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THE REPELLENT EFFECT OF NECROTIC TISSUES ON THE NEMATODE PRATYLENCHUS PENETRANS (COBB, 1917), FILIPJEV AND SCHUURMANS STEKHOVEN, 1941.

A Dissertation Presented

By

Liu-Mei Chang

Submitted to the Graduate School of the
University of Massachusetts in
partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Major Subject Plant Pathology

THE REPELLENT EFFECT OF NECROTIC TISSUES ON THE NEMATODE PRATYLENCHUS PENETRANS (COBB, 1917), FILIPJEV AND SCHUURMANS STEKHOVEN, 1941.

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June 2, 1969
(Month) (Year)

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I INTRODUCTION

Host plant roots are probably the most important single factor in the environment of Pratylenchus penetrans. In addition to providing food to and being the most common habitat of lesion nematodes, roots produce chemicals which may attract or repel nematodes, stimulate or depress their respiration, and regulate reproduction. Nematodes in the soil are also influenced by these compounds, but very little is known about the mechanisms whereby roots influence the behavior of nematodes.

Studies on the movement of nematodes, one aspect of their behavior, have been centered chiefly on the orientation of nematodes in relation to the host plant; the term "attraction" is frequently employed in connection with oriented movement. In a recent review paper Klingler (15) indicates that most evidence supports the conclusion that plant nematodes reach roots and germinating seeds by means of oriented migration. This attraction has been attributed to a number of factors.

In the rhizosphere, root exudates, sloughing off of cell material, and microbial activity lead to increased concentrations of CO_2, amino acids, other organic acids, sugars, reducing and oxidizing agents and other organic compounds (4, 13, 27, 28).

Of the root exudates, carbon dioxide is the most important

quantitatively. Experiments with nematode orientation in CO_2 gradients led to the observation that aggregations do occur around CO_2 sources (2, 12). The hypothesis was then advanced that the higher CO_2 concentrations close to the root inhibit nematode activity, and the aggregations thus occurred by orthokinesis (25). By use of the agar methods of Sandstedt, et al (29) and Rode and Staar (24) it was possible to study more closely the behavior of nematodes in artificially or biologically produced CO_2 gradients, and to analyze more accurately the part played by CO_2 in the finding of the host (14). These works supported the previous hypothesis that CO_2 plays a significant role in attraction.

Bird (1) tested the effect on nematode movement of several amino acids exuded by tomato roots. Of these only glutamic acid attracted the larvae of two root-knot nematode species. In the tests of Oteifa and Elgindi (20) tyrosine proved the most attractive of fourteen different amino acids.

Attractiveness of plant roots to P. penetrans has been demonstrated both in agar and moist sand (17, 37). This oriented attraction has been ascribed to a chemotactic effect of substances diffusing from roots. In addition, Wong (37) indicated that acids or pure CO_2 lowered the pH of the surrounding medium and a medium with low pH was highly attractive under experimental conditions.

Edmunds and Mai (7) reported that in petri dishes containing agar more P. penetrans moved toward alfalfa roots infected with Fusarium

oxysporum than to noninfected roots; P. penetrans was attracted to CO_2 and to gases emanating from germinating alfalfa seedlings; more CO_2 was released from infected than from noninfected roots. They suggested that this may account for the increased attractiveness of fungus-infected roots to nematodes.

El-Sherif and Mai (8) reported recently that heat from germinating alfalfa seeds or an artificial source of heat, attracted P. penetrans. Carbon dioxide emanating from germinating alfalfa seeds failed to attract nematodes in the absence of heat. They concluded that a temperature gradient of 0.03° C increase for each 4 cm in the vicinity of a nematode may be sufficient to initiate a positive thermotactic response under experimental conditions. Theoretically, plant roots liberate heat as a by-product of respiration. This heat along with heat produced by soil microorganisms may set up a temperature gradient around the root system. Therefore, the positive thermotactic response of nematodes may play a vital part in locating plant roots.

A number of changes are known to occur in plants following infection by various pathogens. These include quantitative and qualitative changes in several metabolites which either work against the invading parasite or enhance disease development; plant injury by nematodes is no exception. Increased synthesis or accumulation of phenolic compounds, and increased activity of phenolases are characteristic symptoms of diseased plant tissues. Such metabolic alteration following

exposure to a pathogen may function as a defence mechanism.

Tissue browning from accumulation of melanins produced from oxidized phenols is characteristic of nematode injury. Some nematodes produce browning and necrosis as a typical symptom; other groups such as root-knot and cyst nematodes produce browning only on resistant hosts. Lesions produced by a number of different nematode species have been shown to contain high amounts of phenolic compounds. The highest concentration of phenols in infected roots is usually localized in the endodermis; nematodes are often not able to penetrate the vascular tissues and injury is then confined to the cortex (22, 23, 26, 32, 33).

Observations have been made by several workers as to the effect of browning reactions on nematode activity. Wallace (35) observed that fewer nematodes were found in chrysanthemum leaf tissue that browned rapidly upon infection by foliar nematode than in tissues that browned slowly. Pitcher, et al (23) and Townshend (32, 33) also observed that P. penetrans were found to migrate away from dark-colored lesions on several hosts. Observations by a number of workers in our laboratory and elsewhere on aseptic cultures show that when plant callus tissue infected with P. penetrans becomes necrotic and dark brown, most nematodes leave this tissue and are found in the agar or on the sides of culture tube.

Chlorogenic acid (CA), the major phenol of tomato roots has been

found in higher concentration in variesties resistant to the root-knot nematode Meloidogyne incognita acrita. Brown lesions contain very high amounts of oxidation products of CA, whereas the susceptible variety has very little (22).

Weurman and Swain (36) have demonstrated that chlorogenic acid is one of the substrates involved in enzymatic browning of both apple and pear homogenates and that three new fluorescent compounds were formed during the reaction. In addition, some fluorescent compounds have been reported to accumulate in infected plant tissue which could not be detected in healthy tissue. Yu and Hampton (38) found that two fluorescent compounds accumulate in Nicotiana tabacum leaves infected with Collectotrichum destructivum, a pathogen which induces necrotic lesions. Hampton, et al (10) reported that two ultraviolet-fluorescent compounds accumulated in TMV-infected hypersensitive tobacco and were localized in a narrow band around the necrotic lesion. Castillo (5) found an additional bright blue fluorescent compound with the chemical properties of a phenol to be present in extracts of alfalfa lesions caused by P. penetrans.

This investigation was undertaken to determine the nature of the repellent effect of necrotic plant tissue on nematodes. This effect may be either chemical or physical in nature. Attempts were made to isolate and identify specific compounds formed in necrotic tissue which were most active against nematodes as repellents or as respiratory inhibitors.

II MATERIALS AND METHODS

Culturing nematodes and obtaining test necrotic tissue. Pratylenchus penetrans (Cobb 1917) Filipjev and Stekhoven 1914, was cultured axenically on Dupuits alfalfa callus tissue growing in nutrient agar, using a modification of the technique described by Krusberg (16). Seeds of tomato (Lycopersicon esculentum var. B-5), carrot (Daucus carota var. Imperator) and alfalfa (Medicago sativa var. Dupuits) were surface sterilized and germinated on 2% agar. Seedlings were transplanted into test tubes containing nutrient agar to which 2 mg/l of 2,4-dichlorophenoxyacetic acid was added (16). After five days, the callus tissues were inoculated with P. penetrans and incubated at 20° C.

Infected tissues gradually became dark brown and necrotic while nematodes grew and reproduced in the tissue. Two-months-old necrotic tissues were used as the original materials for determining the repellent effect, because at that time most nematodes had left the tissues and were found in the agar or on the sides of the culture tube.

For each experiment, nematodes were obtained by removing alfalfa callus tissue from the culture tube and placing it on a cotton plug in the upper part of a 15 ml centrifuge tube filled with enough water to wet the plug. The tube containing the water and plug had been previously capped with aluminum foil and autoclaved. The nematodes settled to the bottom of the tube within a few hours. Nematodes were always stored at 5°-10° C to inhibit excessive microbial growth and

reduce nematode motility. In general, infected callus tissue was placed in the centrifuge tube for nematode extraction twenty to twenty-four hours before migration and respiration measurements were to be made.

Measuring nematode movement in agar. The methods used for measuring nematode migration were modifications of the technique described by Lavallee and Rohde (17). Polystyrene chambers (46 x 22 x 5 mm) were marked on the undersurface into 14 mm end sections and an 18 mm center section. Two per cent water agar was added to the depth of 4-4.5 mm and before gelation a small piece of necrotic tissue was placed at one end, the other end remaining undisturbed. These were compared with similar chambers containing a piece of healthy callus tissue.

At least ten hours later, a 7 mm diameter cylinder of agar was removed from the center of the chamber, and a drop of liquid agar was put into the cavity to seal the bottom. About 60-70 randomly selected nematodes were pipetted into the cavity in a small volume of water. The cavity was then filled with cool but ungelled agar to the level of surrounding agar. Nematodes moved readily either on or beneath the agar surface. The procedure used for migration studies was designed to measure the number of nematodes moving from the site of inoculation into the end sections at a given time interval. Modifications of the above procedure were used for other experiments involving crude extracts, eluates of paper chromatograms, and effluents of ion-exchange resin and Sephadex gel filtration in place of necrotic tissue. A 5 mm diameter cylinder of agar

was removed from one of the chamber ends, and the bottom was sealed with a drop of agar before adding the above substances.

The migration chambers and media were disinfested at the beginning of each experiment but axenic conditions were not maintained during the course of the experiments. In general, however, observations were made two to three hours after inoculation with nematodes and the influence of contamination, if any, was not considered significant.

Statistical method used in testing data. The analysis of variance technique as presented by Steel and Torrice (31) was used to test the difference between treated and blank ends of chambers. Modifications of this technique were made only insofar as actual observations underwent logarithmic transformation. To clarify the use of this method, an example is given in Appendix I.

Isolation and purification of substances formed in necrotic tissues. Substances obtained from each of the following steps were tested by the above technique to determine their effect on the migration of P. penetrans; organic solvents used for extraction were always evaporated and the residue was redissolved in water. Healthy callus tissues were subjected to the same procedures and compared with necrotic tissue extract.

1. Preparing crude extracts. Necrotic callus tissue was removed from culture tubes, weighed, dropped in boiling methanol, cooled, and homogenized in a glass tissue grinder. Homogenates

were centrifuged at 3100 x G for ten minutes to remove cell fragments. Supernatants were drawn off and serially diluted. Different concentrations of aqueous extract (200, 100, 50, 25, 5, or 1 mg of necrotic tissue per 1 ml of methanol) were then examined for repellent activity.

a. Membrane barriers between extracts and nematodes. Membranes with different permeability were tested for their ability to block the repellent activity of a methanolic extract of alfalfa. Membranes were formed as small cups and placed in cavities made by removing a cylinder of agar from one of the chamber ends; extract was then pipetted into the cups. Membranes used were collodion film, dialysis tubing and polyethylene, the latter being impermeable to water.

b. Influence of pH gradient. Buffered agar was prepared by adding McIlvaine Standard buffer solutions (11) to 2% agar until a desired pH was reached. Approximate final molarity was 6×10^{-3} and agar concentration was 1.95%. Since autoclaving lowered the pH of the media, one tube of each medium was used to determine the initial pH after autoclaving. pH was determined by a Beckman Zeromatic pH meter.

Experiment A.

(a) The agar in one section was replaced by pH 4.75 buffered agar three hours before nematodes were introduced; the other end containing plain agar was not disturbed. (Fig. 1)

(b) Same as (a) except agar buffered to pH 7.6.

Experiment B.

The agar medium in one end was replaced with pH 7.6 buffered agar three hours before nematodes were introduced. Extract of alfalfa necrotic tissue was placed in the plain agar section 18 hours before nematodes were introduced.

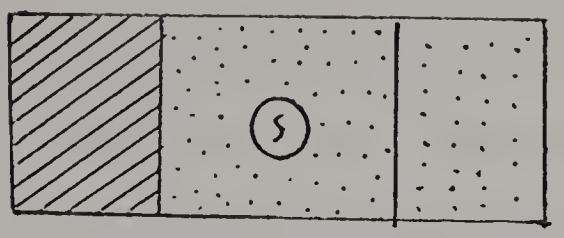
Experiment C.

All agar in the migration chamber was buffered to pH 6.7 or 7.6. Extracts were placed in one of the end sections of each chamber 18 hours before nematodes were introduced.

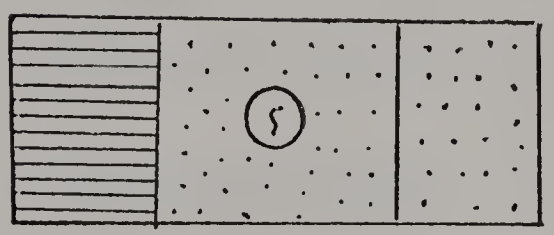
2. Paper chromatography. Crude methanolic extracts of alfalfa, tomato and carrot necrotic tissues were analyzed chromatographically on Whatman paper No. 1, using the methods described by Block et al (3). A number of developing solvents were tested in preliminary studies. The most efficient descending solvents were found to be: 50% ethanol for alfalfa; n-butanol, acetic acid, and water (BAW 4:1L1 v/v/v) for carrot; and BAW (4:1:5 v/v/v) for tomato. Other organic solvents were also used for further

Exp. A

(a)

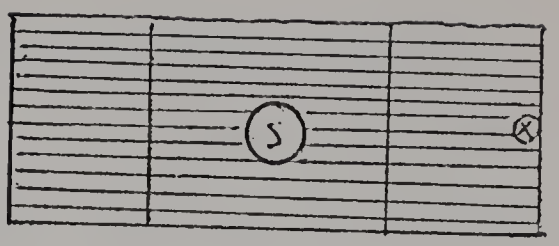


(b)

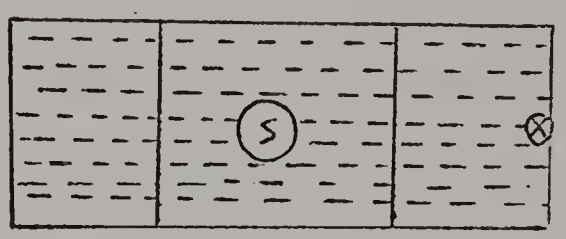


Exp. C

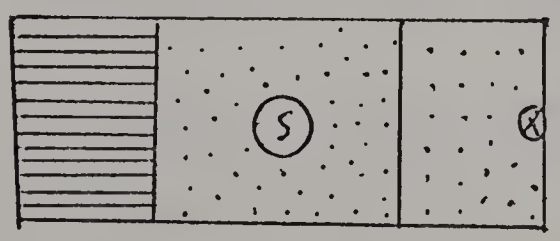
(a)



(b)



Exp. B



plain agar pH 6.2

buffered agar pH 4.75

buffered agar pH 7.6

buffered agar pH 6.7

Fig. 1. Experiments on the influence of pH on migration of P. penetrans. Various combinations of buffered agar, plain agar and extract were placed in migration chambers as shown in the diagrams.

(S) Nematode at start

(X) Extract at start

identification of specific compounds.

Chromatograms were observed under ultraviolet light (320-400 nm) before and after exposure to ammonia fumes. Diazotized sulfanilic acid (DSA), ferric chloride-ferricyanide, ammoniacal silver nitrate and ninydrin were used as detecting reagents (3,30). Areas with the same Rf values were then cut from identical, but unsprayed chromatograms and eluted overnight with distilled water. Each of the eluates was assayed for repellent activity.

3. Solubility. Crude methanolic extracts were oven dried at 75° C and then extracted separately with the following solvents: 95% ethanol, 10% acetic acid, ethyl-ether, ethylacetate, n-butanol and isopropanol. Each supernatant except ether was evaporated to dryness and taken up in 2 ml distilled water. Ether was added to water in a separatory funnel. The ether and water fractions were each evaporated to dryness and residues were dissolved in 2 ml distilled water. Extracts were assayed separately.

4. Use of ion-exchange resin. A 15 cm, 1.1 cm diameter cation-exchange column of Amberlite IRC-50 C.P. resin, H^+ form was prepared. Each methanolic extract was oven dried, redissolved in 5 ml distilled water and quantitatively transferred to the column. Distilled water was used as solvent; the effluent

was collected and concentrated. In addition, 1N HCl was passed through the column to elute materials retained by the resin. The effluent was again collected, neutralized to pH 7 and then concentrated. Both portions were examined for repellent activity.

5. Use of Sephadex gel filtration. A 30 cm, 1.5 cm diameter column of Sephadex G-15 was prepared and allowed to settle for fifteen hours prior to use. The same extract as used in ion-exchange column was quantitatively pipetted into the column, and 140 ml of distilled water was used to wash the extract through the column. Seven-ml fractions were collected and each fraction was tested for repellent activity.

6. Spectrophotometric methods. It was usually necessary to subject the fractions obtained from columns to paper chromatography in order to establish the purity and identity of the highly active fractions obtained from the columns. Those fractions showing highest activity were chromatographed. Spots cut from the chromatogram were eluted with distilled water and assayed for repellent activity. The absorption spectra of active compounds were measured in a Bausch and Lomb Spectromic 505 recording spectrophotometer.

Respirometry. Respiration was measured by Gilson's differential method. Nematodes, glasswares and media were essentially aseptic at the

beginning of each experiment. In addition, a blank was run with each experiment containing the same medium from which nematodes had been removed. Each flask received 10,000 to 15,000 nematodes and each determination was replicated and the entire experiment was repeated. Nematodes were counted and checked for motility before as well as after each experiment and the experiment was discarded, if all nematodes were not recovered alive.

Experiment A.

Ten flasks containing 0.2 ml of 10% KOH in the center well were prepared as described in the following Table. Eight vessels received 0.5 ml nematode suspension and two received 0.5 ml of supernatant from this suspension. Necrotic alfalfa tissue was extracted in boiling methanol. The methanolic extract was evaporated to dryness and redissolved in enough water to give the desired concentrations. Readings were taken every thirty minutes.

Vessel Number	Contents
1 & 2	: 0.2 ml KOH, 0.5 ml supernatant, 0.3 ml H_2O.
3 & 4	: 0.2 ml KOH, 0.5 ml nematode suspension, 0.3 ml H_2O.
5 & 6	: 0.2 ml KOH, 0.5 ml nematode suspension, 0.3 ml extract (10 mg/ml).
7 & 8	: 0.2 ml KOH, 0.5 ml nematode suspension, 0.3 ml extract (50 mg/ml).
9 & 10	: 0.2 ml KOH, 0.5 ml nematode suspension, 0.3 ml extract (100 mg/ml).

Experiment B.

Eight flasks were prepared as in Experiment A, except that No. 5 & 6 contained chlorogenic acid (2000 ppm) and 7 & 8 contained the oxidation product(s) of chlorogenic acid instead of tissue extract.

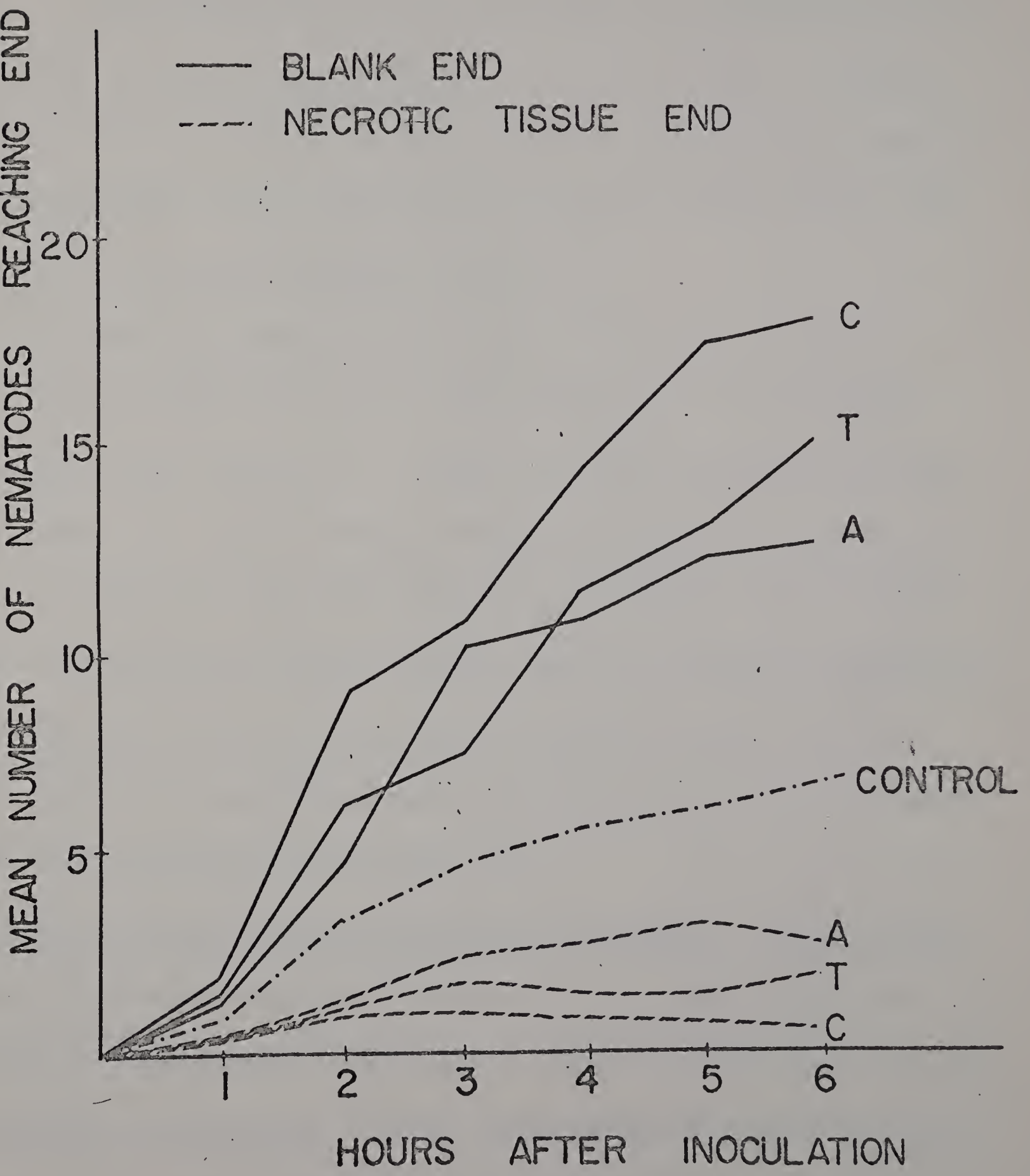
III RESULTS

Preliminary observations. In the absence of necrotic tissue, nematodes were found to move from the inoculation site into the end sections of chambers in approximately equal numbers. When necrotic tissue was placed in the chamber less than ten hours before the nematodes were introduced, there was no significant difference between the numbers of nematodes reaching each end. Therefore, in the following experiments, the test materials were placed in the chamber 12-15 hours before the addition of nematodes.

In the absence of necrotic tissue or other test materials, nematodes were found to move in almost equal numbers to both ends of chambers. In treated chambers, however, if a significantly higher number of nematodes moved away from treatment ends (at the 95% level of probability), the manifestation was referred to as "repellent activity." If a significantly higher number moved toward the treated end, the treatment was considered "Attractive."

Initial migration of nematodes from the inoculation site was significantly greater into blank ends than into necrotic tissue ends in three hosts tested (Fig. 2). The mean number of nematodes reaching the blank end continued to increase after three hours, while the mean number of nematodes going to the necrotic tissue end remained fairly constant. The time interval was based on the time required for nematodes to move to one end and not long enough for them to wander back to the

Fig. 2. Migration of P. penetrans in the presence of necrotic tissue. A significantly higher number of nematodes moved to ends opposite those containing necrotic tissue of the 3 hosts tested. Each point is the mean of 10 replications. C = carrot, T = tomato, A = alfalfa.


BLANK END
NECROTIC TISSUE END
MEAN NUMBER OF NEMATODES REACHING END
20
15
10
5
C
T
A
CONTROL
A
T
C
1
2
3
4
5
6
HOURS AFTER INOCULATION

other.

The oriented movement of nematodes to one direction was considered to be under the influence of an outside stimulus. In these experiments, a negative effect of necrotic tissue resulted in migration of nematodes to ends opposite those containing necrotic tissue. The process was termed "repellent activity."

Crude extract of necrotic tissue in agar. Methanolic extracts of necrotic tissues had the same repellent effect as the tissues themselves (Table I and Fig. 3). Blank ends of the treated chambers did not differ from ends of chambers without any treatment (Appendex II),

Attempts were made to determine the repellent effect of different concentrations of necrotic alfalfa extract on migration of nematodes. High concentrations of extract showed stronger repellent activity. Extracts with concentrations lower than 25 mg of tissue per ml of methanol had no significant effect (Fig. 4).

Membrane barriers between extracts and nematodes. Dialysis tubing and collodion were found to be permeable to repellent substances, whereas polyethylene film appeared to block active materials (Table II).

Influence of pH gradient. In water agar, nematodes appeared to move away from an area of high pH value, and toward an area of low pH. When basic medium and extract were placed in opposite ends of the same chamber, there appeared to be a balance between the negative effects of extract and weak alkali on the movement of nematodes (Table III).

Table I. P. penetrans migration in 2% water agar under the influence of necrotic tissue extracts. Counts were made two hours after nematodes were introduced. AE = necrotic alfalfa tissue extract; CE = necrotic carrot tissue extract; TE = necrotic tomato tissue extract; BL = blank ends.

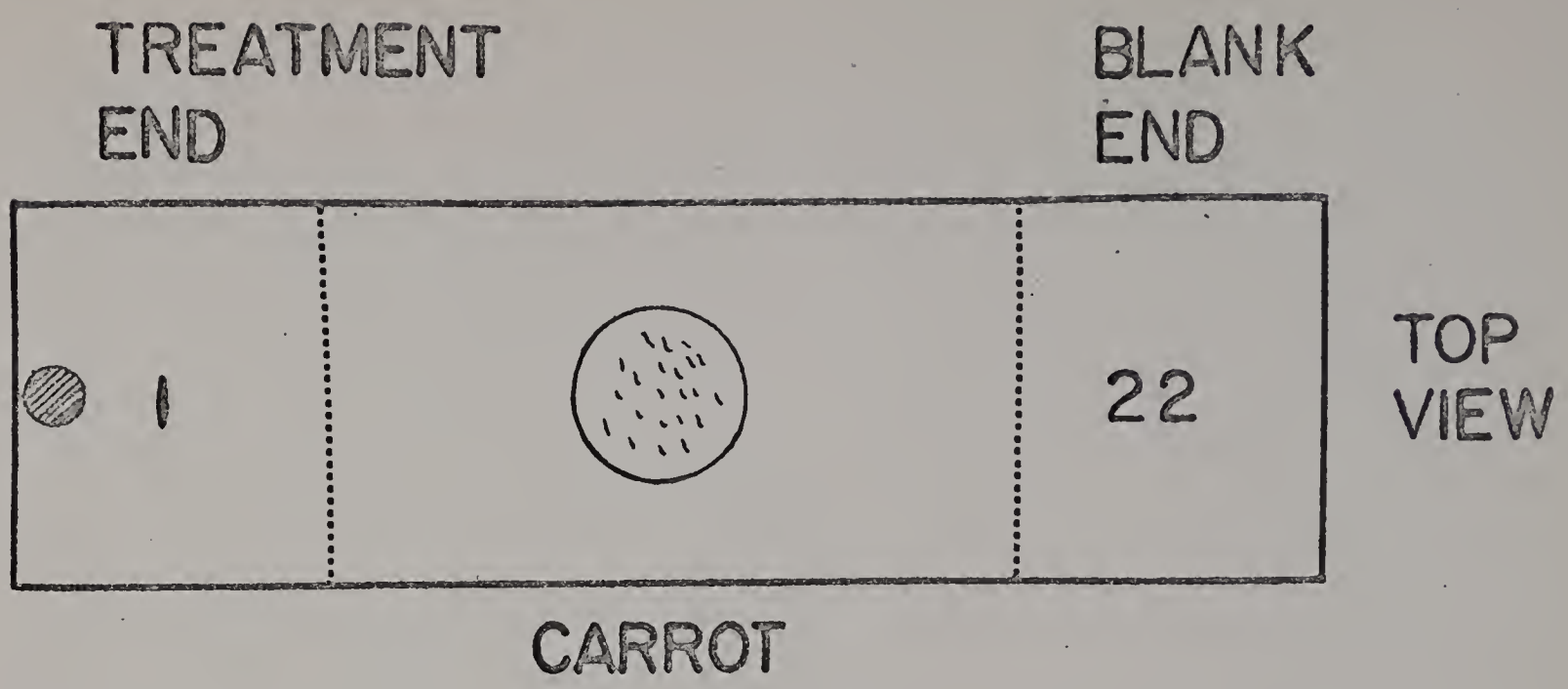
Treatment	Replications								$\bar{X}$	Log (x + 1) $\bar{X}$	.05 L.S.D.
	I	II	III	IV	V	VI	VII	VIII			
AE	2	4	5	3	2	4	2	2	3.00	.585	.1437
BL	10	7	8	17	6	6	8	9	8.87	.975	
CE	1	0	1	2	0	2	1	3	1.25	.309	.1887
BL	7	11	14	13	12	9	12	11	11.10	1.069	
TE	6	4	1	5	0	4	4	3	3.37	.578	.2788
BL	14	10	8	9	4	8	13	12	9.22	1.010	
BL*	3	2	4	3	6	8	2	1	3.62	.619	n.s.
BL*	2	5	4	6	7	8	1	7	5.00	.732	

* Control Chamber without any treatment.

n.s. = Not significant

.05 L.S.D. = 5% Least significant difference

Fig. 3. Migration of *P. penetrans* in agar in the presence of necrotic tissue extract. Nematodes moved from the inoculation site away from rather than toward the extract.



TWO HOURS AFTER INOCULATING
60-70 RANDOMLY SELECTED NEMATODES

Fig 4. Migration of P. penetrans in the presence of different concentrations of extract of necrotic alfalfa tissue. Counts were made three hours after nematodes had been introduced. Differences between blank and extract ends were significant (5% level) at tissue concentrations of 25 mg/ml and above.

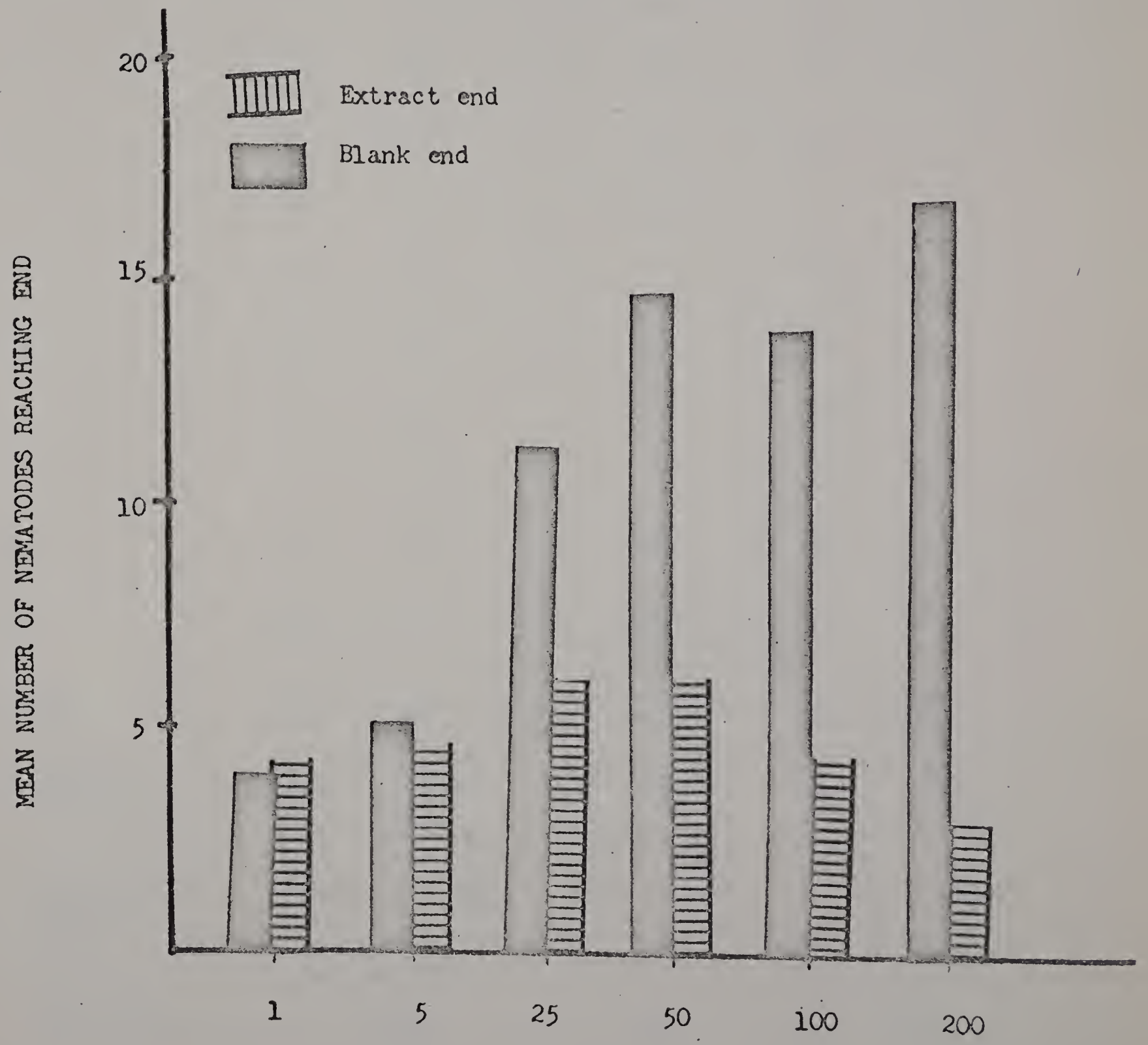

Extract end
Blank end
MEAN NUMBER OF NEMATODES REACHING END
20
15
10
5
1
5
25
50
100
200
CONCENTRATION OF EXTRACT
(mg of necrotic tissue/ ml of methanol)

Table II. Movement of P. penetrans in the presence of membrane barriers between necrotic tissue extracts and nematodes. Counts were made three hours after the addition of nematodes. The repellent effect was not blocked by dialysis tubing or collodion film.
BL = blank end; EX = extract end.

Chamber	Dialysis tubing		Collodion film		Polyethylene	
	EX	BL	EX	BL	EX	BL
1	3	7	0	16	8	7
2	2	18	2	9	5	14
3	2	11	0	6	10	7
4	0	5	1	10	1	2
5	4	14	0	7	9	3
6	3	8	4	10	7	5
7	2	10	2	12	12	7
8	2	6	1	9	6	8
9	1	7	9	29	4	4
10	0	6	0	32	2	5
Total	19	92	19	140	64	62
Mean $\bar{X}$	1.9	9.2	1.9	14.0	6.4	6.2
Log $(x+1)$ $\bar{X}$	.411	.980	.325	1.442	.811	.817
.05 L.S.D.	.046		.111		n.s.	

Table III. Movement of P. penetrans in the presence of pH gradient. Counts were made three hours after nematodes were introduced. Exp. A. -- Higher number of nematodes moved to end sections with the lower pH. Exp. B. -- A balance between the negative effects of necrotic tissue and weak alkali medium resulted in migration of nematodes to both ends in equal numbers.

Experiment A.

	Treatment a		Treatment b	
Chamber	Buffered pH = 4.75	Not buffered pH = 6.2 (plain agar)	Buffered pH = 7.6	Not buffered pH = 6.2
1	7	4	3	21
2	6	1	3	10
3	11	3	4	20
4	10	5	1	9
5	3	0	7	11
6	5	4	11	18
7	9	0	6	10
8	12	5	0	7
Total	63	22	35	106
$\bar{X}$	7.87	2.75	4.37	12.25
Log (x+1) $\bar{X}$	.910	.482	.629	1.126
.05 L.S.D.	.2789		.2801	

Experiment B.

	Buffered pH = 7.6	Extract in plain agar (pH = 6.2)
1	5	6
2	4	2
3	5	11
4	2	3
5	9	6
6	2	5
7	7	9
Total	34	42
$\bar{X}$	4.85	6.0
Log (x+1) $\bar{X}$	.639	.703
.05 L.S.D.	.389 (not significant)	

When all the agar medium in the chamber was buffered to pH 6.7 or 7.6 and there was no pH gradient, extracts were still repellent to nematodes (Table IV).

Paper chromatography. Separation of the methanolic extract by paper chromatography showed that repellent activity was centered around fluorescent compounds. These compounds, when sprayed with several reagents, exhibited the chemical properties of phenols.

The Rf values of compounds present in crude extract and their relative repellent activities are shown in Table V.

Solubility. The active compound or compounds are soluble in water, methanol, 95% ethanol, 10% acetic acid, and isopropanol; partially soluble in butanol, and insoluble in ethyl-ether and benzene.

Purification and isolation of active compounds from crude extract by cation-exchange resin and identification by spectrophotometry.

A. Alfalfa: Paper chromatography of the necrotic tissue extract after passage through cation-exchange column, revealed three spots which fluoresced under U.V. light (320-400nm) (Fig. 5). One compound, which was bright blue under U.V. light showed very high repellent activity and gave positive reactions to various color reagents used for characterizing phenolic compounds on paper. The Rf values of this compound were low in aqueous solvents but high in organic solvents. The ultraviolet absorption spectrum of this compound is shown in Fig. 6. Maximum absorption was at 270nm and lowest absorption was at 250nm. The

Table IV. Movement of P. penetrans in the absence of a pH gradient. Exp. C. -- Counts were made three hours after nematodes were introduced. Significantly higher numbers of nematodes moved away from chamber ends containing extracts of necrotic alfalfa tissue.
BL = Blank end; EX = necrotic tissue extract end.

Chamber	Buffered agar pH = 6.7		Buffered agar pH = 7.6	
	BL	EX	BL	EX
1	19	4	8	3
2	7	4	12	2
3	5	8	4	1
4	24	2	11	4
5	9	1	5	0
6	16	7	8	2
7	12	4	6	1
8	5	1	3	0
9	4	4	1	2
10	10	3	8	2
Total	111	38	66	17
Mean $\bar{X}$	11.1	3.8	6.6	1.7
Log (x+1) $\bar{X}$	1.024	.633	.828	.381
.05 L.S.D.	.0502		.2215	

Table V. Paper chromatography of compounds extracted from necrotic tissues and their relative repellent activity.

Carrot ---- BAW (4:1:1 v/v/v)

Spots	Fluorescense (presence of NH_3)	DSA	Ninhydrin	Rf	Eluate portions	Repellent activity
1	____	-	+	.17	.00- .18	n.s.
2	____	-	++	.21	.18- .22	n.s.
3	Y	+	+	.23	.22- .24	s.*
4	____	-	++	.27	.24- .28	n.s.
5	____	-	++	.31	.28- .35	n.s.
6	lY	+	-	.42	.35- .55	n.s.
7	____	=	+	.51		
8	GY	++	-	.62	.55- .72	s.**
9	lGY	-	-	.78	.72- .80	s.*
10	B	+	-	.85	.80- .87	s.*
11	Br	++	+	.92	.87-1.0	n.s.

Tomato ---- BAW (4:1:5 v/v/v)

1	____	-	+++	.08	.00- .10	s.*
2	____	-	++	.14		
3	____	-	++	.18	.10- .24	n.s.
4	____	-	+	.21		
5	Y	+	-	.26	.24- .35	s.*
6	____	-	+	.45	.35- .50	n.s.
7	____	-	+	.56	.50- .70	s.**
8	lGY	+	-	.62		
9	____	-	+	.67	.70- .85	n.s.
10	B	++	-	.94	.85-1.0	s.**

Alfalfa ---- 50% Ethanol

1	bB	++	-	.05	.00- .10	s.**
2	bB	++	-	.16	.10- .19	s.**
3	Br	++	-	.24	.19- .27	n.s.
4	Y	+	-	.32	.27- .40	n.s.
5	lY	+	-	.50	.40- .62	s.*
6	lGY	+	-	.67	.62- .75	n.s.
7	lB	+	-	.77	.75- .83	n.s.
8	____	-	++	.87	.83-1.0	n.s.

Y = yellow, lY = light yellow, B = blue, Br = brown, lGY = light green-yellow
bB = bright blue, GY = green-yellow.

- \- No color reaction
- \+ Weak color reaction
- ++ Stronger color reaction

- n.s. No significant activity
- s.* Weak repellent activity
- s.** Strong repellent activity

other two compounds showed no significant effect on the migration of nematodes.

B. Carrot: Four compounds were detected whose color of fluorescence varied from yellow to blue (Fig. 5). These compounds were confirmed to be phenolics by their reactions to spray reagents. All of these compounds had a similar degree of repellent activity. None of these compounds showed any specific U.V. absorption peak.

C. Tomato: Three fluorescent compounds are shown in Fig. 5. The blue and yellow-green spots have higher activity than the yellow one. The blue compound had an absorption spectrum very similar to the one found in alfalfa extract (Fig. 6); but their Rf values in a number of solvents were different. The yellow-green compound had similar Rf values and color reactions to the chlorogenic acid but its absorption spectrum proved to be different from chlorogenic acid.

Separation of active compounds from crude extract by Sephadex gel filtration and paper chromatography. Effluent from Sephadex column was collected in twenty 7-ml fractions. The tenth fraction in alfalfa, fifth fraction in carrot, and sixth fraction in necrotic tomato tissue extract had relatively higher activity than other fractions. The most active fraction of each tissue extract was chromatographed on paper and the results are shown in Fig. 7. The spots obtained were similar in every respect to those detected after cation-exchange purification.

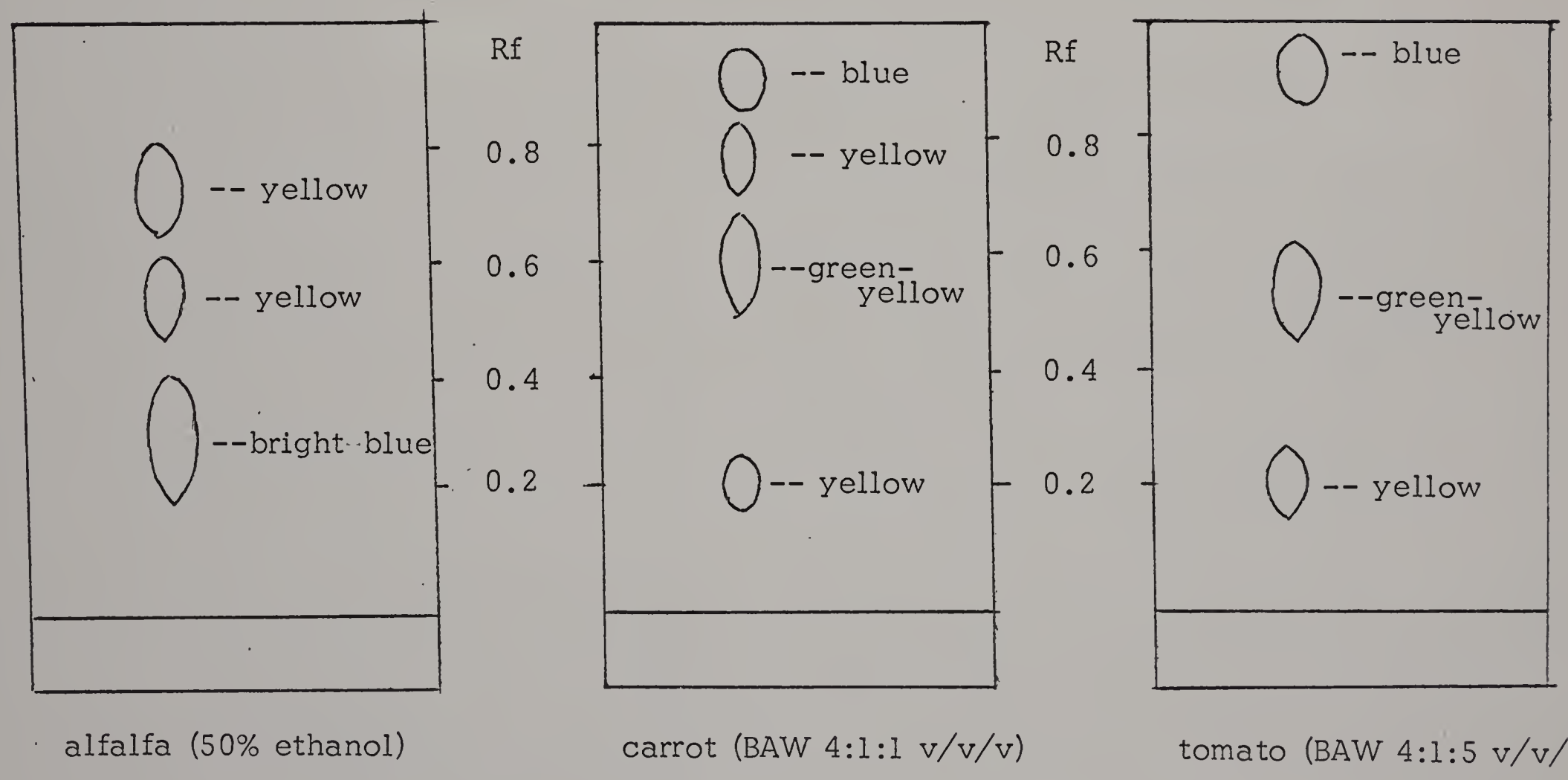
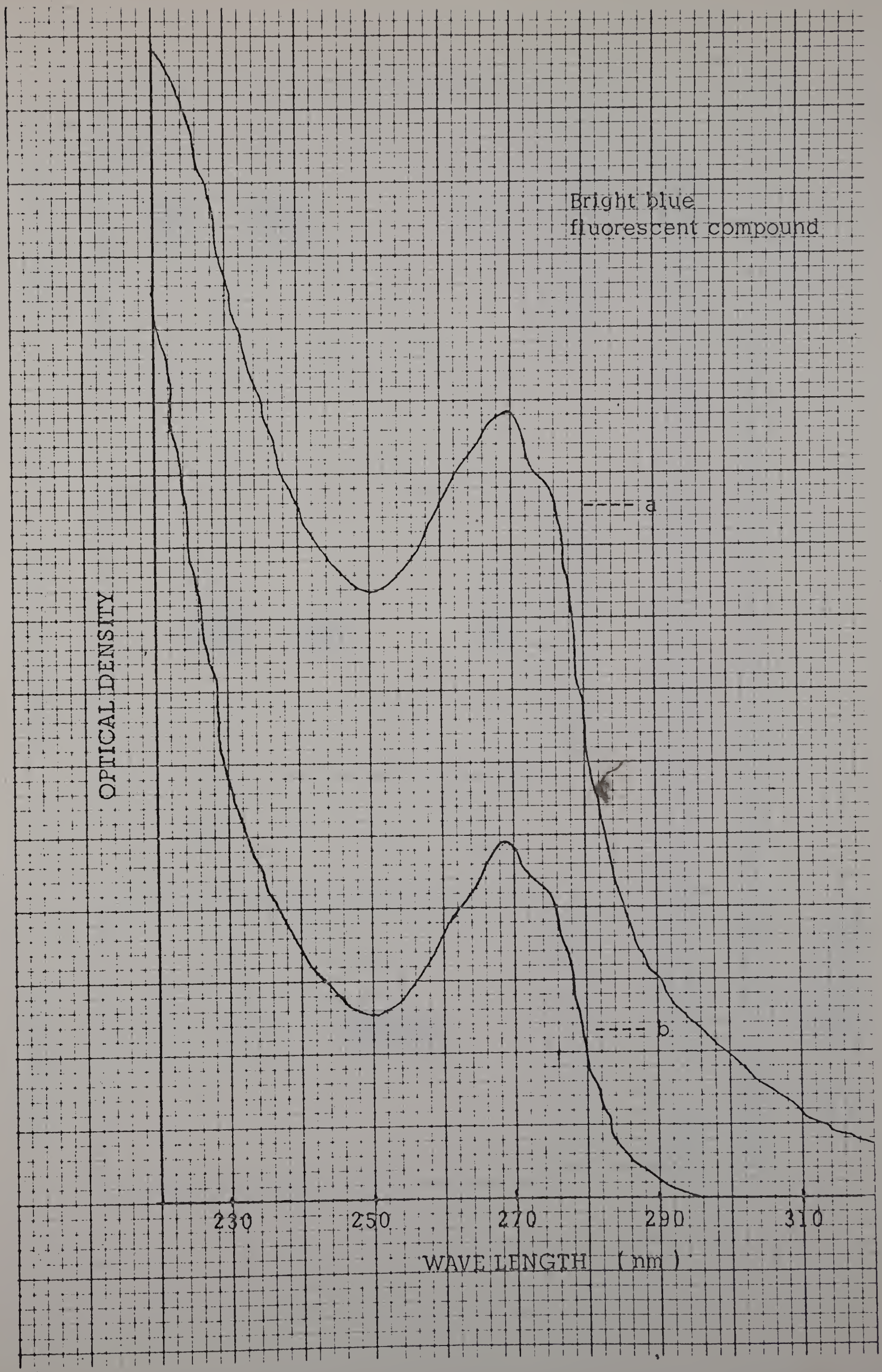


Fig. 5. --- One dimensional paper chromatograms of necrotic tissue extracts showing fluorescent spots under U.V. light. The extracts had previously been passed through a cation-exchange column.

Fig. 6 Absorption spectrum of bright blue fluorescent compound showing a maximum absorption peak at 270 nm and minimum at 250 nm. These compounds were isolated from necrotic tomato and alfalfa tissues by cation-exchange or Sephadex gel chromatography and showed very high repellent activity.

a. Compound from necrotic alfalfa tissue.

b. Compound from necrotic tomato tissue.


Bright blue
fluorescent compound
---- a
---- b
OPTICAL DENSITY
230
250
270
290
310
WAVE LENGTH (nm)

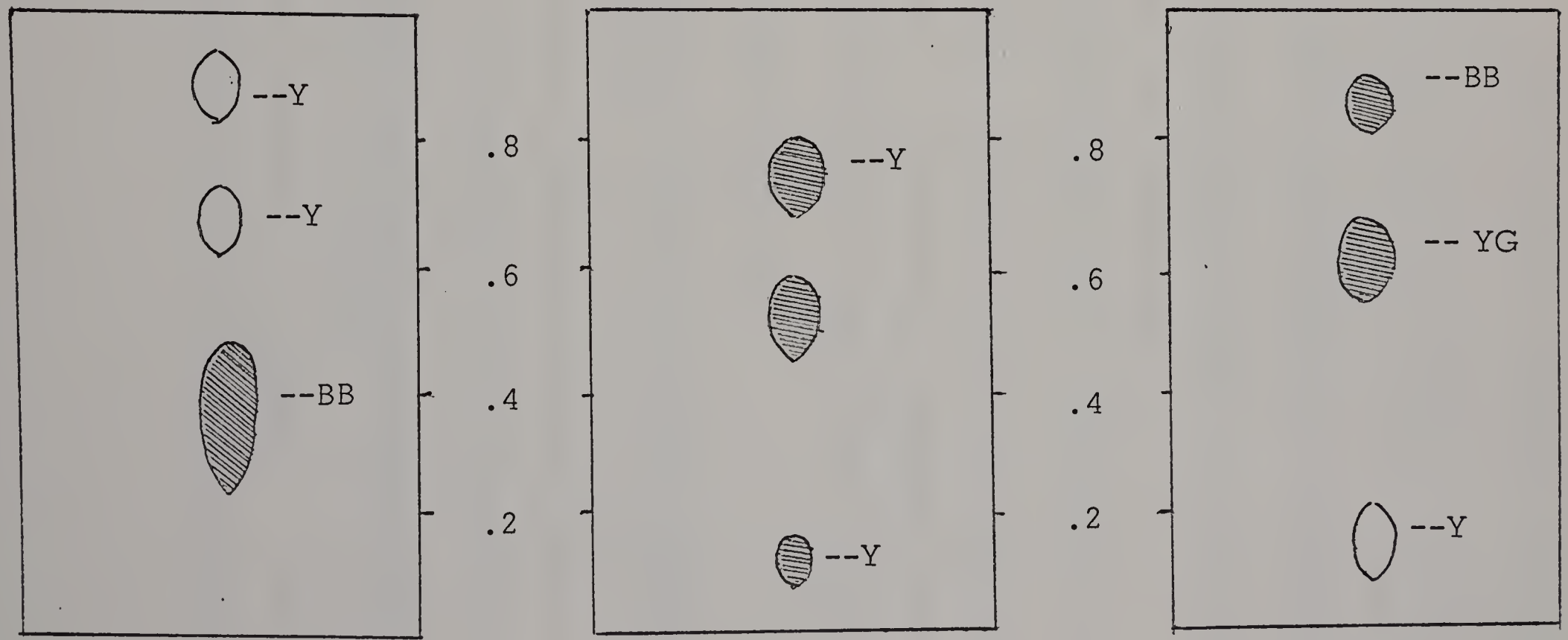


Fig. 7. --- One dimensional paper chromatogram showing fluorescent spots under U.V. light, in which the active fractions collected from Sephadex column were developed.

Shaded spots indicate compounds with relatively high repellent activity.

Y : yellow
BB : bright blue
YG : yellow-green

When oxidized, chlorogenic acid (CA) shows three characteristic spots on paper chromatograms. In chromatographic (Fig.8) and spectrophotometric (Fig.9) comparisons of the fifth fraction of carrot extract with authentic CA and with the oxidation products of CA, it was shown that the carrot extract fraction was quite similar to the oxidation products of chlorogenic acid.

Chlorogenic acid in agar. Chlorogenic acid (CA) had been previously reported as the major phenol in the tomato plant (22). Freshly prepared solution of CA attracted P. penetrans, but when oxidized to a yellowish brown "compound" by adding NaOH or exposing them to the air, they were repellent (Fig.10).

Influence of crude extract of necrotic tissue on the respiration of P. penetrans. The respiration of lesion nematodes was significantly depressed by high concentrations of necrotic tissue extract, whereas lower concentrations of extract reduced respiration only slightly (Fig.11).

Influence of Chlorogenic acid and its oxidation products on the respiration of P. penetrans. Chlorogenic acid has no apparent effect on the respiration of P. penetrans, but when it was oxidized in air, it significantly reduced the rate of respiration (Fig.12).

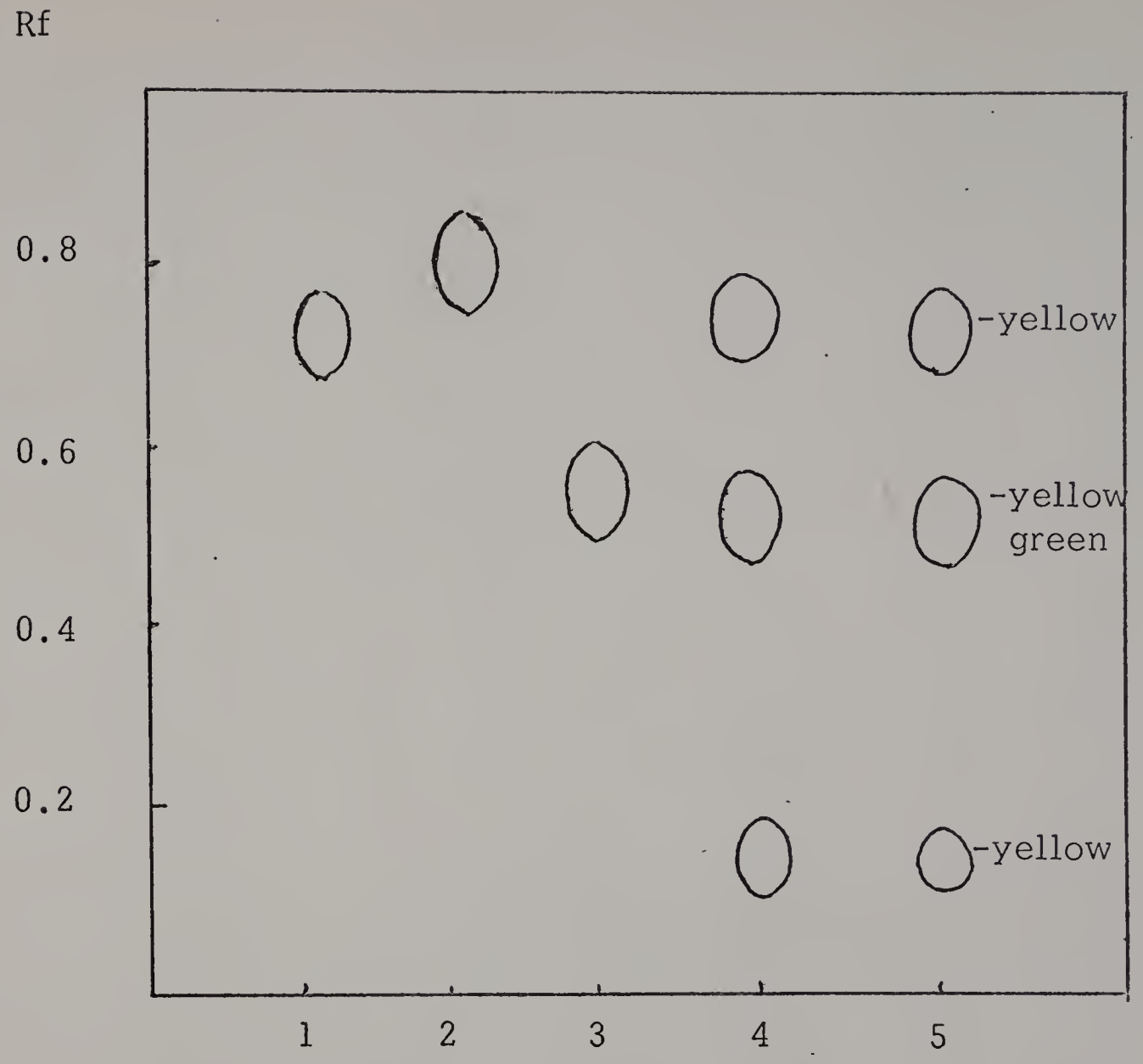


Fig. 8. One dimensional paper chromatogram showing fluorescent spots under U.V. light.
Butanol : acetic acid : water = 4:1:1 v/v/v was used as solvent.

1. caffeic acid.
2. quinic acid.
3. chlorogenic acid.
4. oxidation product of chlorogenic acid.
5. the fifth fraction of carrot extract collected from Sephadex column.

Fig. 9. Absorption spectra of (a) fifth fraction of carrot extract collected from Sephadex column, (b) oxidation product of Chlorogenic acid, and (c) authenic chlorogenic acid.

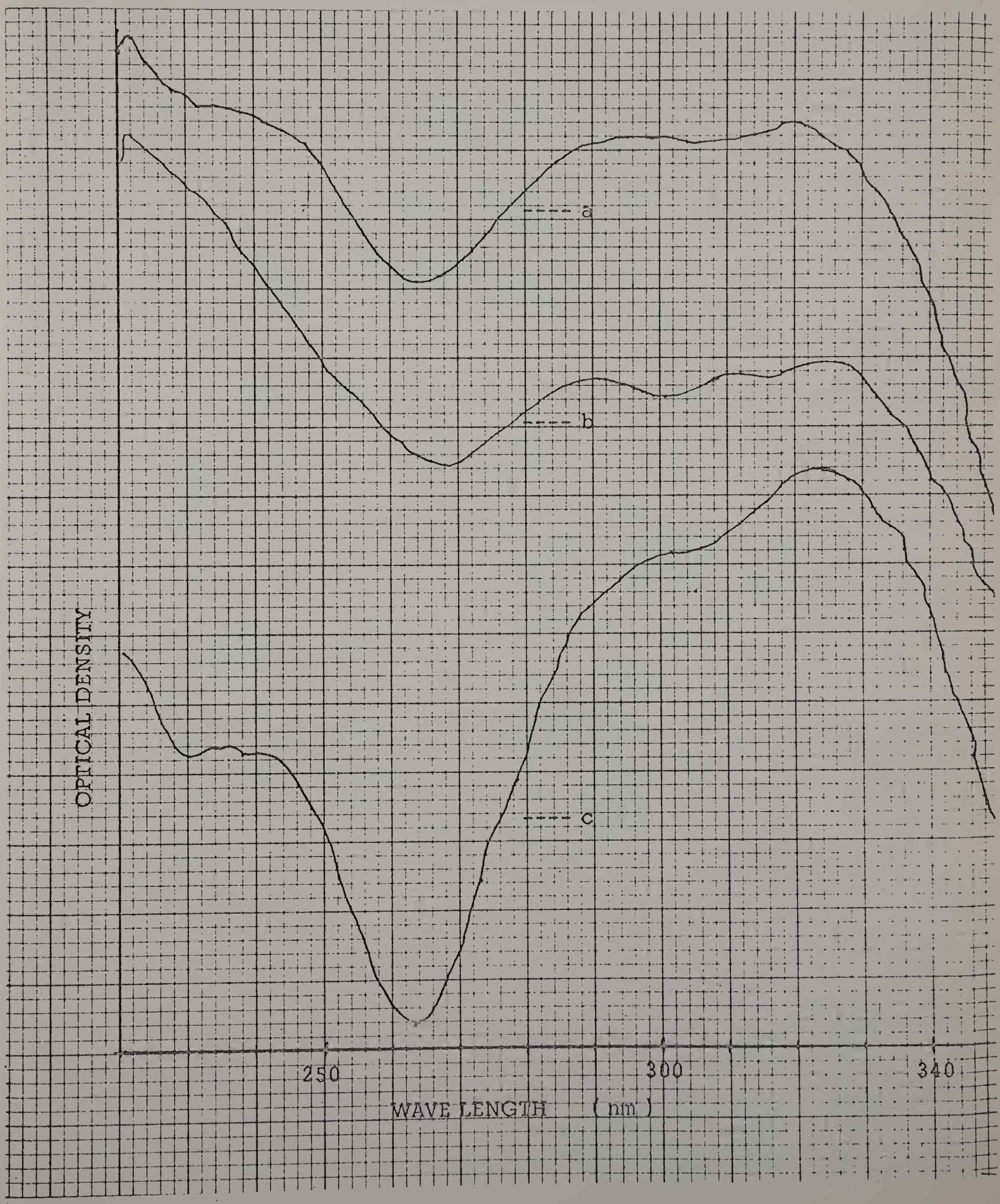
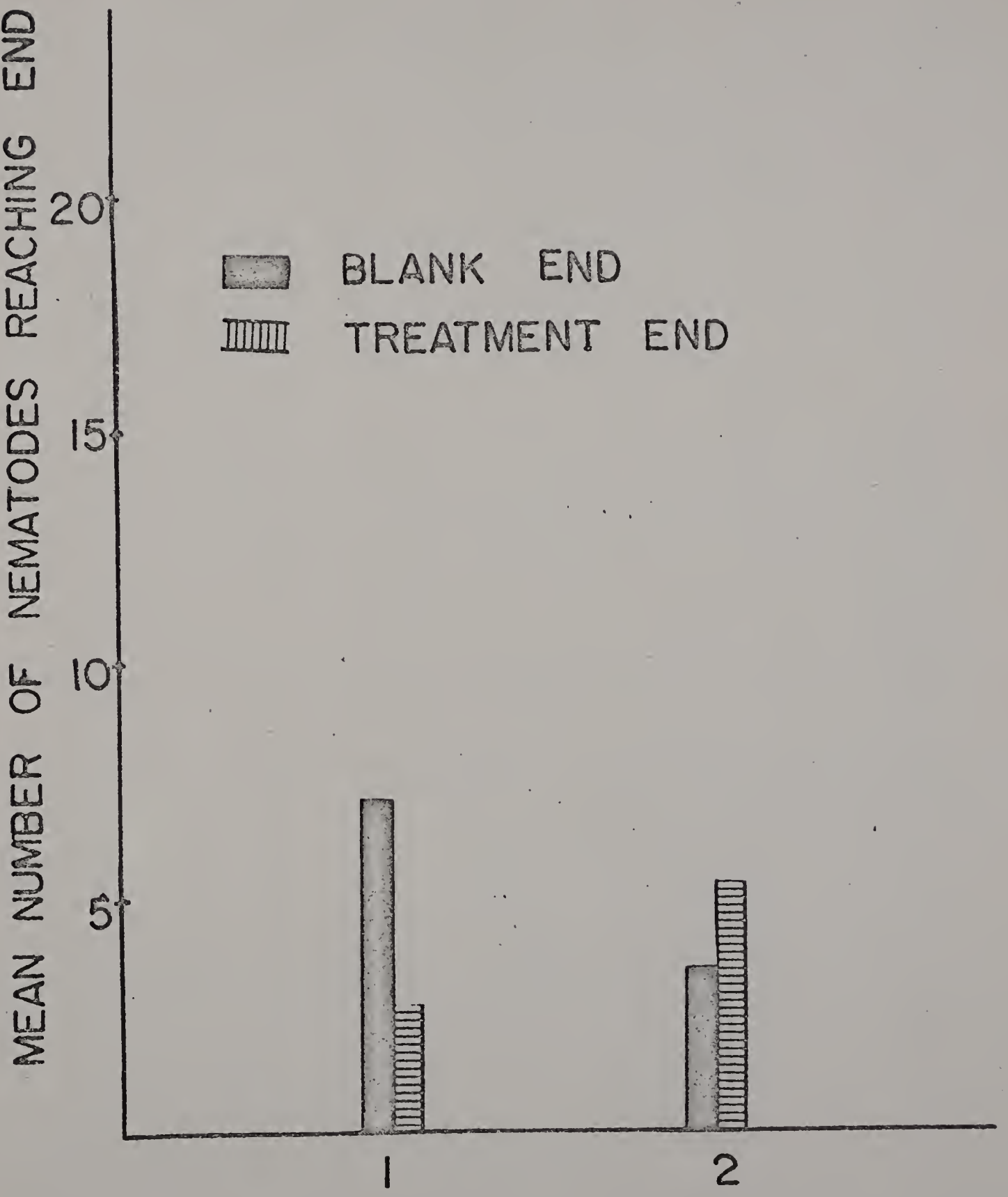

a
b
c
OPTICAL DENSITY
250
300
340
WAVE LENGTH (nm)

Fig. 10. Movement of P. penetrans in migration chambers containing either chlorogenic acid (CA) or its oxidation products. A significantly higher number of nematodes moved toward chamber ends containing CA, whereas a significantly higher number moved away from the ends with oxidation product of CA. Counts were made two hours after nematodes were introduced.



1. OXIDIZED CHLOROGENIC ACID (pH= 8)
2. AUTHENIC CHLOROGENIC ACID

Fig. 11. The effect of crude extract of necrotic alfalfa tissue on the respiration of P. penetrans. Each point represents a determination of two to four replications.

a. Nematodes in water.

b. Nematodes in extract (10 mg/ml).

c. Nematodes in extract (100 mg/ml).

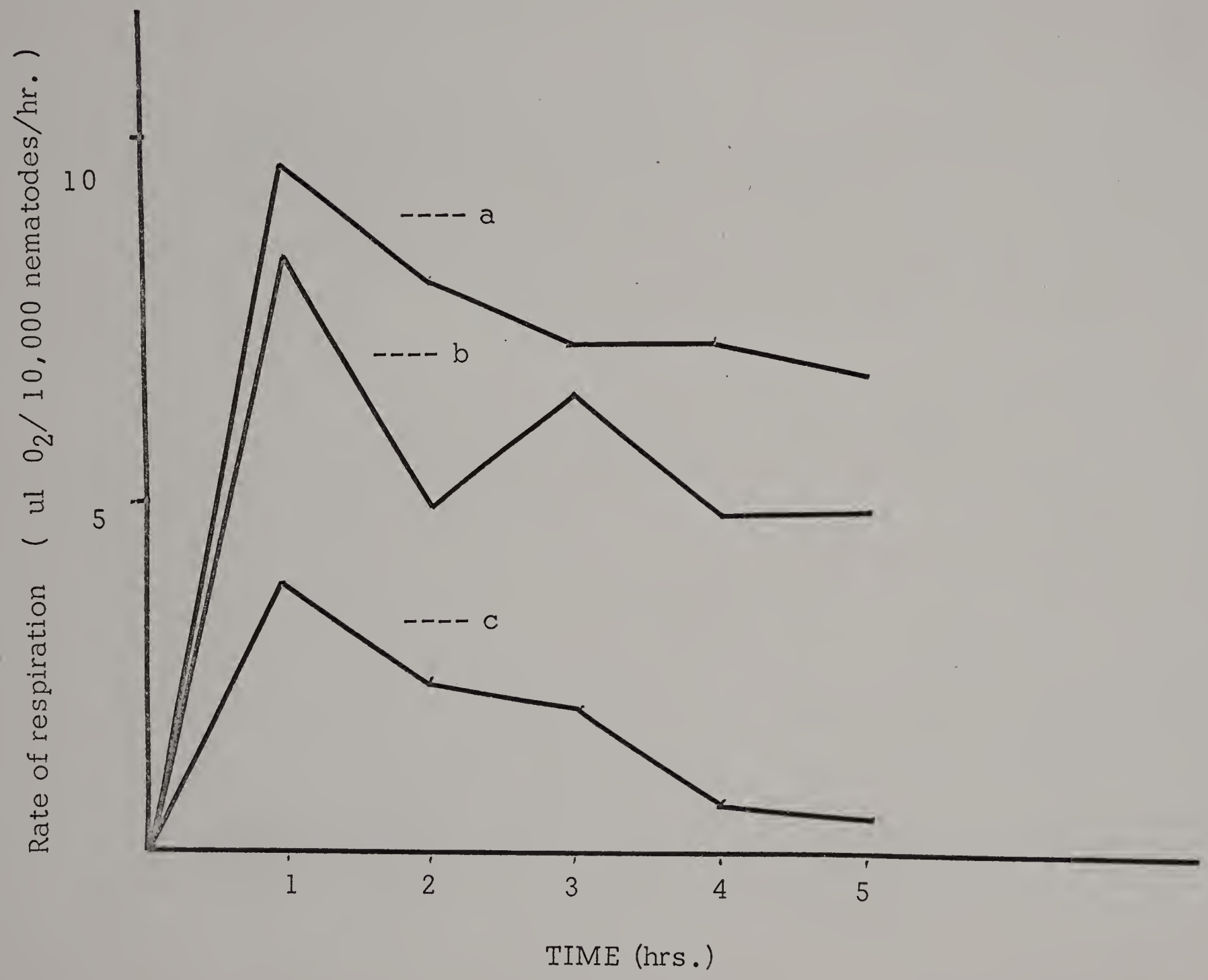
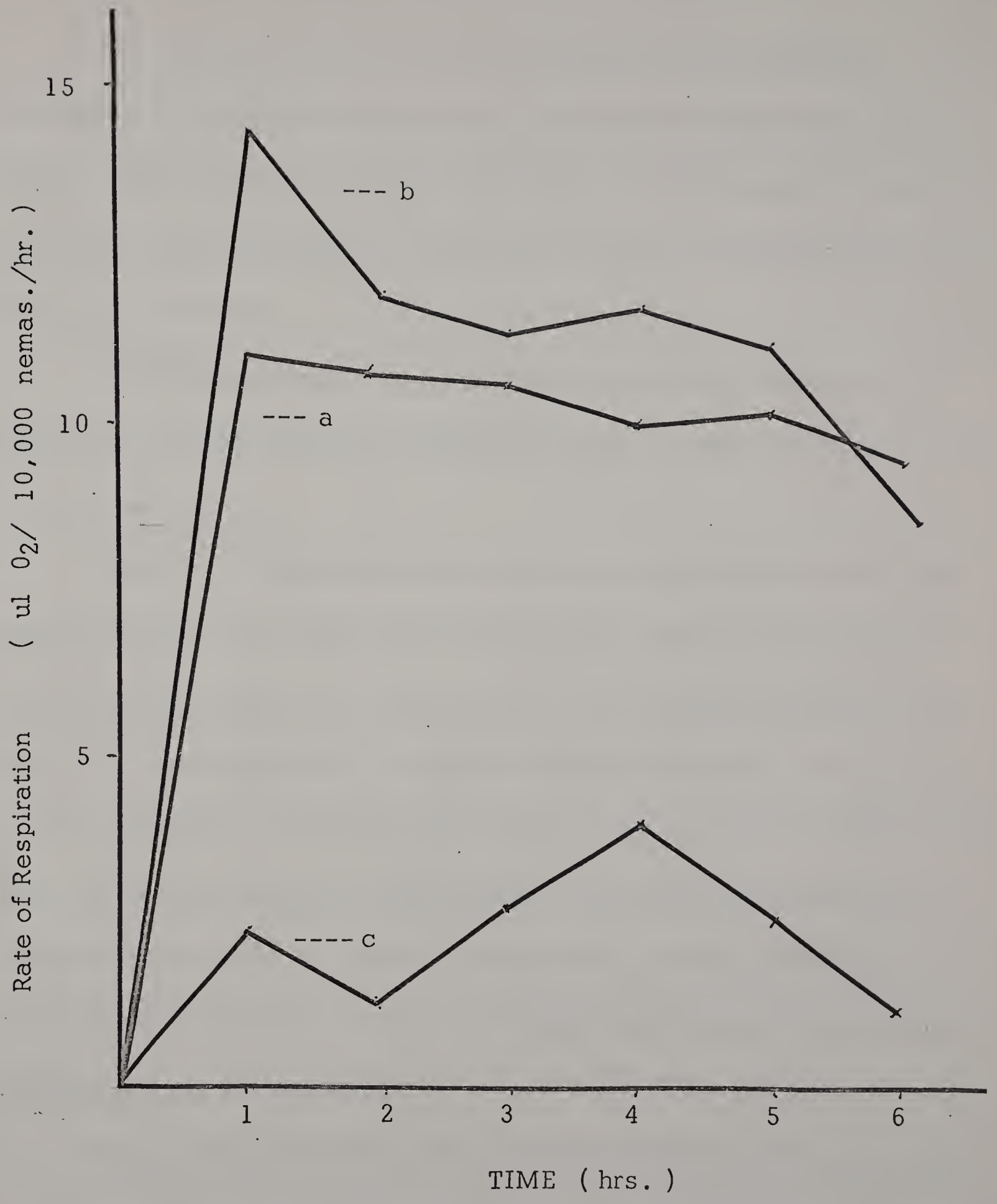

Rate of respiration (ul O_2/ 10,000 nematodes/hr.)
10
5
---- a
---- b
---- c
1
2
3
4
5
TIME (hrs.)

Fig. 12. The effect of chlorogenic acid and its oxidation product on the respiration of P. penetrans. Each point represents a determination of two replications.

a. Nematodes in water.

b. Nematodes in chlorogenic acid solution. (CA)

c. Nematodes in oxidation product of CA, a yellowish brown compound.


15
10
5
--- b
--- a
---- c
1
2
3
4
5
6
Rate of Respiration (ul O_2/ 10,000 nemas./hr.)
TIME (hrs.)

IV DISCUSSION

The repellent effect of necrotic tissues on the nematode Pratylenchus penetrans was observed consistantly during these experiments. The oriented movement of nematodes away from necrotic tissue was apparently a chemotactic response to specific compounds formed in the necrotic tissues.

The repellent effect was not blocked by dialysis tubing or collodion film, suggesting a low molecular weight, water-soluble compound as the active agent.

Nematodes appeared to move away from an area of high pH, and toward an area of low pH in these experiments, which is in agreement with the results reported by others (37). It is doubtful, however, that high pH is responsible for the repellent effect of necrotic tissue under natural conditions. No significant difference of pH could be detected between necrotic and healthy plant tissues. Also, the repellent effect of necrotic tissue still occurred in buffered media, where presumably no pH gradient existed and, in fact, nematodes moved away from necrotic tissue against a pH gradient.

Paper chromatography indicated that repellent activity was centered around fluorescent compounds formed in necrotic tissues. These compounds gave positive reactions to various color reagents used for characterizing phenolic compounds on paper.

An absorption peak at 270 nm, a characteristic of many substituted

phenols, was obtained with active compounds in both necrotic alfalfa and tomato tissues.

The bright blue fluorescent compound extracted from necrotic alfalfa tissue showed high repellent activity. This compound had been previously reported as the only new compound formed following nematode invasion and could not be detected in the healthy alfalfa tissue (5). It was considered to be the primary factor responsible for the repellent activity of necrotic tissue.

Chlorogenic acid (CA) had been previously reported as the major phenol in tomato root (22). Freshly prepared CA solutions had no apparent effect on nematodes, but after oxidation in air they became toxic. Respiration of lesion nematodes was significantly reduced by the oxidation products of CA and nematodes were repelled by them.

Necrotic carrot tissue extracts contained compounds quite similar to those in the oxidation products of chlorogenic acid, as determined chromatographically and spectrophotometrically. Both preparations were repellent to lesion nematodes. Invasion by nematodes may have caused oxidation of CA or similar phenols to new toxic products. It is proposed that the toxic materials are quinones or quinone derivatives.

It has been shown that pehnolics of healthy plants may in some cases be regarded as resistance factors (34). More recently attention has been focused on the accumulation after infection of "induced" phenolic compounds in diseased tissues. Many experimental data support the

contention that the phenolic level is higher in diseased plants than in healthy ones. Particular examples concerning fungal, bacterial, and virus diseases have been summarized by Farkas and Kiraly (9). It has been frequently observed that phenol accumulation takes place in nematode-infected plant tissues as well (22, 23, 26, 32, 33). If a comparison is made between resistant and susceptible combinations, it is apparent that a more rapid accumulation of phenolics takes place in the incompatible host-pathogen combinations than in the compatible ones.

Very little is known about the identity of compounds in nematode-induced necrotic tissues, and whether these compounds occur in the uninfected host, The limited evidence available indicates that fluorescent compounds associated with injury are found in much lower amounts in healthy than in diseased plants and are produced by well known pathways or are released from glycosides at the time of injury (6).

Phenols liberated from glycosidic compounds may be very toxic to the pathogen. Oku (19) has shown that Cochliobolus miyabeanus, the causal organism of Heliminthosporium disease of rice, is able to produce β-glucosidase and liberate phenolic compounds from glucosides in concentrations inhibitory to the fungus. Mountain and Patrick (18) found that when lesion nematodes feed on peach roots, the phenolic glycoside amygdalin is hydrolyzed and hydrocyanic acid is released, much to the detriment of both host and nematodes. The enzyme

demonstrated in this reaction, a β-glucosidase, may be important in other host-parasite interactions since many phenolic compounds in plants occur naturally as less-toxic glycosides. Most of these experiments represent in vitro conditions, and the in vivo role of these processes remains unknown.

Recently several fungitoxic substances, the phytoalexins, have been isolated from diseased plant tissues. These compounds are produced only after infection by pathways related to aromatic biosynthesis. There is no evidence at the present time, however, to show that nematodes induce plants to produce phytoalexins.

V LITERATURE CITED

1. BIRD, A. F. 1959. The attractiveness of roots to plant parasitic nematodes. Nematologica 4: 322-335.

2. BIRD, A. F. 1960. Additional notes on the attractiveness of roots to plant parasitic nematodes. Nematologica 5: 27.

3. BLOCK, R. J., E. L. DURRUM and G. ZWEIG. 1958. Paper chromatography and paper electrophoresis. Academic Press, N. Y.

4. BORNER, H. 1960. Liberation of organic substances from higher plants and their role in the soil sickness problem. Bot. Rev. 26: 393-424.

5. CASTILLO, J. M., and R. A. ROHDE. 1965. Biochemical changes in alfalfa injured by lesion nematodes. Phytopathology 53: 886. (Abstr.)

6. DE-MOTT, H. E. 1965. Observation on utilization of Hexose Monophosphate pathway in nematode-infected root of tomato. Ph.D. Thesis. Univ. of Virginia. Diss. Abstr. 27: 4257-B.

7. EDMUNDS, J. E., and E. F. MAI. 1967. Effect of Fusarium oxysporum on movement of Pratylenchus penetrans toward alfalfa roots. Phytopathology 57: 468-471.

8. EL-SHERIF, M., and W. F. MAI. 1969. Thermotactic response of some plant parasitic nematodes. J. Nematol. 1: 43-48.

9. FARKAS, G. L. and Z. KIRALY. 1962. Role of phenolic compounds in physiology of plant diseases and disease resistance. Phytopathol. Z. 44: 105-150.

10. HAMPTON, R. E., et al 1964. Accumulation of fluorescent compounds in tobacco associated with Tobacco Mosaic Virus hypersensitivity. Phytopathology 54: 1062-1064.

11. HODGMAN, C. D. 1952. Handbook of Chemistry and Physics. 34th ed. Chemical Rubber Publishing Co., Cleveland.

12. JOHNSON, R. N., and D. R. VIGLIERCHIO. 1961. The accumulation of plant parasitic nematode larvae around carbon dioxide and oxygen. Proc. Helminthol. Soc. Wash. 28: 171-174.

13. KATZNELSON, H., J. M. RONATT, and T. M.B. PAYNE. 1956. The liberation of amino acids and reducing compounds by plant roots. Plant and Soil 7: 35-48.

14. KLINGER, J. 1963. Die Orientierung von Ditylenchus dipsaci in gemessenen Kunstlichen und biologischen CO_2-gradienten. Nematologica 9: 185-199.

15. KLINGER, J. 1965. On the orientation of plant nematodes and of some other soil animals. Nematologica 11: 4-18.

16. KRUSBERG, L. R. 1961. Studies on the culturing and parasitism of plant-parasitic nematodes, in particular Ditylenchus dipsaci and Aphelenchoides ritzemabosi on alfalfa tissues. Nematologica 6: 181-200.

17. LAVALLEE, W. H. and R. A. ROHDE. 1962. Attractiveness of plant roots to Pratylenchus penetrans (Cobb). Nematologica 8: 252-260.

18. MOUNTAIN, W. B. and Z. A. PATRICK. 1959. The peach replant problem in Ontario. VII. The pathogenicity of Pratylenchus penetrans (Cobb). Can. J. Botany. 37: 459-470.

19. OKU, H. 1959. Biochemical studies of Cochliobolus miyabeanus. V. β-glucosidase activity of fungus and the effect of phenol-glucoside on mycelial growth. Ann. Rept. Takamine Lab. 11: 190-192.

20. OTEIFA, B. A. and D. M. ELGINDI. 1961. Physiological studies on host-parasite relationship of root-knot nematode, Meloidogyne javanica. Pl. Dis. Rep. 45: 928-929.

21. PATE, J. S. 1962. Root exudation studies on the exchange of C^{14}-labelled organic substances between the roots and shoot of nodulated legume. Plant and Soil. 17: 333-356.

22. PI, C. L. 1966. Phenolic compounds associated with host reaction in tomato to injury caused by root-knot and lesion nematodes. M. S. Thesis, Univ. of Massachusetts.

23. PITCHER, R. S., Z. A. PATRICK and W. B. MOUNTAIN. 1960. Studies on the host-parasite relations of Pratylenchus penetrans (Cobb) to apple seedling. I. Pathogenicity under sterile condition. Nematologica 5: 309-314.

24. RODE, H. and G. STAAR. 1961. Die photographishe darstellung der Kriechspuren (Ichnogramme) von Nematoden und ihre' Bedeutung. Nematologica. 6: 266-271.

25. ROHDE, R. A. 1960. The influence of carbon dioxide on respiration of certain plant parasitic nematodes. Proc. Helminthol. Soc. Wash. 27: 160-164.

26. ROHDE, R. A. 1963. Lesion nematode injury to carrot. Phytopathology 53: 886. (Abstr.)

27. ROVIRA, A. D. 1956. Plant root excretions in relation to the rhizosphere effect. I. The nature of root exudates from oats and peas. Plant and Soil 7: 178-194.

28. ROVIRA, A. D. and J. R. HARRIS. 1961. Plant root excretions in relation to rhizosphere effect. V. The exudation of B-group vitamins. Plant and Soil. 14: 199-214.

29. SANDSTEDT, R., SULLIVAN, THELMA and M. L. SCHUSTER. 1961. Nematode tracks in the stucy of movement of Meloidogyne incognita incognita. Nematologica 6: 261-271.

30. SMITH, I. 1960. Chromatographic and electrophoretic technique. Vol. 1. Interscience Publisher, Inc., N. Y.

31. STEEL, R. G. D., and J. M. TORRIE. 1960. Principles and procedures of Statistics. N. Y. McGraw-Hill, 481 pp.

32. TOWNSHEND, J. L. 1963. The pathogenicity of Pratylenchus penetrans to celery. Can. J. Pl. Sci. 43: 70-74.

33. TOWNSHEND, J. L. 1963. The pathogenicity of Pratylenchus penetrans to strawberry. Can. J. Pl. Sci. 43: 75-78.

34. WALKER, J. C. and K. P. LINK. 1935. Toxicity of phenolic compounds to certain onion bulb parasite. Botan. Gaz. 96: 468-484.

35. WALLACE, H. R. 1961. Browning of chrysanthemum leaves infected with Aphelenchoides ritzemabosi. Nematologica 6: 7-16.

36. WEURMAN, G., and T. SWAIN. 1953. Chlorogenic acid and enzymatic browning of apples and pears. Nature 172: 678.

37. WONG, K. Y. 1963. Factors influencing oriented movement of Pratylenchus penetrans (Cobb). M. S. Thesis, Univ. of Massachusetts.

38. YU, L. M., and R. E. HAMPTON. 1964. Biological changes in tobacco infected with Collectorichum destructium. I. Fluorescent compounds, phenols and some associated enzymes. Phytochemistry 3: 269-272.

VI APPENDIX

I. Necrotic carrot tissue in agar ---- 3 hours after inoculation.

Necrotic tissue end			Blank end		
actual obs.	add 1	logarithm	actual obs.	add 1	logarithm
2	3	.477	14	15	1.176
3	4	.602	15	17	1.230
1	2	.301	11	12	1.079
1	2	.301	15	16	1.204
2	3	.477	10	11	1.041
1	2	.301	15	16	1.204
1	2	.301	11	12	1.079
2	3	.477	9	10	1.000
		3.237			9.013
	$\bar{X}$	0.404		$\bar{X}$	1.127

Sum of observation = 12.250

Correction term = $(12.250)^2 \div 16 = 9.38$

Total sum of squares = $[(.477)^2 + (.602)^2 + \ldots\ldots(1.000)^2] - 9.38$

$= 11.6109 - 9.38 = 2.23$

Group sum of squares = $[(3.237)^2 + (9.013)^2] \div 8 - 9.38 = 2.02$

Individual sum of squares = 2.33 - 2.02 = 0.211

Analysis of Variance Table

Source	Degree of freedom	Mean square
Group	(n-1) = 1	2.02/1 = 2.02
Individual	n(r-1) = 14	0.211/14 = .051
Total	15	

n and r used for the number of treatments and number of replications of each treatment; here $n = 2$, $r = 8$.

A mean square is a mean of squares divided by the corresponding degree of freedom. $[S]^2$

$$\text{L.S.D. } .05 = t_{.05} \left(\sqrt{\frac{2S^2}{r}}\right) = 2.145 \ (.0601) = .1287$$

$1.127 - 0.404 = .723$

$\therefore$ The difference between blank and necrotic tissue ends (= .723) is larger than L.S.D. .05 (=.1287). $\therefore$ significant.

L.S.D. means the least significant difference.

$t_{.05}$ is the tabular value of t for error degree of freedom.

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1. Steel, R. G. D. and J. H. Torrie. 1960. Principle and Procedure of statistics. N. Y. McGraw-Hill. 481 pp. (see P.101-107, 157)

II. The statistical procedure used to test differences between means of the blank ends of treated chambers and blank ends of control chambers is considered by Steel and Torrie in their discussion of unpaired observations and unequal variances. Transformation of actual observations to logarithms occurred in these tests also. The following example illustrates this procedure:

Comparison of blank ends of control chambers and blank ends of necrotic tissue extract treated chambers after 2 hours in agar.

Control blank

actual obs.	add 1	log
3	4	.602
2	3	.477
4	5	.699
3	4	.602
6	7	.845
8	9	.954
2	3	.477
1	2	.302
2	3	.477
5	6	.778
4	5	.699
6	7	.845
7	8	.903
8	9	.954
1	2	.301
7	8	.903

$\Sigma X = 10.8180$

$\Sigma X^2 = 8.0496$

Alfalfa blank

actual obs.	add 1	log
2	3	.477
4	5	.699
5	6	.778
3	4	.602
2	3	.477
4	5	.699
2	3	.477
2	3	.477

$\Sigma X = 4.6860$

$\Sigma X^2 = 2.8700$

Carrot blank

actual obs.	add 1	log
1	2	.301
0	1	.000
1	2	.301
2	3	.477
0	1	.000
2	3	.477
1	2	.301
3	4	.602

$\Sigma X = 2.4590$

$\Sigma X^2 = 1.0908$

Tomato blank

6	7	.845
4	5	.699
1	2	.302
5	6	.778
0	1	.000
4	5	.699
4	5	.699
3	4	.602

$\Sigma X = 4.6240$

$\Sigma X^2 = 3.4509$

	Control blank	Extract blank
M X	10.818	11.769
M X^2	8.0496	7.4117
M $\bar{x}$	.676	.309
M x_1	.7236	x_2 1.6395
s_1^2	.0488	s_2^2 .0683

$$s\bar{d} = \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}} = .06$$

$$t' = \frac{d}{s\bar{d}} = \frac{.676 - .309}{.06} = 6.1$$

$$t'' = \frac{w_1 t_1 + w_2 t_2}{w_1 + w_2} = 2.1 \quad \text{Where } w_1 = \frac{s_1^2}{n_1},\ w_2 = \frac{s_2^2}{n_2}$$

$t_1 = n_1 - 1,\ t_2 = n_2 - 1.$

$t' > t''$

Compare t'' with tabular t for 15 degree of freedom (= 2.13 at 5%) and 23 degree of freedom (= 2.07 at 5%); the value t'' corresponds to a tabulated value.

∴ The statement "blank ends of the treated chambers did not differ from ends of chamber without any treatment" was judged to be true.

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2. Steel and Torrie. pp. 81-83