metabolite in egg yolk has not been determined in the present study. One possible explanation is that 1-naphthyl sulfate ion may be attracted to phospholipids of yolk, or the permeability of the membrane surrounding the yolk in the oviduct may be such that it permits a one-directional movement of 1-naphthyl sulfate into the yolk. Similar factors may also be involved in the higher quantities of peaks S_6 and S_7 in the yolk than in the white.

Unknowns A and B represented a larger percentage of the recovered radioactivity in the white than in the yolk. In particular, Unknown B constituted 46.3% of the white residues, as compared to 7.0% of that in the yolk. Because of the much larger total ^{14}C residues in yolk, the large percentage of Unknowns A and B in the white had relatively little effect on the overall greater amount of total ^{14}C residues in the yolk.

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Chronic Toxicity and Residues from Feeding Nemacide [*O*-(2,4-Dichlorophenyl) *O,O*-Diethyl Phosphorothioate] to Laying Hens

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Technical grade Nemacide [*O*-(2,4-dichlorophenyl) *O,O*-diethyl phosphorothioate] was administered in the feed of laying hens for 55 weeks at 50, 100, 200, and 800 ppm. Excellent control of the housefly, *Musca domestica* L., and the blowfly, *Chrysomya megacephala* (F.), was obtained in droppings from the hens fed 50 ppm but higher levels were required for control of larvae of *Parasarcophaga argyrostoma* (Robineau-Desvoidy) and *Fannia pusio* (Wiedemann). No hen mortality occurred that could be attributed to treatment but there was a direct relationship between Nemacide concentration and blood plasma cholinesterase inhibition. Feed consumption, weight gain, egg production, shell thickness, egg weight, egg quality, and odor were normal but eggs from hens treated with 800 ppm had a less desirable flavor than eggs from untreated hens. A glc electron capture method was developed to measure nanogram amounts of Nemacide and 2,4-dichlorophenol. The limits of detectability were 0.005 to 0.086 ppm for Nemacide and 0.006 to 0.208 ppm for 2,4-dichlorophenol. Appreciable residues of Nemacide were found in the liver, muscle, fat, and yolk of treated hens, while 2,4-dichlorophenol residues were detected only in the liver and yolk.

The ingestion of certain insecticides over a prolonged period of time by means of treated feed or water was found to have little detrimental effect on poultry, yet prevented the development of fly larvae in the droppings (Ross and Sherman, 1960; Sherman and Ross, 1960b; Sherman *et al.*, 1963, 1969). Sherman *et al.* (1967b) found the acute toxicity of Nemacide to 10- to 12-day-old cockerels to be 148 mg/kg and that an admixture of 800 ppm in the feed administered over a 2-week period, although causing 60% plasma cholinesterase inhibition, had no other detrimental effects on cockerels. Droppings from these treated chicks were highly toxic to the larvae of four species of flies (Sherman *et al.*, 1967a). The present paper reports the effects on laying hens of technical grade Nemacide [*O*-(2,4-dichlorophenyl) *O,O*-diethyl phosphorothioate] administered in the feed at 50, 100, 200, and 800 ppm over a 55-week period.

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TOXICOLOGICAL PROCEDURES

One-hundred and fifty pullets of a commercial strain of single-comb White Leghorns were used in this study. At 27 weeks of age, the pullets were leg-banded, weighed, and distributed at random into five treatment groups of 30 birds each. Each group was divided into three replicates of ten birds each. The birds were housed in individual laying cages, two birds per cage. The basal diet was a standard University of Hawaii layer ration (Sherman *et al.*, 1969). Technical grade Nemacide was added to the basal ration at the rates of

50, 100, 200, and 800 ppm and given to four of the treatment groups. The fifth group was designated as the control and continued to receive untreated feed and water. The birds were placed on the insecticide-treated diet for a total of 55 weeks.

Egg production was recorded daily for all birds. For measuring egg weights, albumen height (calculated as Haugh units) and shell thickness, eggs were collected on 1 day every 4 weeks throughout the course of the experiment. More than 900 eggs from treated hens were involved in these determinations. Feed consumption and individual body weights were also obtained every 4 weeks during the course of the experiment.

Manure was collected, approximately biweekly, from dropping pans set beneath the cages and the toxicity to first-instar larvae of the housefly, *Musca domestica* L., an anthomyid, *Fannia pusio* (Wiedemann), a blowfly, *Chrysomya megacephala* (F.), and a flesh fly, *Parasarcophaga argyrostoma* (Robineau-Desvoidy) was determined by methods described by Sherman and Ross (1960a,b) and Sherman *et al.* (1962). In order to determine whether there was any significant difference between larval mortality and adult emergence, the larval mortality was determined after 3 days and then the surviving larvae were replaced in the droppings and held for adult emergence. *F. pusio* mortality was determined after adult emergence only because the larvae were so well-camouflaged in the manure that they were difficult to detect.

Three series of organoleptic tests were held during the experimental period. These tests were conducted after 13, 36, and 54 weeks on experiment. Each series consisted of tests conducted on 3 consecutive days using either 10- or 11-member panels for each test. The panel was asked to evaluate eggs with respect to flavor and odor and to score the characteristics from 6 (best) to 1 (poorest). For each test, five eggs were chosen at random from hens receiving each concentration of Nemacide and five from hens on the untreated diet. These eggs were hard-cooked, allowed to stand overnight, and sliced the following morning just prior to testing.

Five hens from the third replicate of each treatment group were used to determine the effect of treatment on blood plasma cholinesterase activity. Blood was obtained by venipuncture 12 and 5 days prior to treatment: 1, 2, 4, 7, 14, and 29 days after start of treatment; and monthly for the duration of the experiment. Blood was also sampled 1, 2, 5, and 7 days after the hens were withdrawn from treated feed and placed on the untreated control ration. The blood plasma was analyzed colorimetrically as described by Cook (1954) and Fallscheer and Cook (1956).

Statistical analyses were made where applicable using Abbott's formula (Abbott, 1925), the one-tailed *t* test (Cochran and Cox, 1950), analysis of variance (Snedecor, 1946), and Duncan's multiple range test (Duncan, 1955).

Eggs were collected periodically throughout the course of the experiment. Whites and yolks were separated and 3–6 combined whites and the corresponding yolks were placed in plastic bags and quick-frozen. On the termination date of the experiment, 55 weeks after its initiation, five to eight hens from each of the treatments and control were slaughtered; the liver, breast muscle, and peritoneal fat were removed, placed separately in plastic bags, and deep frozen for future analysis. All remaining animals were removed from treated feed and placed on untreated feed. An additional 5–8 hens from each of the treated and control groups were slaughtered 7, 14, and 21 days after removal from the treated feed. During the period that the hens were on untreated feed, eggs were collected from the surviving hens for analysis.

ANALYTICAL PROCEDURES

Nemacide and its metabolite, 2,4-dichlorophenol, were detected by analytical procedures consisting of sample extraction, cleanup, and glc analysis utilizing electron capture detectors and a phosphorus sensitive thermionic detector.

Solvents were reagent grade, redistilled shortly before use; other common chemicals also were reagent grade. The Florisil, 60/100 mesh, obtained from Matheson Coleman and Bell was activated at 600°C for 3 hr. Standardized aluminum oxide was prepared as follows: Baker aluminum oxide for chromatographic work was activated at 800°C for 4 hr. After cooling, 5% distilled water was added and distributed by shaking the bottle until no lumps appeared. The aluminum oxide was used within 2 weeks after standardization. The reference standard solutions were prepared in hexane with analytical standards (99+%) of *O*-(2,4-dichlorophenyl) *O*,*O*-diethyl phosphorothioate and 2,4-dichlorophenol.

Extraction and Cleanup of Nemacide. For analysis of egg yolk, 20.0 g were blended with anhydrous sodium sulfate and extracted with 300 ml of hexane in a Soxhlet apparatus for 3 hr. The hexane extract was evaporated in a Rotavapor to 125 ml and extracted five times with 25 ml of acetonitrile. The combined acetonitrile fractions were diluted with 125 ml of distilled water and back extracted three times with 50, 25, and 25 ml of hexane, respectively. The combined hexane fractions were filtered through a 2–3-cm layer of anhydrous sodium sulfate in a funnel 50 mm top i.d. and the filtrate was evaporated to 5 ml. The residue was chromatographed on 20 g of aluminum oxide in a 450 × 16 mm glass column containing a 3-cm layer of anhydrous sodium sulfate above the aluminum oxide. Hexane (100 ml) was used as an eluent and the eluate was evaporated down to about 5 ml on a Rotavapor and transferred to a 10-ml volumetric flask. This solution or dilutions of this were used for the gas–liquid chromatographic analysis.

For analysis of egg whites, 20.0 g were blended with anhydrous sodium sulfate and extracted with 300 ml of hexane in Soxhlet apparatus for 3 hr. The hexane extract was evaporated to 5 ml and transferred to an aluminum oxide column and chromatographed as for egg yolk.

Peritoneal fat was melted in an oven at 65°C, passed through filter paper (Whatman No. 1), and 10.0 g were dissolved in 20 ml of hexane and transferred to a 125-ml separatory funnel by rinsing three times with 10 ml of hexane. The fat–hexane solution was extracted four times with 20 ml of acetonitrile saturated with hexane. The hexane phase was discarded and 400 ml of 2% sodium sulfate solution was added to the acetonitrile and shaken vigorously for 2 min. After 20 min the water phase was discarded and the hexane layer was passed through a 3-cm sodium sulfate layer in a funnel (5 mm top i.d.) and evaporated to 2–5 ml. The residue was chromatographed on an aluminum oxide column and processed as described for egg yolk.

For analysis of breast muscle, 100.0 g were cut into small pieces and blended for 10 sec in a Waring blender with 50 g of anhydrous sodium sulfate and 50 ml of hexane. An additional 50 g of sodium sulfate and 50 ml of hexane were added and the mixture was blended again for 20 sec. Another 50 ml of hexane was added and the mixture was blended for 30 sec, transferred to a Buchner funnel, and filtered under slight vacuum. The blender was rinsed with 50 ml of hexane and the rinse was poured over the filter cake. The filter cake was

returned to the blender, blended for 30 sec with 150 ml of hexane, and filtered. This treatment of the filter cake was repeated two more times. The combined filtrates were evaporated to 5 ml in the Rotavapor and chromatographed on an aluminum oxide column and processed as described for egg yolk.

For analysis of liver, 25.0 g were ground in a mortar with 200 g of anhydrous sodium sulfate and packed in a 600 × 40 mm glass column. The column was eluted with 400 ml of hexane and the eluate was evaporated to 125 ml. The eluate was extracted with acetonitrile and back extracted with hexane as described for egg yolk. The 5-ml residue was chromatographed on an aluminum oxide column as described for egg yolk, and the eluate was evaporated to 5 ml on the Rotavapor. This residue was chromatographed on 20 g of Florisil in a 450 × 16 mm glass column containing a 3-cm layer of anhydrous sodium sulfate above the Florisil. 5% diethyl ether in hexane (100 ml) was used as an eluent, and the eluate was evaporated to 5 ml on the Rotavapor, transferred to a 10-ml volumetric flask, and chromatographed.

Extraction and Cleanup of 2,4-Dichlorophenol. For analysis of egg yolk, 20.0 g were blended at low speed in a Waring blender with 100 ml of 95% ethanol, 100 ml of distilled water, and 2 ml of 1:1 concentrated sulfuric acid until the mixture was uniform. An additional 100 ml of hexane were added, and the mixture was blended at high speed for 1 min and poured into a 500-ml separatory funnel. The blender was rinsed three times with 5 ml of hexane and the rinses were added to the separatory funnel. After separation, the water phase was washed three times with 20 ml of hexane and then four times with 25 ml of benzene. The benzene and hexane washes were combined and the water phase was discarded. The benzene–hexane solution was evaporated to 10 ml and passed through a Florisil column (20 g of Florisil deactivated with 5% water) into a flask by eluting with 150 ml of hexane–benzene (1:1). The effluent was returned to the separatory funnel and the flask was washed with 10 ml of benzene, which was added to the separatory funnel. This benzene solution was washed five times with 25 ml of 0.1 *N* sodium hydroxide. The benzene was discarded and the aqueous layer which contained the free 2,4-dichlorophenol was collected, returned to the separatory funnel, and acidified with 10 ml of 1:1 concentrated sulfuric acid. This was washed three times with 15 ml of benzene. The water fraction was discarded and the benzene was passed over anhydrous sodium sulfate, evaporated to 3 ml, transferred to a 10-ml volumetric flask, and diluted to volume with hexane for glc analysis.

Peritoneal fat was melted in an oven at 65°C, filtered through filter paper (Whatman No. 1), and 10 g were dissolved in 20 ml of hexane and washed three times with 30 ml of 0.1 *N* sodium hydroxide. The combined water phases were acidified with 20 ml of 1 *N* sulfuric acid and rinsed three times with 30 ml of hexane. The combined hexane phases were filtered through a 3-cm anhydrous sodium sulfate layer in a funnel (50 mm top i.d.). The filtrate was evaporated to about 5 ml in the Rotavapor and transferred to a 10-ml volumetric flask for glc analysis.

For analysis of breast muscle, 100 g were extracted with hexane in a blender as described for Nemacide, except 5 ml of 1 *N* sulfuric acid were added in the blender. The hexane extract was evaporated to 30 ml, washed three times with 0.1 *N* sodium hydroxide, and the procedure described for fat was allowed.

For analysis of liver 25.0 g were ground, combined with anhydrous sodium sulfate, and eluted with hexane as described in the Nemacide procedure. The hexane eluate was evaporated to 50 ml and extracted five times with 10 ml of acetonitrile. The combined acetonitrile fractions were treated with 100 ml of 0.1 *N* sodium hydroxide and back extracted two times with 10 ml of hexane. The hexane fractions were discarded and the polar phase was acidified with 10 ml of 4 *N* sulfuric acid and rinsed three times with 15 ml of hexane. The combined hexane fractions were filtered through anhydrous sodium sulfate as previously described, evaporated to approximately 5 ml, and chromatographed on 20 g of Florisil (5% water deactivated) in a 450 × 16 mm glass column containing a 3-cm layer of anhydrous sodium sulfate above the Florisil. The column had previously been conditioned with 50 ml of hexane; 150 ml of hexane was used as an eluant; the eluate was evaporated, transferred to a 10-ml volumetric flask, and brought to volume with hexane for glc analysis.

Gas Chromatography. Two combinations of Aerograph instruments were used for the quantitative analysis of Nemacide and 2,4-dichlorophenol. Both utilized a Model 550-B oven monitored by a Linear Temperature Programmer Model 326. In one combination, a Model 600 electrometer was used and the recorder was a 1-mV Leeds & Northrup Model Speedomax H (full deflection, 5 in.; chart speed, 0.5 in./min). In the other combination an Autoprep Model 705 electrometer was used and the recorder was a 1-mV Minneapolis-Honeywell Model Elektronik 15 (full deflection, 10 in.; chart speed, 0.17 in./min). The instruments were equipped with tritium foil electron capture detectors using 90 V. The operating parameters for Nemacide analysis were: column, 5-ft × 0.125-in. Pyrex glass packed with Dow Corning Gas Chrom Q, 100–120 mesh; injector, 210–245°C; column oven (isothermal), 180–195°C; detector, 185–195°C; and nitrogen carrier gas, 40–50 ml/min. The operating parameters for 2,4-dichlorophenol were: column, 11.5-ft × 0.125-in. Pyrex glass packed with Gas Chrom Q, 100–120 mesh; injector, 225°C; column oven (isothermal), 100–119°C; detector, 190°C; and nitrogen carrier gas, 30–40 ml/min.

All samples and standard solutions were injected with a constant volume of 2 μl. The retention time for Nemacide was between 2 and 3 min and a linear dynamic range was shown between 0.02–2 ng. The retention time for 2,4-dichlorophenol was between 1.25 and 3.5 min and a linear dynamic range was shown between 0.2–2.0 ng. Peak heights were found to be directly proportional to concentration and were used for quantitation. Peak heights were measured to an accuracy of 0.5 mm.

Each series of analysis was composed of a reference standard, a tissue control sample, a fortified tissue control sample, and four to six unknown tissue samples. Injections of each sample were repeated until the maximum peak height variation was less than 2%. Each series of analysis was initiated and finished with the injection of the reference standard solution and, if more than 5% variation occurred between the initial and final reference standard peak heights, the series of analysis was repeated.

The presence of Nemacide in the tissues was confirmed qualitatively by the use of an Aerograph Autoprep Model 705 with a thermionic phosphorus detector using a cesium bromide pellet and having a detector voltage of 285 V. This Autoprep Model was used as an analytical instrument by plugging the effluent splitter unit so that all the carrier gas went through the detector. The recorder was a 1-mV Minneapolis-Honeywell Model Elektronik 15 (full deflection, 10 in.; chart

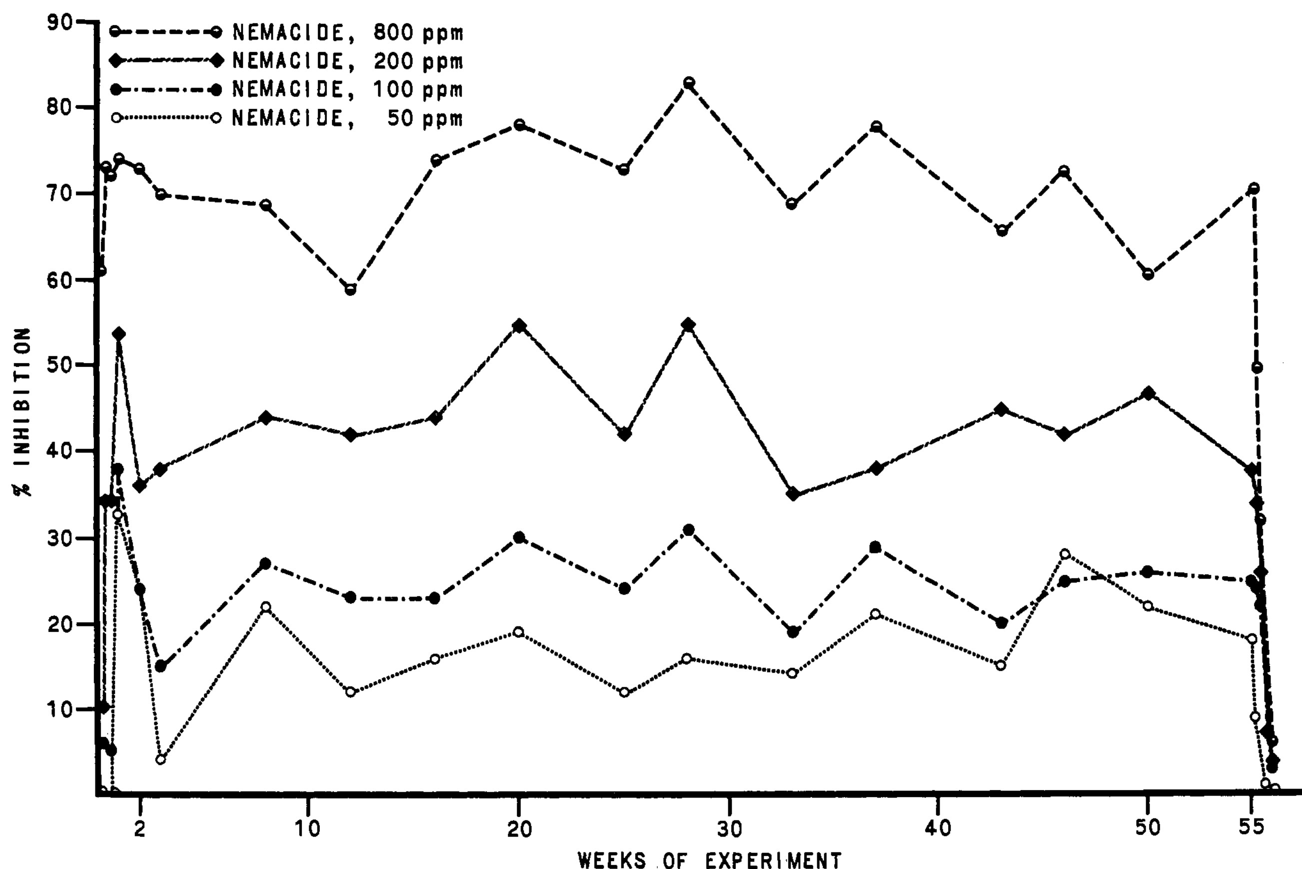

Figure 1. Inhibition and recovery of blood plasma cholinesterase in Nemacide-treated White Leghorn hens. Hens removed from treated feed after 55 weeks

Table I. Toxicity to Fly Larvae of Droppings from Hens Receiving Different Levels of Nemacide in Feed for 55 Weeks

Nemacide concentration in feed, ppm	Corrected % mortality[a]			
	M. domestica	*F. pusio*[b]	*C. megacephala*	*P. argyrostoma*
	After 3 days			
800	100	...	100	100
200	100	...	100	90
100	99	...	96	65
50	91	...	76	47
0	9	...	8	8
	After adult emergence			
800	...	100	...	...
200	...	82	...	97
100	100	45	99	85
50	97	22	91	70
0	23	7	24	22

[a] Mean values based on 78 replications of ten larvae each. [b] Mortality determined only after adult emergence.

speed, 0.17 in./min). The other operating parameters were: column, 5-ft × 0.125-in. Pyrex glass packed with 1% PDEAS (phenyl diethanolamine succinate) on Gas Chrom Q, 100–120 mesh; injector, 220°C; column oven, 170°C; detector, 215°C; nitrogen carrier gas, 20 ml/min; air, 170 ml/min; hydrogen flow was adjusted so that the background current from the flame detector was 1.6×10^{-9} A. A Nupro B-2M Fine Metering valve was used to adjust the hydrogen flow.

RESULTS AND DISCUSSION

Fly Mortality. Table I summarizes the toxicity of droppings from the hens to larvae of *M. domestica*, *F. pusio*, *C. megacephala*, and *P. argyrostoma*, 3 days after placement into the droppings and after adult emergence. Housefly and blowfly control was excellent in droppings from hens fed 50 ppm of Nemacide. Higher levels of Nemacide were required for control of larvae of *P. argyrostoma* and *F. pusio*. There was no significant difference between larval mortality after 3 days exposure to insecticide-containing droppings and after adult emergence.

Hen Mortality. During the experimental period, 23% of the control hens died. Mortality among the treated birds was 20, 17, 28, and 13% for groups of hens receiving 800, 200, 100, and 50 ppm of Nemacide, respectively, in the diet. Autopsy indicated that over one-third of all mortality was due to avian lymphoid leucosis.

***In Vivo* Cholinesterase Inhibition.** Figure 1 summarizes the effect on blood plasma cholinesterase activity of Nemacide treatment and withdrawal from treatment. Inhibition was rapid; a high level was reached within 1 week after placement on treatment. There was a direct relationship between Nemacide concentration in the feed and enzyme inhibition. The concentrations and the resultant mean enzyme inhibition follow: 800 ppm, 71.4%; 200 ppm, 43.7%; 100 ppm, 25.3%; and 50 ppm, 18.3%. Blood plasma cholinesterase of laying hens appears to be more susceptible to *in vivo* inhibition than that of 2-week-old cockerels. In earlier work (Sherman, 1963; Sherman *et al.*, 1967b) it was found that cockerels exposed to similar concentrations of Nemacide in the feed exhibited the following pattern of inhibition: 800

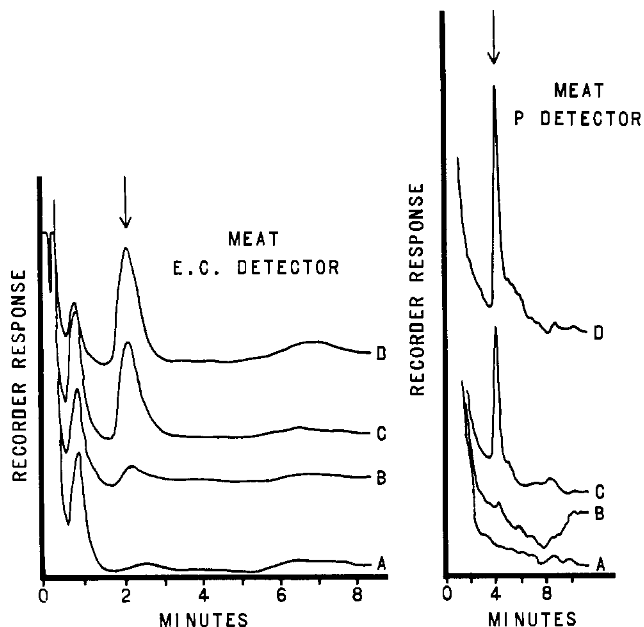

Figure 2. Typical chromatograms of Nemacide in breast muscle using electron capture and confirmatory phosphorus detection. A. From control hen. B. From hen receiving 100 ppm of Nemacide in feed for 55 weeks. C. From hen receiving 800 ppm of Nemacide in feed for 55 weeks. D. Control tissue fortified with 0.1 ppm of Nemacide. Arrow represents the retention time of Nemacide

ppm, 60%; 400 ppm, 31%; 200 ppm, 18%; 100 ppm, 0%; and 50 ppm, 0%. Recovery in laying hens of enzyme activity to normal levels was rapid. Within 5 days of removal of Nemacide from the diet, full recovery of enzyme activity had occurred.

Body Weight. There was no detrimental effect on body weight caused by ingestion of Nemacide. All birds gained some weight during the experimental period, ranging from 96 g in the untreated hens to 236 g in the birds receiving 100 ppm of Nemacide. These differences in weight gain were not statistically significant.

Feed Consumption. Treatment did not significantly affect feed consumption. The mean feed consumed per hen per day was 99.6 g for the control birds and 99.3, 101.3, 100.7, and 102.9 g for birds receiving 800, 200, 100, and 50 ppm of Nemacide, respectively. Because of this similarity in consumption, the mean daily intake of Nemacide was directly proportional to concentration: 79.4, 20.3, 10.1, and 5.1 mg/hen for the hens receiving 800, 200, 100, and 50 ppm of Nemacide, respectively.

Egg Production. Treatment had no significant effect upon egg production. Birds receiving 800, 200, 100, and 50 ppm of Nemacide in the diet produced 57.3, 62.7, 61.7, and 64.5%, respectively, while control hens produced at the rate of 63.3%.

Economic Factors. Table II summarizes the effects of different levels of Nemacide on various factors of economic importance. The continuous administration of the insecticide-treated feed had no detrimental effect on shell thickness, egg weight, or probably interior quality as measured by albumen height. However, eggs from birds receiving 100 ppm of Nemacide exhibited significantly poorer interior quality than eggs from all other birds. This probably was due to factors other than Nemacide, since eggs from birds receiving much higher levels exhibited no deleterious effects.

Feed required per dozen eggs produced was 1.98 kg among control birds and 2.18, 2.01, 2.01, and 1.98 kg among birds receiving 800, 200, 100, and 50 ppm of Nemacide, respectively, in the diet. The birds receiving the highest level of Nemacide were less efficient; however, these differences in feed efficiency were not significant.

Organoleptic Evaluation. Table III summarizes the results of three taste panel sessions. Eggs laid by hens receiving

Table II. Mean Shell Thickness, Weight, and Interior Quality of Eggs Produced by White Leghorn Hens Receiving Different Levels of Nemacide-Treated Feed Over a 55-Week Period

Nemacide concentration in feed, ppm	Shell thickness, 0.00254 cm	Egg weight, g	Albumen height, Haugh units
800	13.5	61.2	78.6
200	13.4	61.0	78.4
100	13.5	61.6	75.8[a]
50	13.5	61.6	78.8
0	13.5	61.2	79.0

[a] Significantly less than the others at $P < 0.05$.

Table III. Organoleptic Evaluation[a] of Hard-Cooked Eggs from Hens Receiving Nemacide-Treated Feed

Nemacide concentration in feed, ppm	Flavor	Odor
	13 weeks on experiment	
800	5.43	5.53
200	5.60	5.77
100	5.70	5.83
50	5.80	5.77
0	5.63	5.73
	36 weeks on experiment	
800	4.63[b]	5.60
200	5.23	5.77
100	5.37	5.73
50	5.33	5.70
0	5.43	5.70
	54 weeks on experiment	
800	4.93[b]	5.73
200	5.60	5.77
100	5.63	5.80
50	5.10[b]	5.83
0	5.63	5.93

[a] Mean values based on 10- or 11-member taste panel scoring 6 for the highest quality and 1 for the lowest. [b] Significantly inferior to control eggs at $P < 0.01$.

Table IV. Recovery of Nemacide and 2,4-Dichlorophenol from Fortified Tissues of Poultry

Tissue	Added		No. of samples	Recovery %	
	μg	ppm		Mean	Range
		Nemacide			
Egg white, 20 g	10	0.5	5	86.9	80.8–89.3
Egg yolk, 20 g	10	0.5	9	90.4	87.0–93.5
Fat, 10 g	10	1.0	6	78.2	71.2–82.8
	5	0.5	9	79.6	70.0–86.9
Muscle, 100 g	10	0.1	5	89.8	85.0–94.5
	5	0.05	5	88.5	84.3–90.6
Liver, 25 g	5	0.2	8	86.1	77.4–90.2
		2,4-Dichlorophenol			
Egg yolk, 20 g	10	0.5	8	85.9	78.5–93.9
Fat, 10 g	10	1.0	3	75.7	65.7–80.8
Muscle, 100 g	10	0.1	4	50.9	46.3–53.8
Liver, 25 g	10	0.4	8	82.3	79.0–86.1

Table V. Apparent Nemacide and 2,4-Dichlorophenol Detectability in Untreated Eggs and Tissues of Laying Hens

	No. of samples	Apparent,[a] ppm		c_{ma}[b] (ppm)	c_{mc}[c] (ppm)
		Mean	Range		
		Nemacide			
Egg white	4	0.028	0.012–0.052	0.114	0.086
Egg yolk	6	0.025	0.016–0.031	0.044	0.019
Fat	10	0.032	0.012–0.072	0.085	0.054
Muscle	9	0.004	0.002–0.007	0.010	0.005
Liver	6	0.005	0.000–0.012	0.021	0.016
		2,4-Dichlorophenol			
Egg yolk	6	0.023	0.000–0.100	0.145	0.122
Fat	4	0.020	0.000–0.082	0.229	0.208
Muscle	4	0.001	0.000–0.002	0.007	0.006
Liver	7	0.014	0.000–0.038	0.065	0.052

[a] From chromatographic response of control sample. [b] Apparent limit of detectability, 99% confidence level. [c] Corrected limit of detectability, 99% confidence level.

Table VI. Residues of Nemacide Found in Tissues of Laying Hens

Nemacide concentration in feed, ppm	Days after removal from treated feed	Residues in tissues, ppm (range)		
		Fat	Liver	Muscle
800	0	3.77 (0.98–6.37)	0.039 (<0.016–0.079)	0.048 (0.007–0.080)
	7	2.10 (1.34–2.99)	0.066 (0.029–0.102)	0.024 (0.017–0.040)
	14	0.16 (<0.05–0.25)	<0.016[a]	<0.005
	21	0.07 (<0.05–0.15)	<0.016[a]	...
200	0	0.96 (0.79–1.23)	0.024 (<0.016–0.035)	0.018 (0.005–0.026)
	7	1.98 (1.43–2.30)	0.085 (0.019–0.180)	0.017 (0.005–0.023)
	14	0.22 (<0.05–0.54)	<0.016[a]	<0.005
	21	0.10 (<0.05–0.16)	0.029[a] (<0.016–0.043)	...
100	0	0.52 (0.47–0.59)	<0.016 (<0.016–0.020)	0.009 (<0.005–0.011)
	7	1.65 (0.89–2.71)	0.071[a] (0.048–0.094)	0.015 (0.010–0.020)
	14	0.15 (<0.05–0.34)	...	...
	21	0.23 (0.18–0.29)	...	...
50	0	0.35 (0.15–0.51)	<0.016 (<0.016–0.016)	<0.005 (<0.005–0.009)
	7	0.49 (0.35–0.55)	0.016[a] (<0.016–0.018)	0.005 (<0.005–0.007)
	14	<0.05 (all <0.05)	...	...
	21	<0.05 (<0.05–0.06)	...	...

[a] Based on analysis of two birds; all other data based on four birds.

800 ppm of Nemacide in the diet were rated by taste panel members in two of the three sessions to have a less desirable flavor than eggs laid by untreated hens. No significant differences in odor were detected among cooked eggs examined by the panel.

Residue Determinations. Nemacide and 2,4-dichlorophenol were added to portions of untreated hens' eggs and tissues prior to extraction and recovery analyses were performed to check the analytical method (Table IV). Good recoveries of Nemacide, ranging from 78 to 90%, were obtained. The recovery of 2,4-dichlorophenol from breast muscle was low probably due to insufficient acidity, preventing complete conversion of salts of 2,4-dichlorophenol into the acid so that they would be hexane-soluble. Increasing amounts of sulfuric acid added to the other tissues allowed for greater recovery. Figures 2 and 3 show typical chromatograms obtained using the electron capture and thermionic detectors.

The corrected limits of detectability based on the chromatographic response of untreated control samples were calculated at the 99% confidence level (Sutherland, 1965) and are given in Table V.

Tables VI and VII summarize the Nemacide residues detected in the fat, liver, breast muscle, and eggs of laying hens on diets containing varying amounts of Nemacide for 55 weeks and at various intervals after removal from these treated diets. No Nemacide residues within the sensitivity of the analytical

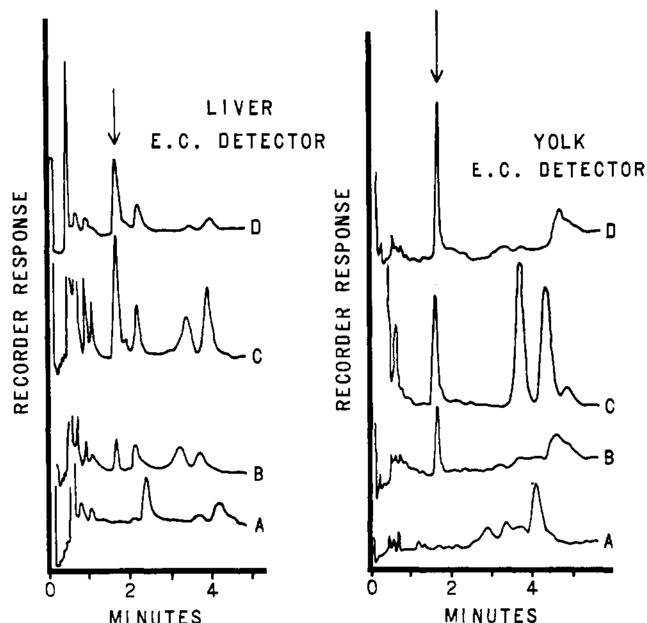

Figure 3. Typical 2,4-dichlorophenyl chromatograms. LIVER A. From control hen. B. From hen receiving 50 ppm of Nemacide in feed for 55 weeks. C. From hen receiving 800 ppm of Nemacide in feed for 55 weeks. D. Control tissue fortified with 0.4 ppm of 2,4-dichlorophenol. YOLK A. From control hen. B. From eggs collected 5 days after removal of hen from diet containing 800 ppm of Nemacide. C. From eggs collected from hen while receiving 800 ppm of Nemacide in diet. D. Control fortified with 0.5 ppm of 2,4-dichlorophenol. Arrow represents the retention time of 2,4-dichlorophenol

Table VII. Residues of Nemacide Found in Egg Yolk

Nemacide concentration in feed, ppm	Days after removal from treated feed	Residues,[a] ppm	Range, ppm
800	0	1.15	1.12–1.28
	5	0.46	0.45–0.46
	10	0.14	0.03–0.26
	13	0.06[b]	. . .
200	0	0.49	0.47–0.51
	5	0.23	0.21–0.25
	10	0.18	0.11–0.24
	13	0.06[b]	. . .
100	0	0.24	0.24–0.25
	5	0.10	0.06–0.15
50	0	0.14	0.12–0.16
	5	0.08	0.05–0.12

[a] Average of two determinations. [b] Single determination.

procedure were detected in egg white. In the other tissues, the amounts of residue detected were determined by the concentration administered in the feed and the time after removal from the treated feed. Particularly high residue levels were found in the fat and egg yolk.

No residues of the metabolite, 2,4-dichlorophenol, beyond the limits of detectability were found in the fat or breast muscle of hens administered Nemacide in the feed. Relatively high levels of 2,4-dichlorophenol, however, were found in the liver and egg yolk from these hens (Table VIII).

Table VIII. Residues of 2,4-Dichlorophenol Found in Laying Hens

Nemacide concentration in feed, ppm	Days after removal from treated feed	Residues in tissue, ppm[a] (range): Liver	Egg yolk
800	0	0.472 (0.140–0.680)	0.613 (0.340–0.750)
	5	. . .	0.267
	7	0.500 (0.250–0.750)	
	10	. . .	<0.122
	14	0.270 (0.056–0.480)	. . .
	21	0.190 (0.111–0.271)	. . .
200	0	0.375 (0.310–0.440)	0.145 (0.123–0.167)
	5	. . .	0.129
	7	0.300 (0.240–0.350)	. . .
	10	. . .	<0.122
	14	0.140 (0.099–0.180)	. . .
	21	0.356 (0.071–0.640)	
100	0	0.260 (0.250–0.260)	0.151 (<0.122–0.210)
	5	. . .	<0.122
	7	0.560	. . .
	10	. . .	<0.122
	14	0.144 (<0.052–0.333)	. . .
	21	<0.052	. . .
50	0	0.310 (0.160–0.460)	<0.122
	5	. . .	<0.122
	7	0.180 (0.140–0.220)	. . .
	10	. . .	<0.122
	14	0.055 (<0.052–0.111)	. . .
	21	<0.052	. . .

[a] Less than 0.006 and 0.208 ppm found in muscle and fat, respectively.

These results suggest that although administering Nemacide at levels as low as 50 ppm in the feed of laying hens will prevent the breeding of houseflies in droppings from the hens with no detrimental effects on the birds or the eggs, the presence of residues of Nemacide and 2,4-dichlorophenol in the yolk and tissues would preclude its use in the feed of laying hens to prevent fly breeding.

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A Low Temperature Cleanup Procedure for Pesticides and Their Metabolites in Biological Samples

Harry A. McLeod* and Patricia J. Wales

A low temperature cleanup procedure was developed to separate in one operation some polar and apolar pesticides from sample lipid, waxes, and water. Samples were extracted by refluxing with acetone–benzene (19 + 1) and extracts were cleaned up by low temperature precipitation at −78 °C. A precipitation time of 30 min followed by filtration through Solka Floc (cellulose) was optimum. The water content of the extract did not adversely affect the recovery of polar residues but the presence of carbon in the filtration did. The cleaned-up extracts were suitable for glc determination with electron capture and flame photometric detectors. Recoveries of thirteen representative insecticides, fungicides, and herbicides (captan, diazinon, dinitrobutyl phenol anisole, 2,4-D acid, 2,4-D isopropyl ester, endosulfan, malathion, malaoxon, parathion, paraoxon, phosphamidon, fenitrothion, and fenitroxon) ranged from 80–116% when added separately or in combination to samples of carrots, peas, wheat, human fat, liver, and kidney.

Low temperature precipitation as a cleanup procedure for separating pesticide residues from sample extractives (lipids, waxes) has been widely reported but does not appear to have found wide acceptance.

Fairing and Warrington (1950) separated methoxychlor from fats and waxes by precipitation at −12 °C. Dicofol, DDT, and methoxychlor were separated from plant waxes at −70 °C (Anglin and McKinley, 1960). Later, McKinley and Savary (1962) and McCully and McKinley (1964a,b) extended the scope of the technique to include dieldrin, endrin, heptachlor, aldrin, isobenzan, and lindane in a variety of biological samples. Organophosphorus pesticide residues were determined in plant extracts following low temperature cleanup by Bates (1965). Recently, Grussendorf *et al.* (1970) described a semimicro apparatus and rapid cleanup method for hexane extracts of cereal grains, soils, and other types of samples. Most procedures use a liquid–liquid partition step interposed between extraction of the sample and low temperature cleanup. In general, apolar compounds are recovered but most polar residues remain in the aqueous fraction and are discarded.

This paper reports the development and evaluation of a low temperature cleanup procedure that separates polar and apolar residues from sample lipids, waxes, and water in a single step. Partition systems and drying agents are not required. Optimum analytical parameters, including recovery data, for pesticides and metabolites representative of insecticides, fungicides, and herbicides added to biological samples are given.

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MATERIALS

Apparatus. Micro-Tek gas chromatograph (MT220) fitted with a Melpar flame photometric detector for S394 and P526. The column was 5% DEGS on 60–80 mesh Chromosorb W, DMCS, packed in 3 ft × 1/8-in. i.d. borosilicate glass column. Operating parameters were as follows: Nitrogen flow, 80 ml/min. Oven temperature, 150 or 195 °C. Hydrogen flow, 200 ml/min. Inlet temperature, 220 °C. Air flow, 15 ml/min. Oxygen flow, 15 ml/min. Melpar temperature, 160 °C. Electrometer settings: S394, Input 10^3, Output 8; P526, Input 10^3, Output 8; FID, Input 10^2, Output 16.

Varian Aerograph 600 gas chromatograph fitted with a Kovar cell (Tritium foil) electron capture detector and a 6% QF-1, plus 4% SE-30 on 60–80 mesh Chromosorb W, AW, packed in 4.5-ft × 1/4-in. i.d. pyrex glass column. Operating parameters were as follows: Nitrogen flow, 120 ml/min. Oven temperature, 195 °C. Inlet temperature, 220 °C. Range, 1 mV. Attenuation, 4.

Cold bath and accessories were custom-made from standard laboratory material and the construction details are described by McLeod (1972).

Vacuum pump with adjustable vacuum control.

Reagents. All organic solvents were pesticide analytical grade or analytical grade solvents redistilled in the laboratory to meet pesticide grade specifications. The extraction solvent was acetone–benzene (19 + 1). Carbon (DARCO