Evaluation of Chemicals for Toxic & Teratogenic Effects Using the Chick Embryo as the Test System-FDA Contract 71-331 (**Sodium Nitrite**) No Date

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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

SODIUM NITRATE: FDA 71-7

WARF Institute, Inc.
Madison, Wisconsin

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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.

3. Post Hatch Trials:

Apparently normal chicks were chosen from one 50 egg experiment for this portion of the study.

Generally 20 chicks (straight-run) were wing banded from each level chosen and were placed in Jamesway electrically heated battery brooders. Central Soya Chick Starter was fed as the sole ration to 8 weeks of age and Central Soya Grower from 8 weeks of age to termination. These diets were non-medicated. The chicks chosen were usually from the approximate LD-50 and no-effect levels for the test compound from each route of administration and time of incubation. Negative control, untreated chicks, were also included. In some cases chicks were chosen from groups where a relatively high incidence of anomalies were seen rather than from the LD-50 or no-effect levels specifically. Body weight data were collected weekly through 4 weeks of age and bi-weekly to termination. Average group feed consumption was recorded periodically.

4. Histopathology:

A random sampling of birds from selected groups were specified for histologic examination. These chicks comprised 5 males and 5 females from the test groups selected and 5 males and 5 females from a negative control group. Groups to be sampled were selected on the basis of observations of specific effects and a judgment made as to what groups would give the most information from the limited histopathologic examination.

The chicks sacrificed were either day old or varying ages in a Post Hatch Trial. The following tissues were collected, trimmed, dehydrated, embedded in paraffin, sectioned and stained with hematoxilin and eosin:

1. Thyroid
2. Liver
3. Spleen
4. Pancreas
5. Lung
6. Heart
7. Kidney
8. Gonad
9. Bursa

The prepared slides were examined and remarkable alterations noted.

Results:

The data developed in the testing of Sodium Nitrate are presented in the following tables:

Sodium Nitrate

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
Table 5 - Body Weight Data - Males
Table 6 - Body Weight Data - Females
Table 7 - Histopathology - Grow-Out Birds

In Tables 1 through 4, the following comments apply:

Column 1 gives the dose administered milligrams per kilogram, respectively. (The milmigrams 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, limbs, 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 sodium nitrate was employed in the test system.

Significant toxicity (P.05) was observed in the 0 hour air cell treatment at 110.0, 220.0, 366.7, 440.0 and 586.7 mg/kg when compared with the solvent treated controls. The data were somewhat erratic with a calculated LD-50 of 4136.6 mg/kg. At 96 hours air cell, the mortality response was significant and generally dose related at 73.3, 110.0, 183.3, 220.0, 366.7 and 440.0 mg/kg. The calculated LD-50 was 117.3 mg/kg.

In 0 hour yolk treatments, toxicity was significant at doses of 73.3, 183.3, 366.7 and 586.7 mg/kg. The LD-50 was calculated to be 380.5 mg/kg. At 96 hours, toxicity was significantly greater than the solvent treated controls at dose levels of 36.7, 73.3, 183.3, 366.7 and 733.3 mg/kg.

Because the slope was minimal, the calculated LD-50 values are meaningless at the 96 hour yolk treatment.

Significant instances of terata were not seen in the 0 hour yolk treatments, however, were observed at both 0 and 96 hour air cell and 96 hour yolk treatments. At 0 hour air cell, the abnormalities were generally limited to the head with exencephaly, malformed skull, flexed and short mandible and poorly developed eyes being encountered at 586.7 and 220.0 mg/kg. Retarded development was also seen. At 96 hour air cell, retarded development (dwarfism) and anomolies of the limbs were the most prevalent terata including flexed limbs, perosis, leg micromelia and missing or fused toes. At 96 hour yolk treatments, the primary terata seen at the upper four dose levels were dwarfism. Also included were clubbed down, short maxilla, microphthalmia, ablepharia, exencephaly, palate atresia, celosomia, perosis, leg rotation, fused toes, edema, bloody yolk, kidney hemorrhage and skull hemorrhage.

Post hatch birds were raised to 12 weeks of age and included birds from the untreated control and various treatment groups as indicated in the attached tables. Body weight and feed consumption were considered within normal limits in all groups and no abnormalities were observed during the grow-out period.

Histopathological examinations were made on tissues from 0 hour air cell and 96 hour yolk treatments - 366.7 mg/kg. The tissues were compared with those of birds of similar age non-treated controls. The tissue alterations were minimal in nature, non-specific and rather randomly distributed between control and test animals.

X-ray examinations did not reveal abnormalities not already noted on gross examination.

Conclusion:

Under the conditions specified for this trial, sodium nitrate was shown to be particularly toxic in the 96 hour air cell administration and teratogenic when administered in the air cell at 0 and 96 hours and in the yolk at 96 hours.

Signed R. M. Bodden

By and For WARF Institute, Inc.

August 30, 1974

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REPORT

Test Sample: Sodium Nitrate

Identification: FDA 71-7

Solvent System: 10% Absolute Ethanol in sterile distilled H_2O

Breeder Flock: N-1

Preliminary Range Finding Experiments

Experiment No.	Initiated
8	12-16-71
19	3-16-72

Dose Response Experiments

Experiment No.	Initiated
26	5- 1-72
39	7-31-72

Table 1

Sodium Nitrate
Air Cell At 0 Hour

Dose mg/kg	Number of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
586.7	50	48.00*	18.00*	10.00*
440.0	20	35.00*	10.00	5.00
366.7	120	22.50*	10.83	6.66
220.0	40	37.50*	20.00*	5.00
183.3	99	13.13	7.07	3.03
110.0	40	27.05*	15.00	2.50
73.3	120	15.00	7.50	2.50
36.7	140	17.14	5.71	.00
14.7	69	7.24	7.24	1.44
10% Ethanol	260	12.30	7.30	2.69
Drilled Control	80	38.75	6.25	.00
Control/ Control	280	8.21	1.42	.35

** LD-50 4136.6 mg/kg

* Significantly different from solvent ($P \leq 0.05$)

Table 2

Sodium Nitrate
Air Cell At 96 Hours

Dose mg/kg	Number of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
440.0	20	100.00*	.00	.00
366.7	119	90.75*	3.36	.84
220.0	39	71.79*	10.25	.00
183.3	99	74.74*	6.06	2.02
110.0	37	62.16*	16.21	2.70
73.3	117	35.89*	16.23*	9.40*
36.7	138	10.14	6.52	1.44
14.7	120	10.83	5.83	3.33
10% Ethanol	260	7.69	7.30	3.07
Drilled Control	80	3.75	5.00	1.25
Control/ Control	280	8.21	1.42	.35

** LD-50 117.35 mg/kg

* Significantly different from solvent ($P \leq 0.05$)

Table 3

Sodium Nitrate
Yolk At 0 Hours

Dose mg/kg	Number of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
586.7	50	60.00*	8.00	2.00
366.7	118	64.40*	9.32	.84
183.3	119	72.26*	10.08	5.04
73.3	117	46.15*	8.54	1.70
36.7	119	35.29	8.40	2.52
14.7	69	23.18	7.24	1.44
10% Ethanol	200	26.50	8.00	2.00
Pierced Control	60	16.66	1.66	1.66
Control/ Control	280	8.21	1.42	.35

** LD-50 380.5 mg/kg

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

Table 4

Sodium Nitrate
Yolk At 96 Hours

Dose mg/kg	Number of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
733.3	50	64.00*	10.00*	2.00
366.7	100	42.00*	11.00*	3.00
183.3	98	36.73*	10.20*	4.08
73.3	98	40.81*	12.24*	3.06
36.7	120	33.33*	6.66	3.33
18.3	20	5.00	5.00	.00
14.7	50	10.00	6.00	.00
7.3	20	5.00	.00	.00
3.7	20	10.00	10.00	.00
1.5	20	10.00	.00	.00
10% Ethanol	198	20.70	2.52	1.01
Pierced Control	60	38.33	6.66	1.66
Control/ Control	280	8.21	1.42	.35

** LD-50 not achieved

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

Table 5 Body Weight Data - Post Hatch Response

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(Males)

Test Dose (1)	Time/Route	Week 1	2	3	4	6	8	10	12
---	Control/Control	72	127	210	297	547	888	1174	1360
366.7	0/AC	73	126	201	292	488	791	989	1200
73.3	0/AC	77	130	226	329	569	965	1227	1414
366.7 & 183.3	0/Y	72	126	209	312	532	806	1086	1289
14.7	0/Y	77	128	248	314	569	903	1175	1351
183.3	96/AC	71	121	201	295	478	785	1007	1205
36.7	96/AC	73	126	213	313	537	1009	1104	1311
366.7	96/Y	67	120	210	301	535	818	1055	1258
36.7	96/Y	75	124	214	311	534	832	1094	1306

(1) Milligrams/Kilogram of body weight.

Table 6 Body Weight Data - Post Hatch Response

FDA 71-7: Sodium Nitrate

(Females)

Test Dose (1)	Time/Route	Week 1	2	3	4	6	8	10	12
---	Control/Control	73	123	206	291	491	742	974	1146
366.7	0/AC	73	120	186	278	464	666	858	1006
73.8	0/AC	69	115	193	280	462	770	973	1131
366.7 & 183.3	0/Y	66	117	175	252	409	618	825	963
14.7	0/Y	69	111	185	239	457	687	810	929
183.3	96/AC	73	119	194	274	460	704	818	1018
36.7	96/AC	78	126	206	288	446	610	830	985
366.7	96/Y	71	113	193	272	442	625	892	991
36.7	96/Y	66	106	186	268	456	664	878	1029

(1) Milligrams/Kilogram of body weight.

Table 7
Sodium Nitrate
Histopathology - Grow Out Birds

Histologic Observations	O/AC 366.7 MG/KG (10 Birds)	96/Y 366.7 MG/KG (10 Birds)
Thyroid/Parathyroid		
degenerate		1
Spleen		
diffuse pigmentation (moderate or mild)	2	2
numerous concretions	1	
degenerate		1
Liver		
diffuse black concretions	3	
focal areas of cellular infiltration	1	1
degenerate		1
degenerate at periphery	1	
pigmentation	4	6
Small Intestine		
degenerate		1
Colon/Bursa		
degenerate		2
Pancreas		
degenerate	1	2
Kidneys		
degenerate	1	2
Testicles		
degenerate	1	2
immature	1	1

Table 7 (Cont.)
Sodium Nitrate
Gross Pathology - Grow Out Birds

Gross Observations	O/AC 366.7 MG/KG (10 Birds)	96/Y 366.7 MG/KG (10 Birds)
Loss of Equilibrium	1	
Spleen		
atrophied	1	
Crop		
enlarged, filled with fluid	1	
Limb		
tarso-metatarsus broken and healed	1	
perosis	1	