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

by

Valerie J Quinn

A thesis
presented to the University of Ottawa
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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 difformites aux pattes et au bec semblables à celles qu'on observe avec les insulines des mammiferes. Ces résultats suggerent que l'insuline endogene peut affecter le developpement des pattes et du bec. Il est cependant difficile de determiner 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 tératogene 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 a cause de la variation marquée mesurée pour les niveaux d'insuline dans differents poulets. De plus, bien que le rapport membre/longueur du corps ait décru du neuvieme au dix-septieme jour et que les niveaux de plasma glucose aient augmenté au septième jour et diminué aux neufieme et onzieme 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.

ACKNOWLEDGEMENTS

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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 carbohydrate 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, 1958; 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 - dimethyl 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 2.0 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

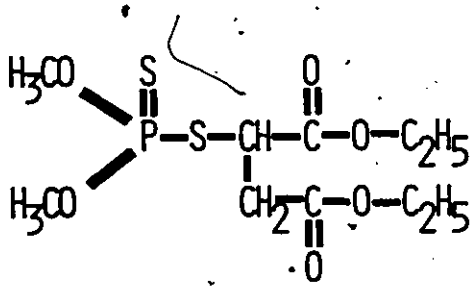
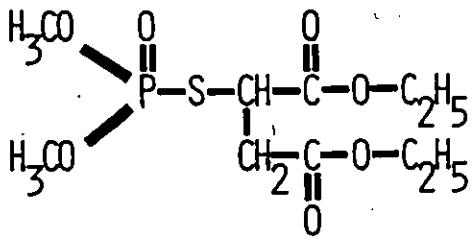


Figure 2: Structural formula of malaoxon



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 endogencus 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 6 (Howand 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

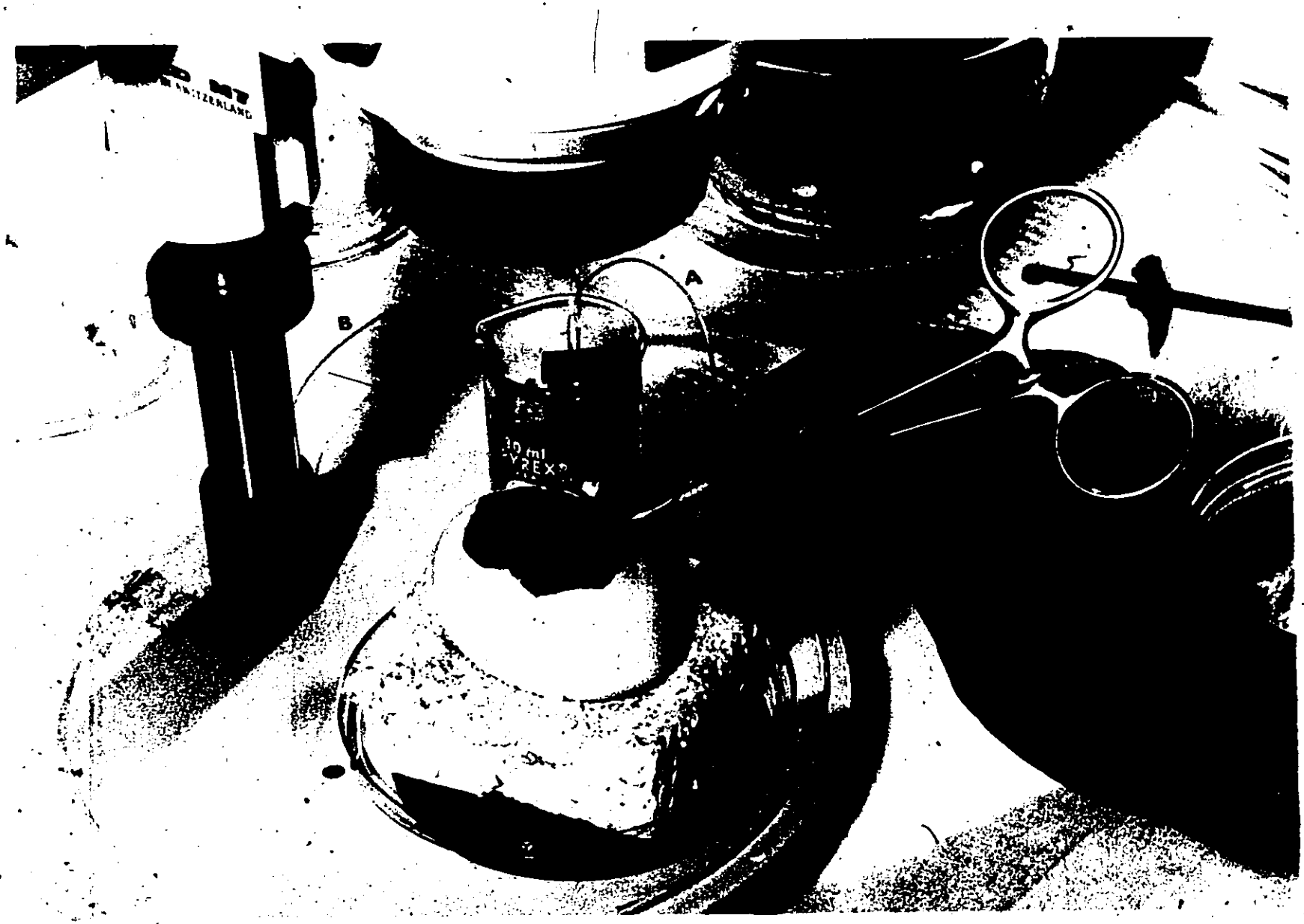
1. Days 7, 9 and 11 of incubation.

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 eppendorf. 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 neparinized 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 occular 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

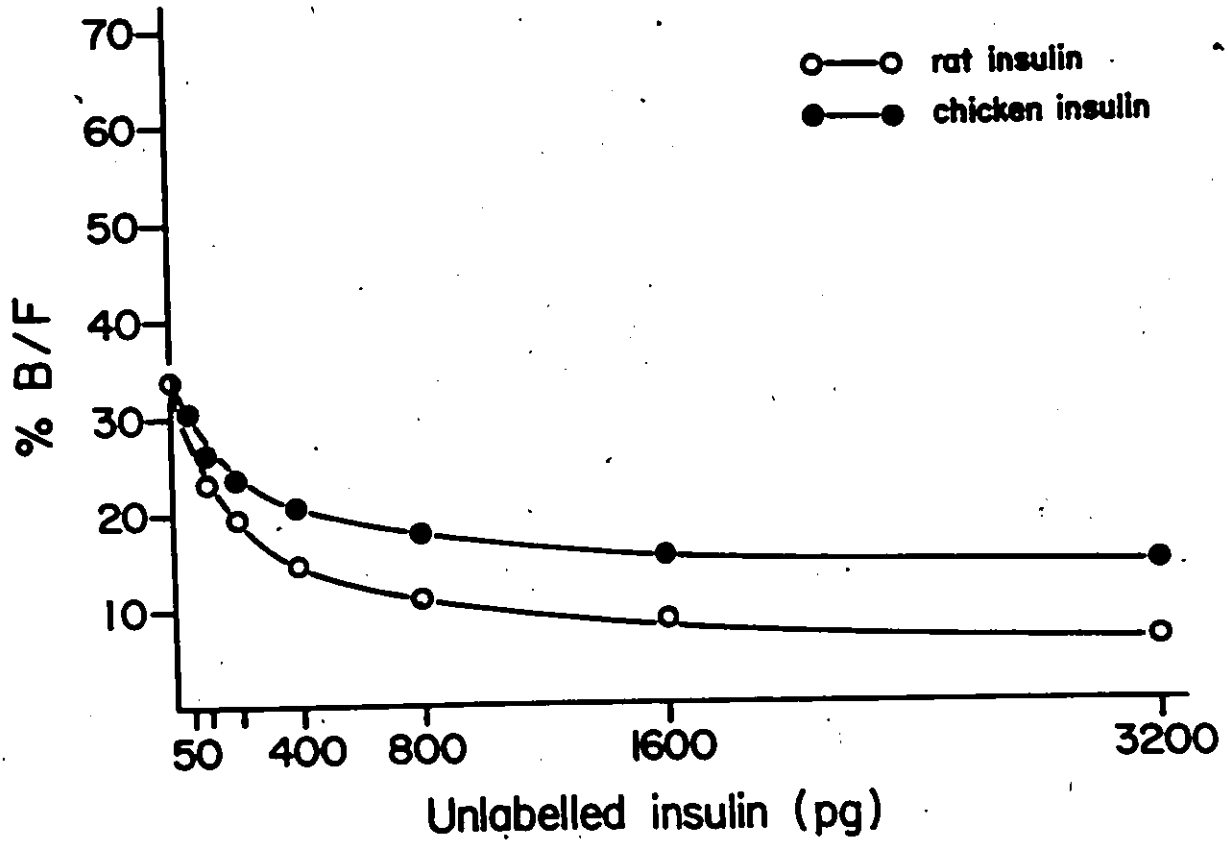
centrifuge, model J-6), decanted, wiped dry above the level of the precipitate and counted using either a Picker, Auto-well 2 or an Amersham, model 1198 gamma counter. Two tubes containing tracer alone were used for the total counts.

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

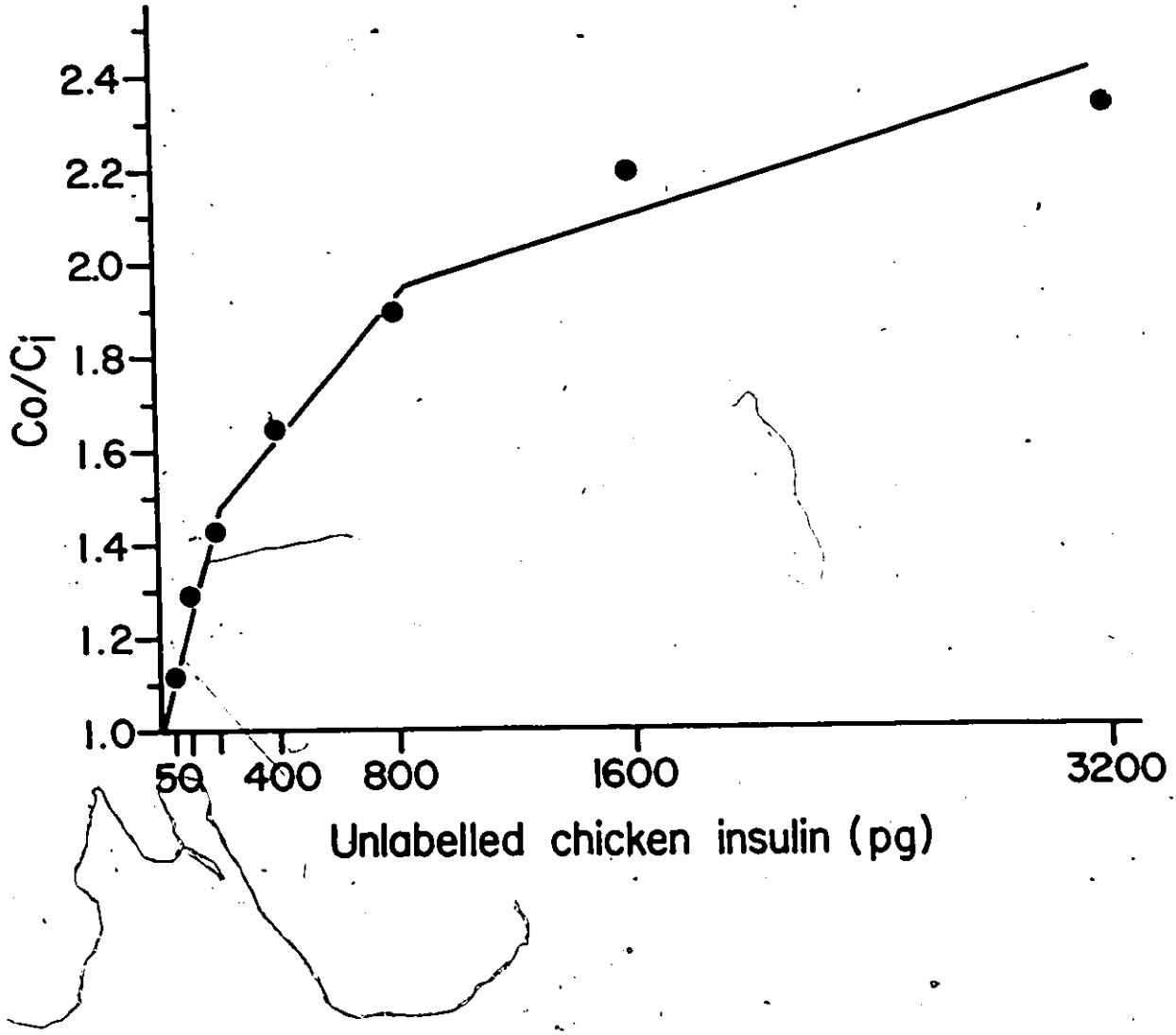

2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
Co/Ci
50
400
800
1600
3200
Unlabelled chicken insulin (pg)

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 Insulin Std[1] (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).

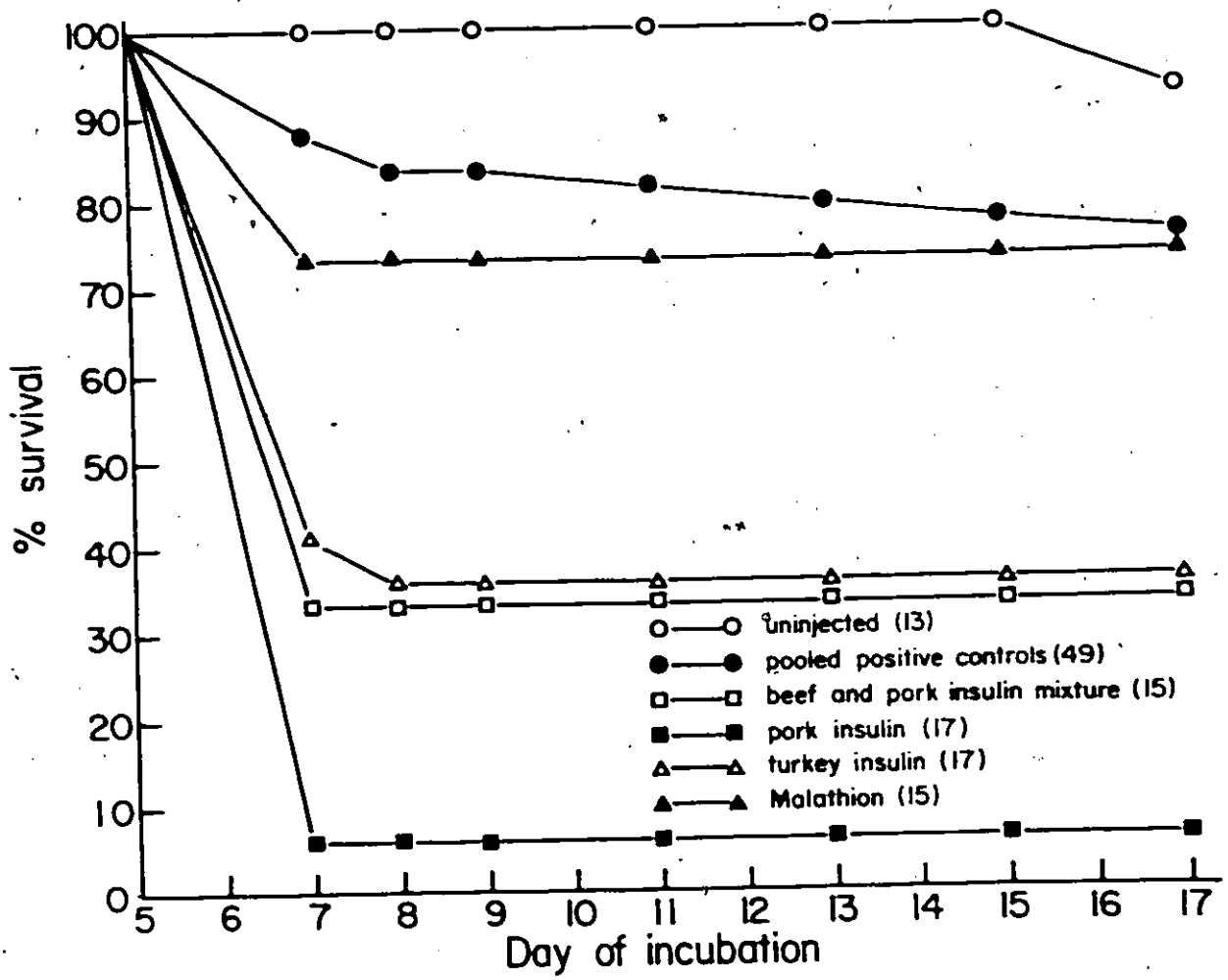
One case of abnormal beak development, cross beak, was reported in the control embryos. This is most likely a random occurrence as cross beak is a known congenital abnormality in chickens (Landauer and Baumann, 1943; Grewal and Singh, 1978).

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.

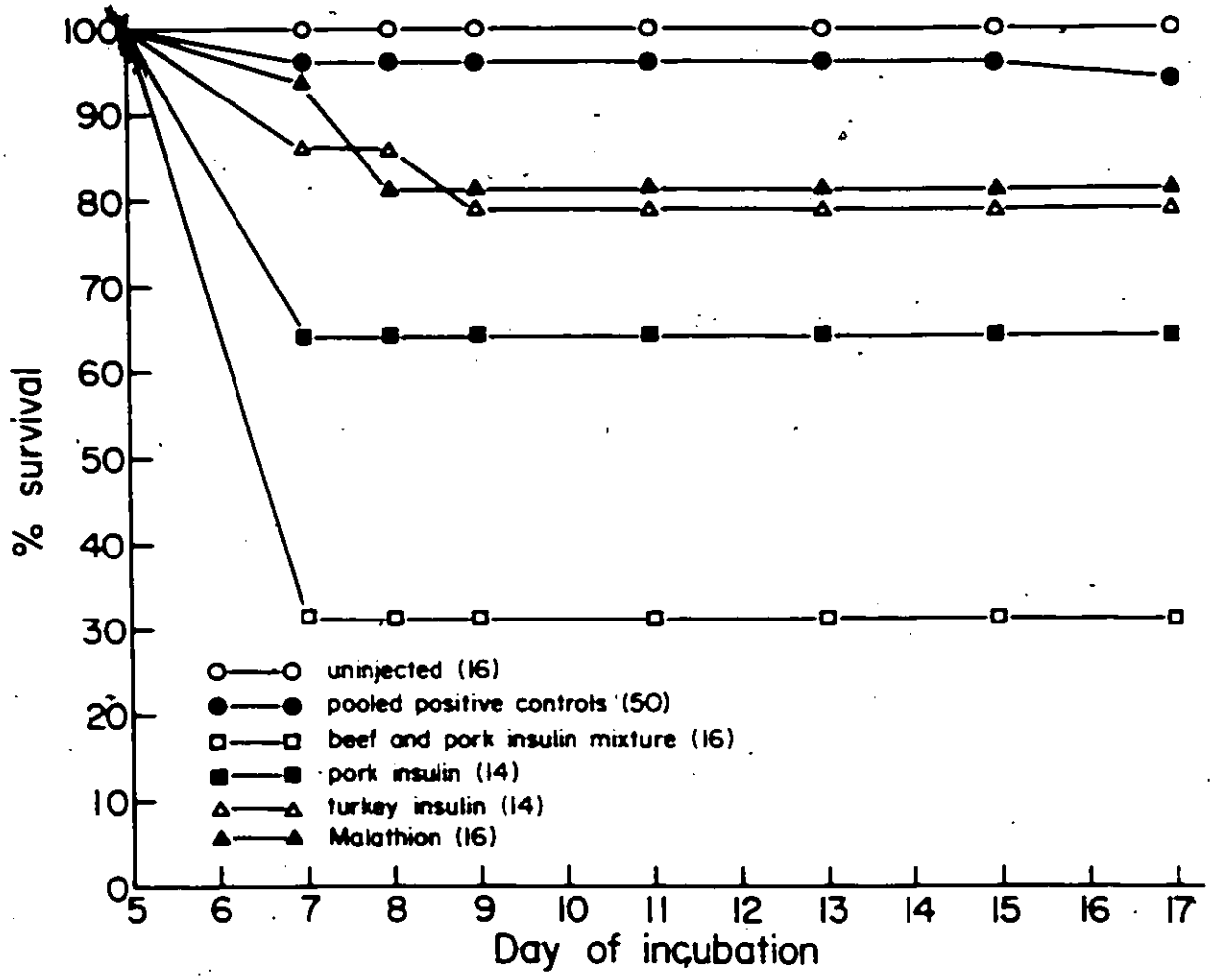
Percent survival of uninjected and injected embryos incubated the first day of a two day incubation period and candled on days 5, 7, 9, 11, 13, 15 and 17.

Figure 6: 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, 8, 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 (Fig 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).

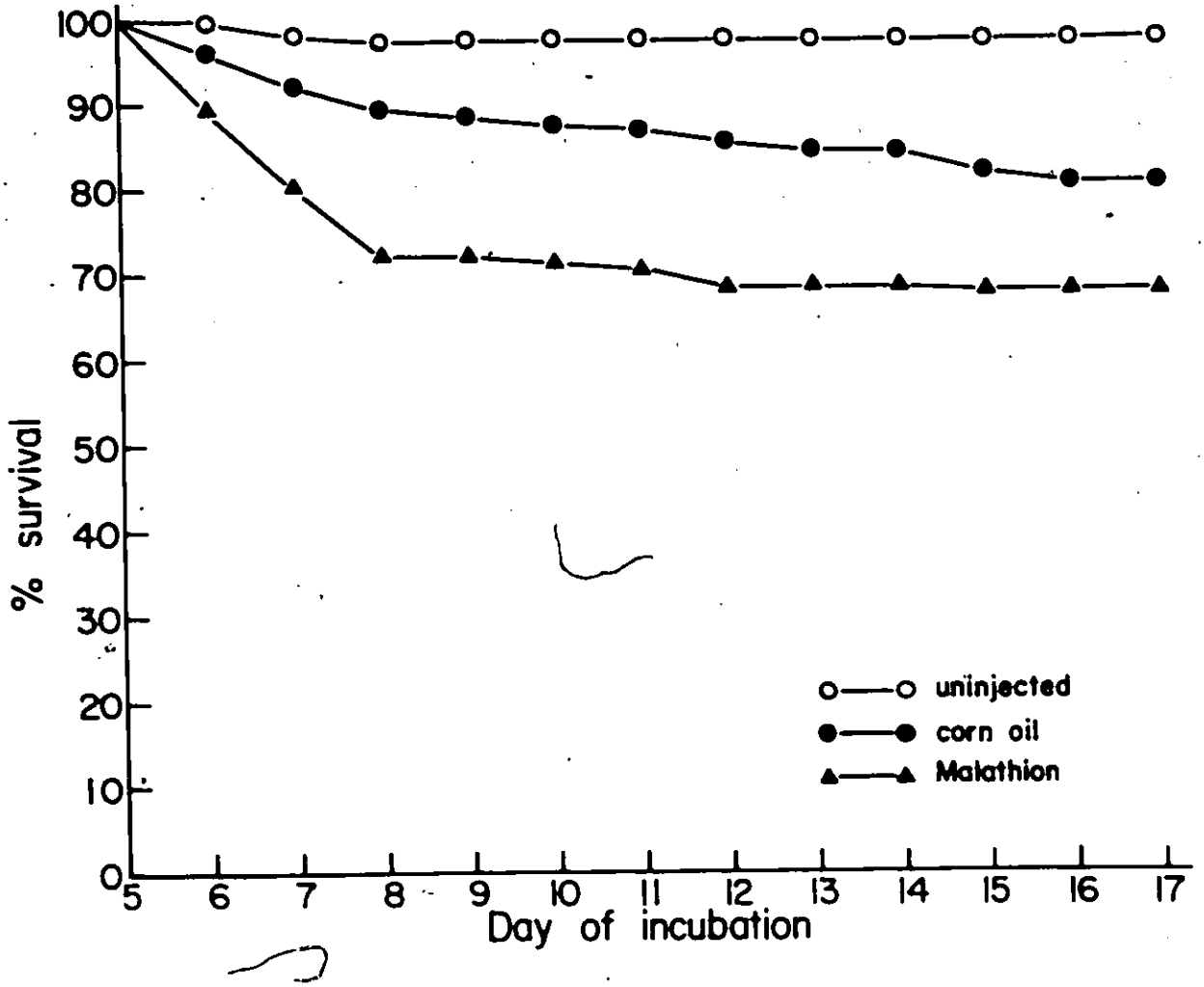
* significant difference from the uninjected group ($p < 0.05$).

** 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.

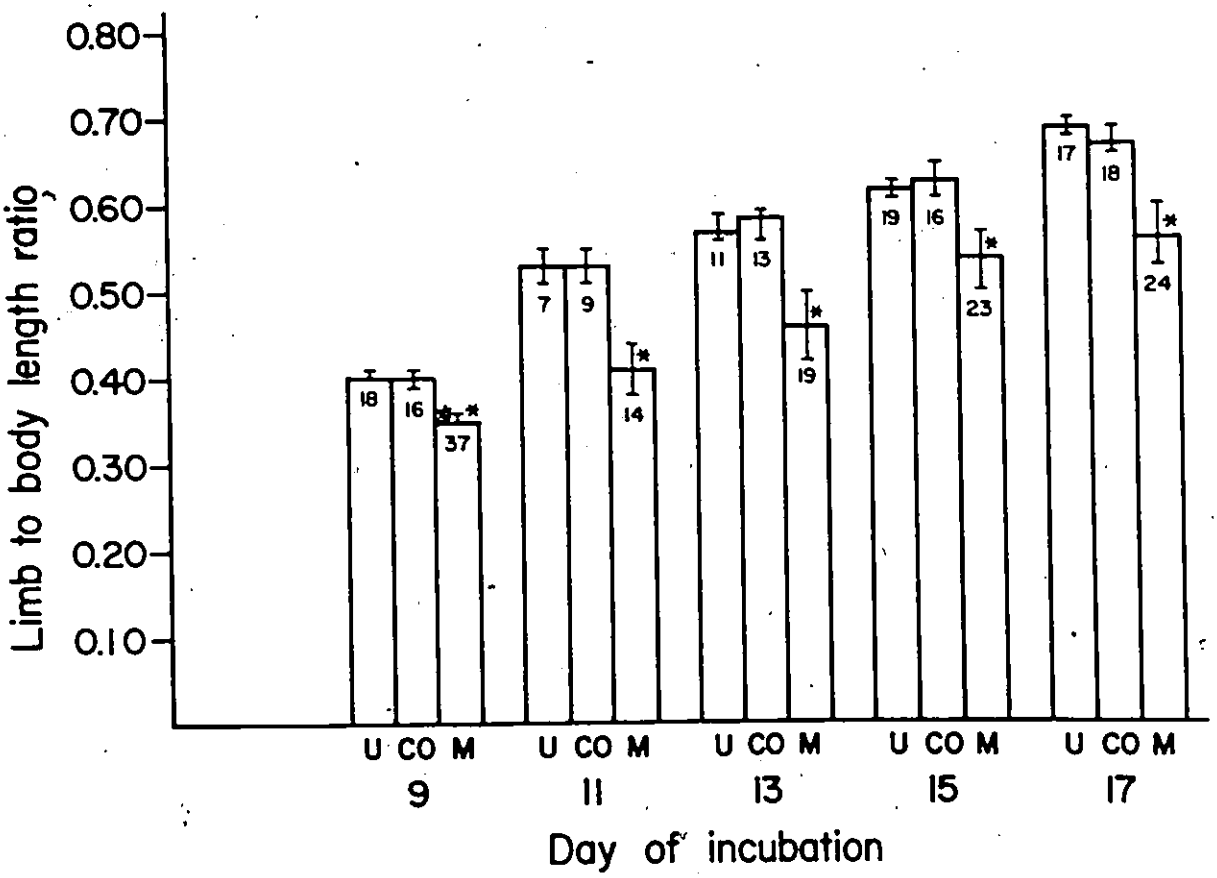
Solution Injected	N	Limb to Body Length Ratio
Corn Oil	9	0.15 (0.14 to 0.16)
Malathion	10	0.14 (0.14 to 0.15)

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

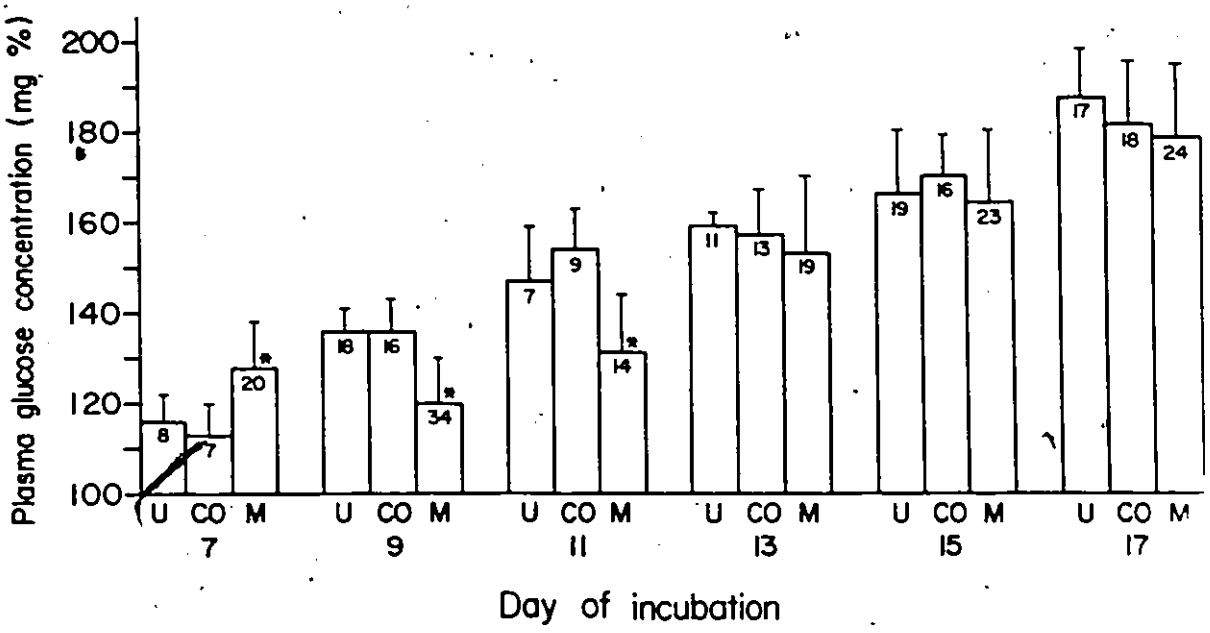

0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
Limb to body length ratio
18
16
37
7
9
14
11
13
19
19
16
23
17
18
24
U CO M
9
11
13
15
17
Day of incubation

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

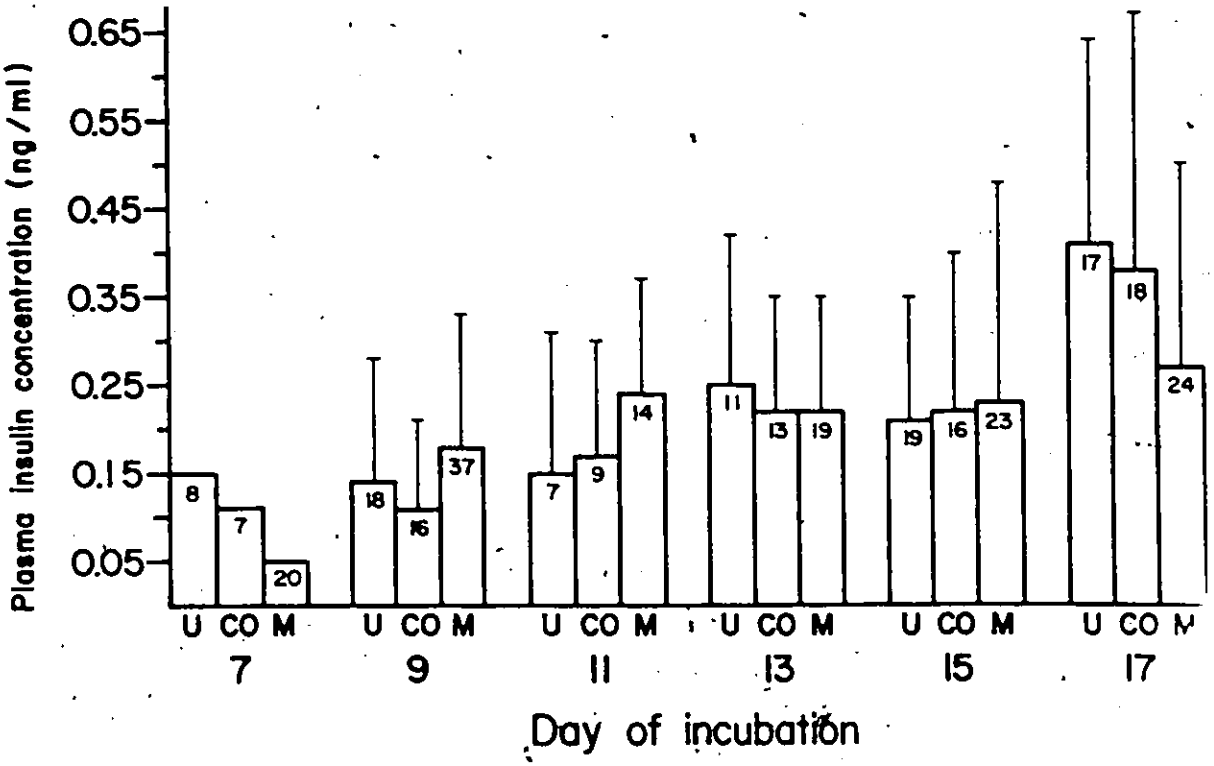

Plasma glucose concentration (mg %)
200
180
160
140
120
100
8
7
20
18
16
34
7
9
14
11
13
19
19
16
23
17
18
24
U CO M
7
9
11
13
15
17
Day of incubation

Plasma 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 (Haley 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 insuline.

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 4) 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; Narbaitz, 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 Singl, 1976), 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

chicken insulin was observed to be more a potent hypoglycemic and glycogenic agent than either sheep or beef insulin (Hazelwood et al., 1968). The authors suggested that the reduced avian response to mammalian insulins probably reflected an inhibition or destruction of the mammalian insulin by the chicken plasma or a decrease in the ability of the mammalian insulin to bind to the appropriate receptor sites. While the latter explanation may be applicable to the present study, the former is unlikely due to the poor development of the embryonic immune system as demonstrated by : 1. the lack of rossette formations in the thymus, bursa of Fabricus, spleen or bone marrow in response to antigen stimulation until the 14th or 15th day of incubation (Glick, 1977), and 2. the inability of embryos to produce antigenic responses until post hatching (Seto, 1980). In addition, it was shown that the half life of bovine insulin injected into adult chickens was identical to that of chicken insulin, this also suggests that specific destruction of mammalian insulin does not occur (Langslow, 1976).

As noted above, different preparations of mammalian insulins from the same species can also have different teratogenic potencies (Greenberg, 1971). It is possible, therefore, that differences in the purification procedure and not the structural differences in the insulins account for the enhanced response of the chicken embryos to the turkey insulin injections. It is evident that further studies are required to resolve this question.

The fact that homologous insulin injections produced a syndrome similar to that which has been described in response to mammalian insulins does, however, support the hypothesis that enhanced secretion of insulin from the embryonic pancreas may be responsible for some congenital abnormalities of the limbs and beak. For comparison purposes, malathion, an organophosphate insecticide, suspected of acting by increasing endogenous insulin levels (Arsenault and Gibson, 1974; Laley and Gibson, 1977) was included in the study with sterile corn oil serving as a control. As no significant differences ($p < 0.05$) were observed in any of the parameters measured (table 5 and 6) between this control group and others, the mortality data obtained for this group was pooled with that of the other injected controls. It should be noted that in the 2nd set of experiments (table 7) corn oil did significantly decrease ($p < 0.05$) mean body weights on the 15th day of incubation. But this was not observed on any of the other days studied and to my knowledge has not been previously reported in the literature.

Although the results of this experiment did not show a significant change in embryonic survival in response to malathion injections (figs 6 and 7) the results of the second experiment did show a small but significant decrease in the malathion injected group (fig 8). The reason for this discrepancy may be that the sample size used in the first experiment was inadequate to detect the small decrease (approximately 10 %) in the survival rates.

A comparison of the anomalies produced in response to malathion or insulin treatment indicated that certain similarities did exist. Malathion decreased embryo weights, as did turkey insulin, and reduced limb to body length ratios similar to that seen following pork insulin and turkey insulin treated groups. Further, all treatments affected beak development. The latter anomalies were however, qualitatively different. Malathion produced parrot beak and insulin produced short upper beak. It should be noted that others (Duraiswami, 1950; Landauer, 1947; Landauer and Rhodes, 1952; Landauer and Clark, 1964) have found both short upper beak and parrot beak following insulin treatment. Two of these authors, Duraiswami (1950) and Landauer and Clark (1964) reported that parrot beak was the primary beak defect whereas Landauer and Rhodes (1952) concluded that the proportions of the beak defects varied from experiment to experiment. Since these proportions were seen to vary even within the same population of White Leghorns, it may be that each of the defects reflect an interference at the same point in metabolism but at a slightly different developmental stage (Landauer and Rhodes, 1952). If this is so, and if malathion acts by increasing insulin levels, then the qualitative differences observed in the beak defects of malathion and insulin injected embryos may reflect a delay in response to malathion due to the indirect nature of this action.

High frequencies of sparse feathering were observed with malathion treatment but not with insulin treatment. The one case of sparse feathering observed in the turkey-insulin injected group is of questionable importance. This anomaly has never been reported as an insulin associated anomaly and it may be that this was a spurious occurrence unrelated to the treatment. On the other hand, it may be a low frequency anomaly which occurs in response to turkey, but not mammalian, insulin. More studies would be required to clarify this point.

Due to the similarities observed between the insulin and malathion induced syndromes this study is in agreement with those of previous authors (Arsenault and Gibson, 1974; Arsenault et al., 1975; Laley and Gibson, 1975) in that it supports the hypothesis that malathion may act by increasing insulin levels.

4.2 MALATHION INJECTIONS

To test the hypothesis that malathion acts to increase endogenous insulin levels, limb to body length ratios, plasma glucose and insulin levels were measured in malathion treated embryos.

Both the uninjected and corn oil injected control groups had values for limb to body length ratios which compared favourably with those of Greenberg and LaHam (1969) for day 15 embryos and Laley and Gibson (1977) for day 11, 15 and 17 embryos. The control values obtained for plasma glucose levels approached those reported by Zwilling (1948) for reducing sugars and by Arsenault and coworkers (1975) for glucose. The values were, however, consistantly higher than those found by Benzo and Green (1974) for the same period of incubation. This may indicate that different populations of White Leghorns have slightly different glucose levels during their developmental stages.

The insulin values obtained in this study agreed substantially with those of Benzo and Green (1974). Both studies used double antibody radioimmunoassays based on the Hales and Randle (1963) method. In the present study, however, normal guinea pig serum (GPS) was added to adsorb non-specific antibodies (Dalpé-Scott et al., 1982) and thus reduce the falsely high values usually associated with the double antibody technique (Malvano et al., 1974; Ashby and McKech-

nie, 1980). One might therefore expect the values presented here to be lower than those of the previous authors. This was not seen, however, and this discrepancy is probably due to the low titer of antibodies present in the chick embryo plasma (Seto, 1981). The values were, however, consisantly lower than those of Leibson and coworkers (1978) who used the 'solid phase' principle to measure insulin levels. In their method the insides of the tubes were coated with insulin antibodies and insulin levels were measured by the ability of a solution to compete with a known amount of radioactive insulin for these antibody binding sites. The discrepancy between the values obtained in this study and those obtained in Leibson's laboratory may be due to either the differences in the methods of assaying insulin or to differences in the populations of embryos used.

The results of the malathion injection studies showed that the injection of a 5 % solution of malathion into the yolk sac of 5 day incubated embryos significantly decreased ($\alpha < 0.05$) the limb to body length ratios from the 9th to the 17th day of incubation. These findings agree with those of Greenberg and LaHam (1969) who also found a decreased ratio in response to malathion treatment on the 15th day of incubation and Ho and Gibson (1972a) and Laley and Gibson (1977) who found decreased limb length on days 8 to 20 and days 11 to 19, respectively. No previous studies have been conducted which examined the effects of malathion on the

limb to body length ratios of day 7 embryos. If, however, one extrapolates the straight line plots for length of tibiotarsus, the long bone found by Bo and Gibson (1972a) to be the most severely affected by malathion treatment, back to the 7th day of incubation it is apparent that the control and treated curves approach each other on day 7. This is consistent with the findings of the present study in which no significant difference ($p < 0.05$) in the limb to body length ratios was found between the malathion treated and control groups on the 7th day of incubation.

The analysis of plasma glucose levels showed that a significant hypoglycemia ($p < 0.05$) was present on the 9th and 11th days of incubation and that decreases in glucose levels can be positively correlated with the extent of the micromelia on incubation days 9, 11 and 13. These findings are similar to, but not as long in duration as, those of Arsenault and coworkers (1975) who found a persistent hypoglycemia from the 9th to the 19th day of incubation following injections of 2 % malathion into the yolk sac of White Leghorns. The reason for this discrepancy may be due to differing genetic backgrounds or to seasonal variations since both of these factors are known to alter the response of chicken embryos to teratogens (Landauer, 1947; Roger et al., 1969).

The distinct hyperglycemia observed in the malathion treated embryos on day 7 has not been reported previously and will be discussed later in this reported.

As mentioned above, a comparison of the plasma insulin levels of the developing embryos showed that there were no significant differences ($p < 0.05$) between levels observed for control and malathion treated embryos on any of the days examined. It should be noted that a wide variation in insulin levels was seen in the normal chick embryos. As the precision and accuracy of the assay was found to be acceptable for the measurement of chick embryo plasma insulin levels (tables 2, 3 and 4), and since the consistancy was checked continually, both on a week to week basis and periodically within a given assay, it must be concluded that these variations represent normal variations in insulin levels. The wide standard deviations makes it difficult to establish if malathion injections altered plasma insulin levels and, within the limits of this experiment, the observation of no significant difference between the treated and control groups indicates that no massive changes in insulin levels occurred. In addition to this no correlations were seen between insulin levels and limb to body length ratios on any of the days examined and no correlation existed between plasma insulin and glucose levels until the 17th day of incubation. Consequently, these results do not support the hypothesis that malathion acts to increase insulin levels. On the other hand, because of the lack of correlation between immunoreactive insulin and blood glucose the possibility of the presence of some protein(s) which interfer with

the assay can not be ignored (e.g. insulin like growth factors or proinsulin, Sonksen, 1976). Therefore, one cannot conclude that malathion does not affect blood insulin levels.

If one does assume, however, that the assay is reasonably specific for insulin the fact that no increases in insulin levels were observed raises an important question. If malathion is not acting at the level of the pancreas then by what mechanism is it exerting its effects on embryonic development? Theories proposing other possible mechanisms of action of organophosphates (OPs) in general, and of another group of insecticides, the methylcarbamates (MCs), which have the same mechanism of action on insects and similar effects on the developing chicken embryo, have been suggested. Before outlining these theories a brief description of the various anomalies produced by these teratogens is in order.

In general, two classes of anomalies are produced, type 1, characterised by anomalies of the limb, beak and feathers as seen in malathion treated embryos, and type 2, characterised by wry neck, arthrogryosis and rumplessness (Moscioni et al., 1977). Most OPs and MCs produce a combination of the two and the extent to which each is produced depends not only on the compound used but also on the dosage (Proctor et al., 1976).

As these compounds are know to act as anticholinesterases studies were undertaken to examine possible relationships

between their type 1 and 2 teratogenic potentials and acetylcholinesterase inhibiting characteristics. In these studies the effects of pralidoxime supplementation of parathion (type 2) or bidrin (type 1 and 2) injections demonstrated that the OP binding agent was effective in suppressing the axial anomalies but had no effect on the type 1 symptoms (Heiniel, 1976). Also, anomalies of the axial skeleton resulting from injections of either parathion or neostigmine were positively correlated with decreases in whole body acetylcholinesterase activity. No type 1 anomalies were observed in the study even when acetycholinesterase innibition exceeded 95 % of the control level (Heiniel, 1978). In addition, attempts to relate the mode of action of two known type 1 reversal agents (tryptophan and nicotinamide) to increases in acetylcholinesterase activities showed that even though they reversed the teratogenic effects of malathion (type 1) they did not alleviate the depressed acetylcholinesterase activity associated with the treatment (Greenberg and LaHam, 1970; Walker, 1971). Later, it was found that nicotinamide had no alleviating effects on the type 2 anomalies produced by parathion and bidrin (Heiniel, 1976).

The theory arising from these studies was that OPs and NCs most probably interfere with the embryonic development at two different levels, the first being acetylcholinesterase inhibition resulting in type 2 anomalies, and the other,

operating at an as yet undefined point in the embryonic metabolism, resulting in decreases in nicotinamide production or utilization.

Several suggestions have been advanced as to the possible mechanism of the latter phenomenon. The discovery of decreased levels of tryptophan in malathion treated embryos led Greenberg and LaHam (1970) to suggest that the decreased levels of tryptophan (a precursor of NAD) was responsible for the depressed NAD levels. Proctor and coworkers (1976), however, found that dipeptidase activity measured by the breakdown of glycyl-tryptophan in yolk sac membrane (YSM) homogenates was similar whether the embryo was treated with either the type 1 teratogens dicrotophos or eserine sulfate or the nonteratogenic compound EPN. These results suggest that proteolysis, at least of glycyl-tryptophan, is not the focal point of embryonic biochemistry where the type 1 teratogen acts.

The theory that the extent of type 1 teratogenesis may be correlated with the ability of the teratogen to inhibit specific YSM esterases was advanced when it was found that certain bands of YSM esterases (separated electrophoretically) were inhited by all 6 teratogens tested but not by any of the non-teratogens examined (Flockhart and Casida, 1972). More extensive studies showed, however, that no one esterase was consistently blocked by teratogens which wasn't blocked by nonteratogenic compounds (Proctor et al., 1976). This

suggested that the YSM esterases are not of primary importance in type 1 teratogenesis.

Studies examining the abilities of various type 1 teratogens to inhibit kynurenine formamidase (KFase), the enzyme which catalyzes the first step in the metabolic conversion of tryptophan to NAD led to the discovery that a good correlation exists between the extent of the inhibition and the decrease of the NAD levels (Moscioni et al., 1977; Seifert and Casida, 1978). These studies, therefore, support the hypothesis that OPs exert their type 1 teratogenic potential by inhibiting the YSM KFase enzyme, and thus the conversion of tryptophan to L-kynurenine in the in vivo synthesis of NAD, resulting in decreased embryonic NAD levels and thus limb, beak and feather defects. It should be noted that the correlations were not linear; in fact decreases in the YSM-KFase of approximately 50 % (49 to 51 %) were associated with decreases in NAD levels of anywhere from 20 to 50 %. The teratogenic indices in these cases varied from no effect to moderate. This may indicate that the extent of the YSM-KFase inhibition is not the only factor involved in decreasing NAD levels or inducing type 1 anomalies.

In the specific case of malathion the dosage used in Seifert and Casidas' (1978) study was insufficient to evoke a teratogenic response. The study did, however, show that small decreases in both NAD levels and YSM-KFase activities were produced in response to the non-teratogenic dose, sug-

gesting that at higher doses malathion may indeed act to inhibit YSM BFKase and decrease NAD levels.

If malathion does act to decrease NAD levels these decreases may be related to the hyperglycemia observed on the 7th day of incubation. During the 1st week of embryonic development the primary sources of energy are monosaccharides (Hazelwood, 1971). Various researchers have identified the enzymes and most of the intermediates of the Embden-Meyerhof anaerobic glycolytic pathway (Stumpf, 1947), and glycogen synthesis pathway (Grillo et al., 1963) on cr before the 7th day of incubation. In addition, the pentose phosphate shut is known tc be active prior to the third day of incubation (Wenger et al., 1967). It is important tc note that the Embden-Meyerhof pathway and the pentose phosphate shunt have at least one step which requires NAD (or NADP) in either its oxidized or reduced form. However, while NAD is not used directly in glycogen synthesis a coenzyme of the pathway (uridine diphosphate, UDP), does require the presence of NAD. It is apparent, therefore, that if NAD levels were decreased during malathion treatment both the metabolism and storage of glucose might be impaired and this could result in the hyperglycemia observed on the 7th day of incubation.

Embryonic metabolism changes during the 2nd week of development and proteins become the major source of energy (Romanoff, 1967). The finding of decreased levels of tryptophan in malathion treated embryos (Greenberg and LaHam,

1970) may indicate a general decrease in protein uptake from the YSM. This could have led to a decrease in the availability of amino acids for gluconeogenesis and may have resulted in the hypoglycemia observed on the 9th and 11th days of incubation. Presumably, the shift in metabolism to an increased utilization of lipids later in development accounts for the return to normoglycemic levels.

As mentioned previously, the extent and duration of the malathion induced hypoglycemia observed in this study was less than that observed by previous authors (Arsenault et al., 1975). The reason for this discrepancy is unclear, but it may be due to more extreme decreases in amino acid uptake from the yolk sac such that no recovery is observed even when lipids become the major source of energy. The morphological changes in the pancreas observed by the earlier authors may in fact have been a secondary effect in response to the more extreme and extended hypoglycemia. These prolonged decreases in glucose levels may have acted to stimulate the proliferation of alpha cells on days 11, 13 and 15 (Arsenault et al., 1975; Laley and Gibson, 1977) and presumably caused increased glucagon levels. The increased glucagon levels may in turn have stimulated the observed increases in the amount of beta tissue (Arsenault and Gibson, 1974; Laley and Gibson, 1977). The latter phenomenon would be analogous to the increases in beta tissue seen when 10 or 12 day embryos were injected with glucagon (Anderson and Gib-

son, 1981). Care should be taken with this interpretation however, since not all dosages of glucagon resulted in increased amounts of beta tissue. In fact, a decrease in beta tissue was observed on days 12 and 13 of incubation after the injection of 150 ug of glucagon on day 10 of incubation.

Previous authors (Acorn and Gibson, 1979) have observed decreases in YSM hemopoietic tissue in response to malathion treatment from the 7th to the 19th day of incubation. Since the YSM is the major hemopoietic site of erythrocyte production from the beginning of erythropoiesis to the 13th day of development (Dieterlen-Lièvre et al., 1976) this may account for the decreased hematocrit values observed in this study on the 7th day of incubation (table 8). The reason for the recovery of the hematocrit values to the control level on days 9 and 11 is, however, unclear. This may be another example, analogous to the observations of plasma glucose levels, wherein malathion had a more extreme effect on the population of White Leghorns used by the previous authors. That is, the duration of the decrease in YSM hemopoietic tissue in this study may be very much reduced, so that recovery occurs by the 9th day of incubation. Another possible explanation for the early recovery of erythrocyte levels is that the decrease in circulating red blood cells acts to stimulate increases in erythropoiesis at other existing hemopoietic sites (Dieterlen-Lièvre, et al., 1976) such as the diffuse loci in the general mesenchyme and the spleen, or it

may induce precocious development of erythropoiesis in the bone marrow.

Another factor which may contribute to the observed decrease in hematocrits on day 7 is edema, since some studies have shown edema to be present in some malathion treated embryos (Greenberg, 1971; present study).

In summary the principle observations and conclusions are:

1. The injection of turkey insulin on the 5th day of incubation resulted in abnormalities of the limbs and beak similar to those observed with the injection of mammalian insulins. This suggests that the abnormalities produced are due to the action of insulin 'per se' and do not result from either steric inhibition or an immunologic response. This is the first time that avian insulin has been shown to have this action.

2. The injection of malathion results in a syndrome similar to that observed with insulin treatment. This observation is in agreement with those of previous authors (Greenberg and LaHam, 1970; Ho and Gibson, 1972a,b; Jackson and Gibson, 1977).

3. Injection of malathion into chick embryos did not produce any measurable effect on plasma insulin levels. However, because of the large variations in the plasma insulin levels no definite conclusions can be drawn at this time concerning the effect of malathion on chick plasma insulin levels.

4. Malathion increases plasma glucose levels on day 7 of development. This may be due to an impairment of glucose metabolism or storage, or both metabolism and storage.

5. Decreases in plasma glucose levels were observed on the 9th and 11th days of incubation in malathion treated embr-

ycs. These decreases may be due an inadequate supply of amino acid substrates required for gluconeogenesis.

6. Day 7 hematocrit levels were decreased with malathion treatment, this is likely to be due to either decreased hemopoietic tissue (Acorn and Gibson, 1979) or edema (Greenberg, 1971; present study, not quantified).

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