Teratogenic and Toxicological Effects of 2, 4, 5— Trichlorophenoxyacetic Acid in Developing Chick Embryos

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INTRODUCTION

Since their development in the mid-forties, the phenoxy herbicides have been instrumental in the control of broad-leaved weeds and brush (WILLIAMS, 1971). Of these, 2,4,5-tricholorophenoxyacetic acid (2,4,5-T) has become important in land and waterway management as well as in agriculture (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 (1962-70). During the Summer of 1969 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). A report by COURTNEY et al. (1970) demonstrated teratogenic and fetotoxic capabilities of 2,4,5-T in mice and rats. Anomalies noted by Courtney as a result of 2,4,5-T administration included cleft palate, cystic kidney, and hemorrhagic gastrointestinal tract. Courtney's report led to widespread interest in the adverse effects of the phenoxy herbicides and, although the 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. (1971) 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). DOUGHERTY et al. (1975) have recently reported that 2,4,5-T containing 0.05 ppm TCDD administered to pregnant rhesus monkeys daily from Day 22 through Day 38 of gestation showed no evidence of toxicity in the mother and no evidence of teratogenicity in the offspring.

Also of interest is the effect of 2,4,5-T ingestion by laying hens. Although no information is available regarding 2,4,5-T, 2,4-D given at a rate of 150 mg/kg daily for twenty weeks produced no reduction in egg production or

Bulletin of Environmental Contamination & Toxicology,
Vol. 15, No. 6 © 1976 by Springer-Verlag New York Inc.

hatchability. It was then the purpose of this study to determine the LD_{50} of 2,4,5-T in two different carrier substances on days zero and five of incubation and thereby project its effect on hatchability and to observe histologically any abnormalities present after 48 hours of incubation.

MATERIALS AND METHODS

Chemicals and Animals

Analytical standard 2,4,5-T (Lot AGR 86187) was acquired from Dow Chemical Company, Midland, Michigan. To facilitate administration and dose control, a 1.5 molar solution of 2,4,5-T in dimethylsulfoxide (DMSO) was prepared for use in the day zero study and a 1.0 molar solution for the day five study. Additionally a 0.75 molar solution of 2,4,5-T in acetone was prepared since high levels of DMSO are known to be teratogenic. The acetone preparation was used in the day zero study only.

Eggs were obtained from KimberCHIK Hatcheries of Dixie (Atlanta, Georgia) and were of the same strain (Shaver-Starcross White-Leghorn).

Procedure

A small hole was bored through the blunt end of each egg to allow access to the air space. The dose of 2,4,5-T in DMSO was injected into the air space with a 50-µl Hamilton syringe. Following treatment each hole was sealed with paraffin. Doses of 12.5, 25, 50, 75, 100 and 125 mg/kg of 2,4,5-T were injected into the air space. The lose of 2,4,5-T in acetone was introduced in a like manner at doses of 25, 50, 75, 100 and 150 mg/kg of 2,4,5-T. Additionally to determine whether or not drill vibration or the addition of some liquid into the air space affected embryo hatchability, six dozen eggs were divided into two dozen groups with the first group being drilled only; the second group drilled and injected with 15 µl of distilled water, and the third group received no treatment. One group of control eggs were injected with DMSO volumes comparable to the level of 2,4,5-T received to determine what toxic effects the carrier might have. On day zero DMSO was administered at volumes corresponding to herbicide doses of 62.5, 93.75, 125 and 156.25 mg/kg, and on day 5 at levels corresponding to 50, 100 and 250 mg/kg. A second group of control eggs were injected with acetone at a volume corresponding to a herbicide doses of 150 mg/kg. Eggs were incubated in the Favorite Incubator, forced-draught unit manufactured by Leahy Manufacturing Company, Inc., Higginsville, Missouri, which had been equipped with a Thermistemp Control (YSI Model 71A).

Twelve eggs for histological evaluation were incubated for 48 hours following the injection of 50 mg/kg 2,4,5-T and processed by a procedure described by HUMASON (1972). A like number of untreated controls were processed. Embryos were fixed in Bouin's, embedded in paraffin (Paraplast), cut at 7-10μ and stained with Delafield hematoxylin and eosin.

Statistical Analysis

Results of the hatchability study were analyzed by the Chi-square test. Using 2 x 2 contingency tables, 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.

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

RESULTS AND DISCUSSION

The results of the hatchability study (Table 1) suggest that the viability of the embryo is not affected by vibration or injection of 15 μl of distilled water into the air space.

TABLE I.

Effects of Drilling and Injection on Egg Hatchability

Treatment	Number of Fertile Eggs	Number of Hatched Eggs	Percent Hatch	X^2
Drilled	22	18	81.8	0.09*
Undrilled	23	18	78.3	0.00003*
Injected	19	15	78.9	

*Not significant, 1 df

Toxicity of 2,4,5-T to chicken embryos injected on the fifth day of incubation revealed a LD_{50} of 68 mg/kg (Table 2). However, a 1.0 molar solution of 2,4,5-T in DMSO was used and while a dose of 100 mg/kg of 2,4,5-T cuased 50% mortality (30% greater than the controls), the equivalent volume of DMSO did not cause an increase in mortality when compared with the controls. It is interesting to note that the administration of DMSO at a volume sufficient to carry an herbicide dose of 250 mg/kg caused 100% mortality. For this reason, in the later day zero study an increased 2,4, 5-T concentration was utilized.

TABLE II.

Day Five 2,4,5-T Toxicity

Treatment (mg/kg)	Number of Fertile Eggs	Number of Hatched Eggs	Percent Hatch
Control	16	13	81
2,4,5-T			
50	10	7	70[a]
75	12	7	58
100	10	5	50
250	18	0	0
DMSO			
50	10	9	90
100	10	8	80
250	18	0	0

[a]Linear regression of percent hatch vs. dose resulted in the following equation $Y = 85.12 - 0.43x$ ($r = 0.9987$). LD_{50} for 2,4,5-T in 1.0 molar solution of DMSO is 68 mg/kg.

Increasing the concentration of 2,4,5-T stock solution to 1.5 molar in the day zero toxicity study decreased by one-third the solvent volume required to introduce the herbicide into the air space. As indicated in Table 3, administration of DMSO did not reduce egg hatchability at volumes corresponding to herbicide doses up to 93.75 mg/kg.

It is not unreasonable to attribute the reduction of egg hatchability of doses less than 100 mg/kg to the toxicity of 2,4,5-T since DMSO toxicity was not apparent at this level in either study. The possibility that the herbicide's toxicity was enchaned by DMSO, however, cannot be overlooked. Administration of DMSO at volumes corresponding to herbicide doses of 125 mg/kg or greater reduced egg hatchability. Because the interaction between herbicide and DMSO is undetermined, 2,4,5-T data points could not be adjusted to allow for the toxicity.

Since acetone at levels equivalent to carry 150 mg/kg revealed no depression in embryo viability, another study using acetone as the carrier was undertaken on day zero. The results of this study are shown in Table III.

The mortality curve calculated for 2,4,5-T by linear regression suggest that the LD_{50} for injection of the herbicide via DMSO into the fertile chicken egg at day zero to be 62 mg/kg. A curve calculated with acetone as the carrier revealed in LD_{50} of 133 mg/kg.

A mortality curve calculated for DMSO alone using the data points from the day zero study suggest that the LD_{50} for DMSO to be a volume sufficient to carry 210 mg/kg of 2,4,5-T. Results from both studies suggest that DMSO alone at levels of 50, 62.5, 93.75 and 100 mg/kg do not decrease hatchability. However, at 125 mg/kg the hatchability was 22% below controls and at 156.25 mg/kg was 25% below controls. By 250 mg/kg no eggs survived.

The difference in the LD_{50}s with DMSO and acetone as the carrier substance points out the liklihood of a syngeristic action between 2,4,5-T and DMSO which, if present with acetone, is not as evident. These differences further point to the highly variable results that can be obtained by a slight modification in procedure. DMSO has been used for years by researchers as a carrier of toxic substances. Information gained in this manner should be carefully reviewed to make sure that toxic levels of substances are not influenced by a seemingly insignificant secondary substance which might highly inflate toxic values and misrepresent the substance in question.

TABLE III.

Day Zero 2,4,5-T Toxicity

Treatment (mg/kg)	Number of Fertile Eggs	Number of Viable Embryos	Percent Live Embryos
Control	67	64	96
2,4,5-T in DMSO			
12.5	11	8	73[a]
25.0	10	6	60
50.0	19	12	63
75.0	10	4	40
100.0	8	3	38
125.0	10	2	20
2,4,5-T in Acetone			
25	24	23	96[b]
50	42	39	93
75	42	35	83
100	18	10	56
150	18	8	44
DMSO			
62.5	21	19	90[c]
93.75	10	9	90
125.0	10	7	70
156.25	12	8	67
Acetone	42	41	98

[a]Linear regression of percent live embryos vs. dose resulted in the following equation $Y = 76.9 - 0.43x$ ($r = 0.9625$). LD_{50} for the 2,4,5-T in 1.5 molar solution of DMSO is 62 mg/kg.

[b]Linear regression of percent live embryos vs. dose resulted in the following equation $Y = 111.2 - 0.46x$ ($r = 0.9573$). LD_{50} for the 2,4,5-T in 0.75 molar solution of acetone is 133 mg/kg.

[c]Linear regression of percent live embryos vs. dose (volume equivalents) resulted in the following equation $Y = 111.2 - 0.29x$ ($r = 0.9275$). LD_{50} for DMSO is a volume equivalent to deliver 210 mg/kg 2,4,5-T.

The teratogenic evaluation revealed no clearly defined results. Comparison between treated (50 mg/kg 2,4,5-T) and control tissues (drilled only) failed to produce evidence of abnormal development in the 24 eggs examined. While this 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). Histological evaluation at later developmental stages need to be completed to determine the exact mode of mortality. Embryos sacrificed for this teratogenic evaluation were still viable at 48 hours of incubation. It must be concluded then that death does not occur early in development.

While the results obtained in this study are of limited value in estimating the health hazard posed by 2,4,5-T to man, 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. Maximum values that would result from 2,4,5-T application to a field would be approximately 41 mg/sq. ft. Assuming an egg occupies 1/10 of a sq. ft., it would be exposed to only 4.1 mg of 2,4,5-T of which not all would be absorbed.

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