Evaluation of chemicals for Toxic & Teratogenic effects using the chick embryo as the test system-FDA Contract #71-331 **Ammonium Saccharin** FDA 71-2
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WARF INSTITUTE, INC.
MADISON, WISCONSIN

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

AMMONIUM SACCHARIN: FDA 71-2

WARF Institute, Inc.
Madison, Wisconsin

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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.

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 Ammonium Saccharin are presented in the following tables:

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 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 retarda-

tion, 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 Ammonium Saccharin was employed in the test system.

Significant toxicity (P.05) was seen in the 0 hour air cell treatments at 36.7, 73.3, 110.0, 220.0, 366.7 and 440.0 mg/kg when compared with the solvent treated controls. The calculated LD-50 was 115.5 mg/kg. At 96 hours air cell, the toxicity was significantly elevated at levels of 55.0, 73.3, 91.7, 100.0 and 183.3 mg/kg with an LD-50 of 57.1 mg/kg.

In 0 hour yolk treatments, toxicity was significant at dose levels of 36.7, 73.3, 220.0 and 440.0 mg/kg, however, the data were somewhat erratic with a calculated LD-50 of 4101.5 mg/kg. In the 96 hour yolk treatment, toxicity was significant only at the highest dose level - 440.0 mg/kg. The slope is minimal and the calculated LD-50 of 191,793 mg/kg is therefore meaningless.

Total percent abnormalities were significantly elevated in the air cell and yolk treatments, at both 0 and 96 hours.

At 0 hour air cell, the primary contributor to the total ab-

normalities was hypopigmentation. This condition was seen at all dose levels and was generally dose related. At the upper dose levels, 110.0, 220.0 and 440.0 mg/kg, all of the chicks which hatched were involved. The severity of hypopigmentation seen was moderate to severe. At dose levels of 36.7 and 73.3 mg/kg the hypopigmentation was moderate to slight and most of the hatched chicks were affected.

At 110.0 and 73.3 mg/kg dose, one occurrance each of small kidney and absence of kidney was seen. These abnormalities are seen at a level of .03% and .06% in the 3,315 egg flock background but were not seen in control eggs for this time and route.

At the 440.0 mg/kg dose level there was a single instance each of limb abnormalities including micromelia, malposition, toe missing, and exostosis on hock. At 73.3 mg/kg a single instance of leg malposition and fused toes was seen. These abnormalities of the limb had not been seen in the solvent or untreated control eggs at this time and route nor had they been observed in the flock background. Similarly at 220.0 mg/kg a single instance of anophthalmia and ablepharia was found which had not been seen in control eggs for this time and route or in the flock background. The flock background involves observations on a total of 3,315 eggs.

In the 96 hour air cell treatment, the major contributor to total abnormalities was hypopigmentation. The severity in hatched chicks was moderate to severe at dose levels of 110.0 and 91.7 mg/kg, slight to moderate at 73.3 mg/kg, moderate at 55.0 mg/kg and slight at 18.3 mg/kg. Retarded development was also seen, however, was not remarkably elevated in its incidence when compared to control eggs or the flock background.

In the 0 hour yolk treatments, total percent abnormalities were significantly elevated at the 6 upper dose levels. Hypopigmentation was the primary anomaly with all of the hatched chicks involved. At 440.0, 220.0 and 366.7 mg/kg, the severity was moderate to severe; moderate at 110.0 and 73.3 mg/kg and slight at 36.7 mg/kg. Retarded development was seen at this time and route but was also seen in the control eggs with similar frequency. Other low incidence abnormalities seen were short mandible and flexed mandible which had not been seen in the control eggs for this time and route but had been seen at low levels in the flock background. One occurrence of small kidney was seen at the 110.0 mg/kg dose, however, one occurrence each of small kidney and kidney absent was seen in the solvent treated eggs for this time and route.

At 96 hour yolk, the total percent abnormalities were significantly elevated at all 6 dose levels. As in the previous times and routes, hypopigmentation was the predominant abnormality which was moderate to severe at 440.0, 366.7 and 220.0 mg/kg; moderate to slight at 110.0 and 36.7 mg/kg and slight at 14.7 mg/kg. All of the hatched chicks were involved at the upper 4 dose levels and a portion of the hatched chicks at the lower 2 dose levels. A low level incidence of abdominal wall fusion failure was seen at dose levels of 440.0, 220.0, 14.7 and 110.0 mg.kg which was not observed

in the control eggs at this time and route. This condition had been observed at a level of .09% in the flock background. At 440.0 and 220.0 mg/kg, a single instance of total or partial kidney agenesis was observed. This condition had not been observed in the control eggs for this time and route but had been seen at a level of .06% in the flock background.

The normal down color for chicks of this breed is pale yellow. In this experiment the chicks with severe hypopigmentation were essentially "chalk white." Moderate hypopigmentation down color was intermediate in paleness and the down of the slight hypopigmentation classification was nearly normal but distinguishable from the down color of untreated control chicks.

Dwarfism or retarded development was an 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.

Post hatch birds were raised to 10 weeks of age and included chicks from the untreated control and various treatment groups as indicated in the attached tables. Body weight gain and feed consumption were considered normal for all groups. As the chicks with light down became older, and their final feathering was established, the feather color was normal.

Five male and 5 female birds from the 0 hour yolk, 440.0 and 110.0 mg/kg, dose levels were sacrificed at approximately 10 weeks of age and examined grossly. No gross abnormalities were seen. Tissues from these chicks were subjected to histopathological examination and were compared with tissues of similar age untreated controls. The tissue alterations were minimal in nature and rather randomly distributed with the exception of mild to moderate atrophy of glomeruli in the kidney. This lesion was seen in 6 of 10 birds at the 440.0 mg/kg dose and in 5 of 10 birds in the 110.0 mg/kg dose but was not seen in the control tissues. In view of the occasional gross kidney abnormalities seen in this experiment, this microscopic lesion may warrant further study.

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

Conclusion:

Under the conditions specified for this trial, Ammonium Saccharin was particular toxic at the 96 hour air cell administration and produced significantly elevated levels of abnormalities in the air cell and yolk treatments at both 0 and 96 hours. The major effect was hypopigmentation, however, in the 0 hour air cell a very low incidence of eye and limb abnormalities were seen which suggest a possible permanent teratogenic effect of the test compound at levels of 73.3, 220.0 and 440.0 mg/kg.

Signed RM Bodden

By and For WARF Institute, Inc.

October 30, 1974

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REPORT

Test Sample: Ammonium Saccharin

Identification: FDA 71-2

Solvent System: 50% Absolute Ethanol in sterile distilled H_2O

Breeder Flock: N-1

Preliminary Range Finding Experiments

Experiment No.	Initiated
24	4-20-72

Dose Response Experiments

Experiment No.	Initiated
29	5-22-72
45	9-11-72

Table 1
Ammonium Saccharin
Air Cell At 0 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
440.0	100	94.00*	8.00	2.00
366.7	9	66.66*	66.66*	.00
220.0	108	80.55*	24.07*	2.77
110.0	110	60.00*	39.09*	1.81
73.3	100	30.00*	75.00*	2.00
36.7	110	22.72*	44.54*	.00
14.7	10	10.00	.00	.00
50% Ethanol	150	10.00	5.33	1.33
Drilled Control	60	11.66	5.00	.00
Control/ Control	240	7.91	4.58	.83

** LD-50 115.5 mg/kg

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

Table 2
Ammonium Saccharin
Air Cell At 96 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
183.3	9	100.00*	.00	.00
110.0	109	89.90*	18.34*	1.83
91.7	100	88.00*	26.00*	4.00
73.3	99	51.51*	59.59*	7.07
55.0	110	57.27*	17.27*	.90
18.3	110	8.18	43.63*	1.81
7.3	10	10.00	.00	.00
50% Ethanol	149	9.39	7.38	4.69
Drilled Control	60	5.00	1.66	.00
Control/ Control	240	7.91	4.58	.83

** LD-50 57.1 mg/kg

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

Table 3
Ammonium Saccharin
Yolk At 0 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
440.0	100	39.00*	66.00*	.00
366.7	9	44.44	66.66*	.00
220.0	110	45.45*	72.72*	4.54
110.0	110	27.27	81.81*	4.54
73.3	100	31.00*	77.00*	1.00
36.7	110	30.90*	45.45*	3.63
14.7	10	20.00	10.00	.00
50% Ethanol	150	18.00	8.00	2.66
Pierced Control	59	15.25	6.77	3.38
Control/ Control	240	7.91	4.58	.83

** LD-50 4101.5 mg/kg

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

Table 4
Ammonium Saccharin
Yolk At 96 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal Total	Percent Abnormal Structural
440.0	100	27.00*	81.00*	12.00*
366.7	10	30.00	100.00*	.00
220.0	110	16.36	91.81*	4.54
110.0	110	15.45	85.45*	.90
36.7	110	6.36	53.63*	2.71
14.7	110	14.54	42.72*	10.90*
50% Ethanol	149	12.08	8.72	.67
Pierced Control	60	16.66	6.66	1.66
Control/ Control	240	7.91	4.58	.83

** LD-50 191,793.9 mg/kg

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

Table 5
Body Weight Data - Post Hatch Response
FDA 71-2: Ammonium Saccharin
(Males)

Test Dose (1)	Time/Route	Average Individual Body Weight - Grams						
		Week 1	2	3	4	6	8	10
-	Control/Control	76	130	209	297	597	793	1077
110.0	0/AC	78	135	224	313	629	864	1202
36.7	0/AC	70	118	205	287	576	757	1147
440.0	0/Y	76	126	213	297	575	749	1117
110.0	0/Y	78	131	218	315	609	828	976
73.3	96/AC	65	109	199	286	565	792	1134
18.3	96/AC	70	116	213	262	586	791	1126
440.0	96/Y	75	125	218	305	583	787	1175
14.7	96/Y	72	126	220	306	612	827	1177

(1) Milligrams/kilogram of body weight

Table 6
Body Weight Data - Post Hatch Response
FDA 71-2: Ammonium Saccharin
(Females)

Test Dose (1)	Time/Route	Average Individual Body Weight - Grams						
		Week 1	2	3	4	6	8	10
-	Control/Control	73	120	190	259	486	644	918
110.0	0/AC	70	115	185	254	491	634	1018
36.7	0/AC	74	117	193	266	494	662	939
440.0	0/Y	68	111	180	251	502	651	948
110.0	0/Y	78	116	185	266	500	520	947
73.3	96/AC	70	112	202	275	511	686	924
18.3	96/AC	67	103	182	235	454	605	824
440.0	96/Y	67	113	194	268	501	655	911
14.7	96/Y	69	124	202	276	518	684	965

(1) Milligrams/kilogram of body weight

Table 7
Ammonium Saccharin
Histopathology - Grow-Out Birds

Histologic Observation	Negative Control (10 Birds)	O/Y 440 MG/KG (10 Birds)	O/Y 110 MG/KG (10 Birds
Thyroid			
focal areas of cellular infiltration	1	3	3
area of cellular infiltration under capsule	1		
less colloid present (but normal)		5	8
diffuse mild congestion		1	
Lungs			
congested	3		
Heart			
focal areas of cellular infiltration	1		
Spleen			
peripheral pigmentation	8	2	2
diffuse mild pigmentation		7	7
diffuse severe congestion		1	
Liver			
diffuse pigmentation	1		
mild/moderate peripheral pigmentation	6	3	1
focal areas of pigmentation			1
focal areas of cellular infiltration	9	3	3
diffuse vacuolization			1
Proventriculus			
focal areas of cellular infiltration	1		
Bursa			
focal encapsulated area (but normal)		2	
small vacuolated areas (but normal)			1
Pancreas			
degenerate	1		
Kidneys			
glomeruli degenerate		1	
mild/moderate atrophy of glomeruli		6	5
focal area of cellular infiltration			1
Gonads			
testicles immature cells	1	1	1
testicles degenerate cells	1		2
Bone Marrow			
numerous fat vacuoles	2		