# A Comparison of Techniques for Extraction and Study of Anhydrobiotic Nematodes from Dry Soils[1]

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*Abstract:* Anhydrobiotic nematodes were fixed and extracted from dry Mojave desert soils with hot and cold fixatives (5% formalin and 4% gluteraldehyde). Morphologically, extracted nematodes were tightly coiled and shrunken in size. Various concentrations of KCl, ethylene glycol, and sucrose solutions were compared for their effectiveness in extracting viable nematodes still in the anhydrobiotic state. Approximately 80-95% of the anhydrobiotic nematodes extracted with 1.25 M and 1.5 M sucrose were tightly coiled and shrunken in a manner similar to those fixed and extracted in formalin. Anhydrobiotic nematodes can be maintained in this form for up to 24 days in 1.25 M sucrose solution and still be revived. All molarities of ethylene glycol and KCl tested were ineffective in recovering and maintaining nematodes in the natural anhydrobiotic state. Straight, air-dried, and active nematodes served as controls and did not coil when placed in hot or cold fixatives or in any concentration of KCl, ethylene glycol, or sucrose. Anhydrobiosis, as represented by the coiled form of nematodes from desert soils, was not confined to any particular life stage or trophic group. *Key Words:* cryptobiosis, nematode survival.

Anhydrobiosis, or survival without water, is common among soil organisms which experience adverse environmental conditions, and it has been the subject of recent reviews (4, 8, 20) and studies (2, 6, 14). Soil animals often undergo a change in morphology in dry environments which results in reduced surface area exposure (5). Nematodes may form a tightly coiled spiral (2, 6, 16), whereas rotifers and tardigrades contract anteriorly to posteriorly (5). The morphological form of anhydrobiotic nematodes, as they exist in the soil, has not been clearly demonstrated because of difficulties in observing and extracting nematodes from soil without the use of water. Historically, nematodes used in studies of anhydrobiosis have been obtained from desiccated plant material (2, 18) or monoxenic cultures (6, 14). Most articles concerned with the survival of nematodes in soils have only described longevity in dry soils (7, 17). Demeure (7) extracted anhydrobiotic *Scutellonema cavenessi* from soils by using 70% (9.4 M) NaCl solutions, but the tightly coiled form, if it existed, was maintained in these solutions for only 2 h. The limiting factor in developing knowledge on the biology and physiology of soil anhydrobiotic nematodes has been the lack of an adequate extraction technique, i.e., one which does not cause hydration of the nematode during processing. In all standard techniques of nematode extraction, the final recovery solution is water.

Nematodes in North American desert soils are often exposed to long periods of extremely dry conditions, and many genera apparently survive by some anhydrobiotic adaptation (9, 12). Soil temperatures of 37 C and soil moistures of 0.8% are frequently recorded during the summer months. Investigations of the productivity of the desert ecosystem, including estimates of metabolic activity and the effect of survival mechanisms on metabolism, require observation of the nematodes under natural conditions (9, 11, 12). This paper reports a means for extracting and maintaining nematodes from soil in their natural anhydrobiotic state for biological studies.

## MATERIALS & METHODS

Soil was collected from an undisturbed Mojave Desert site at Rock Valley, Nevada. This site has been intensively studied under auspices of the United States International Biological Program (US/IBP) Desert Biome (9, 10, 11, 12). Soil samples of approximately 6,000 $cm^3$ were mixed three times on a split sample mixer, and 100-$cm^3$ subsamples were used for all extraction procedures.

*Fixation and extraction:* Nematodes in desert soils were fixed before extraction with 5% formaldehyde or 4% gluteraldehyde at 40 C and 22 C. The 100-$cm^3$ soil subsamples were slowly poured into 200 ml of hot or cold fixatives and continuously stirred with a magnetic stirrer to assure immediate contact of the nematodes with the fixative. The

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soil mixture, with 7.5 μg/ml Separan NP10® (Dow Chemical Company, Midland, Michigan) added to the fixative, was allowed to settle for 30 sec; then the supernatant was decanted onto a 26-μm (500-mesh) sieve and the nematodes were backwashed with the appropriate fixative. Nematodes were examined in the fixatives for morphological characteristics by means of the light and scanning electron microscope.

*Extraction of living anhydrobiotic nematodes:* High concentrations of KCl, sucrose, and ethylene glycol were used as extraction solutions to determine their effects on these nematodes during and after recovery from soil. Molarities of 0, 0.25 M, 0.51 M, 0.75 M, 1.0 M, 1.25 M, and 1.50 M were tested for each compound. There were three replications of each concentration. Subsamples of 100 $cm^3$ soil were mixed with 300 ml of the test solution and 7.5 μg/ml Separan, stirred for 30 sec, and allowed to settle for 90 sec. The suspension was poured through 425-μm (40-mesh) and 44-μm (325-mesh) sieves. Nematodes were collected from the 44-μm sieves by backwashing with the appropriate test solution. The decanting and sieving procedure was repeated three times for each subsample. The volume of the nematode suspension was reduced by pouring the collection through a 38-μm (400-mesh) sieve and backwashing with a small amount of solution (<20 ml). To remove remaining debris and further concentrate the nematodes, the nematode suspension was layered upon 10 ml of 2.0 M sucrose in two conical centrifuge tubes. The suspensions were spun at 340 g for 5 min. Large numbers of nematodes representing all trophic groups recorded previously for Rock Valley soils (9, 12) were concentrated at the interface of the two solutions. This nematode concentrate was removed, poured through a 26-μm (500-mesh) sieve, and backwashed with the extracting solution. The morphological form of nematodes extracted in these solutions was examined (40X) at 30, 60, 90, and 120 min following contact of the soil sample with the solution. The percentages of total nematodes which were either coiled, noncoiled but inactive, or active following extraction were determined for each range of concentrations with each solution at the previously noted time intervals.

A control for comparing the effects of fixatives and nonlethal solutions on nematodes was prepared by extracting living nematodes from desert soil with a modified sugar-flotation-sieving technique (9) and placing approximately 300 of the living nematodes into each of the various solution concentrations. Another control preparation involved drying 500-1,000 living nematodes in open air at room temperature (22 C) and then placing them in each of the solutions. The morphological form of the nematodes in the solutions was then examined (40X) and the percentages of coiled, noncoiled, and active nematodes were determined.

Longevity of extracted nematodes was tested in 1.25 M sucrose because of its lower viscosity and the relatively high rate at which coiled nematodes can be recovered from it. To determine viability, 3-ml aliquots of the nematode/sucrose suspension were placed in 20 ml tap $H_2O$ after 0 and 24 days of storage at 10 C. After 4 h and 24 h, the percentages of active and inactive nematodes were recorded.

The rehydration of nematodes after storage in 1.25 M sucrose was studied with the light microscope (160X). Nematodes in a drop of sucrose solution were placed on a slide, and about 0.1 ml water, which perfused and diluted the sucrose solution, was slowly added to the edge of the coverslip. The time required for rehydration and activity of the nematodes was recorded.

## RESULTS

The chemical solutions used in these extraction studies varied in their effects on the nematodes (Table 1). The total average nematode yield varied from 520-1,272 nematodes/100 $cm^3$ of soil (Table 1). All molarities of ethylene glycol and KCl tested were ineffective in extracting and maintaining the coiled form of the nematodes (Fig. 1-A, 1-B, 2). Nematodes extracted in concentrations of ethylene glycol, a nonionic, low molecular weight compound, were uncoiled and active at 30 min. In fact, there appeared to be increased activity and increased yield of nematodes extracted with ethylene glycol. Nematodes extracted at low

TABLE 1. Percentages of coiled nematodes in total nematodes extracted from desert soils in various molarities of KCl, ethylene glycol, and sucrose, and water controls at different time intervals following contact with extracting solution[a].

| Time (min) | $H_2O$ Control | KCl (M) | | | | | Ethylene glycol (M) | | | | | Sucrose (M) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.51 | 0.75 | 1.0 | 1.25 | 1.5 | 0.51 | 0.75 | 1.0 | 1.25 | 1.5 | 0.51 | 0.75 | 1.0 | 1.25 | 1.5 |
| 30 | 16 | 19 | 15 | 20 | 30 | 27 | 12 | 12 | 12 | 11 | 13 | 33 | 47 | 71 | 85 | 90 |
| 60 | 4 | 10 | 12 | 17 | 23 | 18 | 5 | 7 | 6 | 7 | 8 | 18 | 38 | 65 | 80 | 90 |
| 90 | 1 | 10 | 16 | 11 | 16 | 13 | 1 | 2 | 2 | 5 | 5 | 17 | 35 | 61 | 81 | 85 |
| 120 | <1 | 6 | 11 | 9 | 13 | 9 | 1 | 1 | 0 | 1 | 2 | 18 | 35 | 65 | 78 | 82 |
| Avg.[b] | 4 | 11 | 13 | 14 | 20 | 17 | 5 | 5 | 5 | 6 | 7 | 21 | 39 | 65 | 81 | 87 |
| Mean yield[c] | 1,174 | 536 | 672 | 928 | 824 | 736 | 688 | 928 | 1,112 | 1,272 | 1,256 | 536 | 520 | 704 | 800 | 608 |

[a]Data are averages of three replicates.
[b]LSD ($P=0.05$) = 7. For comparisons between averages.
[c]Avg. total yield of nematodes/100 cm³ soil.

molarities of KCl were active after 90 and 120 min, whereas those extracted with 1.25 M and 1.50 M KCl were uncoiled but inactive at 120 min.

The shape of 80-95% of the nematode population in dry, Rock Valley desert soils, as determined by fixation (Fig. 1-D, 3-A, 3-B) and by 1.25 M and 1.5 M sucrose extractions (Fig. 1-C), was a tight coil. Nematodes in both sucrose solutions remained tightly coiled for 120 min (Fig. 4). Identification of some nematode genera which remained tightly coiled was impossible. Nematodes extracted in water were active after 120 min. (Fig. 5). The controls indicated that the coiled morphological shape was not an experimental artifact. Nematodes which were air-dried, i.e.,

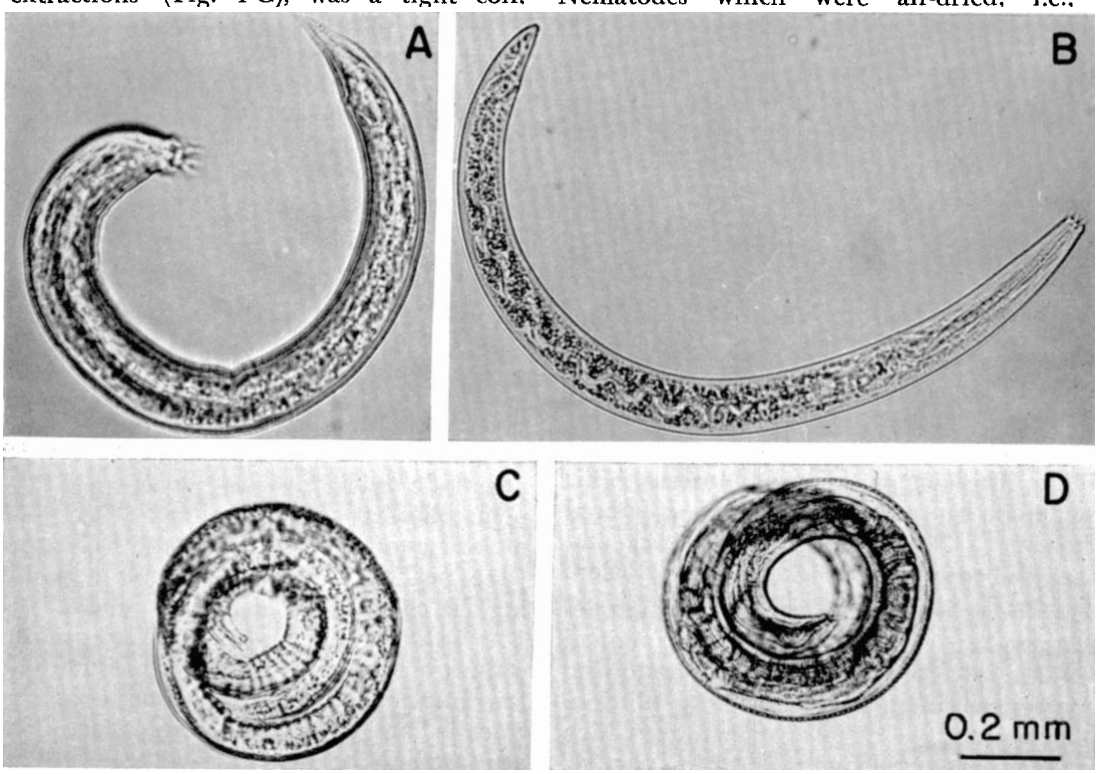


FIG. 1. Anhydrobiotic nematodes (160X) 30 min after extraction with A) 1.25 M KCl, B) 1.25 M ethylene glycol, C) 1.25 M sucrose, D) 5% formaldehyde.

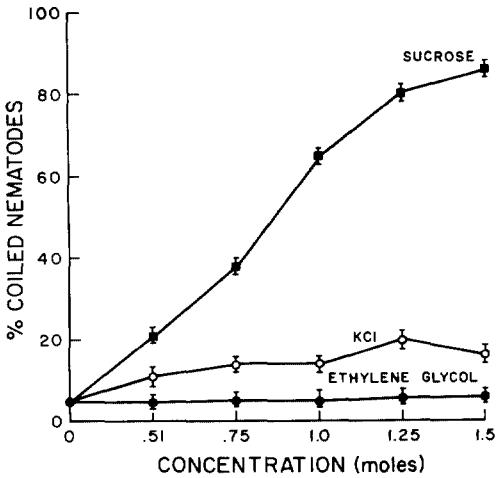


FIG. 2. Average percent coiled nematodes extracted from desert soils in $H_2O$, .51 M, .75 M, 1.0 M, 1.25 M, and 1.5 M, KCl, ethylene glycol, and sucrose, from 0-120 min after extraction. Brackets indicate limits of standard errors. The total average at all molar concentrations: $H_2O$, 1,174; KCl, 739; nematode yield/100 $cm^3$ of soil for each chemical ethylene glycol, 1,051; sucrose, 633.

straight, as well as active nematodes, did not coil when placed in either hot or cold fixatives or in any concentration of KCl, ethylene glycol or sucrose.

The best overall extractant for total nematode yield, coiled shape, and maintenance of anhydrobiotic nematodes from desert soils was 1.25 M sucrose. Although extractions with 1.5 M sucrose yielded more coiled forms of the nematodes, the viscosity of the solution made its use slow and impractical.

Longevity tests indicated that the nematodes could be maintained in the coiled form in 1.25 M sucrose at 10 C for 24 days with 70% of the nematodes reviving upon placement in water.

Anhydrobiosis, as represented by the coiled form of nematodes extracted from desert soils with 1.25 M sucrose, was not confined to any particular life stage or trophic group. Adults and larvae of many genera were observed after coiled nematodes were rehydrated. Rehydration, uncoiling, and expansion usually occurred within minutes, whereas the time required for resumption of activity varied with the nematode species (Fig. 5). During rehydration, an increase in diameter and length occurred (Fig. 5). Reactivation of *Acrobeles*

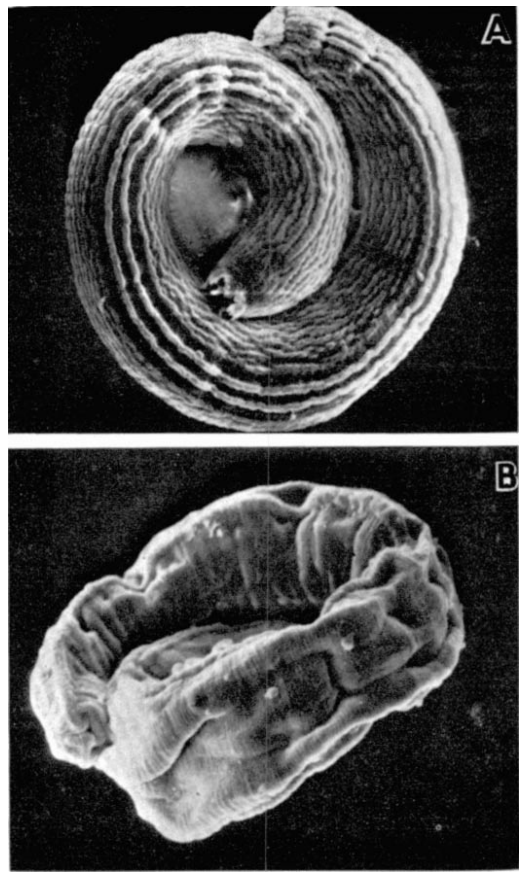


FIG. 3. Anhydrobiotic nematodes fixed and extracted in 5% formaldehyde and photographed with the scanning electron microscope (2,000X). A) Acrobelinae. B) Dorylaimina. These photos illustrate the difficulty in identifying nematodes in the anhydrobiotic state and the transverse and longitudinal folding of the nematode cuticle to reduce surface area.

and *Stegelleta* occurred within 30 min, and was followed by reactivation of *Chiloplacus, Aphelenchoides, Aphelenchus, Paraphelenchus* and *Tylenchorhynchus. Eudorylaimus* and other members of Dorylaimina usually took several hours to resume activity. The viable coiled forms were active 24 h after being placed in water.

## DISCUSSION

Anhydrobiotic nematodes extracted from soil in 1.25 M sucrose appear to retain their shape and physiological state. Morphologically, both formalin-fixed and sucrose-extracted nematodes are tightly coiled, shrunken in size, and folded transversely and longitudinally. Similar

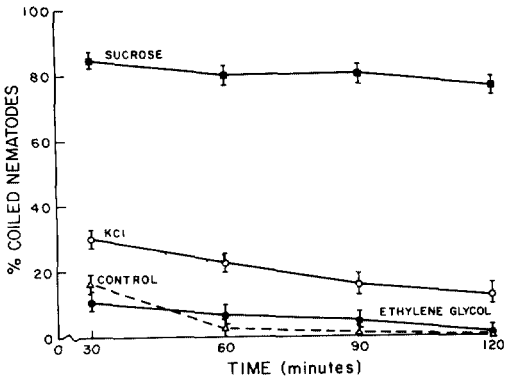


FIG. 4. Percent coiled nematodes extracted from desert soils in $H_2O$ and 1.25 M KCl, ethylene glycol, and sucrose at 30, 60, 90, and 120 min following contact with extraction solution. Brackets indicate limits of standard errors. The average total nematode yield/100 $cm^3$ of soil for each chemical at 1.25 M concentration: $H_2O$, 1,174; KCl, 824; ethylene glycol, 1,272; sucrose, 800.

observations have been made of *Anguina tritici* (2) and *Rotylenchus robustus* (16). Anhydrobiotic nematodes extracted by the sucrose technique rehydrate rapidly in water and behave similarly to active nematodes extracted from moistened soil. The fact that three genera, *Paraphelenchus, Acrobeles,* and *Chiloplacus,* have been cultured on fungi and bacteria in the laboratory after extraction by this technique attests to their viability.

Previous studies designed to extract anhydrobiotic nematodes and other animals from soil have concentrated on hypertonic salt solutions (7) and ethylene glycol (15). In our studies, neither of these solutions maintained desert nematodes in their natural anhydrobiotic state. Stephenson (19) noted that KCl permeated the nematode cuticle at a slower rate than other salt solutions tested, but in our studies, the morphology of the anhydrobiotic nematode was not maintained in KCl.

Nematodes from dry desert soils extracted with 1.25 M sucrose were morphologically similar to coiled *A. avenae* artificially induced into anhydrobiosis under laboratory conditions by Crowe and Madin (6). We attempted to observe nematodes in dry desert soil with the light microscope and the scanning electron microscope, but no coiled anhydrobiotic nematodes or straight nematodes were

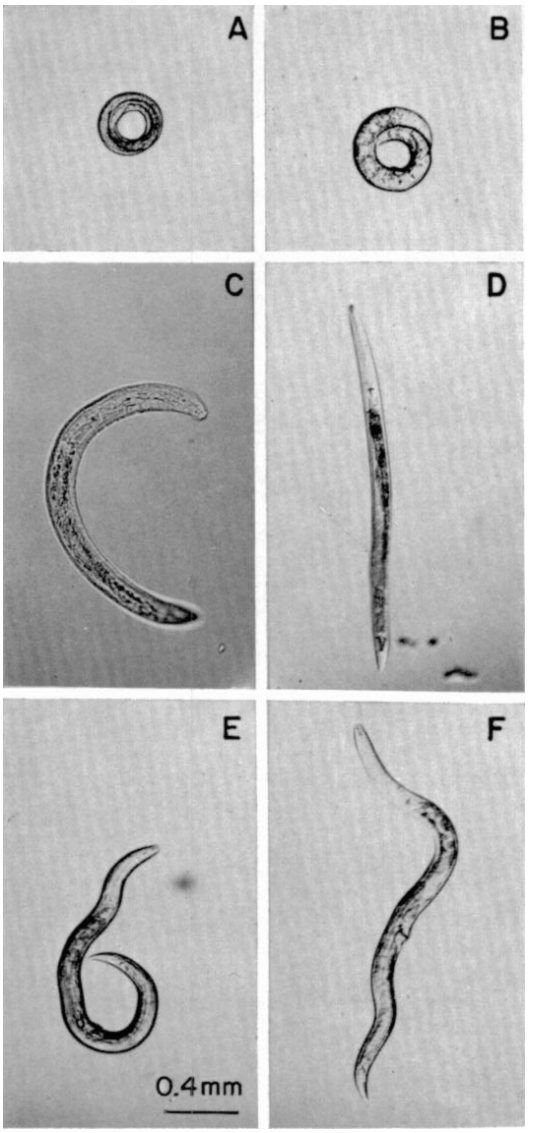


FIG. 5. Rehydration of *Acrobeloides* sp. extracted in 1.25 M sucrose. A) 0 time, B) 1 min, C) 5 min, D) 10 min, E) 25 min, F) 35 min, after addition of water, a demonstration of the increase in diameter and length and return to activity.

recognized. Extraction of nematodes, following the separation of desert soil by dry sieving into three fractions – – 425-$\mu$m (40-mesh), 150-$\mu$m (100-mesh), and <150-$\mu$m, indicated that nematodes were common to all fractions. Workers have suggested previously that dried nematodes may be encased in soil particles (3) which adhere to a lipid covering on the nematode cuticle (2, 13).

Since the form of anhydrobiotic nematodes can be maintained with 1.25 M

sucrose, this extraction method provides a means for the study of nematode survival in dry soils. The implications of these results can be extended to agricultural situations and nematode control. Dry fallow may be ineffective as a means of control since certain plant-parasitic species may enter into anhydrobiosis in slowly drying soils (1, 7) and revives when environmental conditions are favorable. Apt (1) has shown that *Rotylenchulus reniformis* survives best in dry fallow and suggested that moist fallow would be more effective as a means of control.

## LITERATURE CITED


1. APT, W. JR. 1976. Survival of reniform nematode in desiccated soils. J. Nematol. 8:278 (Abstr.).
2. BIRD, A. F., and M. S. BUTTROSE. 1974. Ultrastructural changes in the nematode Anguina tritici associated with anhydrobiosis. J. Ultrastruct. Res. 48:177-189.
3. COOMANS, A., and A. DE GRISSE. 1963. Observations on Trichotylenchus falciformis Whitehead, 1959. Nematologica 9:320-326.
4. COOPER, A. F., and S. D. VAN GUNDY. 1971. Senescence, quiescence and cryptobiosis. Pages 297-318 *in* B. M. Zuckerman, W. F. Mai, and R. A. Rohde, eds., Plant parasitic nematodes. Vol. 2. Academic Press. London and New York, 347 p.
5. CROWE, J. H. 1971. Anhydrobiosis: An unsolved problem. Am. Naturalist. 105:563-573.
6. CROWE, J. H., and K. A. MADIN. 1974. Anhydrobiosis in nematodes: Evaporative water loss and survival. J. Exp. Zool. 193:323-333.
7. DEMEURE, Y. 1975. Résistance à la sécheresse, en zone saheliénne du nématode phytoparasite Scutellonema cavenessi Sher, 1963. Cah. Orstom, sér Biol. 10:283-292.
8. EVANS, A. A. F., and R. N. PERRY. 1976. Survival strategies in nematodes. Pages 383-423 *in* N. A. Croll, ed., Organization of nematodes. Academic Press. London and New York.
9. FRECKMAN, D. W., R. MANKAU, and H. FERRIS. 1975. Nematode community structure in desert soils: Nematode recovery. J. Nematol. 7:343-346.
10. FRECKMAN, D. W., R. MANKAU, and S. A. SHER. 1974. Biology of nematodes in desert ecosystems. US/IBP Desert Biome Res. Memo. 74-35. Utah State Univ., Logan. 10 p.
11. FRECKMAN, D. W., R. MANKAU, and S. A. SHER. 1975. Biology of nematodes in desert ecosystems. Pages 79-89. US/IBP Desert Biome Res. Memo 75-32 *in* Reports of 1974 Progress: Vol. 3, Invertebrate Section, Utah State Univ., Logan. 89 p.
12. FRECKMAN, D. W., and R. MANKAU. 1977. Distribution and trophic structure of nematodes in desert soils. *in* Lohm, U. and T. Persson, eds., Soil organisms as components of ecosystems. Proc. VI. International Soil Zoology Colloquium. Ecol. Bull. (Stockholm) Vol. 25 (In press).
13. LEE, D. L. 1972. Penetration of mammalian skin by the infective larva of Nippostrongylus brasiliensis. Parasitology 65:499-505.
14. MADIN, K. A. C., and J. H. CROWE. 1973. Anhydrobiosis in Nematodes: Carbohydrate and lipid metabolism during dehydration. J. Exp. Zool. 193:335-342.
15. PEASE, D. 1966. The preservation of unfixed cytological detail by dehydration with "inert" agents. J. Ultrastruct. Res. 14:356-378.
16. RÖSSNER, J., and J. PORSTENDORFER. 1973. Rasterelektronmikroskopische Analyse der Oberflächen normal turgeszenter und infolge von Austrocknung geschrumpfter pflanzenparasitärer Nematoden. Nematologica 19:468-476.
17. SIMONS, W. R. 1973. Nematode survival in relation to soil moisture. Meded. Landbouwhogesch. Wageningen 3:1-85.
18. SPURR, JR., H. W. 1976. Adenosine triphosphate quantification as related to cryptobiosis, nematode eggs, and larvae. J. Nematol. 8:152-158.
19. STEPHENSON, W. 1942. The effect of certain inorganic chloride solutions upon the movement of a soil nematode (Rhabditis terrestris, Stephenson), and upon its bodily size. Parasitology 35:167-172.
20. VAN GUNDY, S. D. 1965. Factors in survival of nematodes. Annu. Rev. Phytopathol. 3:43-68.