UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: EPA Identification No. 241-76 - Acute Delayed Neurotoxicity of Malathion in the Hen

MRID NO:
40939301
41371401

TOX Chem No.: 535
Proj. No.: 9-1554
Record No.: 246025

FROM: Brian Dementi, Ph.D., D.A.B.T. Brian Dementi 9/25
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TO: William H. Miller, PM 16
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THRU: Edwin Budd, Section Head
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You will find appended the Data Evaluation Report for the Hen Delayed Neurotoxicity Study of AC6,601 (Malathion).

It will be necessary for the registrant to submit the correct (complete) version of Table II (Individual Body Weight Data).

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Reviewed By: Brian Dementi, Ph.D., D.A.B.T. Brian Dementi 9/5/89
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DATA EVALUATION REPORT 007524

Study Type: Delayed Neurotoxicity - Hen

TOX Chem No.: 535
MRID NO.: 409393-01

Record No.: 246025

Test Substance: AC6,601

Synonyms: Malathion; Phosphorodithioic Acid, S-[1,2-bis(ethoxycarbonyl)ethyl]O,O-dimethyl ester

Study No.: BLAL 87 DN 109

Sponsor: American Cyanamid Company
Princeton, NJ

Testing Facility: Bio-Life Associates, Ltd.
Neillsville, WI

Title of Report: 42-Day Neurotoxicity Study With AC6,601 Technical in Mature White Leghorn Hens.

Author: D.W. Fletcher, B.S.

Report Issued: April 1, 1988

Classification:

Core-Supplementary [can be considered for upgrading upon submission of Table II (Individual Body Weight Data) in a complete form].

Conclusions:

1. AC6,601 LD_{50} = 775 mg ai/kg

2. Atropine sulfate at 30 mg/kg was shown to protect hens from the lethal effects of AC6,601 to the extent that the delayed neurotoxicity study with AC6,601 could be successfully conducted using 1007.5 mg ai/kg (or 1.3 times the LD_{50} dose) for the first 21-day phase of the study and 852.5 mg ai/kg (or 1.1 times the LD_{50} dose) during the second 21-day phase of the study.

Nonetheless, 65 percent of the hens died in the first phase, and 33 percent of those remaining at the end of the first phase, died in the second phase. Thus of 60 hens initially, fourteen survived the full 42 days. Ten of these were examined histopathologically at termination (10 hens in the treated control and in the positive control (TOTP) were similarly examined).

3. AC6,601 did not exhibit evidence of delayed neurotoxicity while the positive control at 500 mg/kg elicited the expected neurotoxicity response in all hens examined.

4. The registrant must submit a complete version of Table II (Individual Body Weight Data).

5. AC6,601 treated and positive control hens lost weight relative to mean body weight values for treated controls.

6. AC6,601 treated hens consumed less food than did treated controls during the first several days following the initial dose of each of the 21-day segments. Subsequently in both segments, surviving hens consumed essentially the same amount of food as the treated controls. During days 28 to 42, AC6,601 hens actually consumed more food than did treated controls.

   Positive controls consumed less than treated controls for the first 6 days, recovered somewhat during days 7 to 9, only to then consume less during days 10 to 16. All positive control hens were sacrificed in extremis on day 16 during the first segment of the study.

A. Materials:

1. Test Compound - AC6,601 (Technical)

Description: Not provided
Lot No.: 5561-92
Purity: 93.6% (p. 69)

2. Test Animals

Species: Hen
Strain: White Leghorn (Gallus gallus)
Age: 12 months (minimum)
Weight: 1.1 to 2.2 kilograms (p. 53)
Source: Gilman's Indian Hill Egg Ranch
Wisconsin Rapids, WI (p. 53)

B. Study Design:

(The following statement is quoted or paraphrased from pages 10 to 19 of the study.)

"The study was conducted in three phases. These included the determination of the approximate LD_{50} for AC6,601; the assessment of the antidotal effect of atropine sulfate in order to determine dosage levels necessary to protect birds from doses of AC6,601 which should be equal to or exceed the LD_{50} when conducting the neurotoxicity assay; and the determination of the potential neurotoxicity of AC6,601.

"All hens selected for the study were observed during an 82-day quarantine period in order to assure that disease-free animals were used in the various phases of the study. All groups of hens were housed in suitable quarters separate from each other. Purina granular meal and water were available to the animals on an ad libitum basis. The light/dark cycle for all birds was approximately 8 hours of light and 16 hours of dark.

"1. Acute Oral Toxicity - LD_{50} Study - This study was conducted in two segments. The first segment was a range-finding study in which initially eight birds were assigned in groups of two to each of four groups which were administered dosages of either 681, 1000, 1470, or 2150 mg ai of AC6,601 per kg of body weight. The test material was administered as single doses via disposable syringes. All but one hen, dosed at 1000 mg ai/kg died during an 8-day observation period. Based upon these results, another range-finding study was conducted in similar manner, testing at dosage levels of 215, 464, or

681 mg ai/kg. In this study no birds died, however, there were clinical signs of severe toxicity, e.g., inability to stand, anorexia, paralysis, etc.

"In the second segment of the LD_{50} study, the above findings were used in selecting dosage levels. Five groups of four birds each were tested as before with either 316, 464, 681, 1000, or 1470 mg ai/kg. Following administration of test material via single dose injection, birds were observed for a 14-day period. Following this, the acute oral median lethal dose (LD_{50}) of the test material was estimated. Twelve additional chickens in three groups of four hens each were later dosed at doses of either 464, 681, or 1000 mg ai/kg in order to further substantiate the true value of the LD_{50}.

"2. Atropine Sulfate Efficacy Study - The purpose of this segment of testing was to determine the dose of atropine necessary to adequately protect birds from the toxicity of AC6,601 in order to permit birds to be dosed with malathion at dosages equal to or exceeding the LD_{50} for purposes of conducting the longer-term neurotoxicity assays.

"Accordingly, 28 birds were selected for the atropine sulfate efficacy study. The birds were divided into seven groups of four birds each. These birds had been acclimatized to laboratory conditions and their suitability as test birds had been established. They were maintained in a manner similar to that of those birds employed in the acute oral toxicity study. All birds were free from any visible signs of ill health or gait abnormalities. On October 1, 1987 six groups of four birds each were dosed with AC6,601 Technical at either the established LD_{50}, 1.1X, 1.5X, 2X, 4X, or 6X the LD_{50}, respectively. All birds in each of the above groups were administered atropine sulfate intramuscularly at 10 mg/kg of body weight at approximately 1 hour prior to dosing with AC6,601 Technical. Also, because of the physical condition of the birds in each of the above groups, atropine sulfate was administered intramuscularly at 30 mg/kg of body weight at approximately each of the following intervals: 1/2, 1, 3, and 5 hours postdosing. All surviving birds were observed daily for a 14-day period following test material administration. Four birds were dosed at the established LD_{50} level on October 14, 1987. One of these birds died and the remaining three were observed until October 20, 1987 when the neurotoxicity study was initiated.

"Following this, the results were used to establish the appropriate level above the established unprotected LD_{50} at which the birds in the neurotoxicity phase could be dosed with AC6,601 Technical and be protected by the antidotal effects of atropine sulfate (pp. 13-14).

"3. Neurotoxicity Study - Ninety birds were selected for the neurotoxicity phase and were divided into three groups as follows: treated control (TC) of 15 birds; positive control group (PC) of 15 birds; and a test group (T-I) of 60 birds. These birds had been acclimatized to laboratory conditions and their suitability as test birds had been established. They were maintained in a manner similar to that of those birds employed in the acute oral toxicity and atropine sulfate efficacy studies. All birds were free from any visible signs of ill health or gait abnormalities (p. 15).

"Following an approximate 17-hour fast with water permitted, birds in the test group were each given a single dose of AC6,601 Technical at a rate of 1007.5 mg ai/kg of body weight (1.3 times the determined unprotected LD_{50} of 775 mg ai/kg of body weight). The dosage level in the test group was determined after assessing the atropine sulfate efficacy study results. Atropinization of the test birds at 10 mg/kg of body weight took place at approximately 1 hour prior to test material administration, and at 30 mg/kg of body weight at approximately 1/2, 1, 3, and 5 hours postdosing. The atropine sulfate was administered intramuscularly (pectoral muscles) to all birds.

"On test day 21, following an approximate 18-hour fast with water permitted, birds in the test group were again each given a single dose of AC6,601 Technical at a rate of 852.5 mg ai/kg of body weight (1.1 times the determined unprotected LD_{50} of 775 mg ai/kg of body weight). The dosage level in the test group was lower than that administered at 0 hour on test day 1 to insure adequate survival for histopathology on test day 42. Atropinization times and rates were again the same as those employed at 0 hour on test day 1.

"Positive control birds each received a single dose of 500 mg/kg of body weight of tri-o-tolyl phosphate (TOTP) at 0 hour on test day 1.

"Treated control birds were atropinized at the same rates and times on test days 1 and 21 as the test birds. Treated control birds each received only 1.87 mL tap water at 0 hour on test day 1 and only 1.15 mL tap water on test day 21. These volumes were selected because they represented the largest quantities of test material that were administered in the test group at these intervals.

"All birds were observed daily for mortality and possible neurotoxicological signs during both 21-day observation periods. Body weights were recorded at 0 hour on test day 1 and on test days 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, and 42, were applicable. Final body weights were obtained for the positive control birds on test day 16. Group food consumption values were determined at each weighing interval.

"The birds in each group were evaluated on a daily basis for possible signs of neurotoxicity. The grading system used was as follows:

Grade	Criteria
0	No neurological signs
1	Generalized weakness with or without slight intermittent ataxia
2	Slight continuous ataxia
3	Moderate to severe continuous ataxia
4	Bird unable to stand, sits on haunches
5	Bird unable to stand, paralysis of legs and wings

"All birds that died during the study received thorough necropsy examinations. At the end of the study, all survivors were sacrificed under the supervision of a board certified pathologist, Donovan E. Gordon, D.V.M. The procedure consisted of intravascular perfusion of the entire carcass of the animal with 10% neutralized formalin. The procedure was performed according to the following steps:

Step 1: The bird was anesthetized by injection of a barbiturate into the wing vein (Bio-Tal, Bio-Ceutic Laboratories, Inc., 2% solution, approximately 1 to 1.5 mL).

Step 2: The bird was placed in dorsal recumbency and positioned in a spread-eagle position.

Step 3: The feathers were wet down, the bird skinned back, incised, and the keel bone deflected toward the head.

Step 4: The pericardial sac was cut, exposing the heart. The apex of the heart was grasped and a small incision was made in the left ventricle. A perfusion needle was inserted into the left ventricle and up into the aorta and - using a hemostat - the needle clamped below the ball end.

Step 5: Perfusion was begun with heparinized buffer solution with a peristaltic pump setting of two to three. The right atrium (right ventricle) was cut, allowing the returning blood and fixative to exit, avoiding any build-up of pressure within the vascular system.

Step 6: When the blood had been flushed, a 10% buffered formalin was used. Perfusion was continued for 10 to 15 minutes. Perfusion produced muscular twitching and a blanching and firmness of the tissues.

"Following perfusion, a thorough necropsy examination was performed on each bird. The entire length of both sciatic nerves was dissected from the legs of each bird, then affixed to a tongue depresser blade to prevent curling and placed in a 10% neutral buffered formalin for fixation. The brain was removed and allowed to fix in the same fixative. The vertebral column was removed, transected into three sections (cervical, thoracic, lumbosacral) and the spinal cord was also fixed in situ. These specimens remained in the fixative prior to further handling. The cord was then removed from the vertebral column.

"The tissue specimens selected from each bird for histological studies consisted of the following:

"a. A midsagittal section of the entire brain (corpus striatum, cerebellum, pons);

"b. A longitudinal and cross section of two segments of cervical, thoracic, and lumbosacral portions of the spinal cord. In an attempt to obtain

specimens from a uniform location, the proximal and distal portions of each of the above spinal cord segments were selected when possible. Those areas of cord which were traumatized during removal from the vertebral column were avoided in selection of the above multiple spinal cord sections; and

"3. Longitudinal (distal) and cross sections (proximal) of the sciatic nerves were selected (both legs).

"The above tissues from sacrificed birds of the treated control (10), positive control (10), and test birds receiving AC6,601 Technical (10) were processed by conventional histological methodology, embedded in Paraplast (Sherwood Industries, St. Louis, Missouri) and sectioned at 6 to 7 microns.

"Sections of all neural specimens were stained with hematoxylineosin (H&E) stain. Replicate neural tissues were stained with Luxol Fast Blue for myelin. All stained tissue sections were examined by light microscopy. Microscopic studies were conducted on birds of the treated control (10), positive control (10), and the test group (10)" (pp. 15-19).

Results:

1. Acute Oral Toxicity-LD_{50} Study - Based upon data provided in Table I (p. 21) of the study, which presents numbers of animal deaths at each dose tested, the LD_{50} for AC6,601 was estimated at 775 mg ai/kg with 95% confidence limits of 610 to 984 mg ai/kg. Data from the range-finding phase of the LD_{50} study were not included in the LD_{50} calculation.

2. Atropine Sulfate Efficacy Study - As explained in the procedure, all birds in the various study groups received 10 mg of atropine sulfate/kg at 1 hour prior to administration of AC6,601. Atropine sulfate was again administered intramuscularly at 30 mg/kg at approximately 1/2, 1, 3, and 5 hours postdosing. Within 9 days after dosing, the following table indicates the percentage of birds in each group of four hens that died:

	$\frac{LD_{50}\ (X)}{100}$	$\frac{1.1X}{25}$	$\frac{1.5X}{75}$	$\frac{2X}{75}$	$\frac{4X}{100}$	$\frac{6X}{100}$
% Dead						

Signs of toxicity included lethargy, stumbling, sitting, anorexia, ataxia, inability to stand, etc.

"All surviving birds appeared to be normal and active within 10 days postdosing. All surviving birds were observed for 14 days following dosing. No signs of delayed neuropathy were noted in the surviving birds (p. 22).

"Subsequently, four additional birds were retested at the LD_{50} (X) in the same manner as above. Only one bird died within 4 days of dosing. However, 25 percent died in this repeat study as opposed to 100 percent as reported above for the LD_{50} dosage group."

In consideration of these findings, the dosage level of 1.3(X) i.e., 1.3 times the established unprotected LD_{50}, was chosen for the neurotoxicity assay. However, during the second 21-day study phase of the neurotoxicity assay, 1.1X was employed to ensure adequate survival for histopathology on test day 42 (p. 23).

3. Neurotoxicity Study - [Note: In Table II, pages 29 and 30 are duplicates. The registrant must submit the complete version of Table II (Individual Body Weight Data)].

   a. Body Weight Data - Body weight data as revealed in Table II (p. 26) show that positive control hens and those treated with AC6,601 lost weight relative to the mean value for treated controls. In the first 21 days, treated control birds lost an average of 72 grams each while positive controls lost on the average of 249 grams each and AC6,601 treated animals lost an average of 234 grams. During the second phase of the neurotoxicity test, i.e., during days 21 to 42, treated controls gained an average of 160 grams each while AC6,601 hens gained an average of 192 grams each. This latter gain relative to gains in the control probably reflects a more pronounced rebound in the AC6,601 group consequential to the more profound loss for this group during days 0 to 21. For the 42-day study period, treated controls gained on the average 88 grams each while AC6,601 on the average lost 42 grams each. The difference for the 42-day study period was clearly statistically significant.

   b. Food Consumption Data - Relative to food consumption in treated controls. Data in Table III (p. 2), indicate that AC6,601 treated hens consumed markedly less food the first several days following dosing at days zero and 21 (the beginning of each phase of the study) and is attributable to the acute cholinesterase inhibition immediately following dosing. At subsequent time points, AC6,601 treated hens consumed

approximately the same as treated controls with hens apparently consuming a little more than controls during days 28 to 42, probably a rebound effect. The positive control group consumed less than treated controls for the first 6 days, appeared to have recovered somewhat during days 7 to 9 only to then consume less during days 10 to 18, probably the result of the onset of more long-term toxicity of TOTP.

c. Mortality-Survival - The following tabulation as reproduced from the study (p. 34) indicates the number of mortalities which occurred in the various groups.

Group	Test Material	Dose Level	No. Birds Tested	No. of Mortal Day 0 to 20	21
Treated Control	Tap Water	*	15	0	
Positive Control[a]	TOTP	500 mg/kg	15	0[a]	
Treated	AC6,601 Technical	1007.5 mg ai/kg (0 Hour)	60	39	
	AC6,601 Technical	852.5 mg ai/kg (Test Day 21)	21	N/A	

a/All positive control birds were sacrificed in extremis on day 16.
*/Treated control birds each received only 1.87 mL tap water at 0 hour on test day 1 while on test day 21, each received only 1.15 mL tap water.

All positive control (TOTP treated hens) were sacrificed in extremis (day 16) before the end of the initial 20-day segment of the study. Of 60 AC6,601 treated hens (those protected by atropine sulfate), 39 died during the initial 20-day segment of the study. Because so many hens died during this period, the laboratory investigators elected to reduce the dose for the subsequent dosing period from 1007.6 mg ai/kg (1.3X the LD_{50}) to 852.5 mg ai/kg (1.1X the LD_{50}) in the hope that sufficient numbers of animals would survive for the duration of the study period. Fourteen animals so survived.

d. Clinical Observations

1) Treated Controls - Data presented in Table IV (p. 37) show that with the exception of one bird that died on test day 33, all treated controls

survived to the end of the 42-day study period without exhibiting any neurotoxicological signs.

2) Positive Controls (TOTP treated) - This group of hens began exhibiting clinical signs of delayed neurotoxicity by test day 10 and from that point forward exhibited increasingly severe neurotoxicological symptoms (Table IV (p. 39)). All animals were sacrificed in extremis on day 16.

3) AC6,601 Treated Hens - Hens treated with AC6,601, even though protected with atropine sulfate, exhibited severe neurotoxicological symptoms by the first day postdosing, with such effects extending to about days 4 to 5. These hens were normal and active by postdosing day 6. Similarly, those animals surviving the initial dosing phase of the study, through day 20, experienced severe neurotoxicological symptoms during the first 4 days postdosing in the second phase of the study. By day 5 postdosing, the surviving hens were normal and active and remained so until the end of the 42-day study period.

Note: The study author describes the signs of delayed neurotoxicity exhibited by positive controls as: moderate to severe ataxia, inability to stand, paralysis of the legs and wings, generalized weakness, and sitting on haunches, while those signs of neurotoxicity in the AC6,601 group are described as: lethargy, anorexia, general weakness, slight to severe ataxia, inability to stand, sitting on haunches, diarrhea, paralysis of legs and wings, and pale comb (p. 35). This reviewer is not certain that the distinctions between delayed neurotoxicity and simply neurotoxicity are that clearly defined.

e. Gross Pathology - Table IV (pp. 37-41) presents individual gross neurotoxicological observations for the three study groups. The 15 hens in the treated control group did not exhibit any neurological signs of toxicity. One hen (#38) died on day 32-33 of the study, but did not exhibit gross evidence of neurotoxicity. All other treated controls survived the full 42-day study period.

All hens in the positive control group exhibited remarkable physical signs of neurotoxicity. However, gross examination of the entire group of 15 on day 16 (in extremis) revealed no abnormal tissues in 10 hens. The remaining five were emaciated.

One of these had a gas-filled cecum and another had a total of four small (1 mm), white foci on the surface of one, large liver lobe (p. 43).

The study report does not provide any table which summarizes or presents individual gross pathology findings. However, with respect of AC6,601 treated hens, the study author indicates that "gross pathological examination of the 39 test birds found dead within 15 days postdosing at 0 hour on test day 1 revealed no abnormal tissue alterations in 20 birds. Findings in the 19 other birds were as follows: nine birds had dilated intestinal vessels (especially the duodenal loops), six birds had hemorrhagic duodenal loops, six birds had enlarged vents (fluid and/or air-filled), two birds had clotted blood on the livers, two birds had a subcutaneous gel-like substance in the breast area, one bird had gas-filled intestines, and one bird had firm matter blocking the gizzard opening.

"Gross pathological examinations of the seven test birds found dead within 7 days postdosing from test day 21 revealed no abnormal tissue alterations in five birds. One bird was emaciated while the other had an extremely enlarged fluid-filled vent and dilated intestinal vessels with diffuse, red discoloration (p. 42).

"Gross pathological examination of AC6,601 treated birds sacrificed on test day 42 revealed no abnormal tissue alterations among 11 of the 14 birds examined. One bird had an enlarged spleen. Another had approximately 115 mL of clear fluid in the oviduct and numerous eggs in all stages of development were present. Finally, one bird had a 3 mm solitary focus of pale discoloration in one lobe of the liver" (p. 43).

f. Histopathology - Histopathologic findings are presented in tabular form in the "Histopathology Incidence Table" (pp. 64 to 68).

In examining this table, this reviewer is of the opinion that none of the 10 AC6,601 treated hens that were examined displayed evidence of neurotoxicity that differed in any test compound-related way from the 10 treated control hens that were examined. It is clear from the table that positive control animals displayed evidence of delayed neurotoxicity including marked axonal degeneration and demyelination of the spinal cord and sciatic nerve.

The study veterinary pathologist concluded that there were no treatment-related neural lesions among birds administered AC6,601. Such lesions were observed among all of the positive control birds which were compatible with those of TOTP induced delayed neurotoxicity (p. 63). Based upon the findings presented, this would appear to be the proper conclusion.