Temperature-Induced Phase Transitions in Nematode Lipids and Their Influence on Respiration[1]

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Abstract: Temperature-induced phase transitions estimated by electron spin resonance (ESR) technique were observed in the lipids of several nematode species. In both *Meloidogyne javanica* and *Caenorhabditis elegans*, there was a phase transition in their phospholipids from a liquid-crystalline state to a solid gel state at about 10 C. *Aphelenchus avenae* also had a phase transition, but at about 20 C. With this species, the spin-label motion parameters indicated the transition was from the liquid-crystalline state below 20 C to a more liquid or disordered state above 20 C. *Anguina tritici* and *Meloidogyne hapla*, in contrast, had no phase transitions over the entire temperature range studied. Each phase transition detected by ESR was reflected in the respiratory rates of the nematodes, and the temperature of the transition coincides with the environmental adaptation of these species.

The existence of temperature-induced phase transitions in the lipid portion of biological membranes has been carefully studied (6, 12, 19, 21, 22, 29). The temperatures at which these transitions occur are closely correlated with the temperature dependency of the activities of several membrane-bound enzymes in mitochondria (22, 25), endoplasmic reticulum (17, 18), and chloroplasts (28). The physiological consequences of such phase transitions limit the survival of chilling-sensitive plants (13, 15) and homeothermic mammals (14, 17, 18, 22, 23) at lowered temperatures. Neither chilling-resistant plant species, nor poikilothermic animals, undergo any such transition in their membranes, and correspondingly these organisms can maintain normal metabolism and survival over a much broader temperature range, down to and including 0 C. Furthermore, eutherian hibernators can alter the physical properties of their membranes such that the animals can function as poikilotherms during hibernation (23). Also, the monotreme echidna can lower the transition temperature to near 10 C when entering a state of torpor (17).

Because a number of nematode species are subject to "chilling injury" (27, 31), it was of interest to expand the observations with chilling-sensitive plants and homeothermic mammals to ascertain if temperature-induced phase transitions could be correlated with nematode survival. A temperature-dependent coincidence between nonlinearities in oxygen uptake and nonlinearities in spin label motion was demonstrated.

MATERIALS AND METHODS

Nematodes:—1) *Meloidogyne hapla.*—*Meloidogyne hapla* was reared on tomato under greenhouse conditions. Air temperature ranged from 20-28 C, and soil temperature from 22-25 C. Sixty days after the plants were inoculated with second-stage larvae of *M. hapla*, roots with egg masses were placed on funnels in a mist chamber (temperature 25 C), and second-stage larvae were collected daily for 8 days. The larvae were washed and stored in chlorine-free tap water at 15 C.

—2) *Meloidogyne javanica.*—*Meloidogyne javanica* was reared on lima beans under conditions similar to those for *M. hapla*, except that the soil temperature was raised to 25-28 C by placing the potted plants on a heated sand bed. Recovery and storage of larvae were as described above.

—3) *Anguina tritici.*—*Anguina tritici* was recovered from infected wheat grain (cockles). The grain was macerated in a food blender for 10-15 sec. Then the second-stage larvae released from the grain were collected on screens and transferred to modified Baermann funnels in the mist chamber. Active nematodes which passed through the Kimwipe® paper tissue on the funnels were washed and stored under conditions similar to those for *M. hapla* and *M. javanica*.

—4) *Aphelenchus avenae.*—*Aphelenchus avenae* was propagated by the technique developed by Evans (7), with *Rhizoctonia solani* as the fungal host. Nematodes recovered after 24-28 days of development in the wheat-*Rhizoctonia* culture were a mixture of adults (primarily females) and larval forms.

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These nematodes were stored at 5 C until used.

—5) *Caenorhabditis elegans.*—*Caenorhabditis elegans* was reared on a peanut butter-agar medium in the presence of contaminating bacteria. Medium was poured into 20 × 100-mm plastic petri plates, and the culture medium then was inoculated with *C. elegans* and contaminating bacteria (on which the nemas fed). The cultures were incubated at 22-24 C for 5 days, after which the agar was removed from the plates and placed on a modified Baermann funnel in the mist chamber. The nematodes obtained (a mixture of adults and larval forms) were washed thoroughly to remove surface bacteria, and then were stored in tap water at 10 C for not longer than 48 h before use.

Caenorhabditis elegans storage test: Suspensions of active nematodes obtained as described above were diluted so that each vial contained approximately 2,200 nematodes in 6 ml of water. Five replicates (1 vial = replicate) each were held at 6, 9, 15, and 21 C for 4, 8, and 16 days. The suspensions were removed from storage on the designated day and placed on in vitro extraction racks (two Kimwipe® filters plus one plastic filter screen) (20). The motile nematodes moved through the filter system into the lower portion of 60 × 15-mm glass petri dishes containing 10 ml of chlorine-free tap water and 1 drop of 0.07% sticker-spreader. The nematodes were on the extraction system 24 h before they were counted.

Respiration studies: Oxygen uptake (as an estimation of the respiratory rate) was measured by an oxygen electrode technique described previously (16). Four Clark-type oxygen electrodes were used simultaneously, each at a different temperature. A 0.5-ml portion of nematode suspension (usually containing 0.5 mg fresh weight) was added to 1.5 ml of water which had equilibrated in the cuvette with air, and had been calibrated for oxygen concentration at the particular temperature. After the system again returned to temperature equilibrium, the reaction chamber was closed and oxygen uptake measured. The absolute rates varied between 0.5 and 12 nmoles 0_2/min per g fresh weight, depending on the species studied. These rates are within the range reported previously for nematodes (2, 8, 10, 26). The data are presented (Fig. 1) as an Arrhenius plot (log rate vs. the reciprocal of the absolute temperature) for comparisons of the effect of temperature on the respiratory rate of several species. Since many factors, e.g. physiological and morphological stages of development, age, and diet, can influence the magnitude of the rate, we have made no attempt to ascribe significance to any differences observed among the actual values obtained for the various species.

ESR studies: Electron spin resonance (ESR) was done with a Japan Electron Optics electron paramagnetic resonance spectrometer, JES ME IX, operating at χ-band and equipped with a laboratory-constructed, variable-temperature unit accurate to better than ± 0.5 C. Temperature was monitored with a thermistor in the cavity near the sample. Preparation and use of the spin-label 2,2-dimethyl-5-dodecyl-5-methyl-*N*-oxyl oxazolidine (2N14) has been described previously (33). The ratio of the first derivative mid-field line height (h_0) to high-field line height (h_{-1}), or the rotational correlation time (τ_c), were used as motion parameters. The line height ratio, h_0/h_{-1}, is valid as long as the integrated areas under the lines are equal; and high, local, spin-label concentrations do not introduce substantial electron-electron dipole or electron exchange effects. τ_c values are valid for approximately the same conditions; motion must be isotropic and in the fast-tumbling range, where τ_c is approximately $\leqslant 10^{-9}$ sec. We believe that the necessary requirements for both h_0/h_{-1} and τ_c are valid, at least for comparative purposes (19).

Lipids were extracted from gram quantities of nematodes by the Folch procedure (9). The extracts were dried under vacuum, redissolved in small quantities of chloroform, streaked on preparative thin-layer plates of silica gel-G_1 and developed in diethylether to move nonpolar lipids to the front. The origin was scraped off, and the polar lipids were eluted with methanol. The polar lipids were dried under vacuum in a small tube and sonicated into a dispersion in water. The phospholipid aqueous dispersion was used for ESR analysis after adding the spin-label to a final concentration of about 10^{-4} M (0.1 μl of SL in ethanol into 60 μl of phospholipid dispersion).

Nematodes in the intact state did not take up spin-label; therefore, they were frozen to dry ice temperature, and then allowed to thaw in the aqueous dispersion of spin-label. In this

way, we adjusted the spin-label concentration until the signal strength was about the same as with the phospholipid dispersions. $K_3Fe(CN)_6$ was added to the frozen preparations as an antireductant.

RESULTS

Arrhenius plots for respiration of the five species of nematodes studied are shown in Fig. 1. Plots for both *A. tritici* and *M. hapla* were linear from about 2 C to above 30 C. In contrast, those of *C. elegans* and *M. javanica* were nonlinear and discontinuous, with the temperature of discontinuity being in the vicinity of 10 C. The plot of *A. avenae* also was nonlinear and discontinous, but the temperature of discontinuity was about 20 C. The appearance of the nonlinear discontinous plots is consistent with the thermodynamics of a phase transition in the system (11).

A more definitive method of demonstrating temperature-induced phase transitions in biological membranes is that provided by ESR spectroscopy (19, 24). In this technique, the lipid phase is permeated with a lipophilic paramagnetic probe, and the effect of temperature on mobility of the probe is determined. In this way, the temperature at which the membrane lipids undergo a transition in physical state can be determined.

In initial experiments, we attempted to have the living nematodes equilibrate with an aqueous dispersion of spin-label compound. In this environment, the nematodes did not take up enough of the compound to spin-label their membranes adequately; hence, we killed the nematodes by freezing. When allowed to thaw in an aqueous dispersion of spin-label, the killed nematodes absorbed enough of the materials to label their lipids and membranes adequately. From these spectra it can be seen in Fig. 2 that the frozen and thawed *C. elegans* and *M. javanica* each had a change in physical state, or discontinuity in the Arrhenius plot, at about 10 C. In contrast, the plots of *M. hapla* and *A. tritici* were both linear over the entire temperature range from near 0 C to above 30

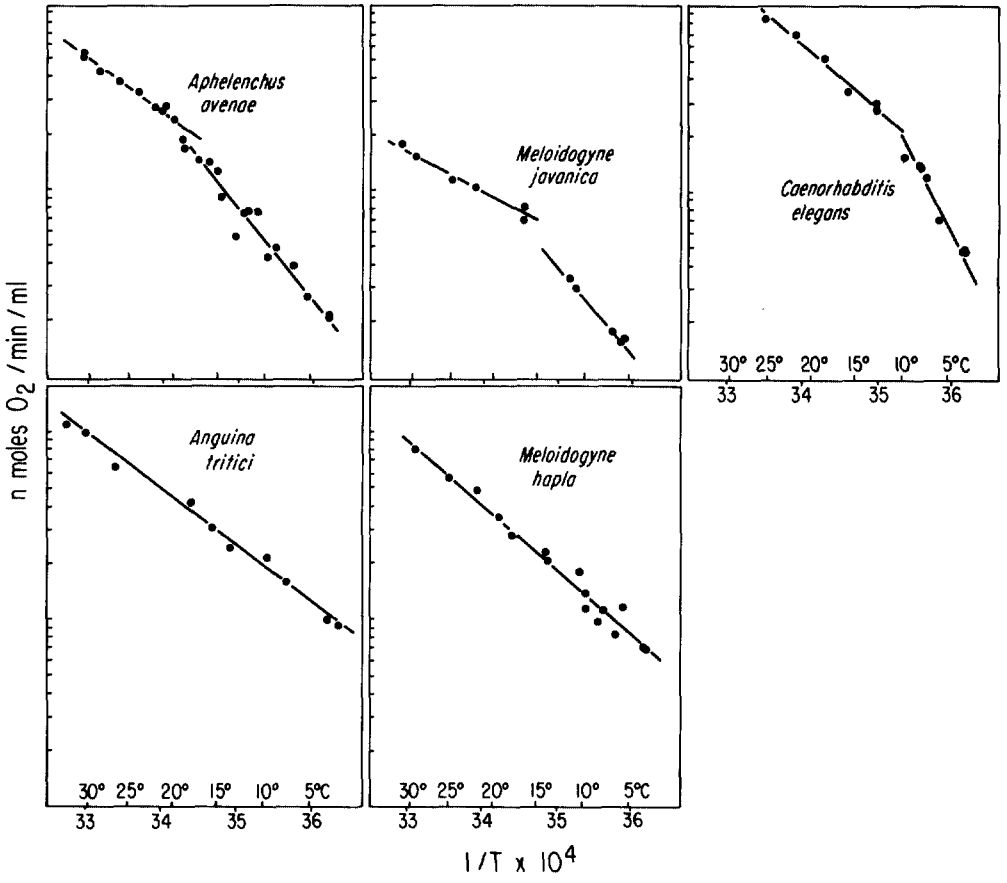

FIG. 1. Arrhenius plots for oxygen uptake as an estimate of respiration rate in several nematode species.

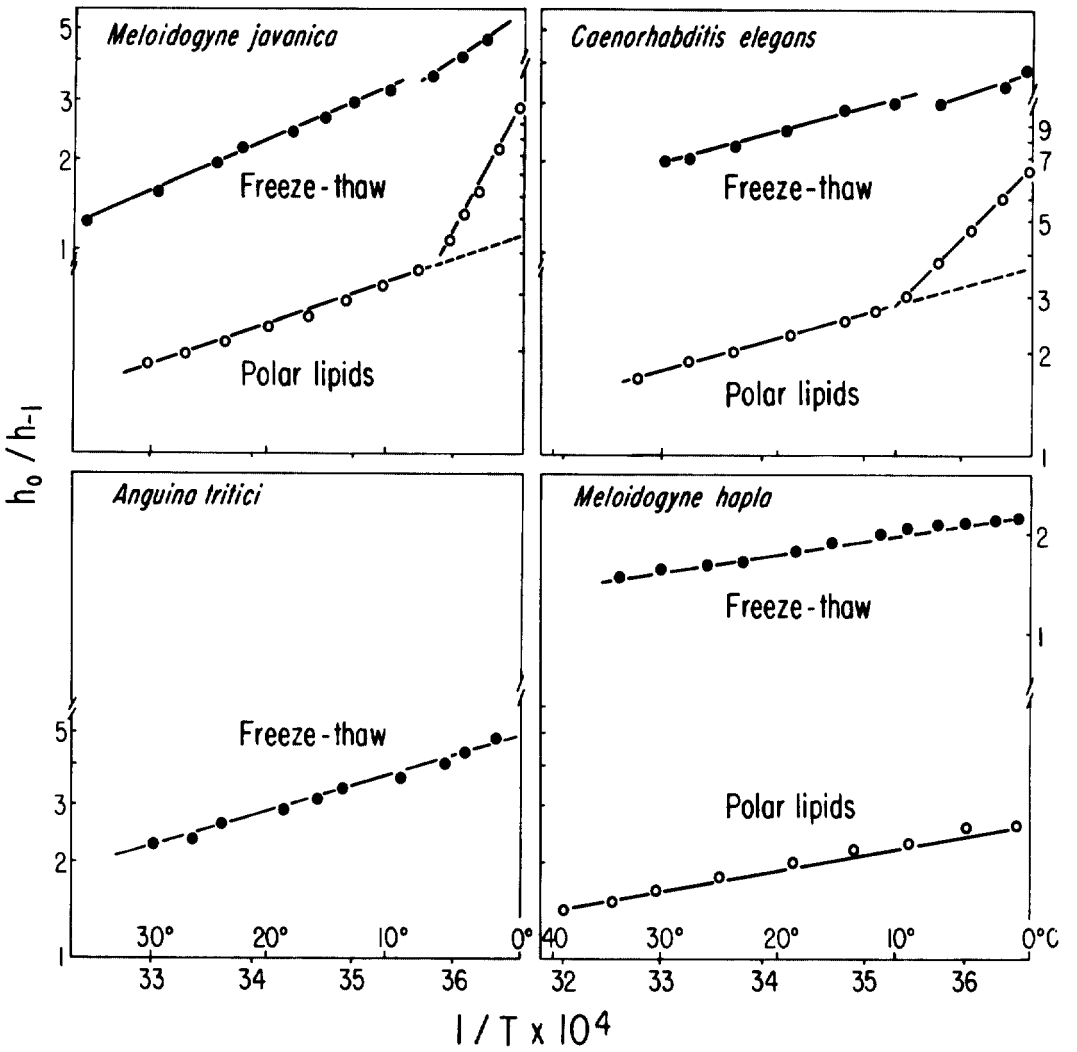

FIG. 2. Arrhenius plots for spin-label mobility [presented as the ratio of the mid-field (h_0) and high-field (h_{-1})] line heights in frozen and thawed nematodes and for extracted polar lipids.

C. Furthermore, when the extracted phospholipids—the lipids predominately associated with cellular membranes—were incubated with spin-label and ESR spectra were obtained, the plots were similar to those of the frozen-thawed material. (There was insufficient material to extract the phospholipids from *A. tritici*.) The motion parameters for the spin-label moiety in *M. javanica* and *C. elegans* indicate that the membrane lipids underwent a transition from a liquid-crystalline state above about 10 C, to a solid gel phase below 10 C. In the other two species, the liquid-crystalline state persisted over the entire temperature range.

A discontinuity and a phase transition are apparent at about 20 C in the frozen and thawed *A. avenae* preparation, using the spin-label, 2N14 (Fig. 3). However, in this case, the motion parameters indicate a transition from a liquid-crystalline state below 20 C to a more liquid or disordered state above the transition temperature. This discontinuity was also apparent in the extracted phospholipids. Both of these correlate with the Arrhenius plot for respiration of the intact organism. When the neutral lipids, which make up over 30% of the dry body weight in *A. avenae* (4), were extracted and spin-labelled, the Arrhenius plot was linear over the entire temperature range, indicating that the bulk lipids differ greatly in physical characteristics from the phospholipids associated with the membranes. This also indicates that the

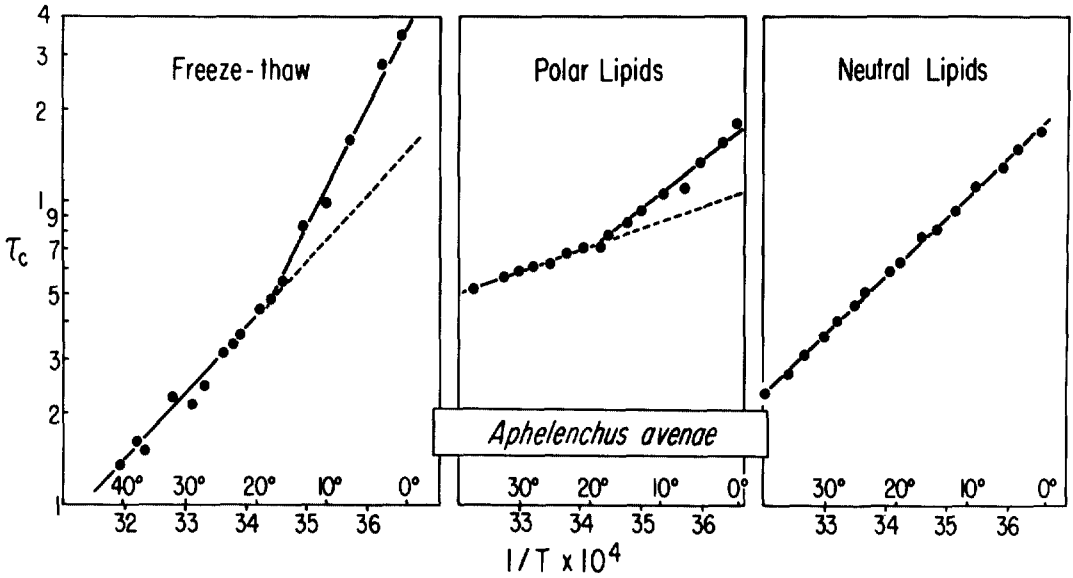

FIG. 3. Arrhenius plots for spin-labeled mobility (presented as the rotational correlation time, τ_c) in frozen and thawed *Aphelenchus avenae* and for extracted polar and neutral lipids.

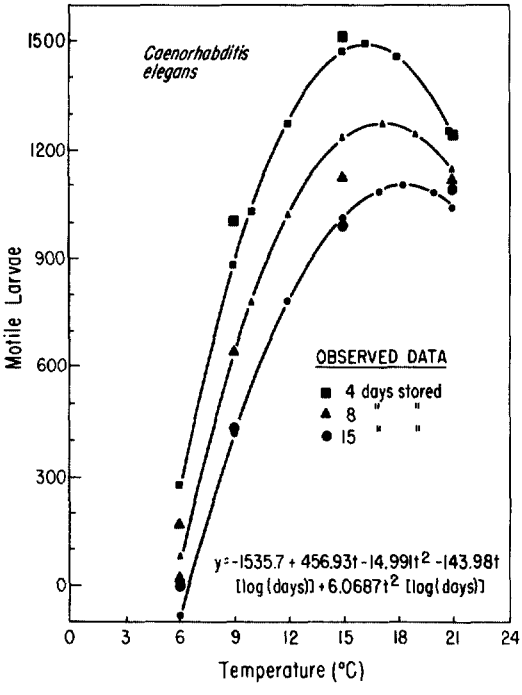

FIG. 4. Survival data for *Caenorhabditis elegans* stored for 4, 8, and 15 days at four temperatures. Large symbols represent actual measurements, small symbols represent calculated values based on the regression equation shown.

physical state of the membrane lipids can exert control over the enzymes of the respiratory chain (22, 25).

DISCUSSION

The interesting question related to our results is whether the phase transitions correlate with the physiology of the intact organism, and, in fact, determine the lower temperature limit for survival, particularly in those species sensitive to "chilling injury". It is evident from previously reported studies (1, 2, 3, 5, 27, 30, 32, 34) that both *M. hapla* and *A. tritici* are adapted to cold environments, i.e., are able to function at or near 0 C. The respiratory data we obtained, as well as the results of our ESR studies, indicate the absence of any phase transition, and the presence of normal metabolism down to or near 0 C, in these two species. On the other hand, *M. javanica,* the Javanese root-knot nematode, is sensitive to chilling temperatures and will not survive prolonged exposure to temperatures below about 10 C (1, 5, 30, 32). In the northern hemisphere, *M. javanica* only occurs south of the 50th parallel, except in greenhouses, or in areas where the climate is moderated by coastal conditions.

In the literature there was no clear-cut evidence as to the temperature limits for *C. elegans,* although indications were that it was sensitive to low temperatures. However, data

in Fig. 4 clearly reveal that *C. elegans* is, in fact, sensitive to chilling, and cannot withstand exposure to temperatures below about 10 C for even a relatively few days. Thus there is a positive correlation between the existence of a phase transition at about 10 C, as indicated by both the ESR data and the Arrhenius plot of respiration, and the lower temperature limit for survival as indicated by the data in Fig. 4.

Recent studies (13, 22) on the significance of temperature-induced phase transitions to cellular metabolism provide insight into the events involved in chilling phenomena. As the temperature is lowered, the membrane lipids in chilling-sensitive species solidify at the critical temperature. This leads to a contraction which might cause cracks or channels increasing permeability and perhaps ionic imbalance. Likewise, a differential effect of reduced temperature on membrane and nonmembrane-bound enzyme systems could lead to a major imbalance in metabolism. Each of the events proposed by Van Gundy (31) as metabolic disruptions leading to injury from low temperature are explainable by the single event of solidification of the membrane phospholipids.

Both the ESR and respiratory data indicated a phase transition in *A. avenae* at about 20 C. But, in contrast to *M. hapla* and *C. elegans,* the ESR spectra indicated that the change was from the liquid-crystalline state below 20 C toward a structure approaching a completely anisotropic liquid above 20 C. Although this phase transition to more disordered conditions in the membrane lipids (phospholipids) causes a suppression in the respiratory rate above 20 C, this event apparently has no marked influence on the function of the intact organism. *A. avenae* can function normally over the entire temperature range from 0 to about 30 C. This would indicate that, in contrast to the situation with those species sensitive to chilling injury described above, at temperatures of 20 C or higher the metabolism rate would be high enough to overcome any inadequacies resulting from disruption or imbalance caused by a phase transition.

It is interesting to speculate concerning the influence of chilling sensitivity on the global distribution of plant parasitic nematodes. In the case of two closely related species, *M. hapla* and *M. javanica,* apparent differences in distribution may be related to the relative sensitivity to chilling in second-stage larvae, correlated here with phase transitions (or lack of) in the lipids. However, until more is known about the temperature sensitivity of other life stages, particularly survival stages such as eggs, it may be premature to assign any overall importance to chilling sensitivity as a determinant of global distribution. There could be an analogy here with seed plants in which chilling sensitivity limits the climatic season for the species. In those plant species sensitive to chilling injury, the seeds (in effect, the plant's survival mechanism) are not affected by chilling temperatures as long as they remain desiccated and dormant (13). As soon as they imbibe water they become sensitive, and hence cannot germinate or complete a life cycle until the temperatures average above 10 C. Similarly, the survival stages of chilling-sensitive nematodes also must be adapted to persist through cold periods, but once the motile larvae are present, low temperature can impose a barrier to survival.

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