A TERATOGENIC AND TOXICOLOGICAL EXAMINATION OF 2,4,5-TRICHLOROPHENOXYACETIC ACID IN DEVELOPING CHICK EMBRYOS

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A TERATOGENIC AND TOXICOLOGICAL EXAMINATION

OF 2,4,5-TRICHLOROPHENOXYACETIC ACID

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

SUMMARY

Due to the widespread use of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in weed control, grazing land and domestic animal feed are sometimes contaminated. Present government regulations require that all farm animals exposed to the herbicide be destroyed. An incomplete knowledge of 2,4,5-T toxicity in chickens has similarly necessitated large economic losses to commercial hatcheries in recent years due to alleged feed contamination.

Practical grade 2,4,5-T, dissolved in dimethyl sulfoxide, was injected into the air space of fertilized chicken eggs prior to incubation. Injected volumes were individually based upon egg weight for a constant mg/kg dose. Doses administered included 12.5, 25, 50, 75, 100, and 125 mg/kg of 2,4,5-T to determine herbicide toxicity. The LD_{50} was estimated to be 62 mg/kg of 2,4,5-T.

Additionally, embryos were exposed to 50 mg/kg of 2,4,5-T on day zero of gestation, sacrificed after 48 hours of incubation, and serial sections were examined for developmental anomalies. None were found.

CHAPTER I

INTRODUCTION

Since their development in the mid-forties, the phenoxy herbicides have been instrumental in the control of broadleaved weeds and brush (Williams, 1971). Of these, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) has become important in land and waterway management as well as in agriculture (MacLeod, 1971). Land management uses include the development and maintenance of rights-of-way, fence rows, roadways, floodways, firelanes, and industrial sites, while aquatic applications include weed control through canals and along shoreline embankments.

Primarily a non-crop herbicide, 2,4,5-T is used to some extent on grains, sugarcane, and hay, but its agricultural importance lies in its widespread use on rangeland and pastures (MacLeod, 1971). The proper control of brush and weeds can increase the carrying capacity of grazing land by enhancing the survival of grass. Grass replaces its space and water competitors as they die, ensuring increased grazing potential (Dow Chemical, 1971).

Aerial application of selective herbicides has drastically reduced the cost of weed control. While brush control utilizing 2,4,5-T costs approximately $6.50 per acre, a substitute chemical or the manual equivalent (hoeing, burning, or mowing weeds) would cost nearly $44.00 per acre. It has been estimated that replacement of the phenoxy herbicides by chemical or manual alternatives would increase the maintenance cost of grazing

lands, rights-of-way, and grain crops by $172 million annually (MacLeod, 1971).

Agent Orange, a combination of n-butyl esters of the phenoxy herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T, was used extensively as a defoliant during the Vietnamese conflict (1962-1970). Although general uses of Agent Orange included defoliation of communication lines, enemy base camps, and undergrowth favoring guerilla warfare, many tactical advantages were not realized because of the restriction of its use to non-crop and unpopulated targets. During the summer of 1969, however, South Vietnamese newspapers reported an increased incidence of human birth defects in some parts of the country and implicated Agent Orange as the cause (Wilson, 1973). The subsequent announcement in October, 1969, by Presidential Science Advisor Lee DuBridge that 2,4,5-T was teratogenic in mice, increased the herbicide's bad reputation and led quickly to a restriction of its use.

The basis for DuBridge's announcement was the study conducted by Courtney et al. (1970) which examined the teratogenic and fetotoxic capabilities of 2,4,5-T in mice and rats (Wilson, 1973). Although Rowe and Hymas (1954) had reported that "hazards to livestock and wildlife associated with the use as recommended of the herbicides containing 2,4,5-T. . . were negligible," the Courtney study was prompted by the results of a 1964 (National Cancer Institute) pesticide screening study which implicated the herbicide as a teratogen. Anomalies noted by Courtney as a result of 2,4,5-T administration included cleft palate, cystic kidney, and hemorrhage of the gastrointestinal tract.

Courtney's report (ibid) led to widespread interest in the adverse

effects of the phenoxy herbicides and, although methodologies and doses greatly varied, the teratogenic capabilities of 2,4,5-T in mice and rats were quickly confirmed (Neubert and Dillman, 1972; Khera and McKinley, 1972; Courtney and Moore, 1971; Bage et al., 1973). Additionally, Sparschu et al. (1971a) noted delayed ossification of the skull bones in rat fetuses, while the U.S. Food and Drug Administration reported poor head fusion and the absence of eyelids in hamsters due to 2,4,5-T administration (MacLeod, 1971).

Early in 1971, the Science Advisory Committee on 2,4,5-T was established by the Environmental Protection Agency to review the teratogenic potential of the herbicide and make recommendations regarding its use (Wilson, 1973). The committee found that present evidence was inadequate to ascertain the hazard posed by the herbicide to human health. More importantly, its report stated that "a decision to restrict the use of 2,4,5-T should not be based on the isolated finding of toxicity but on the expected exposure following recommended use in relation to dose response effects" (MacLeod, 1971). Since the well-being of a developing embryo may be disturbed by virtually any chemical agent applied at the proper dose and time, it is difficult to justify the restricted usage of 2,4,5-T on the demonstration of teratogenicity at dose levels greatly exceeding that resulting from commercial application (0.5 - 8.0 pounds per acre).

The hazard to human health posed by 2,4,5-T following its application is a function of (a) spray drift and (b) environmental persistence. While decomposition in the environment is related to chemical and physical characteristics of the compound, spray drift is a result of application. The extent of spray drift may be increased by aerial application unless caution

is exercised. Droplets from an oil-based mist (100 - 200 microns in diameter) may be dispersed from a target site by more than forty feet when applied from a height of ten feet in a three mile per hour wind (Mullison, 1972). The problem is combatted by spray additives increasing droplet size (200 - 500 microns) and careful application techniques.

The phenoxy herbicides are subject to both photochemical and biochemical degradation. Measurable soil residues may persist for as long as three months following application, and are therefore potentially available for transport to non-target areas (MacLeod, 1971). Known bacterial species primarily responsible for the biochemical degradation of the phenoxy herbicides are Cornyebacterium, Achromobacter, Flavobacterium, Brevibacterium, Mycoplana, and Pseudomonas (Audus, 1964; Winston and Ritty, 1961; Horvath, 1971). Degradation products include carbon dioxide, inorganic chloride ions, and water (Winston and Ritty, 1961).

While the free acid is not readily leached from the soil (MacLeod, 1971), contamination of a non-target area by any means is undesirable. The detection of small herbicidal residues in foraged animals (less than 0.01 ppm), a result of grazing recently treated land, is an example of such contamination.

Economic loss through 2,4,5-T contamination is not as much a result of man's inability to reduce or control it as it is his lack of knowledge about its effects. According to F. G. Coats, manager of Atlanta's Kimber-CHIK Hatcheries of Dixie, present government regulations which require the destruction of exposed animals has caused major stock and feed losses to both private and commercial operations (personal communication, 1973). The dieldrin contamination of chicken feed on March 17, 1974, resulted in

major stock losses to Mississippi and Georgia hatcheries. Only insurance and aid from the regional poultry association prevented bankruptcy of several Georgia hatcheries (personal communication as above).

In a 1969 study for the U.S. Department of Agriculture, Palmer and Radeleff found that six-week old White-Leghorn chickens could ingest as much as 250 milligrams of 2,4,5-T (propylene glycol butyl ether ester) per kilogram of body weight daily for ten days without visible harm. Weight gain was reduced, but no evidence of poisoning or inactivity was noted. Ingestion of 500 mg/kg of 2,4,5-T, however, resulted in weight loss and death. Necropsy revealed congestion of the kidneys and intestinal mucosa.

Histological evidence compiled by Bjorklund and Erne (1971) suggests that the major route of phenoxy acid excretion is via the kidney. Consumption ad libitum of drinking water containing 1,000 ppm 2,4,5-T by day-old broiler chicks for two weeks resulted in spectacular hypertrophy of the proximal tubules. No lesions were noted, however, upon repetition of the experiment utilizing older (eight week) chickens of the same strain.

Similar results were obtained by Whitehead and Pettigrew (1972a) utilizing 2,4-D. Massive distension of the medullary collecting ducts and nephronic tubules was noted. Additionally, four-week old chickens fed a single dose of 2,4,5-T equivalent to 400 mg/kg lost up to 10% of their body weight by two days post-treatment, but recovered within four days.

Also of interest is the effect of herbicide ingestion by laying hens. Although no information is available regarding 2,4,5-T, Whitehead and Pettigrew (1972b) recorded no reduction in egg production or hatchability when hens received 150 mg/kg 2,4-D daily for twenty weeks. While decreased

egg hatchability has been attributed by some investigators to a reduction in egg shell thickness by the action of pesticides on calcium metabolism of the hen, Whitehead and Pettigrew failed to demonstrate similar results utilizing 2,4-D under the conditions mentioned. A study conducted by Sauter and Steele (1972) utilizing feed contaminated with DDT or lindane (10 ppm), however, reported lowered egg production and hatchability and increased embryonic mortality. Smith et al. (1970) injected 10 milligrams of dieldrin into the albumin of chicken eggs prior to incubation and recorded a 45% reduction in hatchability.

Because 2,4,5-T is an economically important herbicide, its use is widespread. Past contamination of feed by spray drift, application error, or common transport, has necessitated the destruction of thousands of hatchery chickens, incurring heavy financial losses to the owners. A more complete knowledge of 2,4,5-T toxicity in chickens might make such losses unnecessary. While ingestion of contaminated feed by broiler chicks might make their consumption undesirable, it is possible that ingestion of sublethal doses by laying hens might not necessitate their destruction. If rapidly excreted from the body, as Erne suggested (MacLeod, 1971), maternally ingested 2,4,5-T might not penetrate to the egg prior to laying in sufficient quantity to represent a health hazard to either the chick as a teratogen or to man as a food residue.

The purpose of this study is to further examine the toxicological effects of 2,4,5-T on the chicken embryo. Specific goals are (a) to determine an LD_{50} for 2,4,5-T in chicken embryos treated on day zero (prior to incubation), and (b) to determine through histological evidence whether or not 2,4,5-T introduced into the egg on day zero induces developmental anomalies in chicken embryos observed after 48 hours of incubation.

CHAPTER II

MATERIALS AND METHODS

Because the toxicity of a compound is influenced by its route of application (Mawdesley-Thomas, 1973), there was some concern as to the method which should be used. Amnionic and intravenous injection, suitable after three and nine days of incubation, respectively, were eliminated. While some investigators (Khera and Lyon, 1968; Gebhardt, 1972) prefer injection of the teratogen into the yolk, its high viscosity reduces the rate of diffusion. Deposition of the herbicide in the air space of the egg, equivalent to its injection into the underlying albumin, was the method finally selected. Gebhardt (1972) recently suggested that this procedure provided the most sensitive method for LD_{50} determination while causing the least stress to the embryo.

Practical grade 2,4,5-T (Lot 701-10) was acquired from Eastman Kodak of Rochester, New York. The dioxin contamination was less than 0.1 ppm. To facilitate administration and dose control, a 1.5 molar solution of the herbicide in dimethyl sulfoxide (DMSO) was prepared. The poor solubility of 2,4,5-T in propylene glycol, corn or mineral oil, and aqueous solutions prevented the selection of a more desirable vehicle.

The doses applied were based upon milligrams of herbicide per kilogram of egg. Volumes of the 1.5M solution, corresponding to each dose, were calculated (Appendix A).

A small hole was bored through the blunt end of each egg to allow

access to the air space. The hole was produced using a high speed (3500 rpm) Dremel hobby drill (Model 260) fitted with a dental bur. The appropriate dose was injected into the air space with a 50-microliter Hamilton syringe (Reno, Nevada). Following treatment, each hole was sealed by paraffin (Paraplast).

All eggs were obtained from KimberCHIK Hatcheries of Dixie (Atlanta, Georgia) and were of the same strain (Shaver-Starcross White-Leghorn). Although eggs were used on the day of collection, embryonic development prior to incubation was inhibited by storage at 55^{o}F. Egg weights (rounded to the nearest gram) ranged from 45 to 72 grams each, but 58 grams represented an approximate median value.

Eggs were incubated in the "Favorite Incubator," a forced-draught unit produced by Leahy Manufacturing Company, Inc. of Higginsville, Missouri. Standard conditions of incubation, as recommended by the manufacturer were maintained (Appendix B).

Eggs were candled on the seventh and fourteenth days of incubation. Infertile eggs were recorded and discarded.

Percent survival based on hatching of viable chicks was used as the index of toxicity. All chicks which emerged from their shells without aid prior to the end of twenty-two days of incubation were ruled viable. Chicks were maintained for a minimum of one week and examined daily for morphological growth defects or abnormal behavior (lack of appetite, absence of startle response, inability to peck accurately). Purina chicken mash and water were provided ad libitum.

In addition to the control eggs used during the toxicity evaluation, placebo eggs, bearing volumes of DMSO comparable to the dose examined

(Appendix A) were used. Control eggs were drilled and sealed with paraffin, but received no injection or additives.

Egg Hatchability

Six dozen eggs were used to determine whether or not drill vibration or the addition of some liquid volume into the air space affected embryo viability. Two dozen eggs were drilled and sealed with paraffin, while two dozen more received no treatment. The remaining two dozen were drilled, injected with 15 $\mu\ell$ of distilled water, and sealed with paraffin. An equivalent volume of the herbicide preparation would have contained a dose of approximately 100 mg/kg of 2,4,5-T for an egg of median weight (Appendix A).

Toxicity Test

Initially, doses of 25, 50, and 100 mg/kg of 2,4,5-T were examined. Doses of 12.5, 75, and 125 mg/kg of 2,4,5-T, however, were later examined to produce more meaningful results. All doses were administered on day zero to a minimum of 12 eggs each.

Teratogenic Evaluation

Because the chick embryo undergoes intense organogenesis at an early stage of its twenty-one day gestational period, fertile eggs used for the teratogenic examination were incubated for two days or less. Histological examination of an embryo incubated for 48 hours reveals development of the following: (a) spinal cord and lobes of the brain (telencephalon, diencephalon, mesencephalon, metencephalon, and myencephalon), (b) three cranial nerves (semilunar ganglion of the trigeminal nerve, V; acoustic ganglion

of the auditory nerve, VIII; and the superior ganglion of the glossopharyngeal, IX), (c) primordia of the palate, pituitary gland, thyroid, pharynx, liver, and kidney, and (d) continued circulatory sophistication (Rugh, 1971).

Fifty mg/kg of 2,4,5-T, a sublethal dose as determined from the toxicity test, was administered to each of 12 eggs on day zero. An additional 12 eggs were drilled and sealed with paraffin, but received no injection. Following incubation periods of 33 and 48 hours, six eggs of each treatment were removed.

Eggs were individually broken and the contents were emptied into baths of warm saline ($100^{\circ}F$). Each embryo was subsequently freed from its yolk and vitelline membrane (Humason, 1972) and fixed for one-half hour in Bouin's solution (75 ml saturated aqueous picric acid; 25 ml concentrated formalin; 5 ml glacial acetic acid).

Embryos were dehydrated and embedded in paraffin (Paraplast) according to accepted histological techniques (Appendix C). Frontal and sagittal series of 10-micron sections were prepared using the Model "820" A. O. Spencer microtome. All slides were stained by Delafield hematoxylin and counterstained by eosin (Appendix D).

Statistical Methods

Results of the hatchability study were analyzed by the Chi-square test. Using 2 x 2 contingency tables (Steel and Torrie, 1960), the drilled and injected groups were tested against the undrilled group for any significant difference.

A "best line" was fitted to the graphed toxicity values using estimating equations (linear regression). Significance of the line was tested

by computation of its correlation coefficient. Allowance for error was imposed over the graphed values through calculation of 95% confidence limits (Croxton, 1959).

No statistical methods were employed in the histological search for terata.

CHAPTER III

RESULTS AND DISCUSSION

The results of the hatchability study (Table 1) suggest that the viability of the embryo is not greatly affected by vibration or injection of 15 microliters of distilled water into the air space of the egg. The hypothesis that no significant difference in percent hatch existed between groups could not be rejected based upon the values obtained (0.09, 1 df; 0.0003, 1 df). Because of these results, no change in procedure was considered necessary.

An earlier study (1973) conducted by the author examined the toxicity of 2,4,5-T to chicken embryos injected on the fifth day of incubation (Appendix E). A 1.0 molar solution of 2,4,5-T in dimethyl sulfoxide (DMSO) was prepared, and eggs received doses based on egg weight as in the present study. While a dose of 100 mg/kg of 2,4,5-T caused 50% mortality, the equivalent volume of DMSO caused only 20% mortality. It is interesting to note, however, that administration of DMSO at a volume sufficient to carry an herbicide dose of 250 mg/kg caused 100% mortality. For this reason, there was some concern over using DMSO as a vehicle in this study.

Because DMSO is readily absorbed through the skin and across a membrane (Wood et al., 1971), its use as a vehicle was desirable in spite of its toxic properties. Increasing the concentration of the 2,4,5-T stock solution to 1.5 molar decreased by one-third the solvent volume required to introduce the herbicide into the air space of the egg at any dose. As

Table 1. Effect of Vibration or Air Space Injection of 15 $\mu\ell$ of Distilled Water on Egg Hatchability

Treatment	Number of Fertile Eggs	Number of Eggs Hatched	Percent Hatch	χ^2
Drilled	22	18	81.8	0.09*
Undrilled	23	18	78.3	
Injected	19	15	78.9	0.0003*

*Not significant, 1 df.

indicated by the results (Table 2), administration of DMSO did not reduce egg hatchability at volumes corresponding to herbicide doses of 62.5 or 93.75 mg/kg.

It is not unreasonable to attribute the reduction of egg hatchability at doses less than 90 mg/kg to the toxicity of 2,4,5-T since DMSO toxicity was not apparent at this level (Figure 1). The possibility that the herbicide's toxicity was enhanced by DMSO, however, cannot be overlooked. Administration of DMSO at volumes corresponding to herbicide doses of 125 and 156.25 mg/kg clearly reduced egg hatchability. Because the interaction between the herbicide and DMSO is undertermined, 2,4,5-T data points could not be adjusted to allow for the vehicle's toxicity.

The mortality curve calculated for 2,4,5-T by linear regression suggests that the LD_{50} for injection of the herbicide via DMSO into the air space of fertile chicken eggs prior to incubation is 62 mg/kg (Figure 2).

The teratogenic evaluation revealed no clearly defined results. Comparison of treated and control tissues failed to produce evidence of abnormal development. While careful examination of the developing tissues revealed no anomalies, it is important to note that the kidney was not sufficiently developed to detect the tubule lesion reported by Bjorklund and Erne (1971). In the 48 hour chick, the mesonephric tubules are just beginning to appear; they may be seen at 72 hours, and the metanephros at 96 hours (Rugh, 1971). No morphological abnormalities were noted among those chicks which survived the doses of the toxicity study (12.5 - 125 mg/kg).

Since membranes prevent organismic penetration by large or electrically charged molecules, it is valid to question whether 2,4,5-T ever

Table 2. Percent Hatch Following Injection of 2,4,5-T or DMSO into the Air Space of Chicken Eggs Prior to Incubation

Dose (mg/kg)	Number of Eggs	Number of Infertile Eggs*	Number of Viable Eggs	Number of Hatched Eggs	Percent Hatch
Control	31	6	25	23	92.0
2,4,5-T					
12.5	12	1	11	8	72.7
25.0	12	2	10	6	60.0
50.0	23	4	19	12	63.1
75.0	12	2	10	4	40.0
100.0	12	4	8	3	37.5
125.0	12	2	10	2	20.0
DMSO					
62.5	24	3	21	19	90.4
93.75	12	2	10	9	90.0
125.0	12	2	10	7	70.0
1256.25	12	0	12	8	66.6

*Eggs were candled on the 7th day of incubation; infertile eggs were removed.

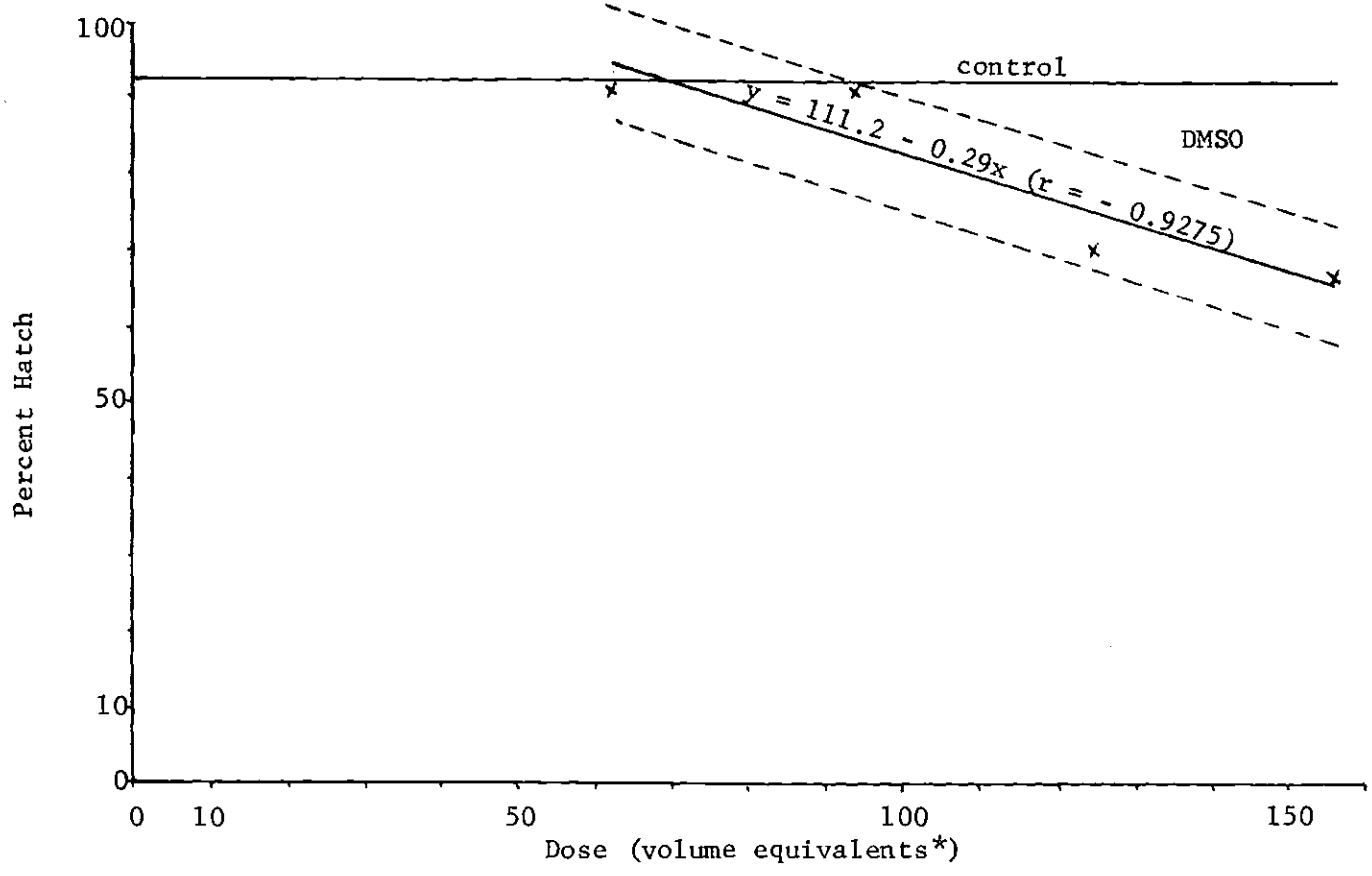

Figure 1. Percent Hatch vs Dose of DMSO

(*Since 80% of the total volume of herbicide preparation is DMSO, a volume of vehicle equivalent to a volume of herbicide preparation contains 25% more DMSO. The values above reflect the vehicular volume necessary to administer herbicide doses of 62.5, 93.75, 125, and 156.25 mg/kg.)

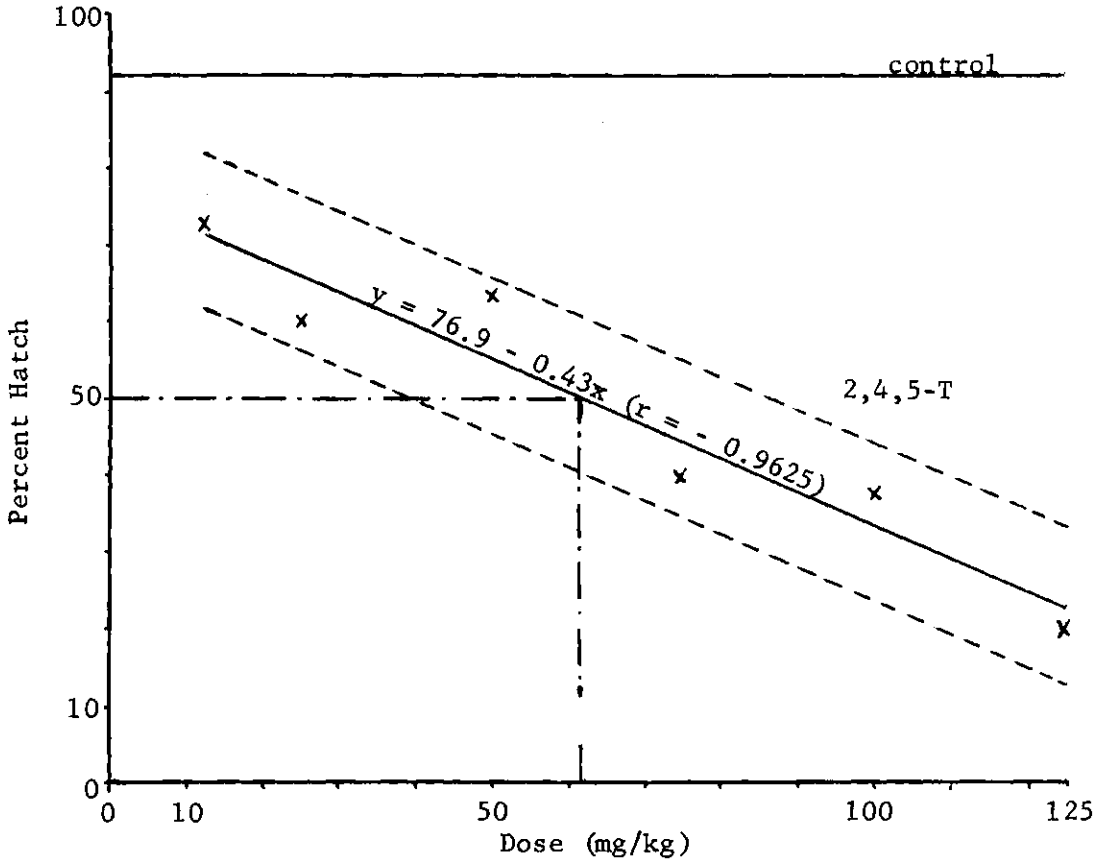

Figure 2. Percent Hatch vs Dose of 2,4,5-T (Dotted lines represent 95% confidence limits.)

reached the embryo. Certainly injection of the solvated molecule into the air space of the egg provided an opportunity for diffusion across the membrane, but the question remains unanswered. Since ingested 2,4,5-T is thoroughly distributed throughout the body prior to its excretion (Lindquist and Ullberg, 1971; Bjorklund and Erne, 1971; MacLeod, 1971), it is not unreasonable to expect absorption of the herbicide into the peripheral blood network of the egg and its subsequent circulation to the embryo.

Lindquist and Ullberg (1971) examined the ability of carbon-labelled 2,4,5-T to cross the placenta in pregnant mice. Using an autoradiographic technique, they found that herbicidal penetration of the placenta followed its distribution throughout the maternal system. While the label may or may not have represented the intact 2,4,5-T molecule, Lindquist and Ullberg suggested that 2,4,5-T crossed the placenta unaltered on the basis that body metabolites of the herbicide have not been found.

The dioxin contaminant of 2,4,5-T synthesis (2,3,7,8-tetrachlorodibenzo-p-dioxin) has been shown to induce terata in mice and rats at doses in excess of 0.001 mg/kg (Wilson, 1973; Sparschu et al., 1971b). While this has presented a problem of no small concern in past years (1969 - 1974), the level of contamination has been reduced from 37 ppm to less than 0.5 ppm by a modification of the synthesis process. Because the 2,4,5-T sample used in this study contained less than 0.1 ppm, the resulting level of dioxin contamination after dilution in DMSO was not considered a significant factor.

While the results obtained are of limited value in estimating the health hazard posed by 2,4,5-T to man (Gebhardt, 1972), they do represent new information concerning 2,4,5-T toxicity to developing chick embryos.

The herbicide is clearly toxic, but at levels greatly exceeding those representative of a residue following application at recommended rates. If used as recommended, increased chicken mortality due to 2,4,5-T contamination appears unlikely.

CHAPTER IV

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Under the conditions of this investigation, the following conclusions are made:

The methodology employed by this investigation did not significantly affect embryo viability.

The use of DMSO as a vehicle for administering weight-based doses of 2,4,5-T to chicken eggs was not entirely satisfactory due to its possible toxicity at levels below 125 mg/kg. Volumes of DMSO capable of delivering doses of 125 and 156.25 mg/kg of 2,4,5-T to the egg clearly reduced hatchability.

2,4,5-T is embryotoxic when introduced into the air space of fertile chicken eggs prior to incubation; using DMSO as a vehicle for administration, the LD_{50} was estimated to be 62 mg/kg of 2,4,5-T.

Injection of 50 mg/kg of 2,4,5-T into the air space of fertile chicken eggs prior to incubation induced no visible histological anomalies in the 48 hour chick.

No gross morphological or behavioral abnormalities were noted in chicks which hatched from eggs injected prior to incubation with sublethal doses of 2,4,5-T.

Recommendations

The methodology presented provides an experimentally acceptable means for examining the toxicological and teratogenic capabilities of 2,4,5-T in developing chick embryos. Because increased information might make unnecessary the mass destruction of chickens exposed to 2,4,5-T, the following recommendations are made:

The teratogenic assay should be modified to include examination of embryos sacrificed after 72 and 96 hours of incubation.

A broadened investigation of the toxicological potential of 2,4,5-T to developing chick embryos should include administration of the herbicide at several stages of gestation. Utilization of the same low dose for each time examined would indicate whether or not one stage of gestation was more sensitive to the herbicide than another.

APPENDICES

APPENDIX A

INJECTION VOLUME

Table 3. Injection Volume (μℓ) by Dose (mg/kg) for a 1.5M 2,4,5-T Solution

Egg Weight (grams)	Dose (mg/kg)					
	12.5	25.0	50.0	75.0	100.0	125.0
	(μℓ)	(μℓ)	(μℓ)	(μℓ)	(μℓ)	(μℓ)
45	1.4	2.9	5.8	8.8	11.7	14.6
46	1.5	3.0	6.0	9.0	12.0	15.0
47	1.5	3.1	6.1	9.2	12.3	15.3
48	1.6	3.2	6.3	9.4	12.5	15.7
49	1.6	3.2	6.4	9.6	12.8	16.0
50	1.6	3.3	6.5	9.8	13.0	16.3
51	1.7	3.4	6.7	10.0	13.3	16.7
52	1.7	3.4	6.8	10.2	13.6	17.0
53	1.7	3.5	6.9	10.4	13.8	17.3
54	1.8	3.5	7.0	10.6	14.1	17.6
55	1.8	3.6	7.2	10.8	14.4	18.0
56	1.8	3.7	7.3	11.0	14.6	18.3
57	1.9	3.7	7.4	11.2	14.9	18.6
58	1.9	3.8	7.6	11.4	15.1	19.0
59	1.9	3.9	7.7	11.6	15.4	19.3
60	2.0	3.9	7.8	11.8	15.7	19.6
61	2.0	4.0	8.0	12.0	16.0	20.0
62	2.0	4.1	8.1	12.2	16.2	20.3
63	2.1	4.1	8.2	12.4	16.4	20.6
64	2.1	4.2	8.4	12.6	16.7	21.0
65	2.1	4.3	8.5	12.8	17.0	21.3
66	2.2	4.3	8.6	12.9	17.2	21.5
67	2.2	4.4	8.7	13.1	17.5	21.8
68	2.2	4.5	8.9	13.4	17.8	22.3
69	2.3	4.5	9.0	13.5	18.0	22.5
70	2.3	4.6	9.1	13.7	18.3	22.8
71	2.3	4.7	9.3	13.9	18.5	23.2
72	2.4	4.7	9.4	14.1	18.8	23.5

Notes: 1. Volumes greater than or equal to 0.05 are rounded up to 0.1.
2. Volumes less than 0.05 are rounded down to 0.0.
3. Eighty percent of each injection volume is DMSO.

APPENDIX B

STANDARD CONDITIONS OF CHICKEN EGG INCUBATION*

1. Incubation period: 21 days
2. Operating temperature: 100°F
3. Wet-bulb reading during turning period: 85-87°F
4. Wet-bulb reading after completion of turning period: 90-94°F
5. Length of turning period: 18 days
6. Eggs turned three times daily during turning period
7. Incubator ventilation required on 10th day
8. Egg positioners used until completion of turning period
9. Eggs candled on 7th and 14th days of incubation

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*Recommended by Leahy Manufacturing Company, Inc., Higginsville, Missouri.

APPENDIX C

TISSUE PREPARATION: SCHEDULE FOR FIXATION, DEHYDRATION, AND INFILTRATION*

1. Fix one-half hour in Bouin
2. Transfer to 50% ethyl alcohol: 1 hour
3. Transfer to 70% ethyl alcohol: 1 hour
4. Transfer to 95% ethyl alcohol: 1 to 1½ hours
5. Transfer to absolute ethyl alcohol Number 1: ½ to 1 hour
6. Transfer to absolute ethyl alcohol Number 2: ½ to 1 hour
7. Transfer to absolute ethyl alcohol/xylene (1:1): ½ to 1 hour
8. Transfer to xylene Number 1: ½ to 1 hour
9. Transfer to xylene Number 2: ½ to 1 hour
10. Transfer to xylene/paraffin (1:1): ½ to 1 hour
11. Transfer to paraffin Number 1: ½ to 1 hour
12. Transfer to paraffin Number 2: ½ to 1 hour
13. Embed

*Humason, 1972

APPENDIX D

DELAFIELD HEMATOXYLIN STAINING SCHEDULE*

1. Xylene: 2-3 minutes
2. Xylene: 2-3 minutes
3. Absolute alcohol: 2-3 minutes
4. 95% alcohol: 2-3 minutes
5. 70% alcohol: 2-3 minutes
6. 50% alcohol: 2-3 minutes
7. Running water: 3 minutes
8. Delafield hematoxylin: 4 minutes
9. Running water: 3-5 minutes
10. Scott solution: 3 minutes
11. Running water: 3-5 minutes
12. Eosin: 1 to 1½ minutes
13. 70% alcohol: dip
14. 95% alcohol: dip several times
15. Absolute alcohol: 3-5 minutes
16. Absolute alcohol: 2-3 minutes
17. Absolute alcohol/xylene (1:1): 2-3 minutes
18. Xylene: 2-3 minutes
19. Xylene: 2-3 minutes or longer
20. Cover (Permamount)

*Humason, 1972

APPENDIX E

2,4,5-T TOXICITY

Table 4. 2,4,5-T Toxicity: Injection into the Air Space on Day Five*

Treatment (mg/kg)	# Hatch	# Eggs (fertile)	% Hatch
Control	13	16	81
2,4,5-T			
50	7	10	70
100	5	10	50
250	0	18	0
DMSO			
50	9	10	90
100	8	10	80
250	0	18	0

*Kerr, 1973

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