to incubation, eggs were candled and those with cracks, poor calcification or displaced air spaces were discarded. The remainder were stored and placed in a Jamesway single-stage incubator (38°C) over a period of 2 to 4 days. The eggs were rotated every two hours.

2.2 INJECTION PROCEDURE

At 120 hours of incubation the eggs were candled and non-fertile or dead embryos were discarded. Viable eggs were punctured above the air space using a sterilized dissecting needle fitted with a stopper such that approximately 4 mm of the needle was exposed. Through this hole 0.1 ml of solution was injected into the yolk sac using a 1 ml tuberculin syringe fitted with a 23 gauge, 1 inch needle. At the end of each injection the bevel of the needle was checked. If yolk was present (indicating damage to the vitelline membrane and

a possibility of the presence of yolk in the albumen, McLaughlin et al., 1963) the egg was not included in the study. The remainder were sealed with paraffin wax and returned to the incubator. An untreated group was included in each batch of eggs.

2.3 INSULIN INJECTIONS

2.3.1 Solutions

1) Turkey Insulin

Purified turkey insulin (TI) 26 IU/mg (mouse convulsion test, Blundell, 1981) was obtained from Dr. T. L. Blundell, Birkbeck College, University of London. The injection solution was prepared by dissolving insulin and sodium chloride in hydrochloric acid, pH 3.0. Sodium hydroxide was then added drop by drop so that the solution turned from clear to cloudy and then cleared again. When the solution had cleared, indicating a pH greater than 7.0 (Windholz, 1976), the sodium hydroxide additions were stopped. Distilled water was then added to bring the total concentrations of insulin and sodium chloride to 2 IU/0.1 ml and 0.9 %, respectively.

A control solution (saline-TI) consisting of 0.9 % sodium chloride solution was prepared in a similar fashion.

ii) Mammalian Insulins

Two regular Iletin insulin solutions (100 IU/ml), one a beef and pork mixture and the other made from pork only, (compliments of Eli Lilly Co., Toronto) were used. Each was diluted using a 0.9 % sodium chloride solution to give a final concentration of 2 IU/0.1 ml.

A control consisting of saline + glycine, was prepared by diluting a 1.6 % solution of glycine which is normally found in the commercially prepared insulin solutions, in a similar manner to that of the above mammalian insulin solutions.

iii) Additional Controls

In addition to the above mentioned controls one group was injected with 0.9 % sodium chloride solution (saline) and another was left untreated.

Each of the above solutions was sterilized using a 0.22 um nitrocellulose filter and stored for not more than five days in a vacutainer at 4°C. The solutions were warmed to room temperature prior to injection.

iv) For comparison purposes groups of malathion and corn oil treated embryos (see below) were included in this study.

2.3.2 Assessment of Toxicity

In order to assess embryo mortality, the eggs were candled on incubation days 7, 8, 9, 11, 13, 15 and 17, and all dead embryos were recorded and discarded. The remaining embryos were collected on incubation day 17, weighed, examined for gross anomalies and measured for body length (distance from crown to rump) and limb length (distance from the proximal end of the femur to the proximal end of the phalanges).

2.4 MALATHION INJECTIONS

2.4.1 Solutions

A 0.1 ml volume of 5 % malathion (95 % technical grade, compliments of Cyanamid of Canada Ltd., Pt Claire, Québec) in sterile corn oil was used for all malathion injections. The solution was stored at 4°C for no longer than 24 hours or at room temperature for up to 12 hours. Uninjected and corn oil injected embryos were used as controls.

2.4.2 Sampling Techniques

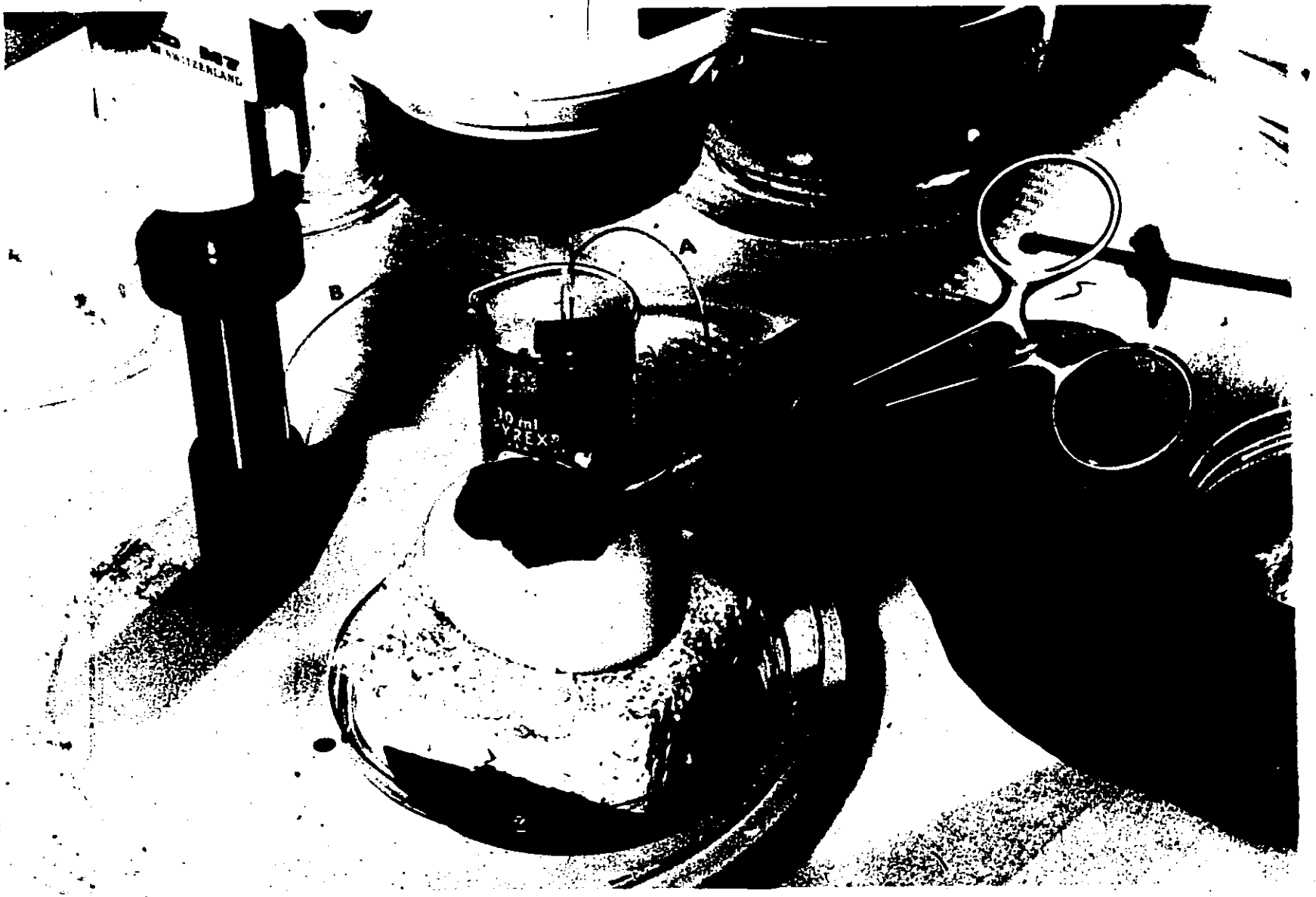
1. Days 7, 9 and 11 of incubation.

On the day of sampling the egg was candled and marked above the vitteline vessels, and then the shell and chorionic membrane above the vesssels were removed. Blood was collected using a 12.5 cm long piece of Tygon tubing, with an inner diameter of 0.25 mm (Cole-Parmer Instrument Company, Chicago, Illinois). This tubing was fitted at each end with a 26 gauge, 1/2 inch syringe needle (tubing A). One end of the tubing was placed through a vacutainer stopper which fit tightly on a chilled 0.5 ml eppendorf centrifuge tube. To create suction a second needle was passed into the vacutainer top, the distal end of this tubing (tubing B) was connected to a 1 ml tuberculin syringe (see fig 3). Prior to

sampling tubing A and the eppendorf tube were heparinized by passing 0.1 ml of 20 USP/ml ammonium heparin solution through the tubing to the eppenderf. The solution was rolled around the sides of the tube and expelled. Any remaining solution was evaporated at room temperature.

Surgical technique used in the collection of blood samples from embryos on days 7, 9 and 11 of incubation.

Figure 3: Surgical technique for blood collection.


A
B

ii) Days 13, 15 and 17 of incubation.

Blood was collected from the allantoic artery directly into a heparinized 1 ml tuberculin syringe fitted with a 23 gauge, 1/2 inch needle and was immediately transferred into a 0.5 or 1.5 ml eppendorf tube and placed on ice. Heparin concentrations did not exceed 4 USP/ml of blood.

Samples from all days were kept on ice for a maximum of 2 hours. They were then centrifuged in a Brinkmann eppendorf centrifuge at 12,000 x g (model 5412) for one minute and the plasma was stored for no longer than a year at -70°C. Due to mechanical problems with the storage freezer, the samples were transferred to a -20°C freezer for a period of approximately 2 weeks. At no time were the samples thawed prior to the insulin assay.

2.4.3 Assessment of Toxicity

i) Gross Morphological Examinations

Immediately after blood collection the embryo was freed of its membranes, weighed, measured for body and right limb length (in all except day 7 embryos) and examined for gross anomalies. Due to difficulties in distinguishing the long bones from the the surrounding tissues, day 7 embryos were fixed in 95 % alcohol and stained with alizarin red S and

Alcain blue (McLeod, 1980). Limb measurements were then made using a stereoscope equipped with an ocular micrometer.

In order to assess the embryonic mortalities the eggs were candled daily from the 6th to the 17th day of incubation.

ii) Insulin Measurements

a) Materials

Guinea-pig anti-insulin serum (AIS; prepared by Dr. J. Braaten, Department of Endocrinology and Metabolism, Civic Hospital, Ottawa), rabbit anti-(guinea-pig globulin) (AGG), normal guinea-pig serum (NGS) and rat insulin standards were generously provided by M. Dalpé-Scott, Dr. H.M.C. Heick and Dr. N. Begin-Heick (Department of Biochemistry, University of Ottawa, Ottawa). Purified chicken insulin (Kimmel et al., 1968), lot number 615-1082B-249 was donated by Dr. R. E. Chance (Lilly Research Laboratories, Indianapolis, Indiana). Bovine serum albumin, fraction V, RIA-grade was purchased from Sigma Chemical Co. (St. Louis, Mo), sodium merthiolate was obtained from BDH and ^{125}I-labelled pork insulin (specific activity of 100 uCi/ug) was purchased from New England Nuclear Corporation (Lachine, Québec).

b) Preparation of Standards

Purified chicken insulin (225 ug) was dissolved in 2.5 ml of distilled water. An aliquot of this solution was further

diluted with sodium phosphate buffer (0.577 % $Na_2 HPO_4 2H_2O$; 0.105 % $NaH_2PO_4 H_2O$) containing 6 % bovine serum albumin (BSA), 0.6 % sodium chloride and 0.24 % merthiolate, pH 7.4. This solution was stored (-20 °C) in 1 ml aliquots. Final dilutions were made as needed with borate buffer (8.25 g boric acid, 2.7 g sodium hydroxide, 10.0 mg merthiolate and 3 ml of concentrated HCl per liter) containing 0.5 % BSA, pH 8.0. These solutions were also stored at -20 °C. The concentrations of insulin used in the standard curve ranged from 50 to 3,200 pg/0.3 ml and 0.5 % BSA borate buffer was used as the zero standard.

c) Preparation of Samples

Immediately prior to their addition to the insulin, assay samples were thawed and centrifuged in a Brinkmann eppendorf centrifuge (model 5). Samples were pooled when necessary. On all days, except day 7, pooling was based on the limb to body length ratios and no embryos were pooled if their ratios differed by more than 0.02. Pooling of day 7 embryos was based on blood volumes. The amounts of plasma to be used in the insulin assay for each day sampled was chosen based on preliminary assays showing the minimum volumes required to obtain between 75 to 125 pg of insulin per assay tube. This range was selected as it is within the most sensitive portion of the standard curve (50 to 200 pg) and kept the number of embryos per pool to a minimum. As the maximum allowable sample volume per assay tube was 300 ul, it was

not possible to obtain mean insulin values within the 75 to 125 pg range for samples from incubation days 7 and 9. The values were, however, above 50 pg/tube and thus, were still in the most sensitive portion of the standard curve. When possible, usually for samples from day 15 and 17 incubated embryos, assays were performed in duplicate but only single assays were made of pooled samples. Table 1 shows the number of samples pooled for each day of incubation sampled.

It should be noted that a total 'sample volume' of 300 ul was added to each assay tube and 0.5 % BSA borate buffer, pH 8.0 was added as required, to bring the samples up to volume.

TABLE 1

Number of plasma samples pooled

Number of embryos used to obtain the minimum required volume of plasma from embryos of different days.

Day of Incubation	7	9	11	13	15	17
Number of Embryos Pooled	3 to 5	3 to 4	1 to 2	1 to 2	1	1

d) Preparation of Tracer

^{125}I-labelled pork insulin was diluted with 300 ul of distilled water, divided into 25 ul fractions and stored at -20 °C. A further dilution using 0.5 % BSA borate buffer was made such that the concentration of ^{125}I-insulin was reduced to 50 pg/ul. Immediately prior to the assay a final dilution was made (with 0.5 % BSA borate, pH 8.0) such that 2 ul of tracer (50 pg/ul) in a volume of 100 ul was added to each tube.

The original 25 ul fractions were stored up to 1 month and further dilutions were stored at 4 °C for up to one week.

e) Insulin Assay

Insulin was measured using the Hales and Randles (1963) double antibody radioimmunoassay method modified by Dalpé-Scott and coworkers (1982). In this method 300 ul of AIS, diluted to give 30 to 40 % precipitation of radioactivity when no insulin standard is present (1:720,000 final dilution) and 100 ul of rabbit AGG (1:90 or 1:72 final dilution depending on the batch) were incubated in the presence of 100 ul of NGS (1:900 final dilution) at 4 °C for 24 hours. To this mixture 300 ul of chicken insulin standard or sample was added and the preparations were incubated for 6 hours. ^{125}I-labelled pork insulin (100 ul) was added at the end of this time and the tubes were returned to 4 °C for 19 hours. They were then centrifuged at 3,000 rpm (Beckman

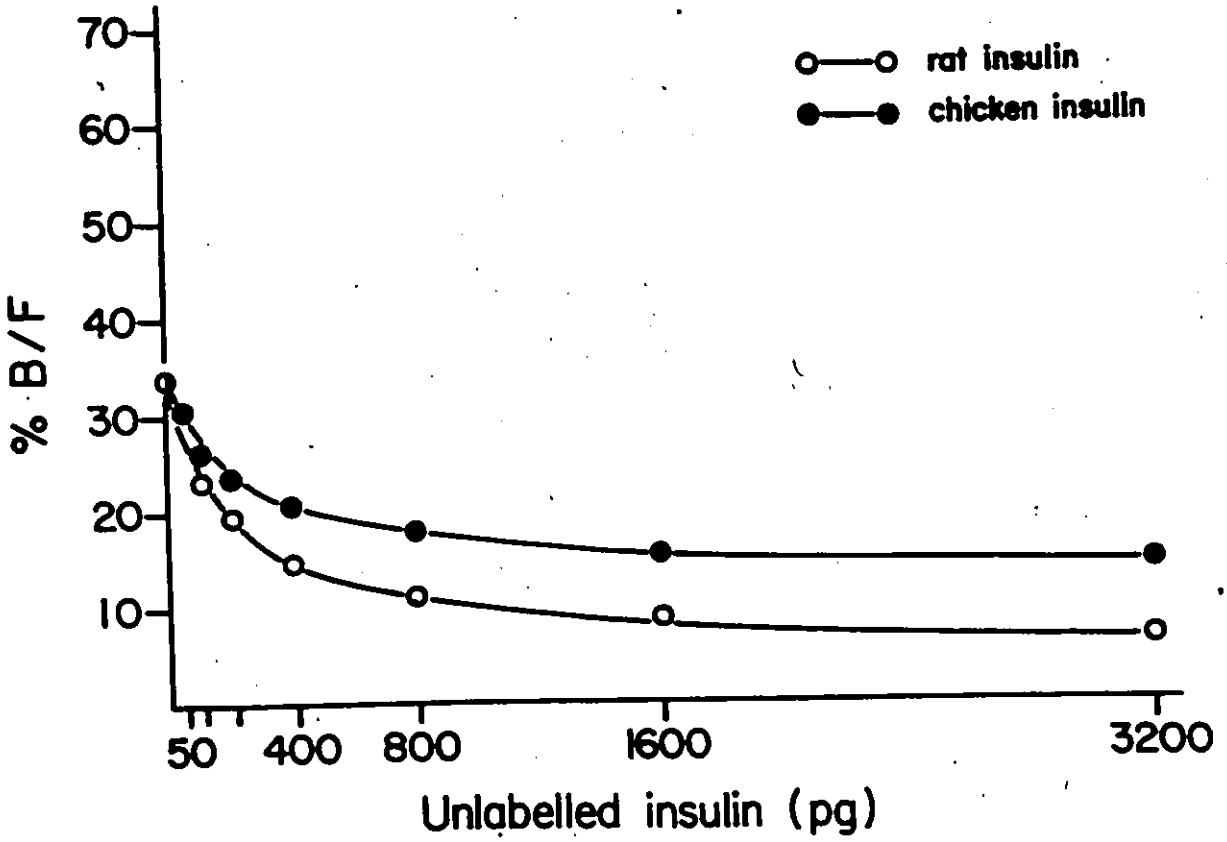
centrifuge, model J-6), decanted, wiped dry above the level of the precipitate and counted using either a Picker, Auto-well 2 or an Amersham, model 1198 gamma counter. Two tubes containing tracer alone were used for the total counts.

A comparison of the chicken and rat insulin standard curves is shown in figure 4. By comparing these one can see that the assay is more sensitive to the rat insulin, as greater decreases in the % bound to free ^{125}I- insulin were seen in response to the increasing rat insulin levels than with increasing chicken insulin levels. Decreases in the % bound to free ^{125}I-insulin observed with the low concentrations of chicken insulin were, however, reasonably large and the Co/Ci curve (where, Co and Ci are the concentrations of radioactivity in the anti-body complex when the amount of unlabelled insulin is zero and 1, respectively, Hales and Randle, 1963) shows that linearity is obtained over the range from 0 to 200 pg (fig 5). In order to verify that the standard curve was sensitive enough to measure chicken plasma insulin levels several studies were conducted.

The effects of unlabelled rat and chicken insulins on the recovery of ^{125}I-labelled rat insulin.

B represents the amount of radioactivity (cpm) present in precipitate.
F represents the total radioactivity (cpm)

Figure 4: Recovery of rat and chicken insulins.


70
60
50
40
30
20
10
% B/F
rat insulin
chicken insulin
50
400
800
1600
3200
Unlabelled insulin (pg)

The effect of unlabelled chicken insulin on the recovery of ^{125}I-labelled rat insulin.

Cc represents the radioactivity (cpm) present in the precipitate when no insulin is added to the assay mixture.

Ci represents the radioactivity (cpm) present in the precipitate. A know concentration of insulin standard is added to the assay mixture.

Figure 5: Effect of chick insulin on the recovery of labelled insulin

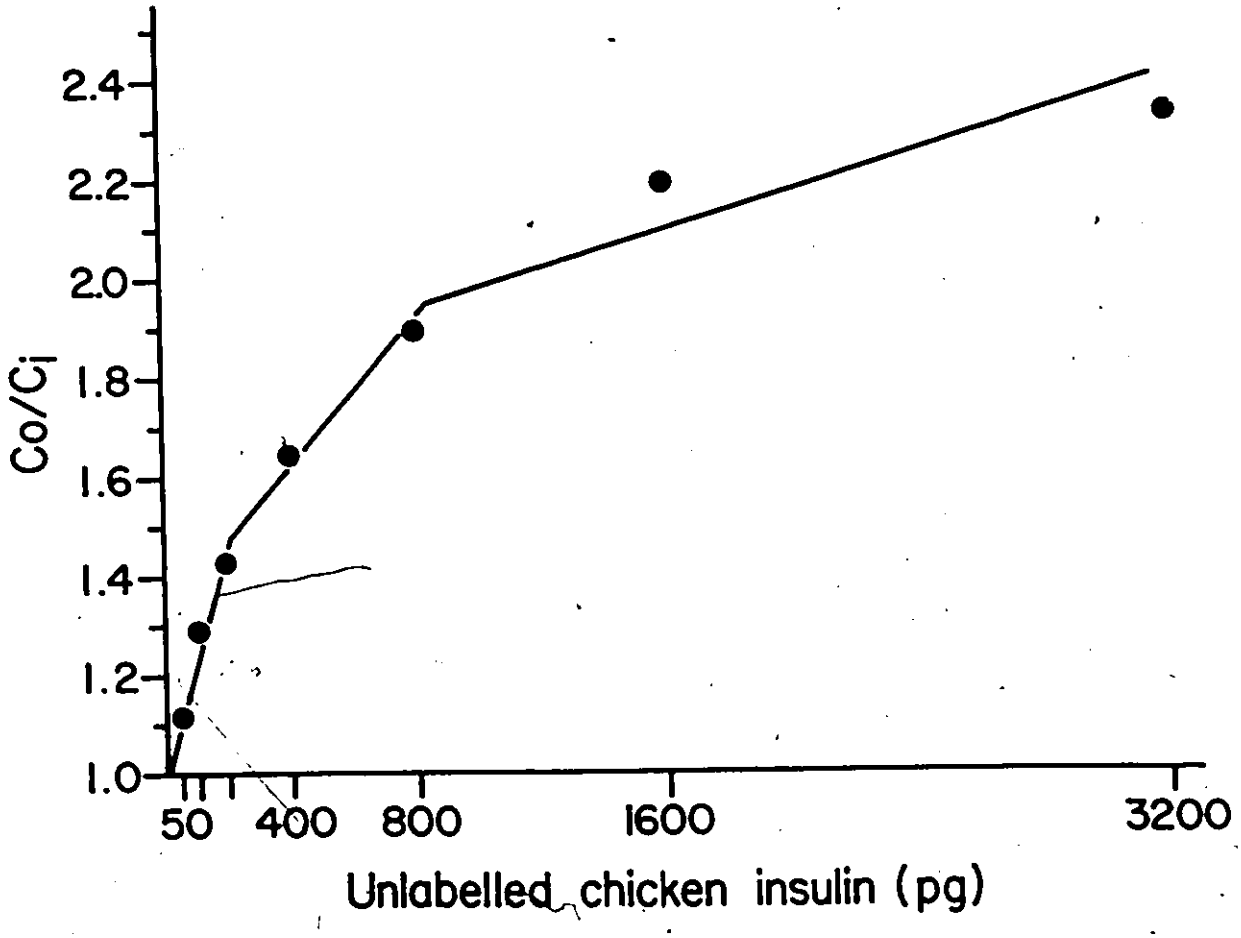

2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
Co/Ci
50
400
800
1600
3200
Unlabelled chicken insulin (pg)

Table 2 gives the data collected to test the effect of using different volumes of plasma in the insulin assay. From the values obtained it was concluded that the measurement of insulin was not affected by sample volume.

Table 3 shows results of a study on the reproducability of insulin measurement in which single samples were divided into two aliquots and insulin levels measured in separate assays. The findings indicated that a high degree of precision was obtained in successive assays.

In addition, to ensure that there were no factors present in the plasma which interfere with the assay, samples or standards were assayed separately and together. The rationale was that if no interfering factors were present then the calculated value of the standard (standard and plasma minus plasma alone) would equal that of the actual measured value. Table 4 shows that this is indeed the case. Therefore, it can be concluded that the embryonic chicken plasma did not contain substances which interfere with the measurement of insulin levels.

TABLE 2

Effect of plasma volume on insulin measurement

Data showing the effects of variations in the plasma volume used on the measurement of embryonic chicken insulin levels.[1]

[1] All volumes from a given day of incubation were obtained from a single pooled sample of plasma.

Day of Incubation	Volume of Plasma Used	Plasma Insulin	Mean Plasma Insulin
13	100 125 150 200	0.42 0.43 0.53 0.44	0.46 ± 0.049
13	100 125 150 200	0.30 0.30 0.22 0.25	0.27 ± 0.039
15	100 125 150	0.55 0.63 0.57	0.58 ± 0.042
17	125 150	0.42 0.37	0.40 ± 0.035

TABLE 3

Aliquots of chicken plasma assayed on separate days

Data showing the precision achieved when insulin levels were measured in aliquots of chicken plasma on separate assay days.[1]

[1]Values shown are ng of insulin per ml of plasma

Day of Incubation	1ST Assay Plasma Insulin	2ND Assay Plasma Insulin	Calculated Difference
7	0.22	0.19	0.03
9	0.38	0.19	0.19
11	0.26	0.34	0.08
13	0.16	0.13	0.03
15	0.25	0.25	0.00
15	0.55	0.51	0.04
17	0.33	0.33	0.00

TABLE 4

Effect of chicken plasma on insulin measurements

Precision of insulin measurements when insulin standards (std) were measured alone and in the presence of chicken embryo plasma.

Insulin std alone - (plasma + std - plasma insulin alone)

Plasma insulin alone - (plasma + std - insulin std alone) expressed as concentration of insulin per ml of plasma.

Day of Incubation	Plasma Volume (ul)	Plasma Insulin Alone (pg)	Insulin Std Alone (pg)	Plasma plus Std Combined (pg)	Actual minus Calculated[1] Insulin Std (pg)	Actual minus Calculated Plasma Insulin[2] (ng/ml)
9 and 11 (Pooled)	250	45	24	72	3	0.01
			48	107	14	0.06
			72	115	2	0.01
15	225	55	210	244	21	0.09
15	225	51	219	256	14	0.07

In addition to a standard curve several other controls were tested in the assay. These controls included tubes containing no 1st or 2nd antibody and no 1st antibody. This procedure ensured that the background level of counts in the assay were no greater than that of the gamma counter. Also, extra standards containing 75 to 200 pg of insulin were assayed in duplicate every 20 to 25 tubes; thus the precision of the assay was monitored continually. In addition, to ensure that the assay was consistent from week to week tubes containing aliquots of two plasma pools (made from day 16 incubated chicken embryo plasma), one a "high pool" spiked with chicken insulin, containing approximately 515 pg and another a "low pool", containing approximately 58 pg, were included in assays. The assay was found to be consistent, as the means and standard deviations for the high and low pools in the eight assays used were 10.3 ng/ml ± 1.05 and 0.29 ng/ml ± 0.06 ng/ml, respectively.

iii) Glucose Measurements

Plasma glucose levels were determined in duplicate from measurements of 10 ul of either undiluted or two fold diluted plasma using a Beckmann glucose analyser #2. This method is based on the reaction of B-D glucose and oxygen in the presence of glucose oxidase and water producing gluconic acid and hydrogen peroxide. An oxygen electrode within the reaction chamber measures the rate of oxygen consumption. This rate is proportional to the amount of glucose present and is scaled to give the glucose concentration.

2.5 NOTE ON STATISTICS

All comparisons, except as indicated below, were made using a Student-Newman-Keuls test corrected for sample size (Sokal and Rohlf, 1969). Comparisons of day 7 limb to body length ratios were made using a t-test (Zar, 1974). Since the data for the day 7 plasma insulin levels was not normally distributed a Kruskall-Wallis test was used for its analysis (Seigel, 1956). All mortality data was analyzed using a Chi Squared test (Sokal and Rohlf, 1969). Correlations between parameters were made using regression analysis (SPSS manual, 1975).

Limb to body length ratios were normalised prior to their analysis using an arcsine transformation (Zar, 1974). The significance level used for all analysis was the 95% confidence level.

Chapter III

RESULTS

3.1 INSULIN INJECTIONS

Table 5 shows that there were no significant differences ($p < 0.05$) between body weights or between limb to body length ratios in the uninjected controls, injected controls or the beef and pork insulin-mixture injected groups. The latter group did, however, contain one case of moderate micromelia (limb to body length ratio of less than 90 % of the control mean, Laley and Gibson, 1977). Micromelia was never observed in the control animals. The pork insulin treated embryos showed a significant decrease ($p < 0.05$) in body weight relative to the untreated and saline-glycine injected embryos. They were not, however, significantly different from the other control groups. The turkey insulin injected group weighed significantly less ($p < 0.05$) when compared to all of the control groups. Embryos treated with either turkey or pork insulin had significantly ($p < 0.05$) lower mean limb to body length ratios. The effect was greater in the turkey insulin injected group, as the mean limb to body length ratios of this group was significantly less ($p < 0.05$) than the control groups and groups injected with other

insulins (table 5). All three insulin solutions had similar effects on beak development, treated animals having a higher incidence of short upper beak being observed in each of these groups (table 6).

The effects of turkey insulin on the embryonic development of chickens was similar to those observed with the mammalian insulins except that the decreases observed in the limb to body length ratio was more pronounced with the turkey insulin injections.

The effects of 0.1 ml of 5 % malathion (table 5) were similar to those described for insulin in that malathion significantly decreased ($p < 0.05$) day 17 embryo weights, as did turkey insulin, and limb to body length ratios, as did turkey and pork insulins. Malathion had an extreme effect on the latter parameter, as the decrease observed was not only significantly different ($p < 0.05$) from the controls but also from all of the insulin injected groups.

The effects of malathion on beak development, however, differed from that of the insulin solutions in that parrot beak and short lower beak were produced whereas short upper beak was not (table 6). Further, whereas malathion produced a high incidence of sparse feathering, only one case of sparse feathering was observed with insulin injections. This was seen in the turkey insulin group. It should be noted that sparse feathering is difficult to quantify and only embryos demonstrating obvious cases were recorded.

TABLE 5

Insulin or malathion treatment - weights and limb/body ratios

Effect of injecting various control solutions, mammalian insulins, turkey insulin or malathion on body weights (gm)[1] and limb to body length ratios[2] of day 17 embryos.

length ratios of day 17 embryos.

Embryos were injected on day 5 of incubation.

1 mean ± standard deviation.

2 mean (95 % confidence interval).

* significantly different from all control groups, $p < 0.05$.

** significantly different from all control and mammalian insulin groups, $p < 0.05$.

*** significantly different from all other groups, $p < 0.05$.

Solution Injected	N	Body Weight	Limb to Body Length Ratio
None	28	20.5 ± 1.87	0.71 (0.70 to 0.71)
Saline	21	19.2 ± 2.36	0.70 (0.69 to 0.71)
Saline + Glycine	22	19.8 ± 1.52	0.71 (0.70 to 0.71)
Saline - TI	20	18.7 ± 2.64	0.70 (0.69 to 0.71)
Corn Oil	21	20.1 ± 2.87	0.71 (0.69 to 0.72)
Beef and Pork Insulin Mixture	10	18.2 ± 2.21	0.67 (0.65 to 0.69)
Pork Insulin	10	17.1 ± 3.00	0.62 (0.56 to 0.67)*
Turkey Insulin	17	16.2 ± 3.64*	0.58 (0.51 to 0.65)**
Malathion	24	14.9 ± 3.01*	0.51 (0.47 to 0.55)***

TABLE 6

Gross morphology of insulin and malathion treated embryos

Effect of injecting various control solutions, mammalian insulins, turkey insulin or malathion on the gross morphology of day 17 incubated embryos.[1,2]

[1] Embryos were injected on day 5 of incubation.

[2] Values shown are the % of injected embryos (n) which demonstrated the abnormality.

[3] Pooling of the results obtained with saline, saline + glycine, saline-TI and corn oil injections.

Solution Injected	N	Short Upper Beak	Parrot Beak	Short Lower Beak	Cross Beak	Sparse Feathering
None	28	--	--	--	--	--
Pooled Controls [3]	84	--	--	--	1	--
Beef and Pork Insulin Mixture	10	30	--	--	--	--
Pork Insulin	10	20	--	--	--	--
Turkey Insulin	17	18	--	--	--	6
Malathion	24	--	33	4	--	38

In summary then, the effect of 0.1 ml of 5 % malathion injections on embryonic development was similar to that produced by 2 IU of insulin with the exception that malathion had a more marked effect on the limb to body length ratio and sparse feathering. Further, at least in this study, the effects of malathion on beak development were qualitatively different from those of insulin. However, insulin has been reported as producing a higher incidence of parrot beak or short lower beak, similar to those produced by malathion (Duraiswami, 1950; Landauer and Clark, 1963).

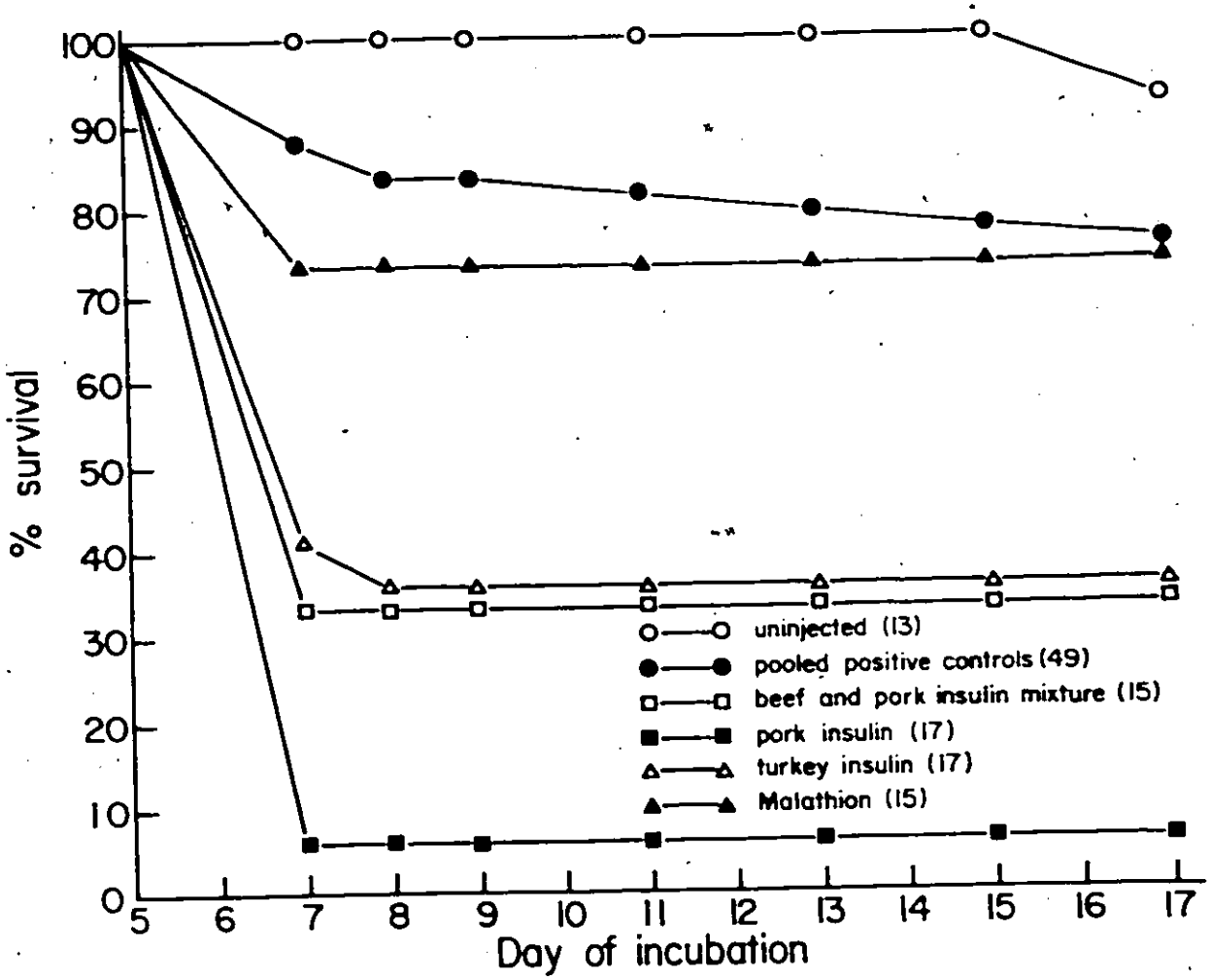
One case of abnormal beak development, cross beak, was reported in the control embryos. This is most likely a random occurrence as cross beak is a known congenital abnormality in chickens (Landauer and Baumann, 1943; Grewal and Singh, 1978).

Figures 6 and 7 show the mortalities for each of the two days of initial incubation. A comparison of these two figures shows that, in general, the day 7 mortalities for embryos injected on the first day of incubation were higher than the day 7 mortalities for embryos injected on the second day and this was particularly evident for those in the turkey insulin injected group. This phenomenon was not observed in any of the other experiments. Indeed, no differences in mortalities were seen between the various days of incubation when eggs were incubated over a 4 day period. The large differences in intra group mortalities for the two

incubation days makes it difficult, to make inter-group comparisons. Consistent trends between the two days do exist, however, and it is apparent that injections of the various control solutions results in a small but not significant decrease in embryonic survival rates between the 5th and 7th days of incubation. The injections of various insulins resulted in decreases in embryonic survival compared to that of the pooled saline control injected groups. These decreases were significant ($p < 0.05$) for all three insulins examined in the group of eggs which was incubated on the first day of the two day incubation period, and for the pork insulin, and the beef and pork insulin mixture groups in the eggs incubated on the second day. In this experiment no significant difference in mortalities were seen between the malathion and corn oil injected groups.

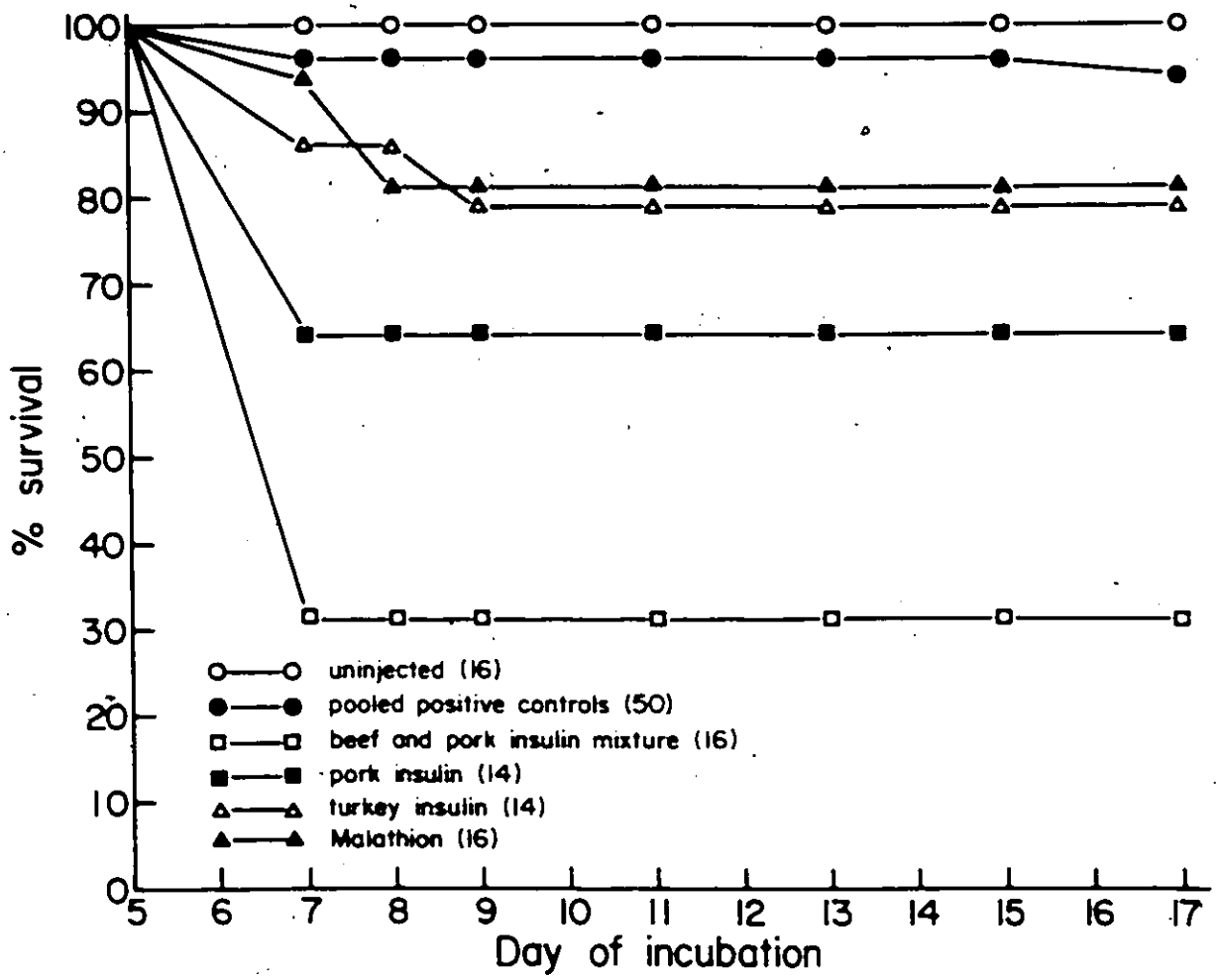
Percent survival of uninjected and injected embryos incubated the first day of a two day incubation period and candled on days 5, 7, 8, 9, 11, 13, 15 and 17.

Figure 8: Survival curve for embryos incubated on the 1st day.


100
90
80
70
60
50
40
30
20
10
0
% survival
5
6
7
8
9
10
11
12
13
14
15
16
17
Day of incubation
uninjected (13)
pooled positive controls (49)
beef and pork insulin mixture (15)
pork insulin (17)
turkey insulin (17)
Malathion (15)

Percent survival of uninjected and injected embryos incubated on the second day of a two day incubation period and candled on days 5, 7, 9, 11, 15, and 17.

Figure 7: Survival curve for embryos incubated on the 2nd day


100
90
80
70
60
50
40
30
20
10
0
% survival
5
6
7
8
9
10
11
12
13
14
15
16
17
Day of incubation
uninjected (16)
pooled positive controls (50)
beef and pork insulin mixture (16)
pork insulin (14)
turkey insulin (14)
Malathion (16)

3.2 MALATHION INJECTIONS

Table 7 shows that the mean body weights of corn oil injected embryos were significantly less than ($p < 0.05$) those of the uninjected embryos on day 15. This finding is contrary to that of Greenberg (1971) who found that the injection of 0.1 ml of corn oil into the yolk sac of chicken embryos on any day from the 4th through to the 12th day of incubation produced no difference in the day 15 body weights when compared to an uninjected group.

The malathion injected group had mean body weights less than both the uninjected and corn oil injected groups on days 9, 11, 13, 15 and 17. These decreases in body weights were significantly different from the corn oil group on days 9 and 17 and this suggests, as has been found previously in this report and by Greenberg and LaHam (1969), that malathion injections produce decreases in embryonic weights. In addition, a decrease in the survival rates of the chick embryos was observed with either corn oil or malathion treatment (11; 10). It was reported earlier (Greenberg and LaHam, 1969), and substantiated by the present study that the exent of the decrease in survival is significantly greater ($p < 0.05$) in the malathion treated embryos than in those receiving corn oil.

TABLE 7

Body weights of malathion treated embryos

Body weights (in grams) of untreated, corn oil-treated and malathion-treated embryos sampled on alternate days from day 7 to 17 of incubation.[1]

[1] Mean ± standard deviation (n).

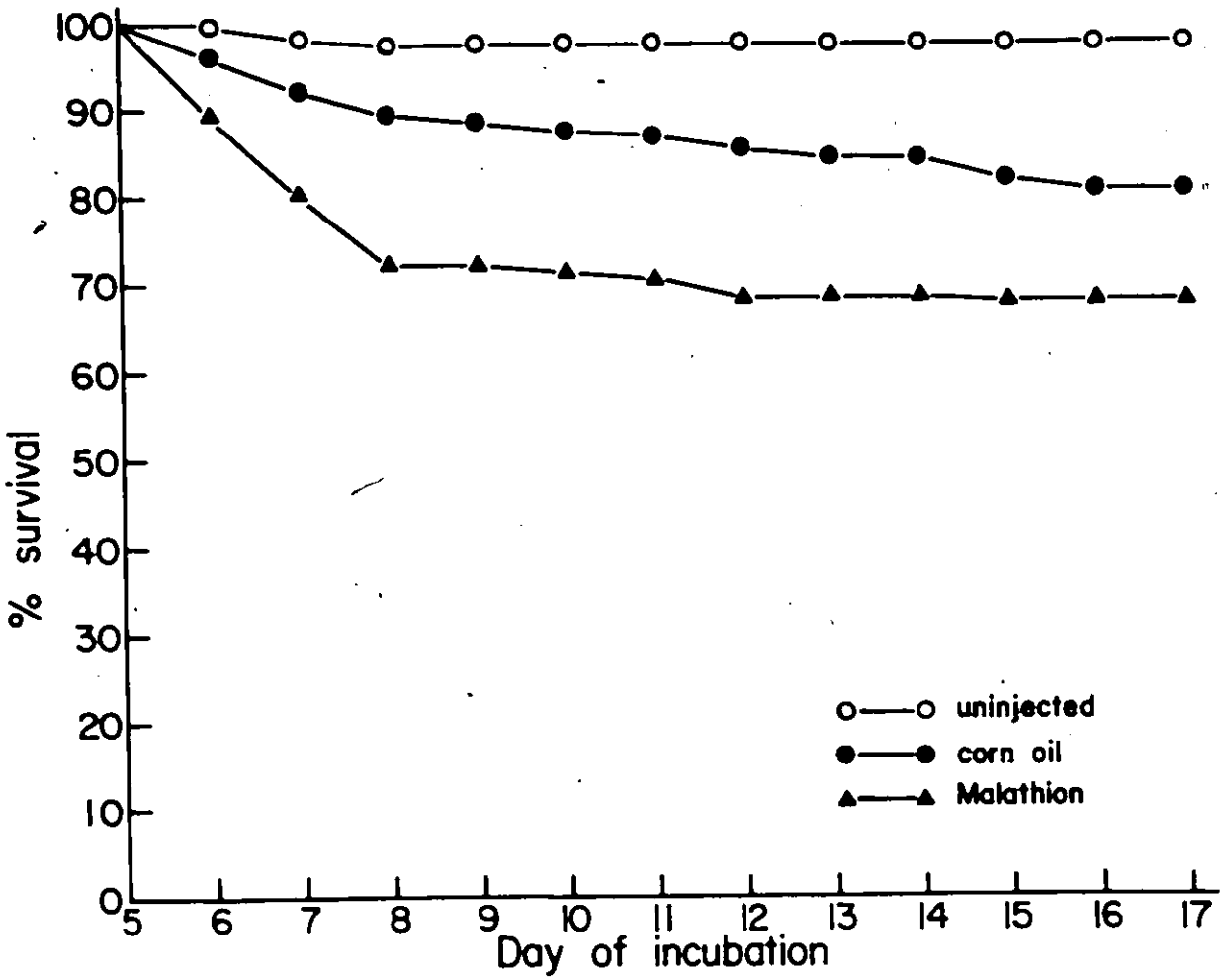
* significant difference from the uninjected group ($p < 0.05$).

** significant difference from both the uninjected and corn oil injected groups.

Day	Uninjected	Corn Oil Injected	Malathion Injected
7	0.74 ± 0.07 (37)	0.76 ± 0.08 (37)	0.78 ± 0.09 (78)
9	1.69 ± 0.12 (38)	1.68 ± 0.16 (41)	1.57 ± 0.17** (100)
11	3.62 ± 0.47 (13)	3.50 ± 0.33 (14)	3.18 ± 0.41* (26)
13	6.76 ± 0.57 (12)	6.25 ± 0.94 (14)	5.55 ± 1.05 (25)
15	12.58 ± 1.31 (19)	11.16 ± 1.97* (18)	10.65 ± 1.73* (32)
17	18.70 ± 1.23 (17)	17.90 ± 2.41 (18)	15.35 ± 3.01** (24)

percent survival of uninjected (U) and day 5 corn oil (CO) or 5 % malathion (M) injected embryos candled daily from the 5th to the 17th day of incubation.

Figure 8: Survival curve for malathion treated and control embryos


100
90
80
70
60
50
40
30
20
10
0
% survival
5
6
7
8
9
10
11
12
13
14
15
16
17
Day of incubation
uninjected
corn oil
Malathion

Figures 9, 10, and 11 show the mean limb to body length ratios, plasma glucose and plasma insulin values for the embryos used. There were no significant differences between the uninjected and corn oil injected embryos in any of these parameters. Since malathion's effects on the limb to body length ratio of chicken embryos were already well established (Lalley and Gibson, 1977), these measurements were taken primarily to assess correlations between the extent of micromelia and the plasma glucose and insulin levels. The limb and body length measurements were not performed on day 7 embryos as preliminary results showed that no significant difference exists between uninjected, corn oil injected or malathion injected embryos at this stage of development (table 8). Fig 3 shows that significant decreases ($p < 0.05$) in limb to body ratios were observed in response to malathion treatment on the 9th, 11th, 13th, 15th and 17th days of incubation and this was true regardless of whether the limb to body length ratios were compared as individual data points (not shown) or as average limb to body lengths used in the embryo pools.

TABLE 8

Limb to body length ratios for day 7 embryos

Data showing the limb to body length ratios for day 7 incubated embryos injected with 0.1 ml of corn oil or 5 % malathion on the 5th day of incubation.[1]

[1] Values shown are means with the 95 % confidence interval in parentheses.

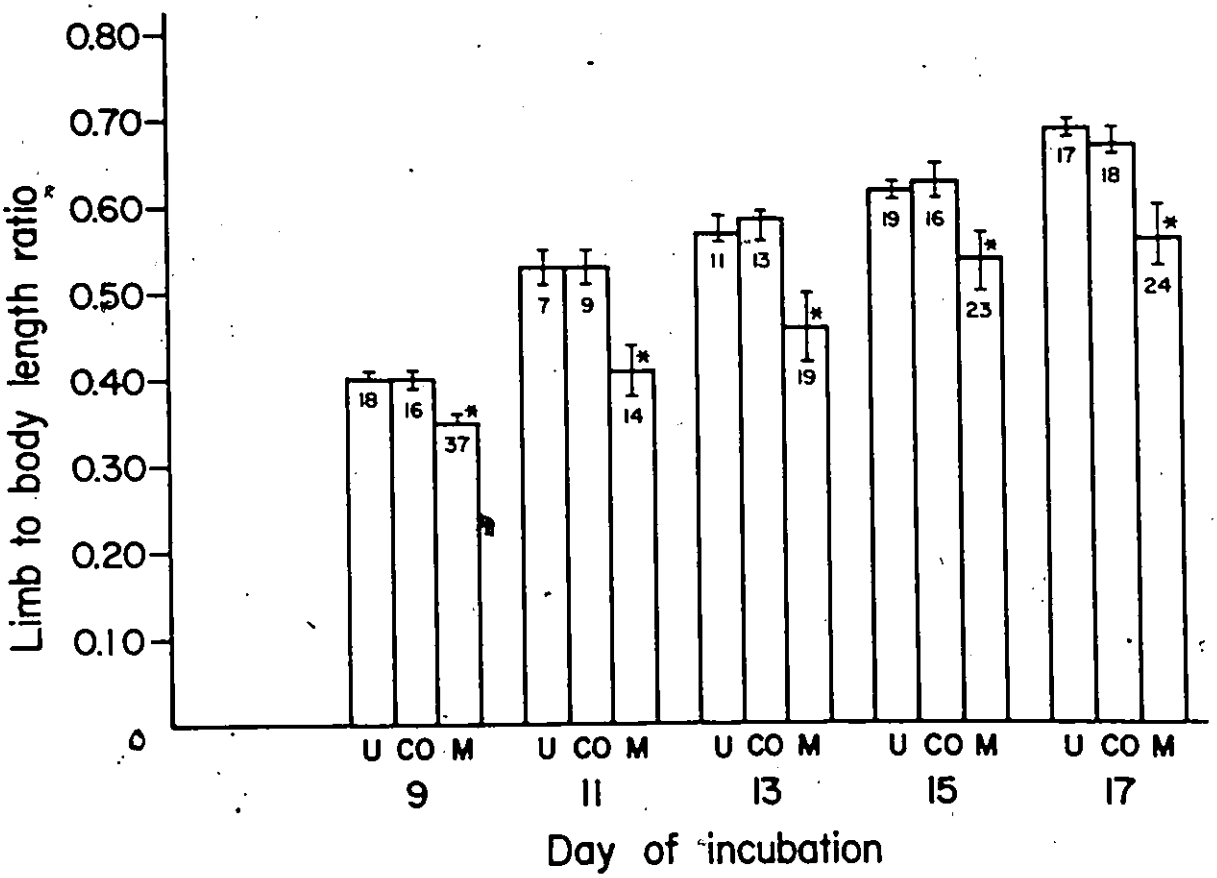
Solution Injected	N	Limb to Body Length Ratio
Corn Oil	9	0.15 (0.14 to 0.16)
Malathion	10	0.14 (0.14 to 0.15)

Limb to body length ratios of uninjected and day 5 corn oil or 5 % malathion injected embryos examined on alternate days from day 9 to day 17 of incubation.[1]

[1] Values shown are means and standard deviations.

* Indicates a significant difference from uninjected and corn oil injected embryos, $p < 0.05$.

Figure 9: Limb to body length ratios of malathion treated embryos

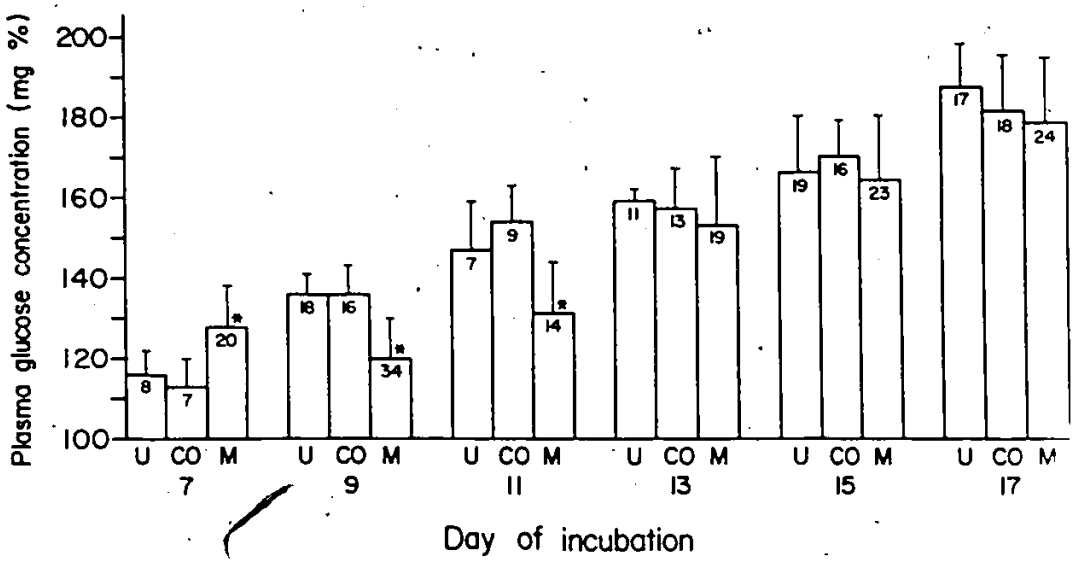

Limb to body length ratio
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
18
16
37
*
7
9
14
*
11
13
19
*
19
16
23
*
17
18
24
*
U CO M
U CO M
U CO M
U CO M
U CO M
9
11
13
15
17
Day of incubation

Plasma glucose levels of uninjected and day 5 corn oil or 5 % malathion injected chicken embryos sampled on alternate days from the 7th to the 17 day of incubation.[1]

[1] Values shown are means and standard deviations.

* Indicates a significant difference from uninjected and corn oil injected embryos, $p < 0.05$.

Figure 10: Plasma glucose levels of malathion treated embryos

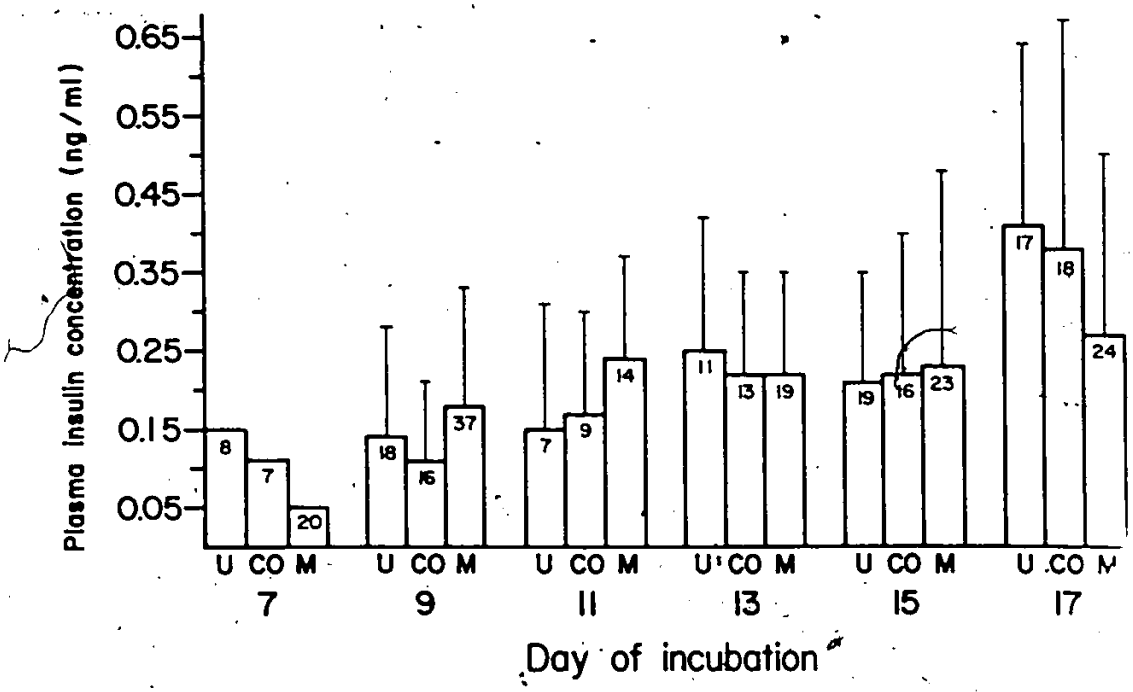

Plasma glucose concentration (mg %)
200
180
160
140
120
100
U CO M U CO M U CO M U CO M U CO M U CO M
7 9 11 13 15 17
Day of incubation
8 7 20 18 16 34 7 9 14 11 13 19 19 16 23 17 18 24

Plasma insulin levels of uninjected and day 5 corn oil or 5 % malathion injected chicken embryos sampled on alternate days from the 7th to the 17th day of incubation.[1,2]

[1] Values shown for incubation day 7 are means (n).

[2] Values shown for incubation days 9 to 17 are means ± standard deviations (n).

Figure 11: Plasma insulin levels of malathion treated embryos


Plasma insulin concentration (ng/ml)
0.65
0.55
0.45
0.35
0.25
0.15
0.05
8
7
20
18
16
37
7
9
14
11
13
19
19
16
23
17
18
24
U CO M
7
9
11
13
15
17
Day of incubation

Plasma glucose measurements showed that hypoglycemia was present in malathion treated embryos on days 9 and 11 of incubation. The plasma glucose levels were positively correlated with the limb to body length ratios on days 9, 11 and 13 (not shown). These finding generally concur with those of previous authors who also found hypoglycemia to be present in malathion treated embryos on the 9th to the 19th day of incubation (Arsenault and Gibson, 1972; Haley and Gibson, 1977). A positive correlation was also found to exist between the degree of hypoglycemia and the extent of micromelia on the 11th, 15th, 17th and 19th days of incubation Haley and Gibson, 1977). Plasma glucose levels of day 7 embryos were examined. The results showed that hyperglycemia was present in the malathion treated embryos at this stage of development (fig 7).

One predominant theory of the mechanism of action of malathion on limb development is that malathion increases pancreatic insulin secretion (Arsenault and Gibson, 1974; Haley and Gibson, 1977). To test this, plasma insulin levels were measured in the three groups of embryos on alternate days from the 7th to 17th day of incubation (fig 11). These levels were quite variable and no significant differences were observed between the uninjected, corn oil injected or malathion treated embryos. An attempt was made to correlate the insulin levels with the limb to body length ratios but no correlations were observed on any of the incubation days examined.

Since the extent of the hypoglycemia observed with malathion treatment has been positively correlated with increased quantities of B cells on the 11th to the 17th day of incubation (Laley and Gibson, 1977) an attempt to correlate plasma insulin and glucose levels was made. A positive correlation between these two parameters was seen on the 17th day of incubation. No other correlations were observed. As plasma insulin and glucose levels may be affected by the hematocrit values, these values were also measured. Day 7 hematocrits (measured as red blood cell to whole blood weight) of malathion treated embryos were significantly decreased compared to the uninjected and corn oil injected embryos. Hematocrit levels returned to normal on days 9 and 11 (table 9).

TABLE 9.

Hematocrit values for malathion treated embryos

Hematocrit values of untreated and day 5 corn oil or 5 % malathion injected chicken embryos sampled on the 7th, 9th and 11th days of incubation.[1]

[1] Values shown are means ± standard deviations (n).

* Indicates a significant difference from uninjected and corn oil injected embryos, $p < 0.05$.

Solution Injected	Red Blood Cell Weight to Whole Blood Weight Ratio		
	Day 7	Day 9	Day 11
Uninjected	25.3 ± 5.2 (21)	24.1 ± 2.1 (30)	21.6 ± 8.9 (8)
Corn Oil	25.4 ± 2.5 (25)	24.3 ± 3.6 (29)	25.5 ± 2.9 (9)
Malathion	19.8 ± 3.1*(30)	24.9 ± 5.0 (85)	23.9 ± 3.1 (26)

Chapter IV

DISCUSSION

4.1 INSULIN INJECTIONS

This study showed that turkey insulin injected into the yolk sac of 5 day incubated chick embryos resulted in anomalies of the beak and limbs similar to those produced by injections of mammalian insulins (Landauer, 1947; Duraiswami, 1950; Landauer and Rhodes, 1952; Rabinovitch and Gibson, 1972a). In addition, the study showed that the turkey insulin had a significantly greater effect on limb development than did either of the two mammalian insulin preparations studied. This is the first demonstration, that the anomalies produced in response to day 5 insulin injections can be produced by homologous insulin.

The study also confirmed the observations of previous authors who demonstrated that malathion injections produce a syndrome similar to that produced by insulin (Greenberg and LaHam, 1970; Ho and Gibson, 1972a,b; Jackson and Gibson, 1977). By themselves, these data support the hypothesis that malathion may produce the observed anomalies by triggering an increase in endogenous insulin levels (Arsenault

and Gibson, 1974; Arsenault et al., 1975; Laley and Gibson, 1977).

In the past, homologous chick insulin was not available in sufficient quantities and for this reason bovine insulin preparations were used in chicken embryo injection studies. But as there are differences between the amino acid sequences of bovine and chicken insulin (Smith, 1966) in both the A chain (bovine alanine, serine and valine residues occupying the 8th, 9th and 10th positions are replaced by histidine, asparginine and threonine, respectively, in the chicken embryo) and the B chain (bovine phenylalanine, valine and threonine occupying the 1st, 2nd and 27th positions being replaced by alanine, alanine and serine, respectively) it was impossible to determine if the observed anomalies were due to the natural effects of insulin or the result of steric or kinetic differences between the exogenous and endogenous insulins.

In this study an attempt was made to minimize this problem by using turkey insulin, which has the same amino acid sequence as chicken insulin (Markussen and Sundby, 1973) and can therefore be considered as an homologous hormone. For comparison purposes the effects of pork insulin and a beef-pork insulin mixture were also tested. To ensure that the observed anomalies were not due to either the trauma of the injection procedure, or to compounds in the solutions other than insulin, appropriate control groups were included.

Due to the large numbers of eggs used in the study (table 6) the start of incubation was staggered over a two day period. Figures 4 and 5 show the survival curves from the 5th to the 17th day of incubation for each of the two days of initial incubation. A comparison of the figures shows that the mortality was higher at day 7 in all but the beef and pork mixture group, incubated on the first day of the two day period. The reason for this discrepancy is unclear since all eggs were from the same flock of hens. Further, the present author and others (Romanoff, 1967; Nartaitz, 1981; LaHam, 1981) have not observed differences in mortalities when groups of eggs were stored under similar conditions (room temperature) and placed in the incubator over a four day period. The high intragroup differences in mortalities between the two days of initial incubation makes it difficult to make intergroup comparisons but some general trends were observed. The injection of various control solutions resulted in a small but not significant decreases in embryonic survival rates. In addition, the injection of insulin resulted in decreased survival beyond that observed with the injection of the control solutions. These results are consistent with those of Rabinovitch and Gibson (1972a).

There were no significant differences between any of the control injected groups in any of the parameters measured (tables 5 and 6) and only one anomaly, cross beak, was observed. The frequency of cross beak, a known congenital de-

fect in chickens (Landauer and Baumann, 1943; Grewal and Singh, 1970), was low enough to suggest that its occurrence was unrelated to the injection procedure.

A summary of the results of injecting mammalian insulins singly into the yolk sac revealed that neither pork insulin nor the beef-pork insulin mixture produced changes in the day 17 embryo weights. Both treatments, however, did increase the incidence of short upper beak. On the other hand, only the pork insulin produced a significant decrease ($p < 0.05$) in the limb to body length ratio although one incident of moderate micromelia was observed in the beef and pork insulin mixture injected group. These findings differ from those of most other authors who reported decreased body size and limb to body length ratios and increases in the incidence of short upper beak and parrot beak in response to bovine insulin treatment (Duraiswami, 1950; Landauer 1947; Landauer and Rhodes, 1952; Landauer and Clark, 1964; Rabinovitch and Gibson, 1972). This, however, has not been a universal finding as a study comparing the teratogenic potential of 4 IU of four different bovine insulins, Toronto, Iletin, protamine zinc or crystalline zinc, injected into the yolk sac on day 5 of incubation showed that normal development occurred in all but the Iletin insulin group. In this group 20 % of the embryos had short hind limbs and some of the embryos were smaller than the controls; no beak defects were reported (Greenberg, 1971). It would seem there-

fore, that the extent of the response depends, at least in part, on the insulin preparation used.

Genetic variability may also have contributed to the observed differences in response. It was shown that the anomalies produced by various stocks of fowl in response to insulin injections are qualitatively similar but differ in frequency and severity (Landauer, 1947; Landauer, 1951; Landauer and Rhodes, 1952). Indeed, Landauer and Rhodes (1952) reported seasonal variations in response to insulin treatment. Specifically, they observed a gradual decline in the insulin induced production of micromelia and beak defects from early spring to mid summer.

Which of the above factors resulted in the differences in embryonic response to the insulins used in this study and those found by previous authors is unknown.

Injecting 2 IU of turkey insulin into the yolk sac of the 5 day incubated embryos resulted in significant decreases in body weights and limb to body length ratios, and increases in the incidence of short upper beak (fig 7 and 8). Indeed, the fact that the decrease in the limb to body length ratio was significantly different not only from the controls but also from the two mammalian insulin injected groups suggests that the homologous insulin was a more potent teratogen than the mammalian insulins.

The increase in potency of the homologous insulin is analagous to the situation found in juvenile chickens where

as blackfly and midge larvae was recorded by Kingsbury (1978). Holmes (1979) reported increased drifting of blackfly larvae and reductions in mayfly nymph populations, and Holmes and Kingsbury (1980) observed increased drifting of mayfly nymphs, blackfly larvae, chironomid larvae and caddisfly larvae, as well as population reductions for these organisms, dragonfly nymphs and stonefly nymphs.

This increased sensitivity of aquatic larvae and nymphs of terrestrial insects may be influenced by a number of factors, both environmental and physiological. Identification of these may provide information which could lead to reduced non-target impact. Also, the effects of the formulation additives, nonylphenol and diluant oil 585, should be investigated with respect to toxicity of Matacil to terrestrial insect larvae and aquatic invertebrates in light of the work by Philogene and Labaky (1982) which demonstrated that the additives were as toxic as the active ingredient to Aedes atropalpus larvae.

Two of the three benthic aquatic invertebrates so far examined (isopods and crayfish) respond, acutely, to a similar concentration of aminocarb (approximately 30 mg/L). This observation may add some support to the existence of some basic difference between benthic invertebrates and aquatic larvae of terrestrial invertebrates.

The increased toxicity of aminocarb to C.r.racovitzai with increased temperature is not surprising. This phenomenon is likely related to increased uptake of the toxicant with increased temperature (Figure 4). Metabolic rate of poikilotherms is regulated by the temperature of their environment. Increased metabolic rate results in increased respiratory rate causing a direct increase in toxicant uptake across gill surfaces when exposure is via water.

4.1.2 Uptake and Clearance

Uptake of aminocarb was proportional to duration of exposure (Figure 3). It was observed that uptake was occuring into two compartments with one approaching steady-state within 12 hours and the slower uptake compartment linearly increasing its aminocarb content at least to 72 hours. This biphasic behavior is also reflected in clearance of aminocarb residues from isopods (Figure 5).

Both concentration and temperature of exposure have a direct effect on uptake of aminocarb by C.r. racovitzai (Figure 4). This has been observed for other contaminants and organisms (Jessiman, 1981; Trudel, 1980; Lloyd, 1979) and, as stated previously, the temperature effect likely reflects an increase in metabolic rate with increasing temperature resulting in increased absorption. This may indicate that uptake of aminocarb by this isopod is an active process.

Clearance of aminocarb following exposure to 0.4 mg/L is represented in Figure 5 and analysis indicated that clearance patterns from the fast-clearing compartments of the two different exposure temperatures was not significantly different. Clearance from the slow-clearing compartments, however, was significantly different between 12°C and 20°C (Table 3). Therefore, clearance of aminocarb residues from the fast-clearing compartment of C.r.racovitzai appears to be passive. Subsequent to clearance to most residues from the fast com-

partment, however, clearance becomes proportional to temperature and is probably metabolically controlled.

The metabolic control over clearance of aminocarb from the slow compartment is emphasized by the effect of temperature on the biological half-life for the elimination of residues from that compartment (Table 3). Elimination of carbamate residues is generally via the excreta subsequent to metabolic degradation (Kuhr and Dorough, 1976). As metabolic rate increases with temperature, clearance of aminocarb residues would also increase, thereby reducing the half-life of residues within the organism.

4.1.3 Environmental Impact

To determine the impact of aminocarb application on this species of isopod, it is necessary to consider the factors of acute toxicity, uptake and clearance under environmental conditions. Data collected at 12°C for C.r.racovitzai will be used for purposes of discussion since the mean aquatic environmental temperature recorded by Holmes and Kingsbury (1980) was 12.7°C and, therefore, 12°C is likely representative of water temperatures during typical spraying periods (May-June). Sundaram et al. (1976) reported a rate of decrease of aminocarb from environmental waters (streams) of 42% over a 4 day post-spray period. Holmes and Kingsbury (1980) reported a maximum water level of aminocarb of 24.2 ug/L following an application at the maximum allowable level

(175 g AI/ha). They also noted that this level corresponded well with a level of 24.0 ug/L found following application of formulated Matacil in Newfoundland.

Assuming, therefore, a water level of aminocarb of 24 ug/L and a 42% drop over 4 days, the average exposure level for that 4 day period would be approximately 19 ug/L. This level of exposure would result in no appreciable acute lethality or knock-down. Considering the patterns for uptake represented in Figures 3 and 4, uptake of aminocarb would not be rapid enough nor would exposure be long enough to cause any appreciable accumulation of residues. Also, clearance is rapid (Figure 5; Table 3) and, therefore, no chronic effects would likely occur. However, dietary exposure to aminocarb for predators of the isopod, such as the brown bullhead, might occur if sufficient organisms were consumed within the 4 day post-spray period.

4.1.4 Predator Impact

If uptake parameters at environmentally significant exposure levels were similar to those observed in this study, then, by extrapolating Figure 4 to 19 ug/L, the isopod tissue burden of aminocarb after 24 hours exposure would be 1.31 ug/g dry weight. Extrapolating Figure 3 to 96 hours results in an increase in tissue burden, caused by extending the exposure duration from 24 to 96 hours, of 2.74 fold. Therefore, isopods exposed to 19 ug/L for 96 hours would be expec-

ted to have an average tissue burden of 3.60 ug/g (dry weight).

Catfish of approximately 5 g weight must consume about 3% of their body weight per day (wet weight of fish: dry weight of feed) to sustain proper functioning and growth (Ralston Purina Co., 1980). Rubec (1975) reported isolated instances when isopods constituted 100% of the stomach contents of brown bullheads. Considering a 5 g fish, this would amount to approximately 0.15 g (dry weight) of isopods ingested for a single day of feeding. This would result in an ingested dose of about 0.54 ug aminocarb/fish. If assimilation efficiency was considered as 100%, this would correspond to a tissue load of 0.11 ug aminocarb/g fish after one day. This level of fish contamination would cause no toxic effects since 4 days exposure of brown bullhead to 0.1 mg/L produced a mean tissue load of 0.32 ug/g fish and no noticible toxic effect.

Of course 100% assimilation will not occur, nor will all the detected residues be aminocarb because of the rapid metabolism of carbamates. However, the potential exists for the metabolic alteration of aminocarb within the isopod to compounds more toxic than the parent chemical. Basic variations of the metabolic pathways for carbamates between vertebrates and invertebrates could result in production of significant quantities of 4-methylamino-3-methylphenyl N-methylcarbamate (MAM) and 4-amino-3-methylphenyl N-methylcarbamate (AM) in invertebrates, both of which are about 10

times more toxic than aminocarb (Kuhr and Dorough, 1976).

In order, then, to estimate the overall impact of consumption of aminocarb-contaminated isopods on the brown bullhead we must consider the amounts of the various toxic metabolites within the isopod and the bullhead's assimilation efficiency for those toxic components. Using as assimilation efficiency for aminocarb, MAM and AM of 10.5% in the brown bullhead (calculated from the data of Korn (1973) for assimilation of carbaryl by the channel catfish (Ictalurus punctatus)), and assuming an average proportion of the detectable residues within the isopod as aminocarb of 0.17 ($\pm$0.06), MAM of 0.83 ($\pm$0.06), and AM of 0.007 ($\pm$0.005) (as found by Sundaram and Szeto (1979) in the crayfish), then the levels of these 3 toxic compounds within the tissues of the bullhead after consumption of contaminated isopods can be calculated as 0.002 ug aminocarb/g fish, 0.009 ug MAM/g fish and 0.0001 ug AM/g fish. Allowing for the ten fold increase in toxicity of MAM and AM over aminocarb, then the total tissue burden of toxic compounds, in 'aminocarb equivalents', would be 0.093 ug/g fish. Again it is observed that this is well below a level sufficient to cause appreciable toxic response in this fish species.

In any case, ingested carbamate pesticides undergo considerable first-pass metabolism since blood flow from the intestinal region enters the liver where most metabolism of carbamates occurs in vertebrates (Kuhr and Dorough, 1976).

Figure 15 shows the levels of aminocarb and MAM found in the brown bullhead during exposure via water to 9.0 mg aminocarb/L. Since absorption in this case would be primarily through the gills and skin, first-pass metabolism would not occur. It is likely then that consumption of isopods exposed at a given level of aminocarb would result in a fish tissue load of toxic components less than that caused by exposure of this fish to aminocarb in water.

Therefore, environmental exposure of the isopod, C.r. racovitzai, to aminocarb following application of formulated Matacil would cause no appreciable acute toxic response and, once exposed, this species of isopod would constitute no threat to brown bullhead as a dietary source of aminocarb.

4.2 Brown Bullhead

4.2.1 Acute Toxicity

From the data collected, exposure of brown bullhead to aminocarb at expected environmental levels (approximately 19 ug/L) would appear to pose no acute toxic threat to this species. The relatively high 96 hour LC50 estimated for this species is not surprising since studies by Macek and McAllister (1970) showed that the catfish family, as a whole, is less susceptable than other fish families to acute toxicity by Zectran and Carbaryl. Also, Murphy et al. (1968) showed the black bullhead (Ictalurus melas) to be extremely tolerant of cholinesterase inhibition which is the primary mode of action

of aminocarb (Kuhr and Dorough, 1976).

Due to variations between metabolic pathways in vertebrates and invertebrates, vertebrates will, in general, be less susceptible to acute toxicity by aminocarb. However, variations in susceptibility between species, genera, families, etc. will affect the overall distribution of toxic response, as demonstrated by Macek and McAllister (1970). Salmonids appear relatively sensitive to aminocarb (Penney, 1971; Woodward and Mauck, 1980) and Matacil spraying can result in significant acute toxic response in this group of fish, as observed in trial spraying operations in Maine by Marancik in 1975 (Holmes and Kingsbury, 1980).

Responses following pesticide spraying must be considered in terms of the entire formulation and not just the active ingredient. Data contained in Holmes and Kingsbury (1980) shows that fish are more susceptible to formulated Matacil than to aminocarb alone. For the channel catfish, formulated Matacil was 43 times more toxic than aminocarb. This factor could have great significance in the overall impact of a pesticide on non-target organisms. Assuming a similar relationship for toxicity of Matacil in the brown bullhead, an increase in toxicity of 40 times could result in some form of acute toxic response by the species at environmentally significant levels.

The acute toxic response of spinal dislocation in the caudal region ('kinked' tail) has also been observed in tad-

poles exposed to acutely toxic concentrations of aminocarb (Lyons et al., 1976) and in various species of fish exposed to other toxins (McCann and Jasper, 1972; Couch et al., 1977; Buckler et al., 1981; Mehrle et al., 1981). It is attributed to neural disorders caused by the toxicant which result in severe muscle contractions (Couch et al., 1977; Mehrle et al., 1981). This condition did not directly affect survival of brown bullhead under test conditions but the possibility does exist that such a condition could interfere with prey capture or predator avoidance. To be environmentally significant it would have to occur at environmental levels of 25 ug/L or less. Although only observed in test fish exposed as low as 1.0 mg aminocarb/L, if this form of toxic response was increased by a factor of 40 in the presence of formulation additives, formulated Matacil could possibly cause a 'kinked' tail at levels corresponding to 25 ug/L. Further investigations of the effects of formulated Matacil on this response in this and other species of fish should be conducted.

4.2.2 Uptake and Clearance

As observed by Korn (1973), for the clearance of carbaryl by channel catfish, the bullhead clears aminocarb rapidly except for a small persistent load. Since gas chromatography showed that aminocarb and primary toxic metabolites are eliminated within 2 days post-exposure, and considering the normal metabolic fate of carbamates in vertebrates, it is likely

that this persistent residue represents a phenolic metabolite which has been incorporated into cellular components. Since the carbamate moiety has been removed from the molecule, the primary toxic action would be eliminated and the residue likely offers no danger to the fish or to organisms which might consume it.

Since environmental levels of aminocarb can persist at relatively significant levels for approximately 4 days and aminocarb and its primary toxic metabolite (MAM) may persist in the tissues of brown bullhead up to 2 days post-exposure it would be beneficial to ban fishing in treated areas for at least 6 days following Matacil application. Although the application of the maximum seasonal quantity of aminocarb (175 g/hectare) would not likely result in sufficient contamination of tissues to cause problems for persons consuming fish caught in this period, accidental spillage or over-spraying to the amounts used in Maine in 1975, which caused the acute response in fish reported by Marancik (Holmes and Kingsbury, 1980), could possibly result in acute symptomology in persons consuming exposed fish.

From an evaluation of the various data collected, it appears that the compartmentalization occurring during the investigation of kinetics may distinguish between the parent compound and its metabolites. At least two compartments are obvious during clearance of residues by the fish (Figure 8). The presence of more than one compartment during uptake is also apparent (Figure 7) although insufficient data was collected within the first 24 hours of exposure to mathematically define the fast uptake compartment. During clearance, levels

of aminocarb were not detectable beyond day 1 of clearance. Similarly, clearance from the fast compartment (Figure 8) was nearly complete by day 1 of clearance and beyond day 2 this compartment did not contribute significantly to overall tissue levels of residues.

Clearance of residues from the tissues most commonly associated with metablolism of carbamates (liver, kidney, stomach/intsetine - Figure 13) was very rapid with more than 90% of the residues detected during pesticide exposure being released within 2 days post-exposure. Also, no residues were detectable in the liver or stomach/intestine after 4 days post-exposure and no residues were detectable from the kidney during the entire clearance period, indicating very rapid clearance from this tissue. Clearance from the less metabolically active tissues (skeletal muscle, bone, skin - Figure 12) showed a persistent residue but this was not identifiable as aminocarb (Table 7). The decrease in aminocarb levels within the first 24 hours of clearance was 99.6% (Table 7) which corresponds with the amount of residue clearance which occurred from the fast-clearing compartment and also with the amount of residue clearance for the liver, kidney and stomach/intestine.

Uptake of aminocarb is relatively rapid. The pesticide tissue level reaches steady-state as uptake from water reaches equilibrium with metabolism of the compound. Production of the metabolic residues is represented by the slow uptake compartment which would reach steady-state when residue production equals residue elimination. Once exposure to aminocarb ends,

clearance of the parent compound would be rapid, reflecting the rate of metabolism of this compound. Clearance of the metabolic residues would appear slow because remaining traces of aminocarb would be converted to metabolites, producing a prologation of metabolite levels. This relationship is apparent from Table 7 where, in the first day of clearance, aminocarb levels dropped by more than 99% but MAM levels dropped by only 43.6%, the metabolism of MAM compensated by the production of MAM from aminocarb.

Metabolism of aminocarb can go through various hydrolytic and oxidative pathways eventually leading to conjugation and release of the phenolic portion of the molecule through urine and faeces. The conjugation and release in excreta would be slower than the primary detoxifying steps of hydrolysis and/or oxidative transformation, thereby prolonging clearance of the final metabolites.

Further research is needed to define kinetics within the first 24 hours of uptake and clearance, to identify and quantify metabolites in individual tissues during uptake and clearance, and to develop and utilize methods to identify and quantify metabolites which do not contain the carbamic acid moiety and which are not extractable by the method used herein.

4.3 Gas Chromatography

The extraction technique used here was quantitative, relatively uncomplicated and required no specialized equipment. The micro-filtering step used by Szeto and Sundaram (1980) to remove coextractives was attempted and found

unsuccessful at eliminating the interfering contaminant shown in Figure 16. Therefore, this procedure was discarded for the simpler process of hexane partitioning used by Sundaram and Szeto (1979). It was unnecessary to use centrifugation of separatory funnels to separate phases, as done by Stanley and Delphia (1981), in order to get a quantitative recovery of aminocarb or MAM but the use of such equipment would likely increase the overall efficiency of recovery by reducing the loss of residual solvents adhering to the sides of glassware.

Florisil clean-up was attempted on the final extract as well as on the derivatized residue in an attempt to eliminate the interfering contaminant. The methods described by Lawrence (1976), Wong and Fisher (1975) and Hesselberg and Johnson (1972) were tried as well as modifications of these using various solvents and combinations of solvents but no effective method was found. Identification of this contaminant may provide the information required to enable separation of this compound from those of interest but such work was beyond the scope and resources of this study.

The problem of contaminant interference was also encountered by Sundaram et al. (1976). Their procedure permitted a sensitivity for detection of aminocarb from a 10 g sample of only 0.5 ug/g fish. The procedure presented here had a lower limit of detectability of 0.02 ug aminocarb/g fish from a nominal 5 g sample and, by increasing the quant-

ity of fish used, could be reduced below 0.01 ug/g. MAM was only detectable to 0.3 ug/g using approximately 5 g of tissue.

The derivatization procedure used was simple and required no additional heating, as used by Sundaram et al. (1976) and Seiber (1972), or prolonged reaction time, as used by Wong and Fisher (1975). There was no problem encountered in terms of moisture inhibiting the derivatization reaction, as implied by Stanley and Delphia (1981). The reagents were stable for more than 6 months provided that the containers were kept tightly closed and the stability of the TMA solution was further enhanced by addition of anhydrous sodium sulphate to the flask.

The described procedure offers the advantage of derivative stability on-column and enhanced sensitivity of detection due to the presence of 7 fluorine atoms in the derivatized molecule. The derivatization precedure could also be used with phenolic metabolites of aminocarb (Seiber, 1972) provided a suitable extraction procedure was developed. These metabolites are formed by the removal of the carbamic acid moiety resulting in the loss of a nitrogen atom from the molecule (Kuhr and Dorough, 1976). Flame-ionization gas Chromatography depends significantly on the presence of nitrogen atoms for detection and this results in a lowered sensitivity for these metabolites when this method is used. Also, the extraction and derivatization technique could be modified to assess tissue contamination by aminocarb (and other carbamates) in

combination with persistent contaminants such as organochlorine pesticides and PCB's which are also evaluated by electron-capture gas chromatography.

5.0 CONCLUSIONS

Aminocarb levels normally occurring in the environment following application of formulated Matacil would not likely be sufficient to cause any significant acute effect in the isopod, C.r.racovitzai, or in the brown bullhead. Furthermore, this isopod would not constitute a significant threat to the brown bullhead in terms of a dietary source of aminocarb. This is due to the rapid clearance of aminocarb by the isopod combined with the rapid clearance and metabolism of aminocarb by the brown bullhead. Although clearance of aminocarb residues is relatively rapid in the brown bullhead, levels of aminocarb and the toxic metabolite MAM reached 51.5% of the total accumulated dose after 4 days of exposure (Figure 15). It would therefore be beneficial to prevent harvesting of this fish species, and others, at least 6 days post-spray in order to permit elimination of aminocarb from the environment and to permit elimination of aminocarb and toxic metabolites from fish tissues. Since significant acute response has been observed in fish following application of Matacil (Holmes and Kingsbury, 1980) it is conceivable that consumption of such fish could occur and could lead to acute symptoms in the persons eating them.

Although environmental levels of aminocarb are low and far below those levels found in this study to cause acute toxic response, there is sufficient evidence that further investigation of the formulated pesticide is needed to identify any possible synergistic effect of the additives on acute toxicity of aminocarb and uptake and clearance of pesticide residues in non-target organisms. Also, the additives should be studied individually to determine if they constitute an environmental threat.

From the acute toxicity, uptake, clearance and metabolism data presented above for aminocarb, and that information available from other studies, any environmental perturbation involving these organisms, and possibly others, resulting from Matacil application, would not likely be solely the result of aminocarb levels. However, the formulation additives nonylphenol and diluant oil 585, either on their own or in conjuction with aminocarb, might cause some sublethal effects. This possibility should receive further attention in light of the extent to which Matacil is used in Canada.

It is apparent that the pharmacokinetics of this pesticide, in the brown bullhead, may be intricately associated with the in vivo metabolism which occurs during and after exposure. Further research is needed to identify all metabolites, tissue distribution of those metabolites and how these factors interplay with pharmacokinetics.

The analytical technique employing chemical derivatization

and gas chromatographic analysis presented herein was simple and rapid. Further research is needed to identify and eliminate interfering contaminants, to ascertain the extent of tissue contamination with other metabolites of aminocarb and to investigate its applicability to other organisms and other carbamate pesticides.

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