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Effects of Cyanide Ion and Hypoxia on the Volumes of Second-Stage Juveniles of *Meloidogyne incognita* in Polyethylene Glycol Solutions

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Abstract: Changes in the volumes of second-stage juveniles of *Meloidogyne incognita* were monitored in aqueous solutions of polyethylene glycol supplemented with dilute balanced salts. At key points within a 48-hour cycle of fluctuating water potential, nematodes were placed under hypoxic conditions or exposed to the respiratory inhibitor, sodium cyanide, to detect any respiration-dependent process that regulates volume. Aerobic respiratory arrest at −500 kPa induced pronounced water loss, lateral and dorsoventral collapse of the body wall, and abnormal failure to shorten longitudinally. Durations of hypoxia that were innocuous in dilute solutions were lethal during 500 kPa increases and decreases in water potential; the same water potential changes under aerobic conditions had no effect on viability. Data are consistent with the hypothesis that respiration is essential to survive water potential changes.

Key words: cyanide, hypoxia, *Meloidogyne incognita*, osmoregulation, oxygen, polyethylene glycol, respiratory inhibitors, volume regulation, water potential.

Most nematodes are highly permeable to water (3) and yet possess body turgor as an essential part of structural and behavioral integrity (5). Little is known about the extent to which plant-parasitic nematodes can actively regulate their volumes, and thereby turgor, in response to changes in external water potential. Soil water potential is highly variable which suggests an ecological need for such a capability. If plant parasitic nematodes actively regulate their volumes, several kinds of energy-requiring processes could be involved, such as ingestion of water, contraction of the somatic musculature, or control of internal osmotica concentrations through ion pumping and free-amino acid pool regulation. Before determining how a given plant-parasitic nematode might regulate its volume, however, it needs to be established that the nematode can control its volume. Also, the time intervals over which internally regulated responses occur as a result of external changes need to be studied. The objective of this research was to examine second-stage juveniles (J2) of *Meloidogyne incognita* (Kofoid and White) Chitwood for energy-dependent processes that result in the gain and loss of water. Effects of exposure to a respiratory inhibitor and of oxygen deprivation on nematode volume and viability were measured during a 48-hour cycle of fluctuating water potential.

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Materials and Methods

Effects of respiratory arrest with sodium cyanide on nematode volume: M. incognita J2 were obtained by incubating eggs in a modified Baermann apparatus with continuous aeration. All aqueous solutions used experimentally were prepared by adding 8,000 molecular weight polyethylene glycol (PEG) and (or) 0.25 mM sodium cyanide (NaCN) to a dilute balanced salt solution (DBSS) consisting of glass distilled water and 6 mM NaCl, 1 mM KCl, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$. By adjusting the concentration of PEG, water potentials of −20, −500, −1,000, −2,000, and −4,000 kPa were achieved and verified with a dewpoint osmometer. We wanted to control water potential and impose respiratory arrest by exposure to NaCN to provide an opportunity to detect positive and negative volume regulation. At the same time, we wanted to distinguish between discrete responses to abrupt changes in external water potential and processes that might occur continuously at a constant external water potential. A discrete response, for example, might be a sudden change in intracellular free amino acid or extracellular urea concentration, whereas a continuous process might be ion pumping or water ingestion. We resolved this problem as follows. Nematodes were transferred from DBSS to DBSS or PEG solutions at −500 and −2,000 kPa with and without NaCN and then from PEG solutions to DBSS or PEG solutions with and without NaCN at 24-

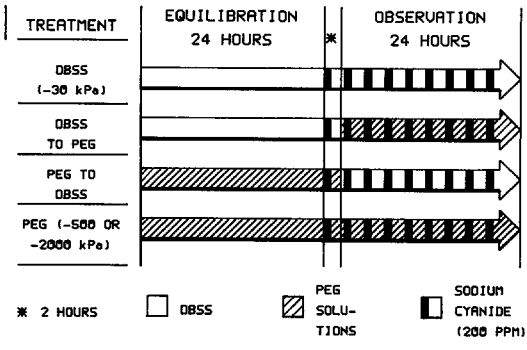


FIG. 1. Protocol for the sodium cyanide (NaCN) experiment described in text. Second-stage juveniles (J2) of *Meloidogyne incognita* were held continuously for 24 hours in a dilute balanced salt solution (DBSS) or in a solution of polyethylene glycol (PEG) with a water potential of −500 or −2,000 kPa; 0.25 mM NaCN (200 ppm) was then added. After 2 hours, nematodes in DBSS were kept in DBSS or were transferred to a PEG solution with a water potential of −500 or −2,000 kPa; this PEG solution also contained 0.25 mM NaCN. Nematodes that initially had been in a PEG solution were kept in that PEG solution or were transferred to DBSS containing 0.25 mM NaCN. A control consisting of nematodes that were never exposed to NaCN was included for each DBSS and PEG solution combination. During the next 24 hours, nematodes were photographed periodically to obtain length and volume measurements.

hour intervals (Fig. 1). In each case, exposure to NaCN was initiated 2 hours before changing solution water potential. The 2-hour pre-exposure to NaCN was based on the observation that 2 hours was sufficient to render 100% of a J2 suspension immotile. It was assumed that other energy-requiring events were similarly inhibited by DBSS-NaCN. Exposure for 24 hours to 0.25 mM NaCN in DBSS was lethal. At various intervals after each transfer of nematodes from one solution to another, 35–40 nematodes were individually photographed at 340× as described previously (7) to obtain volume and length estimates by image digitization. Control nematodes that underwent the same water potential changes as nematodes treated with NaCN were examined in DBSS at the end of the experiment to verify that more than 95% were spontaneously motile.

Effects of oxygen deprivation on nematode survival: These experiments paralleled the NaCN experiment and were designed to test the hypothesis that energy-requiring processes affecting nematode volume may be essential to survival during otherwise innocuous changes in external water potential. Nematodes were incubated in PEG solutions at −500 or −2,000 kPa or in DBSS, each in equilibrium with atmospheric oxygen, for 24 hours and then transferred to the same or a different solution with or without oxygen. Oxygen deprivation was imposed as in the previous experiment (Fig. 1), with two exceptions. The 2-hour pre-exposure to NaCN was changed to 4 hours in a nitrogen atmosphere, and survival of 4 hours hypoxia at each water potential was determined. Nematodes so exposed in PEG solutions at −500 and −2,000 kPa were oxygenated in PEG solution for an additional 20 hours before final transfer back to DBSS.

Simultaneous changes in water potential and dissolved oxygen concentration were achieved by displacing water with oxygen-free grade molecular nitrogen inside an inverted 9-liter polycarbonate jar. After the initial purge, oxygen diffusion through the water barrier sealing the jar opening was offset by a constant slow passage of nitrogen. A plastic raft containing two 10-ml beakers that could be tilted by magnets from outside the jar was placed on the water surface within the jar. In one beaker were placed a nematode suspension and a floating magnetic stirring bar. By placing DBSS, flake PEG, or PEG solutions of the proper concentrations in the second beaker, the water potential of the nematode suspension could be altered at any time by tilting one beaker to mix the two solutions. When fourth-stage juveniles of *Orrina phyllobia* were used as a bio-indicator, dissolved oxygen concentrations lower than 0.5 ppm were obtained (8). After exposure to desired conditions within the jar, the *M. incognita* J2 were removed, examined, oxygenated for 24 hours, and returned to DBSS for 24 hours; the percentage of 300 nematodes that moved spontaneously within 5 seconds was then measured.

RESULTS

Effects of respiratory arrest with NaCN on nematode volume: Effects were detected for six of the seven water potential–NaCN regimes imposed. The following notation is used to simplify the description of those effects. Symbols for the two solutions in which nematodes were held for consecutive 24-hour intervals are separated by

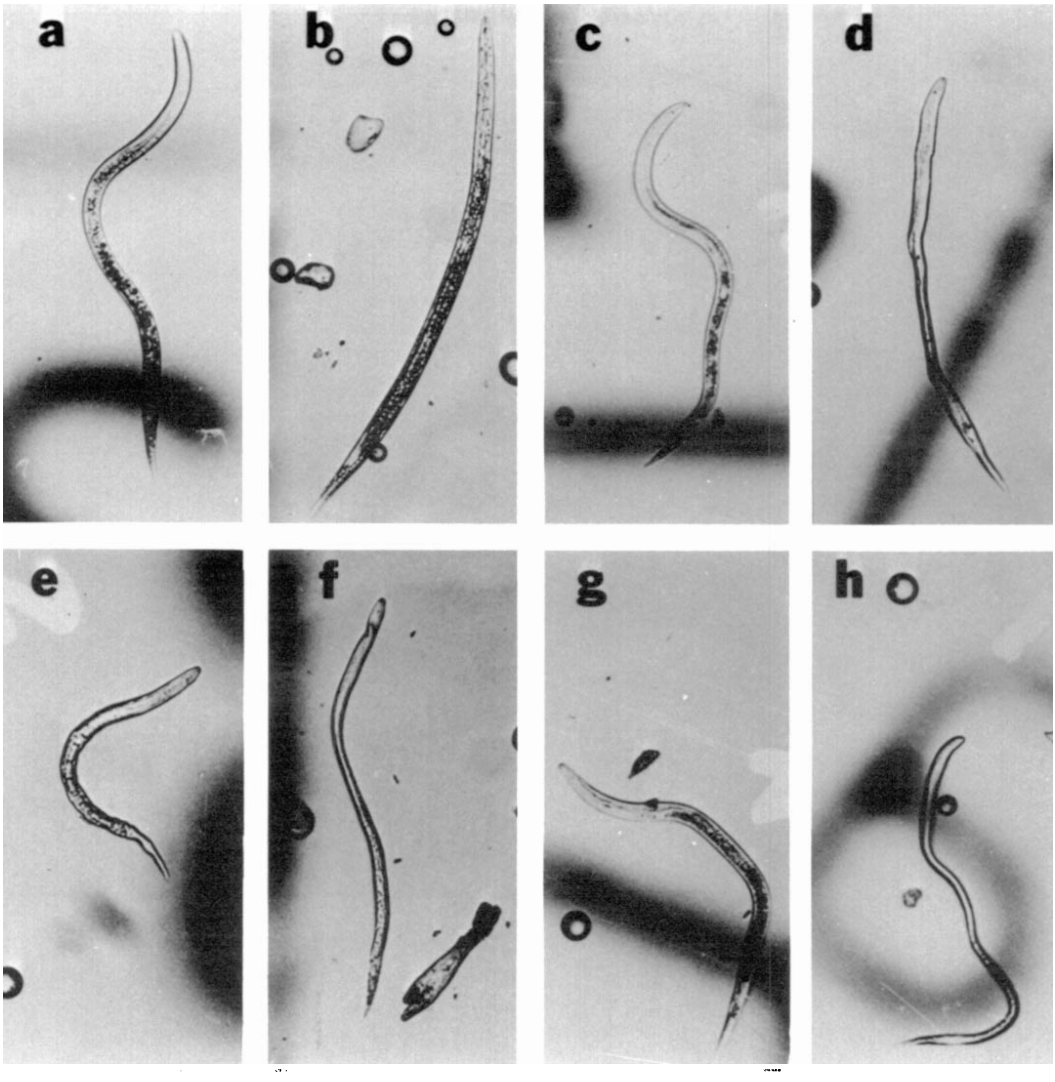
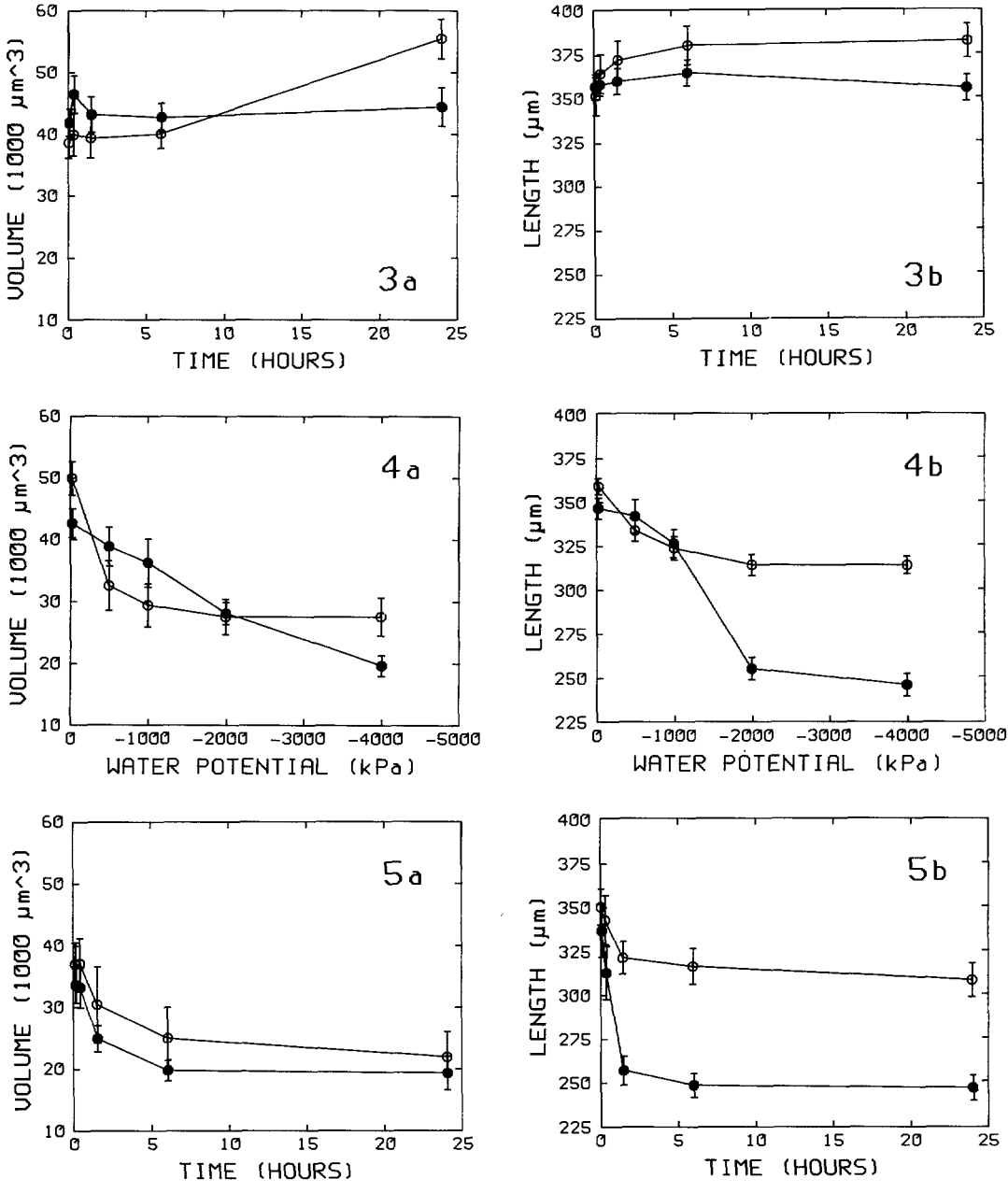


FIG. 2. Changes in the appearance of second-stage juveniles (J2) of *Meloidogyne incognita* (216×) in a dilute balanced salt solution (DBSS) and in polyethylene glycol (PEG) solutions with and without 0.25 mM sodium cyanide (NaCN). a) Motile nematode after 24 hours in DBSS. b) Dead nematode after 24 hours in DBSS containing NaCN. c) Live nematode with slightly reduced motility in PEG solution at −500 kPa 24 hours after transfer from DBSS. d) Dead nematode in PEG solution at −500 kPa containing NaCN 6 hours after transfer from DBSS. e) Live nematode in PEG solution at −2,000 kPa 24 hours after transfer from DBSS. f) Dead nematode in PEG solution at −2,000 kPa containing NaCN 24 hours after transfer from DBSS. g) Dead nematode in PEG solution at −500 kPa containing NaCN 6 hours after transfer from PEG solution at −500 kPa without NaCN. h) Same as g, but 24 hours after transfer.

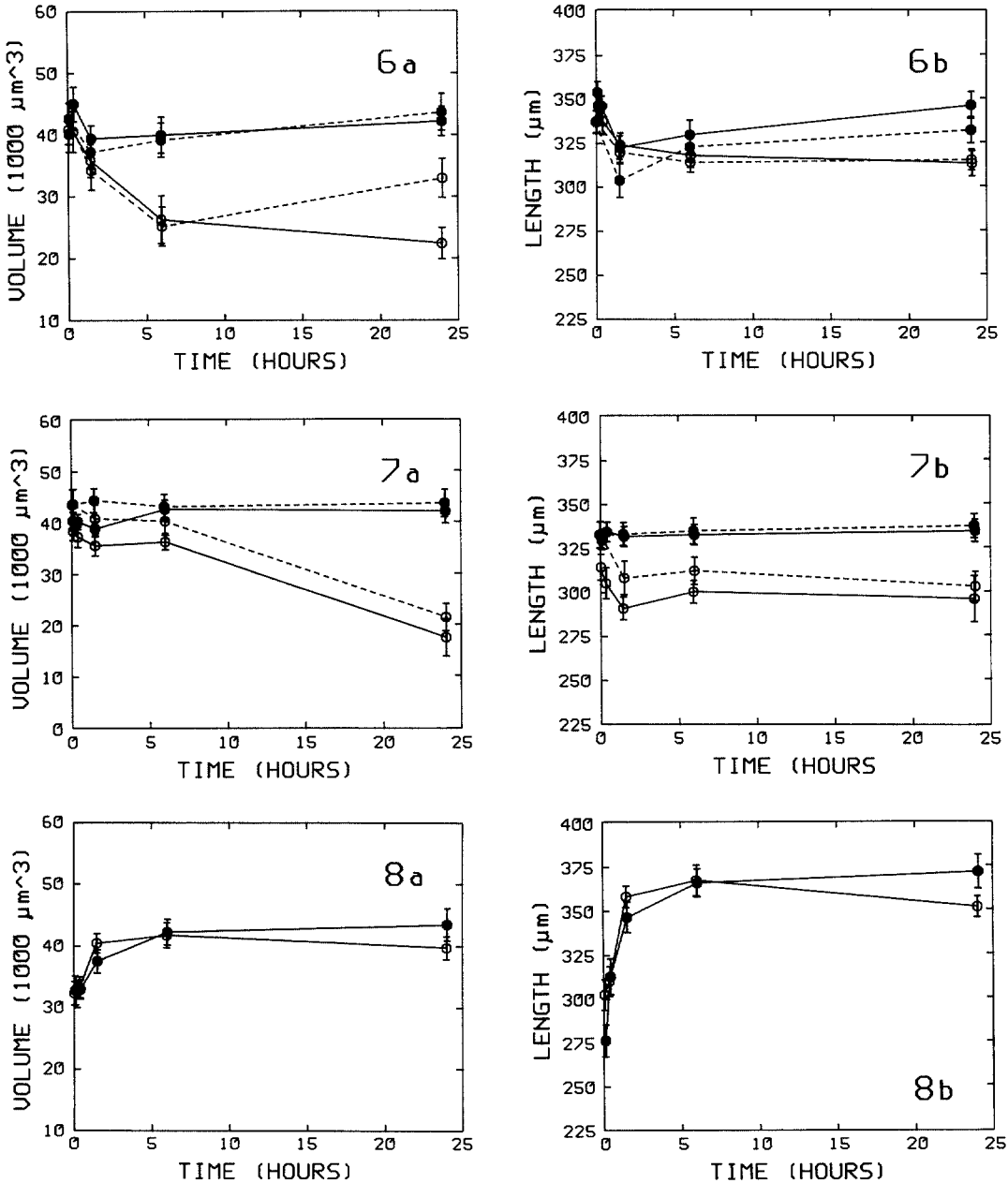
a hyphen where DBSS denotes DBSS, PEG500 denotes PEG solution at −500 kPa, and PEG2,000 denotes PEG solution at −2,000 kPa. A second hyphen followed by CN or CK denotes whether the second solution was treated with NaCN (CN) or not treated (CK).

Nematodes in DBSS-DBSS-CN assumed a straight posture and increased in length 5% within several hours (Figs. 2b, 3b); after 24 hours, the volumes of these nematodes were 15% greater than volumes of the controls (Figs. 2a, 3a). The volume of J2 in DBSS-PEG2,000-CN decreased 40%, similar to those in DBSS-PEG2,000-CK (Fig. 5a); however, almost all NaCN treated nematodes were laterally or dorsoventrally collapsed, and they retained 90% of their fully hydrated lengths (Figs. 2e, f, 4b, 5b). Control nematodes primarily lost volume by shortening longitudinally ca. 30%. J2 in DBSS-PEG500-CK underwent an initial



FIGS. 3–5. Changes in the volumes (a) and lengths (b) of second-stage juveniles of *Meloidogyne incognita* in the presence of 0.25 mM sodium cyanide (open circles) and in its absence (closed circles). 3) In dilute balanced salt solution (DBSS) after 24 hours equilibration in DBSS. 4) After 24 hours in DBSS and various concentrations of polyethylene glycol (PEG). 5) In PEG solution at −2,000 kPa after 24 hours equilibration in DBSS. Each datum is the mean for 35–40 nematodes. Brackets denote fiduciary limits at $P = 0.05$.

rate of volume and length loss similar to those in DBSS-PEG2,000-CK for ca. 1 hour; however, this event was reversed during the next several hours, and most of the original volume and length were eventually recovered (Figs. 2c, 6a, b). Ultimately, these nematodes were only slightly less motile than those in DBSS-DBSS-CK. Nematodes in DBSS-PEG500-CN, by contrast, irreversibly underwent pronounced volume loss through dorso-ventral and lateral collapse similar to those in DBSS-



FIGS. 6–8. Changes in the volumes (a) and lengths (b) of second-stage juveniles of *Meloidogyne incognita* in the presence of 0.25 mM sodium cyanide (open circles) and in its absence (closed circles). 6) In polyethylene glycol (PEG) solution at −500 kPa after 24 hours equilibration in a dilute balanced salt solution (DBSS). 7) In PEG solution at −500 kPa after 24 hours equilibration in PEG solution at −500 kPa. 8) In DBSS after 24 hours equilibration in PEG solution at −2,000 kPa. Each datum is the mean for 35–40 nematodes. Brackets denote fiduciary limits at $P = 0.05$. Dashed lines in Figures 6, 7 indicate data from a replicate experiment.

PEG2,000-CN (Figs. 2d, 6a). The technique used for measuring volume assumed that nematodes were circular in cross section; therefore, volume measurements (Fig. 6a) probably were overestimates wherever pronounced flattening occurred in the absence of a curled body posture. This was true for most J2 in DBSS-PEG500-CN. Nematodes in PEG2,000-PEG2,000-CN, PEG2,000-PEG2,000-CK, and PEG500-

TABLE 1. Combined effects of aeration (O_2) and hypoxia (N_2) within and during transfers between a dilute balanced salt solution (DBSS) and polyethyelene glycol (PEG) solutions on the survival of second-stage juveniles of *Meloidogyne incognita*.

	Nematode transfer and aeration sequence*			Water potential of PEG used (kPa)	Percentage survival
Solution	DBSS + N_2			Not	88† (70–98)‡
Time (hours)	24			used	
Solution	DBSS + N_2	PEG + N_2	PEG + O_2	−500	7§ (3–10)
Time (hours)	4	24	24	−2,000	8§ (0–23)
Solution	PEG + O_2	PEG + N_2	PEG + O_2	−500	72§ (57–89)
Time (hours)	24	4	24	−2,000	32§ (14–48)
Solution	PEG + O_2	PEG + N_2	PEG + O_2	−500	42‖
Time (hours)	24	24	24	−2,000	30¶ (18–42)
Solution	PEG + O_2	DBSS + N_2		−500	88¶ (78–98)
Time (hours)	24	24		−2,000	87¶ (82–92)
Solution	PEG + O_2	PEG + N_2	DBSS + N_2	−500	20¶ (9–30)
Time (hours)	24	4	24	−2,000	1¶ (0–2)

* For each series, nematodes were held in aerated DBSS for 24 hours before and after treatment.
† Mean based on six time replicates.
‡ Range of replicate means.
§ Mean based on three time replicates.
‖ Mean based on one replicate.
¶ Mean based on two time replicates.

PEG500-CK changed little or none during the second 24-hour interval. Nematodes in PEG500-PEG500-CN, by comparison, slowly flattened in a dorsoventrally curled posture (Fig. 2g, h); this occurred about one-sixth as fast as flattening of those in DBSS-PEG500-CN (Fig. 6a). Nematode volume and length recovery in PEG2,000-DBSS-CK and PEG2,000-DBSS-CN (Fig. 8a, b) was completed during the first 1.5 hours in DBSS. Final body postures of NaCN treated nematodes were straight.

Effects of oxygen deprivation on nematode survival: Effects were detected for eight water potential–oxygen regimes (Table 1) and a modification of the previous notation is used to describe them. The symbol N2 denotes hypoxia, and the parenthetical suffix (4 hours) denotes that hypoxia was imposed for 4 rather than 24 hours.

Water potential change under oxygenated conditions had no measurable effect on nematode survival. Low mortality also occurred among nematodes in DBSS-DBSS-N2 (Table 1); however, high mortality (70–100%) occurred whenever water potential was changed directly after 4 hours pre-exposure to N2 atmosphere. Dead nematodes in DBSS-PEG500-N2 and DBSS-PEG2,000-N2 were flattened similarly to those in DBSS-PEG500-CN and DBSS-PEG2,000-CN. Dead nematodes in PEG500-DBSS-N2 and PEG2,000-DBSS-N2 were straight and appeared swollen. About 30% and 70% mortality occurred among nematodes in PEG500-PEG500-N2 (4 hours) and PEG2,000-PEG2,000-N2 (4 hours), respectively, indicating that part of the mortality observed for nematodes in PEG500-DBSS-N2 and PEG2,000-DBSS-N2 occurred before transferring them to DBSS. Transfer of nematodes from PEG solutions to deoxygenated DBSS with no pre-exposure to hypoxia within PEG had no measurable effect on their survival.

DISCUSSION

The abrupt increases in length and volume of *M. incognita* J2 in DBSS following treatment with NaCN suggest that living nematodes continuously expend energy to prevent water uptake. Continuous active transport of water out of the body would explain this observation. Alternatively, hydrostatically forced stretching of the body wall upon loss of muscle tonus, as should occur upon respiratory arrest, could be responsible. The latter explanation would imply that in dilute solutions, such as DBSS, the turgor within J2 of *M. incognita* has a magnitude approximately equal to the osmotic potential of cellular and interstitial

fluids. Direct measurements of osmotic potentials within the tissues of plant-parasitic nematode juveniles have not been made (9).

The recovery of volume and length initially lost by active nematodes upon transfer from DBSS to PEG solutions at −500 kPa indicates an ability to regulate volume in hypertonic PEG solutions. Partial volume recoveries by J2 of *Globodera rostochiensis* and *Heterodera schachtii* also have been observed in sucrose and trehalose solutions; however, the effect of respiratory inhibition on volume regulation was not examined (2,6). The failure of NaCN treated *M. incognita* J2 to recover volume confirms the occurrence of some active process. Membrane-bound ion pumps probably failed upon respiratory arrest, causing net leakage of osmotically active solutes—particularly sodium, potassium, and chloride ions. This event would have contributed to water loss by NaCN treated nematodes. The following evidence suggests that more than ion pump failure is involved. Nematodes equilibrated 24 hours in PEG solution at −500 kPa without NaCN were morphometrically and behaviorally similar to nematodes in DBSS; however, their volume responses upon transfer to PEG-NaCN solution at −500 kPa were markedly different. Volume changes in DBSS-equilibrated nematodes was essentially finished before appreciable volume reduction even began among nematodes equilibrated in PEG solution. Eventual water loss by the latter nematodes may have resulted from solute leakage; the big difference between the rates of water loss by DBSS and PEG solution equilibrated nematodes indicates osmotic adjustment.

The possibility of solute uptake by ingestion or active transport should be considered. If PEG was taken up by *M. incognita* J2, it had no observable effect on viability after nematodes were transferred back to DBSS. The large molecular weight (8,000) of the PEG used possibly precludes passive uptake. Alternate explanations for volume recovery would be inward active transport of water or passive movement of water in response to internal osmotica accumulation. Evidence for active transport of water in animals is controversial (4). Rate constants obtained for permeation of tritiated water into and out of *Aphelenchus avenae* (1) at various osmotic pressures in glucose solutions indicated the absence of active transport of water. Various osmotica might be involved—in particular, extracellular sodium and chloride ions, balanced intracellularly by potassium ions and the free amino acid pool. Other biologically important osmotica exist, such as urea in elasmobranchs and polyols in plant cells and microflora (4). Monitoring changes in osmotica concentrations within *M. incognita* J2 when placed under water stress should be done. Our results provide a basis for defining the conditions and time intervals that might be imposed.

Failure of *M. incognita* J2 to shrink longitudinally and pronounced lateral and dorsoventral collapse of the body wall during water loss following treatment with NaCN could have resulted from rigor of the somatic musculature caused by ATP depletion. It may be useful to examine somatic muscles of these nematodes for evidence of mechanical damage. The failure of NaCN-free nematodes to collapse laterally suggests that body turgor may be maintained over a wide range of water content. It may be instructive to determine the importance of muscular excitability in this regard.

The similarity of the morphometric responses occurring under hypoxia to those occurring after NaCN treatment supports the interpretation that aerobic respiratory arrest caused volume regulation failure. Our data indicate that lethal effects can occur, particularly during water loss and during water uptake. The processes affected in *M. incognita* J2 also may be important to survival in other nematode species. Further research will be required to determine causes of death at the tissue level.

Literature Cited

1. Castro, C. E., and I. J. Thomason. 1973. Permeation dynamics and osmoregulation in *Aphelenchus avenae*. Nematologica 19:100–108.

2. Clarke, A. J., R. N. Perry, and J. Hennessy. 1978. Osmotic stress and the hatching of *Globodera rostochiensis*. Nematologica 24:384–392.

3. Crofton, H. D. 1971. Form, function, and behavior. Pp. 83–113 *in* B. M. Zuckerman, R. A. Rohde, and W. F. Mai, eds. Plant parasitic nematodes. New York: Academic Press.

4. Eckert, R., and D. Randall. 1978. Animal physiology. San Francisco: W. H. Freeman and Company.

5. Marks, C. F., I. J. Thomason, and C. E. Castro.

1968. Dynamics of the permeation of nematodes by water, nematicides and other substances. Experimental Parasitology 22:321–337.

6. Perry, R. N., A. J. Clarke, and J. Hennessy. 1980. The influence of osmotic pressure on the hatching of *Heterodera schachtii.* Revue de Nematologie 3:3–9.

7. Robinson, A. F. 1984. Comparison of five methods for measuring nematode volume. Journal of Nematology 16:343–347.

8. Robinson, A. F., C. C. Orr, and C. E. Heintz. 1981. Effect of oxygen and temperature on the activity and survival of *Nothanguina phyllobia.* Journal of Nematology 13:528–535.

9. Wright, D. J., and D. R. Newall. 1980. Osmotic and ionic regulation in nematodes. Pp. 143–164 *in* B. M. Zuckerman, ed. Nematodes as biological models, vol. 2. New York: Academic Press.