Behavioral Effects of Carbofuran and Phenamiphos on Pratylenchus vulnus. I. Motility and Dispersion

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Abstract: The motility and dispersion of *Pratylenchus vulnus* was affected by lower concentrations of phenamiphos (an organophosphate) than of carbofuran (a carbamate). At the higher concentrations in the active ranges, somatic musculature activity was inhibited while the activity of internal-organ musculature persisted. Treated nematodes recovered when placed in aerated distilled water, but recovery decreased as treatment concentrations increased. Second-stage larvae dispersed more slowly than later stages or adults. Dispersion from a point source followed a natural log pattern under ideal conditions, similar to a diffusion pattern. *Key Words:* root-lesion nematodes, non-volatile nematicides, mode of action.

Studies of toxicants affecting nematodes *in vitro* provide information on the range of doses that will provoke particular responses and enable direct observation of behavioral reactions. A series of *in vitro* experiments was conducted to establish the concentrations of two selected nematicides that cause immobilization (defined as the inability to respond to mechanical stimulation) of *Pratylenchus vulnus* Allen and Jensen, 1951.

The ability to disperse has important survival value for any organism. Nematodes may disperse passively, by drifting in water, in air currents, and on moving objects, or actively, by swimming or creeping (6). Active dispersion is critical to nematode distribution. Schematic models have been developed of nematode movement (26,27),

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and the behavioral analysis and factors affecting locomotion were reviewed by Croll (6). Many researchers have used impairment of nematode movement by nematicidal compounds as a principal criterion in *in vitro* screening methods for assessing nematicial activity on nematodes. In water screening tests (16,22,24), nematodes exposed to a toxicant were either classified as motile or nonmotile; lack of motility was equated with lack of infectivity or death, an assumption that was not necessarily valid. The ability of treated nematodes to move through sieves has also been used as a criterion for toxicant action (17,18,21); however, the passage of dead nematodes through sieves and a Baermann funnel is easily demonstrated. Also used to assess nematicidal activity has been a nematode's ability to move vertically in sand columns (12).

Nematicide effects on nematode body undulation rates, either in solution or on agar plates (14,18), may not necessarily show nematicidal influence on nematode migration. It would be preferable to assess nematicidal influence on nematode dispersion directly by the use of their inherent vagility in a non-oriented environment after the method of Croll and Blair (7), which uses displacement on agar plates, and adopted by Keetch (14) to assess recovery of *Aphelenchus avenae* pretreated with nonfumigant nematicides.

Current interest in nonvolatile nematicides has demonstrated a need for more information on their modes of action; therefore, a laboratory study was conducted to determine the effect of carbofuran (a carbamate) and phenamiphos (an organophosphate) on the lateral dispersion of *P. vulnus*. Since *P. vulnus* moves poorly in agar, lateral dispersion (heretofore unassessed) was studied in thin horizontal sand discs. Use of this method established the effect of time, moisture, and sand particle size on the rate of lateral dispersion of *P. vulnus* in a nondirectional environment and selected appropriate conditions for routine nematicide bioassays. Succeeding experiments explored two aspects of nematicidal treatments: 1) the effect of increasing concentrations of carbofuran or phenamiphos on nematode motility as measured by *in vitro* immobilization and by lateral dispersion; and 2) the reversibility of the treatments inhibiting motility of nematodes, by removal of the active nematicide and assaying *in vitro* immobilization and lateral dispersive power.

MATERIALS AND METHODS

Nematicides: Technical-grade carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl-carbamate) and phenamiphos (ethyl 4-(methylthio-m-tolyl isopropylphosphoramidate) were used in this study. As an aid to dissolution of chemicals, an aqueous 0.86 M acetone solution was used to make up stock from which treatment solutions were subsequently prepared by dilution with distilled water. Preliminary tests showed that exposure of *P. vulnus* to aqueous acetone solutions as high as 3.4 M for 1 week had no visible effects on the nematode, but acetone was included in all controls as a precaution.

In vitro treatments were 0.86 M acetone; 0.0005, 0.005, 0.05, and 0.5 mM phenamiphos; and 0.005, 0.05, 0.5, and 1.0 mM carbofuran. Nematodes were incubated at room temperature (about 20 C), and treatments were replicated five times.

For dispersion studies, nematodes were pretreated in nematicide solutions for 12 h and then concentrated and inoculated onto sand discs, prewet with fresh solutions of the same concentrations, for another 12 h. Treatments were 0.86 M acetone and 0.001, 0.005, 0.01 mM carbofuran and phenamiphos. During both pretreatment and dispersion periods the tests were incubated at 20 C in the dark. In the recovery portions of the experiments, nematodes were pretested by immersion in solutions of carbofuran (0.24, 1.25, and 6.25 mM), phenamiphos (0.005, 0.05, and 0.5 mM), and 0.86 M acetone for 24 h in the dark at 20 C. After the exposure period the nematodes were washed three times with 15 ml of aerated distilled water (ADW), with the nematode suspension in the final wash being divided into two portions, both kept in 50-mm petri dishes in the dark at 20 C, but one for a 24-h recovery period, and the second for 96 h. The experimental design in all tests was a randomized block, with four and five replicates. Data were subjected to analysis of variance and Duncan's multiple-range tests.

Nematode: Pratylenchus vulnus, originally collected from a walnut orchard near Winters, California, was obtained from stock populations reared on axenized carrot discs. Nematodes of all stages were extracted by placing the carrot discs on milk filter paper in the mist chamber. Preliminary tests showed that *P. vulnus* second-stage larvae (L_2) appeared to be more sensitive to both nematicides than did the older stages but that their response to mechanical stimulation for the bioassay could not be ascertained with reliability. Therefore, the L_2 stage was eliminated from these tests by passing the mixed-stage nematode suspension through a sieve (74-μm aperture). The nematodes remaining on the sieve, accounting for about 40% of the initial population, consisted of about 8% L_2 and 92% third-stage (L_3), fourth-stage (L_4), and adults. The L_3, L_4, and adults used in these tests were obtained by this method, and the few undesirable L_2 remaining were ignored in the response evaluation.

Constituting a replicate in the *in vitro* tests was a 15-ml centrifuge tube containing a 6-ml suspension of 300 ± 13 *P. vulnus* (L_3, L_4, and adults). Before use, the nematode suspensions were aerated with a stream of air for 3 h and then passed through wet double tissue paper to eliminate most dead and inactive nematodes. This nematode stock, retained no longer than 1 week, was kept viable by storing in ADW at 15 C until needed. Fifteen tubes were used for each chemical concentration. These were divided randomly into three groups of five tubes each. One group was used to evaluate nematode immobilization in a nematicide solution; the second group was similarly treated and then allowed a 24-h recovery in ADW. The third group was allowed a 48-h recovery. After centrifugation (2 min/1000 *g*), the supernatant was decanted and replaced with the appropriate volume of treatment solution. To avoid dilution errors the procedure was repeated twice with fresh solution before the tube was incubated on a reciprocating shaker. Treatment durations varied from 1 to 168 h (Figs. 1, 2). Throughout the experiment, chemical solutions were replaced every 48 h to prevent undesirable variations due to possible chemical degradation. For the recovery portions of the experiment the procedure was repeated with ADW substituted for the treatment solutions. The bioassays were conducted by transferring 0.5-ml aliquants, containing about 25 nematodes, from the incubation tube to a 50-mm petri dish, followed by observation with a microscope of the nematode response to mechanical stimulation of touching the nematode body with a dissection needle. The numbers responding to stimulation with movement were recorded and the percentage of immotile nematodes was calculated.

The dispersion studies were conducted with the modified slide-ringing apparatus shown in Fig. 3. A metric rule was attached above the plate in a horizontal position. A steel cutter plate (12.5 × 2.0 × 0.02 cm) with a 90° bend at one end was held in place with a clamp on the rule at the desired distance from the disc center. The dispersion matrix was a layer of quartz sand, 1 to 1.5 mm thick and 150 to 250 μm in particle size. This was placed on glass discs, 100 mm in diameter, with three square plastic pads attached by epoxy resin in a triangle to the underside so as to fit tightly around the horizontal metal wheel of the slide ringing apparatus. The appropriate nematicide solution (2.0 to 2.5 ml per sand disc) was applied through a 15-ml separatory funnel. The treated nematode suspension was concentrated by centrifugation, and then 400–1400 nematodes were inoculated at the disc center through a 10-μl disposal pipet (Microcaps, Drumond Scientific Company).

Inoculated sand discs were kept in the dark over moist filter paper in a closed petri dish at 20 C throughout the dispersion period. The sand from successive 5-mm concentric annuli was transferred with a spatula into a 50-mm petri dish. To separate nematodes, the sand in each petri dish was suspended by agitation in 30 ml of water and allowed to settle momentarily, and then the supernatant containing the nematodes was decanted into a 100-ml beaker. After the process was repeated the sand was discarded and the suspension of nematodes was allowed to settle overnight before being fixed in 2.5% formalin. The nematodes in each annulus were counted with the aid of a microscope and the results were expressed as a percentage of the inoculum found per cm^2 in each annulus.

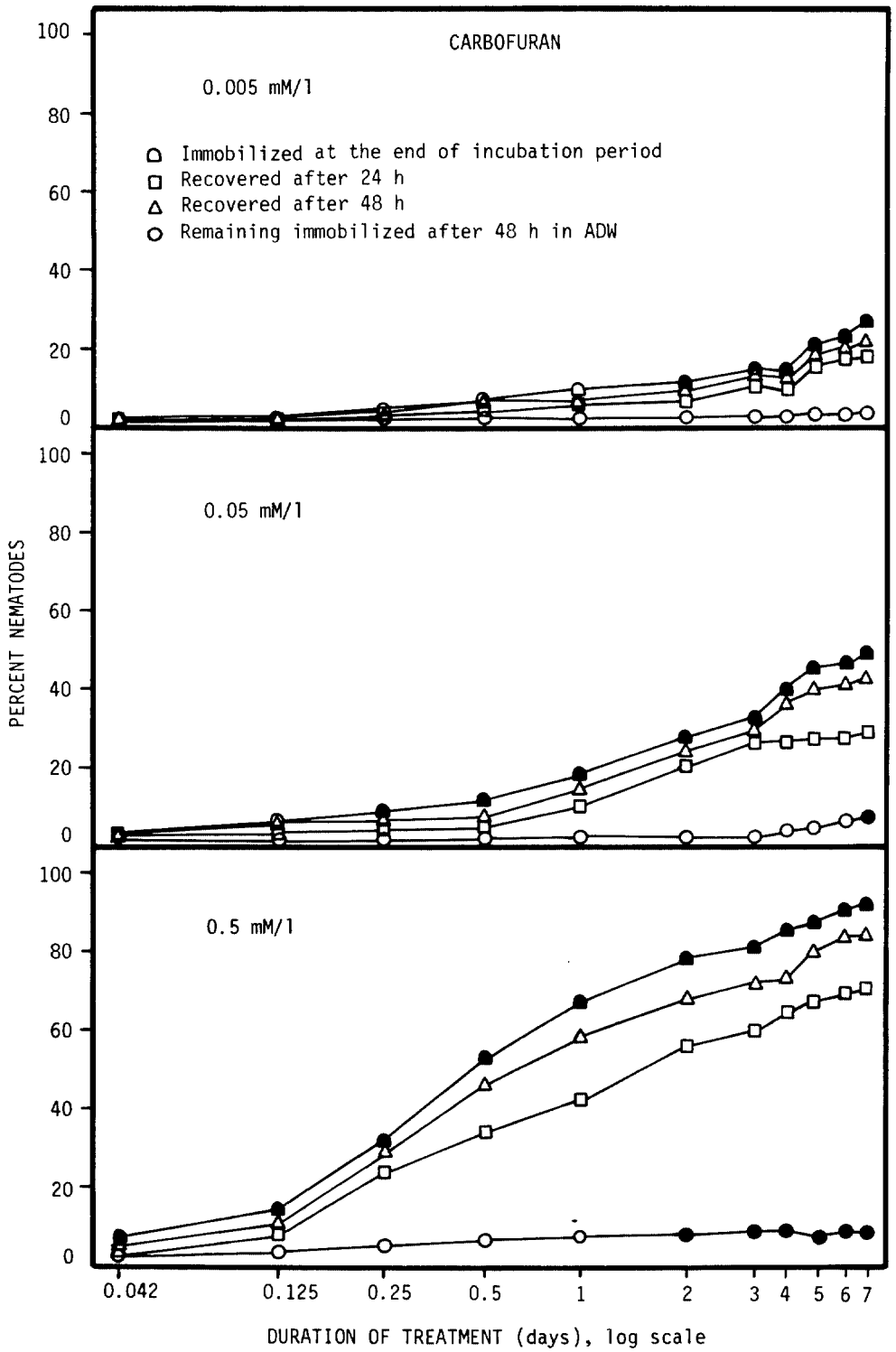

Fig. 1. *P. vulnus* responding (%) to carbofuran in relation to exposure and recovery times. Mean of five replicates, 25 ± 3 nematodes each. Immobile nematodes = no response to mechanical stimulation. Filled symbols indicate internal-organ musculature activity (stylet, esophageal, vaginal, spicular) in 20% or more of the immobile nematodes.

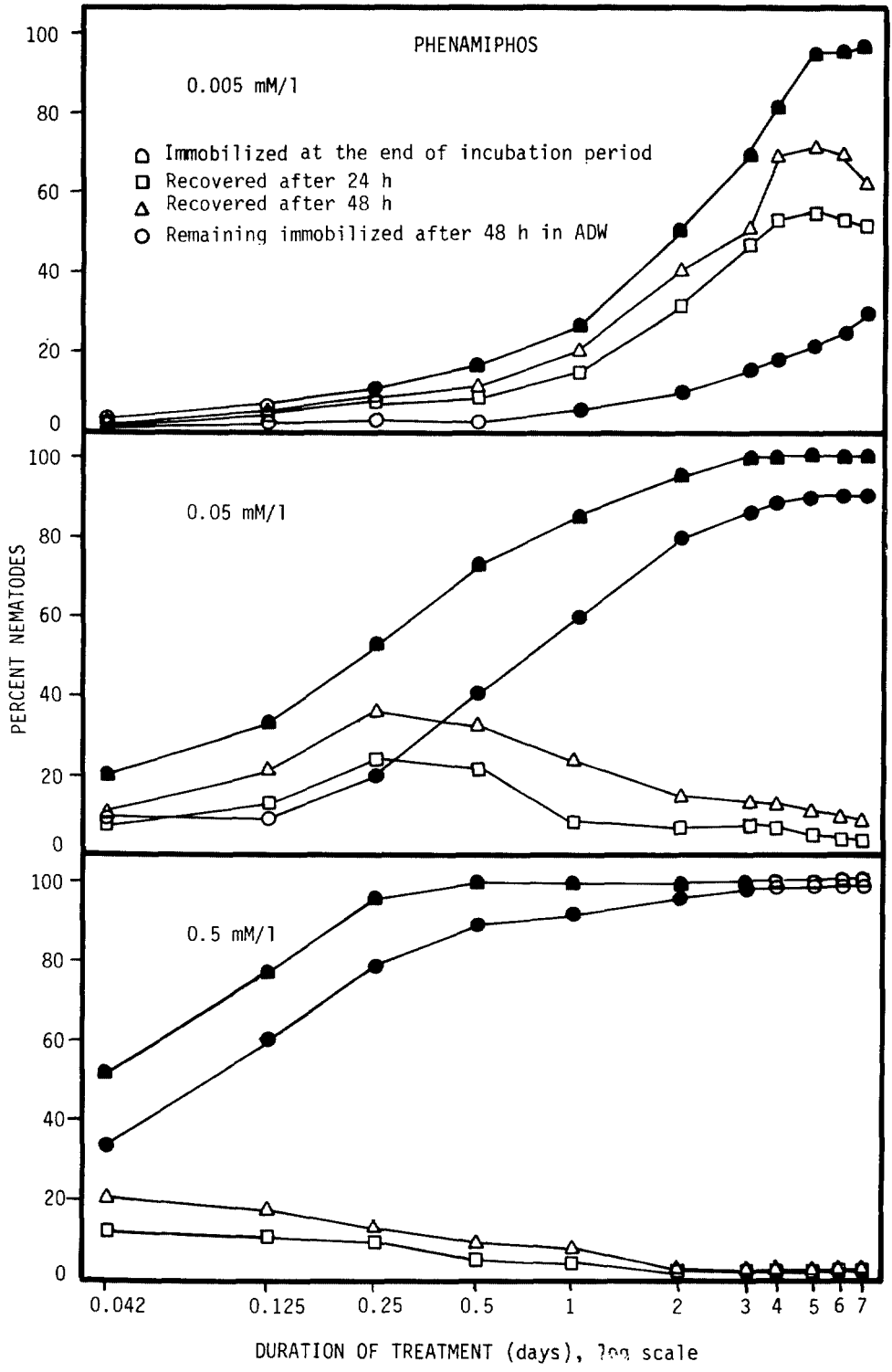

Fig. 2. *P. vulnus* responding (%) to phenamiphos in relation to exposure and recovery times. Mean of five replicates, 25 ± 3 nematodes each. Immobile nematodes = no response to mechanical stimulation. Filled symbols indicate internal-organ musculature activity (stylet, esophageal, vaginal, spicular) in 20% or more of the immobile nematodes.

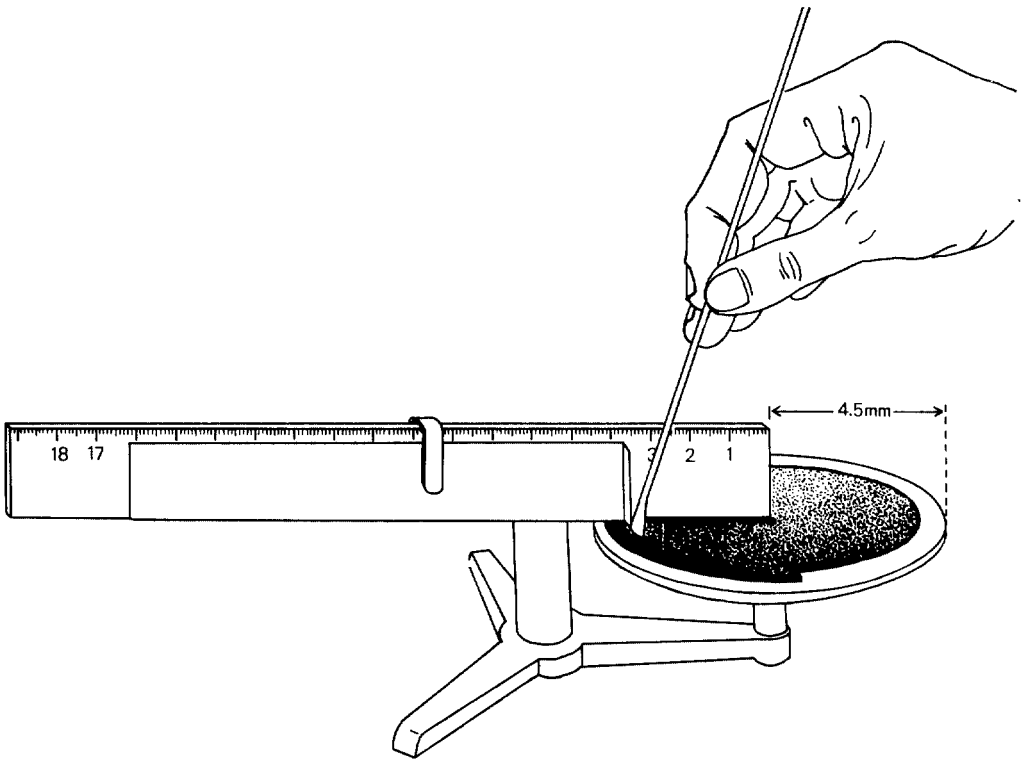

Fig. 3. Schematic diagram of apparatus used to evaluate dispersion of *P. vulnus*. Portion of first assay (0.5 cm) annulus removed.

To reduce activity lags, nematodes were pretreated in nematicide solutions for 12 h and then concentrated and inoculated onto sand discs wet with fresh solutions of nematicides at the same concentrations for another 12-h dispersion period. Treatments were 0.86 M acetone and 0.001, 0.005, and 0.01 mM carbofuran and phenamiphos. During both the pretreatment and dispersion periods, the tests were incubated at 20 C in the dark.

For the nematode recovery tests, nematodes were pretreated by immersion for 24 h in the dark at 20 C in solutions of carbofuran (0.25, 1.25, and 6.25 mM), phenamiphos (0.005, 0.05 and 0.5 mM), and 0.86 M acetone. After the exposure period, the nematodes were washed three times with 15 ml of ADW with the nematode suspension in the final wash being divided into two portions, both kept in 50-mm petri dishes in the dark at 20 C, but one for 24 h and the second for 96 h as recovery periods. The experimental design in all tests was a randomized block, with four or five replicates. The data were subjected to analysis of variance and Duncan's multiple-range test.

RESULTS

In vitro tests. The motility of *P. vulnus* (L_3, L_4, and adults) incubated in carbofuran or phenamiphos solutions decreased with longer treatment duration and increased concentration (Figs. 1, 2). At the same concentrations, fewer nematodes remained motile in phenamiphos solutions than in carbofuran. More than 90% of the nematodes were immobilized with 0.005 mM phenamiphos after 6 days of exposure, whereas carbofuran required a concentration about 100 times as great to get a similar response in that time. Complete immobilization was detectable with 0.05 mM phenamiphos after 96 h, but immobilization was not complete with carbofuran at the maximum concentration and exposure period used in these experiments. Depending upon nematicide concentration and exposure period, *P. vulnus* showed variable degrees of immobilization after 24 and 48 h in the recovery bioassays.

The recovery of phenamiphos-treated nematodes was dependent on treatment concentration and exposure period, since nematode recovery decreased as exposure period or concentration increased. When nematodes became permanently inactivated (no recovery response), their bodies assumed the posture and internal characteristics associated with dead nematodes. On the other hand, carbofuran-treated nematodes showed almost complete recovery after 48 h in ADW.

From microscope observations it appeared that carbofuran and phenamiphos solutions induced effects on *P. vulnus* other than impairment of motility; e.g., an initial increase of body undulatory activity followed by a decrease, leading to spasmodic movements ending with a coiled posture, and finally immotility. The body became thicker and shorter, and at 0.5 mM phenamiphos some nematodes displayed abnormal stylet protrusions beyond the lip margin, seldom seen in untreated *P. vulnus*.

When nematodes became partially to fully immotile, some of their internal organs began to show abnormal activities: increased median bulb pulsation, stylet movements, vaginal-vulval contractions in females and spicular movements in males. The frequency and vigor of such activities decreased with time. This unusual internal-organ activity disappeared in nematodes treated with phenamiphos at 0.5 mM for more than 72 h, regardless of recovery periods.

Dispersion Studies. Dispersion patterns of different stages of *P. vulnus* from an inoculum point on horizontal sand discs differed ($P = 0.01$) among certain stages and also varied according to dispersion time (Figs. 4, 5). The dispersion curve for the L_2 at 24 h was significantly different from that of the L_3, L_4, and adult stages, but there were no significant differences among the older stages. Dispersion was slower for L_2 than for older stages, as indicated by the slopes of the corresponding curves.

When a population of mixed L_3, L_4, and

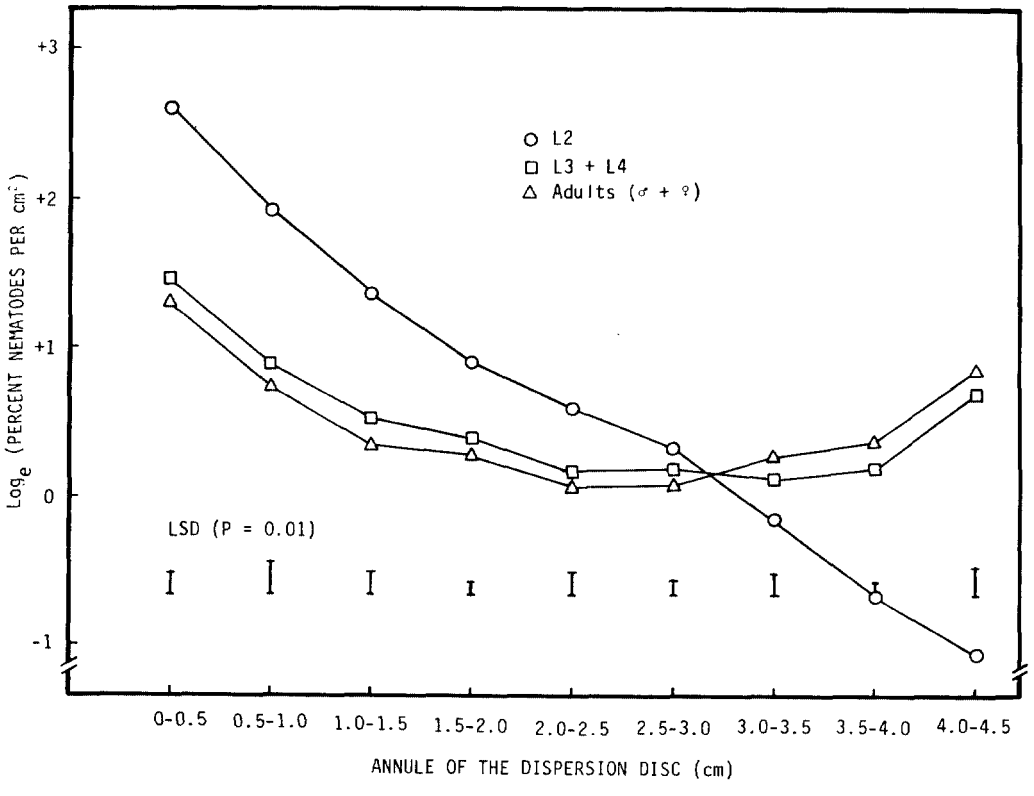

Fig. 4. Dispersion of *P. vulnus* life stages on sand discs for 24 h at 20 C. Vertical bars indicate LSD values at $P = 0.01$.

adults of *P. vulnus* dispersed at 20 C, the dispersal curves at 3 and 6 h were similar but different ($P = 0.01$) from the curves obtained for 12 and 24 h, which also were similar. In a seminatural log plot a straight line was obtained with the L_2 dispersal data at 24 h, and with the L_3, L_4, and adults at 3 h.

P. vulnus (L_3, L_4, adults) pretreated with carbofuran or phenamiphos for 12 h and then allowed to disperse on horizontal sand discs at 20 C for 12 additional hours in the same solutions showed a progressive inhibition with increasing concentrations (Fig. 6). At all concentrations, phenamiphos showed greater ($P = 0.01$) inhibition of nematode dispersion than carbofuran, whereas in control dispersion tests the nematode distribution approximated the hypothetical equilibrium distribution ($P = 0.01$). The percentages of *P. vulnus* remaining at the inoculation point at concentrations of 0.01, 0.005, and 0.001 mM were 75%, 55%, and 18% for carbofuran, and 100%, 81% and 60% for phenamiphos (after using the Abbott's correction formula, 1). The requirement for 100% inhibition was 0.01 mM phenamiphos or 0.05 mM carbofuran.

Nematode recovery, as measured by the ability to disperse during a 12-h period at 20 C, depended on the concentrations of nematicide and the length of the recovery period in ADW ($P = 0.01$, Figs. 7, 8). There were no differences ($P = 0.01$) in dispersion of nematodes between controls (24, 96 hours) and the hypothetical equilibrium distribution. No nematode dispersion was obtained after treatment with 0.5 mM phenamiphos regardless of recovery period, whereas dispersion, indicating partial recovery, was obtained with 0.05 mM phenamiphos at 96 h but not at 24 h. With 0.005 mM phenamiphos dispersion was good at both 24 and 96 h, although less than in controls ($P = 0.01$). Almost complete recovery was obtained with carbofuran treatments at 1.25 mM or less after 96 h in

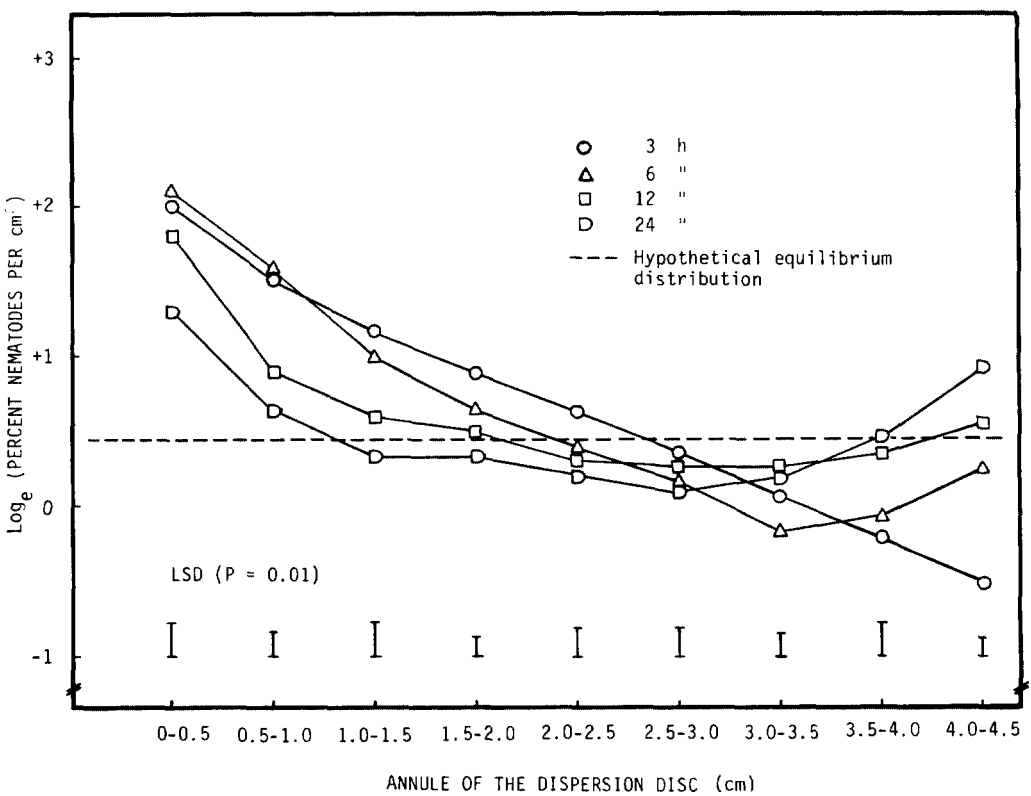

Fig. 5. Dispersion of *P. vulnus* (L_3, L_4, adults) as a function of time in sand discs expressed as $\log_e$ of the percent of nematodes/cm^2. Vertical bars indicate LSD values at $P = 0.01$ against the theoretical equilibrium distribution (horizontal broken line) for each 0.5-cm interval (5 replicates).

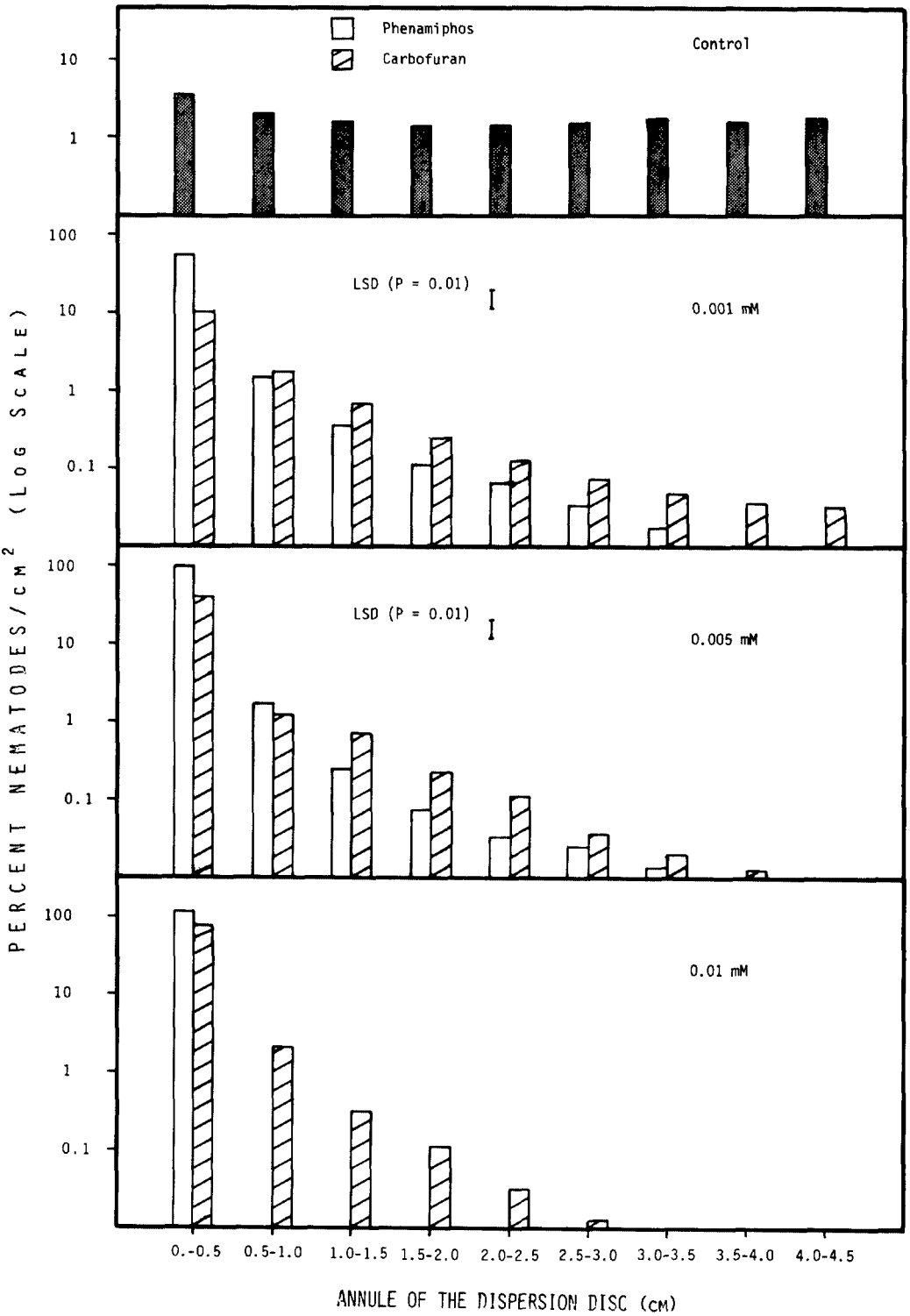

Fig. 6. Dispersion of *P. vulnus* (L_3, L_4, adults) as a function of carbofuran or phenamiphos concentration in sand discs after 12 h at 20 C. Nematodes were pretreated for 12 h before bioassay (5 replicates).

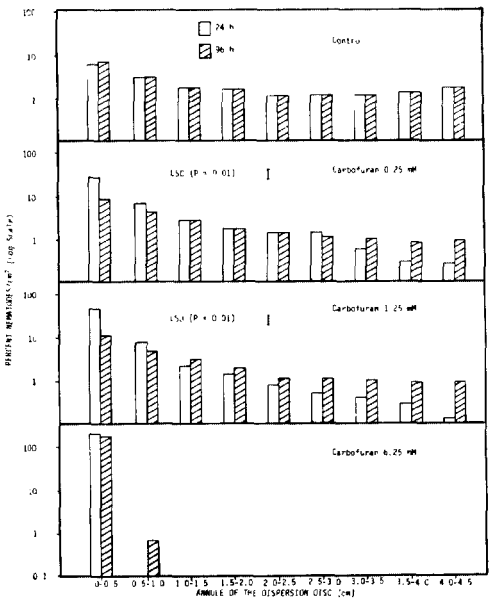

Fig. 7. The dispersion of *P. vulnus* (L_3, L_4, adults) in sand discs for 12 h at 20 C as a function of a 24-h or 96-h wash in ADW subsequent to 24-h treatments with different concentrations of carbofuran.

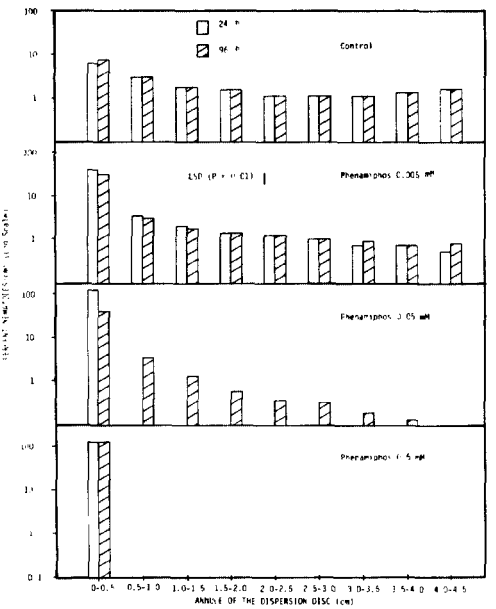

Fig. 8. The dispersion of *P. vulnus* (L_3, L_4, adults) in sand discs for 12 h at 20 C as a function of a 24-h or 96-h wash in ADW subsequent to 24-h treatments with different concentrations of phenamiphos.

ADW; however, nematodes treated at 6.25 mM carbofuran were able to recover only slightly after 96 h in ADW.

DISCUSSION

In these studies, carbofuran and phenamiphos were physiologically active on *P. vulnus* over a wide range of concentrations; phenamiphos was active at lower concentrations than was carbofuran. It is generally accepted that the activity attributed to the carbamate and organophosphate insecticides is based on the inhibition of acetylcholinesterase and cholinesterase function. This inhibition disrupts the transmission of impulses through the neurosystem (4,20). Carbofuran and phenamiphos are potent acetylcholinesterase inhibitors in insects and mammals (25,29). Evidence of cholinesterase activity has been reported from different species of nematodes (9,10,28) and it is believed to be the enzyme involved in the transmitter destruction at cholinergic synapses (6,28). Our experiments do not provide evidence of biochemical mode of action, but there are many similarities in symptoms seen with *P. vulnus* and those observed with insects and vertebrates treated with cholinesterase inhibitors (irritability, lack of coordination, convulsions, contractile paralysis, and death), which supports the hypothesis that these nematicides act by interfering with the transmission of nerve impulses to somatic musculature. However, *immotile P. vulnus,* with the exception of those individuals exposed to 0.5 mM phenamiphos for 96 h or more, continued to manifest internal-organ activity (pulsation of the median bulb, stylet movement, vaginal-vulval contraction, and spicular movement) for extended periods during continuous exposure to these chemicals. This behavior was observed with nematicide concentrations of both carbofuran and phenamiphos 100 times the amount necessary to inhibit somatic musculature activity. Other data suggest that comparable behavior responses occur with different species of nematodes when exposed to other organophosphate or carbamate insecticide-nematicides (3,14,18).

Croll (5) observed similar behavioral responses with other nematodes treated with biologically active substances and explained the differences in the two kinds of muscular activity by hypothesizing two different chemical nerve coordinating systems: an exogenous system coordinated by acetyl-

choline, controlling primary somatic musculature, and an endogenous system, possibly coordinated by serotin or epinephrine, controlling the musculature of internal organs. Data from our tests indicated that *P. vulnus* treated with carbofuran solutions would regain normal activity if allowed to recover in ADW for 48 h, whereas phenamiphos-treated nematodes showed negligible recovery in the same period. These results strongly suggest that phenamiphos affected the function of physiological systems other than the neuromuscular coordinating system of *P. vulnus*.

There are indications in the literature that may support that contention. Carbamates and organophosphate pesticides including phenamiphos inhibited cytochrome oxidase of *Panagrellus redivivus* and *Rhabditis oxycerca* (13). Proteases of *Panagrellus silusiae* and *Turbatrix aceti* were inhibited by organophosphate pesticides (11). Respiration of aldicarb-treated *P. redivivus* and *R. oxycerca* showed a steady decline with increasing dose (23). Despite the apparent similarity of the behavioral responses of the nematodes treated in our experiments to those previously reported, the evidence is insufficient to establish whether, and to what degree, the effects of carbofuran and phenamiphos on *P. vulnus* are explicable by differences in the impulse-transmission mechanisms, differences between the musculatures, or differences in the rate of uptake, metabolism, and elimination of nematicides.

The dispersion of *P. vulnus* would have more nearly approximated that occurring in nature had a practical three-dimensional method been available. The two-dimensional method adopted was rapid, reliable, and easily ammenable to the maintenance of favorable moisture and oxygenation levels. The straight lines obtained in dispersion patterns at 20 C for a 3-h period with L_3, L_4, and adult stages and in a 24-h period with L_2 indicated that *P. vulnus* outward dispersion initially followed a seminatural logarithmic curve when theoretical requirements were most nearly met. Under ideal conditions, nematode dispersion therefore appeared to be governed by the mass-movement principle governing diffusion of gases and solutions. A gaussian diffusionlike curve (8) could be obtained from the data by a plot of the distance from the inoculation source as abscissa and the nematode relative abundance as ordinate (Fig. 9).

Under our experimental conditions, the rate of dispersion of *P. vulnus* differed between certain nematode stages, as indicated by the slope of the corresponding lines; L_2 is slower than the older stages. This difference is explicable in part by Wallace's (26) suggestion that nematode mobility is max-

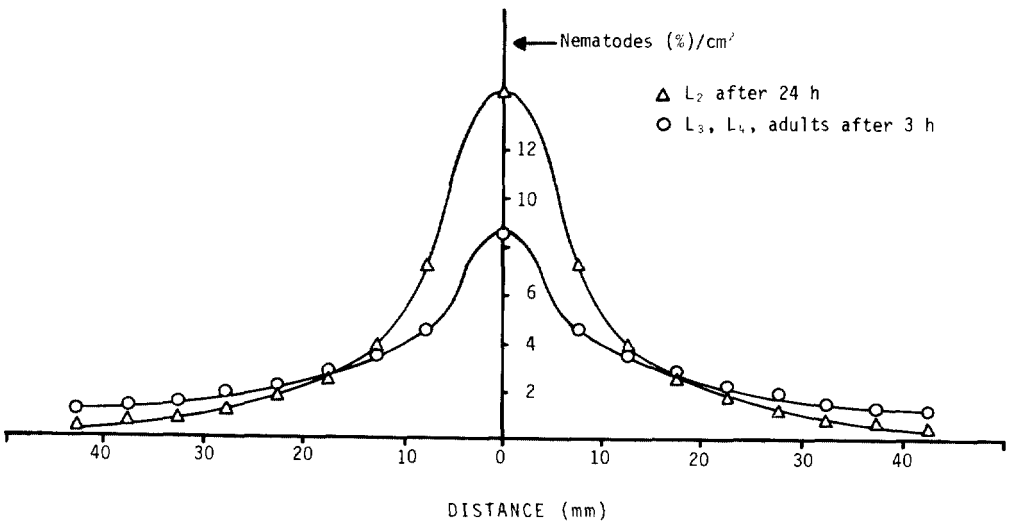

Fig. 9. Gaussian curve obtained as a consequence of a nematode density plot through the inoculum point of control dispersion tests under conditions where theoretical requirements were most nearly met. See text for details. Abscissa: distance (mm to annulus midpoint) from inoculation point. Ordinate: average nematode density (%/cm²) of successive annuli.

imum when the nematode length is about three times the soil-particle diameter. The average lengths of *P. vulnus* L_2 alone and of L_3, L_4, and adults were respectively about 180 μm and 580 μm. Thus, the L_2 was about the same size as the soil-particle diameter (150-250 μm) used in this experiment, whereas the L_3-L_4-adult group had a length about three times the particle-size diameter.

The results of the dispersion studies indicated that sublethal concentration rates of carbofuran and phenamiphos inhibited dispersion of *P. vulnus,* much as aldicarb has been shown to do with other nematodes. Laboratory experiments with aldicarb at 1 to 5 ppm have shown inhibition of movement of *Heterodera rostochiensis, Meloidogyne incognita, H. schachtii, Aphelenchus avenae,* and *Panagrellus redivivus* (2,12,18,19). Concentrations below 6.25 mM of carbofuran may not be lethal to *P. vulnus,* for nematodes so treated recovered their ability to disperse in sand discs upon recovery in ADW after 96 h of exposure to the nematicide.

P. vulnus can be treated with carbofuran solutions 125 times that required to impair dispersion under continuous incubation and recover in ADW given sufficient time, while phenamiphos solutions five times that needed to impair dispersion under continuous incubation also permit recovery. While recovery increased with time in ADW, the reversibility of the reaction disappeared with increasing concentrations of either nematicide. The threshold at which irreversibility occurred after 24 h of incubation was about 12.5 times as great for carbofuran as for phenamiphos using a 96-h recovery period. The disruption of the dispersion pattern of *P. vulnus* at low doses or time exposures suggests that these chemicals have the potential to change the normal response behavior of nematodes, possibly by impairing a nervous-system coordinating function. This is consistent with the generally accepted notion that most carbamates and organophosphates have little or no direct killing effect on nematodes at concentrations achieved in commercial field applications (3,12,15,19).

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