Evaluation of chemicals for Toxic & Teratogenic Effects Using the chick embryo as the test system FDA Contract #71-331 (**Manganese Sulfite**) FDA Compound #71-71
12/7/74

N19

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS USING THE CHICK EMBRYO AS THE TEST SYSTEM

MANGANESE SULFATE: FDA 71-71

12/7/74

WARF INSTITUTE, INC.

MADISON, WISCONSIN

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS USING THE CHICK EMBRYO AS THE TEST SYSTEM

Objective: To determine the toxic and teratogenic effects of GRAS List compounds when injected into the air cell and yolk of fertile chicken eggs.

Procedure:

A. Test System and Incubation Procedures:

Fertile hatching eggs were chosen from a single comb white leghorn breeder flock. The eggs were candled and graded to eliminate internal and external defects; blood and meat spots, tremulous air cells, rough or cracked shells. The eggs chosen for injection weighed from 23 - 26 ounces/dozen and were not washed or dipped. The eggs were gathered within 48 hours of the injection or incubation and were held at 50 - 60°F. and 60 - 80% relative humidity. The breeder ration fed the flock was formulated by the breeder to meet or exceed the recommendations of the Nutrient Requirements of Poultry, Number 1 - 1971, National Academy of Sciences, and contained no additions of antibiotics, arsenicals, nitrofurazones or similar chemical additives. The breeder flock was blood tested and negative for pullorum-typhoid and mycoplasm gallispecticum.

Eggs were incubated in Jamesway 1080 forced air incubators equipped with automatic controls to regulate temperature, humidity and egg turning. Temperature and relative humidity were maintained at 99.5°F. and 86°F.* wet bulb respectively for the first 18 days of incubation and eggs were turned each two hours. The eggs were then transfered to the hatcher in 3½" x 5" x 25" covered hardware cloth hatching baskets for the hatching period. Temperature in

* 86°F. wet bulb refers to temperature of wet bulb apparatus in standard incubator hatching equipment and is equivalent to approximately 56% relative humidity.

the hatcher was maintained at 98.5oF. and relative humidity at 86oF. wet bulb. When the humidity had risen to 88 degrees as a result of moisture generated by hatching the hatcher was adjusted to hold 88oF. wet bulb relative humidity until the chicks were removed on the morning of the 23rd day of incubation.

Prior to incubating or hatching each setting of eggs and following each hatching the incubator and its metal parts were thoroughly cleaned by vacuuming and washing with a 200 ppm solution of "ROCCAL" which contains 10% alkyl (C12, C14, C16 and related alkyl groups from C8 to C18) - dimethyl benzyl ammonium chloride. The sanitized surfaces were allowed to completely air dry prior to the introduction of eggs. Following each transfer of eggs to the hatching compartment, and when temperature and humidity had returned to normal levels, the hatcher and the eggs it contained were fumigated by combining 10 grams of potassium permanganate crystals and 20 grams of 37% formaldahyde solution.

B. Test Sample Preparation and Administration:

The test sample was taken up in an appropriate solvent to facilitate administration at the levels chosen. Sterile glassware, syringes and needles were employed to prepare and administer the test sample or solvent. Eggs previously selected were candled and the location of the air cell marked with pencil. The eggs were then randomized into the experimental groups. Injection of solvent or test sample dilution was accomplished by placing the material on the air cell membrane or by injection into the yolk sac. These administrations were made at both 0 and 96 hours of incubation. For the 0 hour experiments the eggs were assumed to be fertile; however, in the 96 hour experiments the eggs were candled as previously described and only those eggs with a well developed 96 hour embryo were selected for use.

1. Air Cell Administration:

The eggs were wiped at the injection site with 70% ethanol and allowed to air dry. A hole measuring approximately 6mm was then drilled over the air cell in each egg using a "Dremel Moto-tool", model 270. The cutter employed deflected the shell fragments upwards and outwards. Remaining shell membrane fragments were removed with a small

forceps and the surface of the egg membrane visually examined for damage. The solvent or test sample was then deposited on the egg membrane with a model SB2 Syringe Microburet. Immediately following, the hole was sealed with ½" Scotch Brand transparent tape. Two additional groups of eggs were normally included with each air cell experiment; a group which had been drilled, shell membrane fragments removed, and sealed only and a group of control eggs which had received no treatments whatsoever.

2. Yolk Administration:

The eggs were placed within a Fisher Scientific "Isolator/Lab" equipped with plastic irises through which the hands and forearms were placed during injection. Prior to injection the eggs and miscellaneous required equipment were submitted to a fumigation of 1.8 grams of potassium permanganate crystals and 3.6 grams of 37% formaldahyde. The eggs were held in this atmosphere for 30 minutes prior to further handling.

Each egg was then wiped at the injection site with 70% ethanol and allowed to air dry. A small hole was engraved directly over the air cell with a Burgess model V-13 Vibro-Graver. Care was taken not to damage the membrane attached to the shell. The surface of the egg at the engraved site was vacuumed to remove the shell particles produced. The egg was then slid onto the needle of the Syringe Microburet with the egg horizontal on its long axis until the top of the egg reached the hub of the 1" - 25 ga. hypodermic needle. Following the injection of the material into the yolk sac, the egg was carefully withdrawn from the needle and the hole sealed with transparent tape. The hypodermic needle was carefully wiped with a sterile gauze pad prior to the next injection. As in the air cell administration, normally two additional groups of eggs were included in each yolk experiment; a group which had been drilled, pierced with the hypodermic needle and sealed only and a group of control eggs which had received no treatment other than fumigation.

Following the air cell and yolk injections the eggs were identified as to experiment and group with a No. 3 lead pencil and were then incubated as described above.

C. Test Profile:

The work was divided into one or more Preliminary Range Finding Experiments, two Dose-Response and Teratogenic Experiments, and Ancillary Investigations (Post Hatch Trials).

1. Preliminary Range Finding Experiments:

The objective of these trials was to locate the approximate LD-50 of the test sample. This data was used to design the dose levels for the Dose-Response trials. The test sample and solvent were administered by two routes; air cell and yolk, and at 0 and 96 hours of incubation. In general, at each route and time of incubation, 5 volumes of test sample dilution were administered together with 5 levels of solvent at the same volumes. Control eggs were also usually included as described above. Normally 10-20 eggs were used per group in these trials. When necessary these trials were repeated in an effort to locate the approximate LD-50 for the test compound.

Beginning on the 6th day of the incubation, the eggs set in the Preliminary Range-Finding Experiments were candled daily and non-viable embryos removed. These embryos were examined grossly for determination of developmental age and evidence of teratogenic effect, however, mortality was the main parameter in these trials. The remaining eggs were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The resultant chicks and non-viable embryos were examined grossly for teratogenic effects and all pertinent data was recorded. An estimate of the LD-50 for the test compound was then made.

2. Dose-Response and Teratogenic Experiments:

Based upon information from the Preliminary Range-Finding Experiments, the Dose-Response Experiment was designed, employing 5 levels of sample dilution expected to produce mortality from the background level up through approximately 90%. Five volume levels of solvent were included as solvent controls at each route and time of administration. Normally 10 eggs/group were used in the solvent series with 50 eggs/group for the test sample dilutions. Twenty eggs/group were normally included for the drilled or pierced and non-treated controls. Two such experiments were conducted for each sample so that ultimately 100 eggs were tested on each test dilution at each route and stage of incubation.

The eggs set in these experiments were candled daily beginning on the 6th day of incubation and the non-viable embryos removed for examination as previously described. Where necessary, embryos were examined with the aid of a dissecting microscope. Remaining embryos were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The apparently normal chicks were then removed from the hatching trays and examined externally for anomalies.

Remaining non-viable embryos and chicks which were alive but unable to hatch were individually examined externally for abnormalities. These non-viable embryos, chicks which were alive but unable to hatch, and a portion of the normal chicks were examined in one aspect by x-ray. The chicks and embryos which had been x-rayed and all remaining normal chicks were then examined internally for possible anomalies of the viscera. All pertinent data were recorded.

Results:

The data developed in the testing of Manganese Sulfate are presented in the following tables:

Manganese Sulfate

Table 1 - Air Cell At 0 Hours
Table 2 - Air Cell At 96 Hours
Table 3 - Yolk At 0 Hours
Table 4 - Yolk At 96 Hours

In Tables 1 through 4, the following comments apply:

Column 1 gives the dose administration milligrams per kilogram, respectively. (The milligrams per kilogram figure is based on an average egg weight of fifty grams).

Column 2 is the total number of eggs treated.

Column 3 is the percent mortality, i.e., total non-viable divided by total treated eggs.

Column 4 is the total number of abnormal birds expressed as a percentage of the total eggs treated. This includes all abnormalities observed and also toxic response such as edema, hemorrhage, hypopigmentation of the down and other disorders such as feather abnormalities, significant growth retardation, cachexia, ataxia or other nerve disorders.

Column 5 is the total number of birds having a structural abnormality of the head, viscera, limb or body skeleton expressed as percentage of the total eggs treated. Toxic response and disorders such as those noted for column 4 are not included.

Column 2 through 5 have been corrected for accidental deaths if any occurred. Included in these columns are comparable data for the solvent-treated eggs and the untreated controls.

The mortality data in column 3 have been examined for a linear relationship between the probit percent mortality versus the logarithm of the dose. The results are indicated at the bottom of each table.

The data of columns 3, 4 and 5 have been analyzed using the Chi Square test for significant differences from the solvent background. Each dose level is compared to the solvent value and levels of percent mortality or percent abnormalities that are significant (probability of being the same is 5% or less) are indicated by an asterisk in the tables. All values so indicated have a higher incidence than the solvent values.

Discussion:

The comments and data which follow concern the results obtained when Manganese Sulfate was employed in the test system.

Significant mortality (P.05) was seen in the 0 hour air cell treatments at dose levels of 200.0, 300.0, 400.0 and 500.0 mg/kg. with a calculated LD-50 of 168.1 mg/kg. Solvent mortality for this time and route averaged 32% which would considerable modify the actual LD-50 for the test material. In 96 hour air cell treatments, the mortality response was significantly elevated at all dose levels between 10.0 and 400.0 mg/kg. The calculated LD-50 was 13.9 mg/kg. The average solvent mortality for this time and route was 17%.

In 0 hour yolk treatments, mortality was significantly elevated at dose levels of 200.0, 300.0, 400.0 and 500.0 mg/kg with a calculated LD-50 of 175.5 mg/kg. The average solvent mortality for this time and route was 47%. Therefore, the actual LD-50 for this series of dosages would appear to be in excess of 500.0 mg/kg. At 96 hour yolk the mortality was significantly increased at dose levels ranging from 100.0 to 400.0 mg/kg with an LD-50 of 68.6 mg/kg. The average solvent mortality at this time and route was 25%.

Significantly increased numbers of abnormal birds were seen in some air cell treatments at 0 hours and in some yolk treatments at both 0 and 96 hours.

In the 0 hour air cell treatments, percent total abnormal birds

was significantly elevated at dose levels of 25.0, 50.0, 200.0, 300.0 and 500.0 mg/kg. Dwarfism (retarded development) was the primary contributor to the total. There were a considerable number of other anomalies seen at this time and route. Included in the approximately 600 eggs dosed were the following anomalies at the frequencies indicated: hair-like down (3), short mandible (3), long mandible (2), flexed mandible (24), parrot beak (11), crossed beak (6), mandible absent (1), microphthalmia (2), anophthalmia (5), exophthalmia (3), buphthalmia (1), exencephaly (1), anencephaly (1), acrania (2), small brain (1), celosomia (3), leg duplication (1), light down (44) and edema (14). Of the above abnormalities, mandible absent, exophthalmia, anencephaly and small brain, had not been seen either in the solvent control for this time and route or in the flock background. Most of the remaining abnormalities mentioned had been seen either in the solvent control or flock background with similar frequencies. Flexed mandible, parrot beak and light down, were observed at considerable higher levels than found in solvent controls or flock background. (The flock background includes observations on a total of 1,580 drilled, pierced and untreated control eggs from flock N-2 which were used in 50 egg studies).

At 96 hour air cell, a scattering of low incidence anomalies were seen. For the most part these conditions had been found in the solvent control eggs or in the flock background for this time and route. Exceptions were sparse down (1), cataract (1) and anencephaly (1).

In the 0 hour yolk treatments, a scattering of anomalies were seen in the approximately 600 eggs dosed with the test material. Generally the anomalies had been encountered in the solvent control or flock background. Exceptions were the following abnormalities at the frequencies indicated: one head-2 bodies (1), fetal monster (1), triocephalus (2), short maxilla (2), short vertebral column (1), enlarged heart (1), small liver (1), wing micromelia (1) and edema (12), which had not been found in solvent control eggs for this time and route or in the flock background. A scattering of other anomalies were found, which were also seen in the solvent control eggs or in the flock background.

At 96 hour yolk, percent abnormal birds was significantly elevated at dose levels of 100.0, 150.0 and 200.0 mg/kg. Dwarfism (retarded development) contributed heavily to the total anomalies seen. In approximately 600 eggs treated with the test sample, the following important anomalies were seen: malformed body (1), flexed mandible (11), short mandible (1), cleft palate (1), parrot beak (2), crossed beak (3), microphthalmia (2), anophthalmia (3), buphthalmia (1), ablepharia (1), acrania (4), exencephaly (2), malformed head (1), celosomia (6), edema (3) and light down (8). Many of these anomalies had been seen at similar levels in the solvent controls or flock background. Malformed body, short maxilla, buphthalmia, ablepharia, edema and light down had not been seen in either the solvent control for this time and route or in the flock background.

Several embryos described as "fetal monsters" were found in this experiment. In general, this term was used to describe an embryo whose features were not readily discernable. One such embryo was found in a non-treated, pierced, control egg. It was a 6 day embryo with bilateral anophthalmia, large head, left wing absent, severe growth retardation and features of head not discernable. Another 6 day embryo was seen in the 500.0 mg/kg, 0 hour air cell treatment, with bilateral anophthalmia, moderate growth retardation and features of the head which were not readily discernable. A third 6 day embryo was found in the 0 hour yolk treatment, 400 mg/kg which was described as a "fetal monster" due to its physical appearance.

The light down seen in this experiment tended to be dose related both in its frequency and severity. Most, but not all, of the hatched chicks were involved.

Dwarfism (retarded development) was a frequent observation in this experiment. To clarify our designation of dwarfism we will explain our classification procedure. At the first candling, day 5 or 6,, we did not classify any embryos as retarded unless they were alive and definitely younger in development than 5 or 6 days. In subsequent candling any dead embryo judged by size to be 3 days behind in development was labeled as slight dwarfism, 4 days behind was labeled moderate dwarfism and 5 days or more behind was labeled severe dwarfism. If embryos were removed alive, 1 day behind was labeled slight, 2 days behind was labeled moderate and 3 days behind was labeled severe dwarfism. At hatch time an 18 day embryo was classified slightly dwarfed, a 17 day embryo was classified as moderate dwarfism and a 16 day embryo was classified as severe dwarfism. One might suspect that at the toxic levels administered, embryo development could be delayed due to metabolic or nutritional alterations that produced temporary growth depression which would not result in a permanent growth defect. Chicks which hatched were of normal size and no evidence of permanent growth retardation was observed.

X-ray examination did not reveal any abnormalities not already noted during daily examination of embryos.

Conclusion:

Under the conditions specified for this trial, Manganese Sulfate was particularly toxic in 96 hour air cell and 96 hour yolk administrations and produced significantly elevated numbers of abnormal embryos at 0 hour air cell and 96 hour yolk treatments.

Temporary growth retardation during incubation was a major contributor to the total abnormalities seen. A relatively high level of anomalies of the head were seen. Similarly, edema was a frequent observation. Some of the anomalies had been seen in the solvent control eggs or in the flock background, however, many had not. For this reason and because of the serious nature, these findings suggest that further investigation with the test

material is indicated.

Signed R M Bodden

By and For WARF Institute, Inc.

December 7, 1974

Test Sample: Manganese Sulfate

Identification: FDA 71-71

Solvent System: Sterile distilled H_2O

Breeder Flock: N-2

Preliminary Range Finding Experiments

Experiment No.	Initiated
59	12/18/72
61	1/15/73

Dose Response Experiments

Experiment No.	Initiated
68	3/05/73
74	4/02/73

Table 1

Manganese Sulfate
Air Cell At 0 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
500.0	110	92.72*	31.81*	13.63
400.0	119	89.07*	24.37	9.24
300.0	99	89.89*	25.25*	6.06
200.0	118	66.94*	29.66*	3.39
100.0	109	33.02	23.85	2.75
50.0	10	10.00	60.00*	20.00
25.0	10	30.00	70.00*	.00
Water	130	32.30	13.84	6.92
Drilled Control	50	22.00	18.00	12.00
Control/ Control	200	13.50	7.50	3.00

**LD-50 168.1 mg/kg

*Significantly differenct from solvent ($P \leq .05$)

Table 2

Manganese Sulfate
Air Cell At 96 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
400.0	10	100.00*	.00	.00
200.0	10	100.00*	.00	.00
100.0	10	100.00*	.00	.00
50.0	10	100.00*	.00	.00
30.0	99	83.83*	12.12	4.04
25.0	20	80.00*	5.00	.00
15.0	99	65.65*	13.13	4.04
10.0	106	50.00*	17.92	2.83
5.0	110	25.45	4.54	1.81
2.5	109	19.26	11.92	11.00
1.0	10	.00	.00	.00
Water	178	17.41	9.55	3.93
Drilled Control	60	10.00	13.33	5.00
Control/ Control	200	13.50	7.50	3.00

**LD-50 13.9 mg/kg

*Significantly different from solvent ($P \leq .05$)

Table 3

Manganese Sulfate
Yolk At 0 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
500.0	100	85.00*	35.00	5.00
400.0	120	74.16*	16.66	2.50
300.0	99	70.70*	22.22	9.09
200.0	118	63.55*	17.79	6.78
100.0	118	48.30	11.86	3.39
50.0	20	35.00	15.00	.00
25.0	20	20.00	15.00	5.00
Water	127	47.24	13.38	3.14
Pierced Control	50	40.00	12.00	8.00
Control/ Control	200	13.50	7.50	3.00

**LD-50 175.5 mg/kg

*Significantly different from solvent ($P \leq .05$)

Table 4

Maganese Sulfate
Yolk At 96 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
400.0	20	100.00*	15.00	5.00
300.0	10	100.00*	10.00	.00
200.0	70	94.28*	32.85*	2.85
150.0	49	93.87*	51.02*	14.28
100.0	120	67.50*	35.83*	7.50
50.0	120	26.66	10.00	3.33
25.0	110	21.81	13.33	5.45
10.0	50	32.00	18.00	2.00
5.0	50	18.00	4.00	.00
Water	180	25.55	8.88	2.77
Pierced Control	60	23.33	10.00	6.66
Control/ Control	200	13.50	7.50	3.00

**LD-50 68.6 mg/kg

*Significantly different from solvent ($P \leq .05$)