Journal of Nematology 16(1):14–21. 1984.
© The Society of Nematologists 1984.

Transport of Palmitic Acid across the Tegument of the Entomophilic Nematode *Romanomermis culicivorax*[1]

ROGER GORDON AND IAN R. BURFORD[2]

Abstract: *Romanomermis culicivorax* juveniles, dissected out of *Aedes aegypti* larvae 7 days after infection, were incubated under controlled conditions in isotonic saline containing ^{14}C-U-palmitic acid to investigate the nature of the transport mechanism(s) used by the nematode for transcuticular uptake of palmitic acid. Net uptake of the isotope by the nematode was of a logarithmic nature with respect to time. Uptake of palmitic acid was accomplished by a combination of diffusion and a mediated process which was substrate saturable and competitively inhibited by myristic and stearic acids. Both 2,4-dinitrophenol and ouabain inhibited uptake of palmitic acid and thus supported the hypothesis that the carrier system is of the active transport variety and is coupled to a Na^+K^+ ATPase pump.
Key words: active transport, *Aedes aegypti*, diffusion, fatty acid, lipid, mediated transport, mermithid, nutrition, transtegumentary uptake.

The mermithid nematode *Romanomermis culicivorax* (Enoplida: Mermithidae) has considerable potential for the biocontrol of mosquitoes (16). Juvenile stages of this nematode develop in the hemocoel of larval mosquitoes and then kill the host as they egress through the latter's exoskeleton.

As with other mermithids, *R. culicivorax* feeds only during the parasitic phase of its life cycle. The nutrition of mermithids is considerably modified from the basic nematode pattern. The parasitic juveniles absorb nutrients from the host's hemolymph across their outer cuticle and store them in a trophosome for subsequent utilization by the free-living stages.

Attempts to culture *R. culicivorax* in vitro, a necessary prerequisite for its commercial deployment in mosquito abatement programs, have been thwarted by inadequate knowledge of the parasite's nutrition. The nematode has been cultured through a portion of its parasitic development in vitro (3,6), but the trophosomes of cultured nematodes were deficient in lipids (6) which constitute the predominant types of storage metabolite (11).

Recent studies have shown that the parasitic juveniles can absorb exogenous sources of triacylglycerols, monoacylglycerols, and free fatty acids (9), the three categories of saponifiable lipids present in the hemolymph of its laboratory host, *Aedes aegypti* (8). The present study was done to obtain information on the nature of the process(es) used by *R. culicivorax* to transport free fatty acids across its outer cuticle. Palmitic acid was chosen for this investigation because it is prevalent in the trophosome of the mermithid as well as in the hemolymph of its host (8).

MATERIALS AND METHODS

Source of biological material: A laboratory colony of *R. culicivorax* was maintained by propagating the mermithid through *A. aegypti* larvae (1).

Seven-day-old nematode juveniles required for lipid uptake studies were obtained from experimentally infected *A. aegypti* larvae maintained at 27 C (9). Under these conditions, postparasitic nematodes emerged from nondissected hosts 8–10 days after infection.

Experimental design: Emulsions of palmitic acid-U-^{14}C (403 μCi/μmole; Amersham/Searle Corporation, Canada) were prepared by vigorous manual shaking in half-strength Dulbecco's phosphate buffered saline (½ DPBS; Grand Island Biological Company, New York, USA). Groups of five nematodes were transferred to covered glass dishes (3 cm d) and incubated (27 C) in 1 ml of the saline containing the requisite amount of the radiolabelled fatty acid for the time period (5–60 minutes) appropriate to the experiment. For experiments designed to examine uptake of

Received for publication 3 February 1983.
[1] Continuing financial support from the Natural Sciences and Engineering Research Council of Canada (Grant No. A6679) is gratefully acknowledged.
[2] Department of Biology, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9.

radiolabelled palmitic acid in the presence of another labelled fatty acid, sodium taurocholate (0.1 μmoles/ml) was included in the incubation medium to provide an evenly dispersed emulsion. A series of preliminary experiments revealed that, when used at such concentrations, the emulsifying agent did not affect the degree of uptake but facilitated a more consistent pattern of uptake among similarly prepared replicates. In all other experiments, the emulsifying agent was found to be unnecessary and was not used.

At the end of the incubation period, the nematodes were washed free of radioactive medium in three consecutive washes of 1 minute, 2 minutes, and 2 minutes duration with 10 ml ½ DPBS. Assay for radioactivity of a 0.1-ml sample of the third wash using a liquid scintillation counter showed counts only slightly (< 50 cpm) higher than background. Washed nematodes were oven-dried (60 C), weighed on an electrobalance, and stored frozen (−20 C) in ½ DPBS until analysis. The groups of five worms were transferred to scintillation vials, then dissolved at 60 C in 0.4 ml of 'NCS' solubilizer (Amersham/Searle). The contents of each vial were taken up in 13 ml 'Aquasol' liquid scintillation cocktail (New England Nuclear Corp., Boston, Mass., USA) and acidified with 0.5 ml 1 N hydrochloric acid.

The relationship between uptake of palmitic acid and time was determined by measuring the absorption of isotope (2.0×10^{-3} μmoles/ml incubation medium) at varying times up to 1-hour incubation. With a 5-minute incubation period, the uptake of palmitic acid as a function of substrate concentration was determined over a range from 0.1 to 2 μM. The absorption of palmitic acid (1.0 μM) was also investigated in the presence of 1.0 mM potassium cyanide (KCN), 2,4-dinitrophenol (DNP), and ouabain over time intervals of 5 and 60 minutes and compared to controls (without inhibitor) run alongside the test samples. Competitive inhibition of palmitate uptake was examined using a fixed concentration (0.5 μM) of substrate in the presence of increasing concentrations (0–10 μM) of stearic or myristic acid. Conversely, the capacity of nematodes to absorb varying concentrations (0.1–2 μM) of palmitic acid in the presence of a fixed (1 μM) concentration of stearic or myristic acid was also measured. A 5-minute incubation time was used for all experiments designed to examine palmitic acid uptake in the presence of other fatty acids. To ascertain the degree to which passive diffusion accounted for uptake of the palmitic acid, nematode juveniles dissected out of hosts were first placed in distilled water, a solution in which the nematodes were killed within a few seconds due to osmotic intolerance, and then incubated (5 minutes) in ½ DPBS containing radiolabelled palmitic acid at concentrations ranging from 0.1 to 2 μM.

Fate of absorbed palmitic acid: To ascertain the extent to which the palmitic acid absorbed by the nematodes had been incorporated into storage lipids, groups of 20 nematodes were incubated (27 C) for either 5 or 60 minutes in 4 ml of ½ DPBS containing 7.96×10^{-3} μmoles palmitic acid-U-^{14}C. After incubation, each group of nematodes was washed free of radioactive medium, dried (15 minutes) in a desiccator containing calcium chloride, weighed, and then homogenized in a tissue grinder with 300 μl ½ DPBS. The homogenate was transferred to microtubes and 100 μl chloroform:methanol (2:1) was added to extract lipids (7). After 15 minutes, 100 μl 9% sodium chloride solution was added, and after centrifugation (13,500 *g*; 30 seconds), 40 μl of the lower lipid-containing layer was spotted on a heat-activated thin-layer chromatography (TLC) plate. Polar lipid fractions were separated according to the procedure of Mangold (13) and visualized with I_2 vapor. The areas of the plate-coating corresponding in size and position to that of lipid standards (monoacylglycerols, diacylglycerols, triacylglycerols, free fatty acids, sterols, sterol esters) were scraped into separate scintillation vials along with 13 ml 'Omnifluor' liquid scintillation cocktail (New England Nuclear). Polar lipids remained at the origin on the TLC plate.

All samples prepared for liquid scintillation counting were counted using a Beckman LS–3150T Liquid Scintillation Counter and corrected for quenching and counting efficiency by adding a ^{14}C-toluene internal standard.

Analysis of data: Statistical significance of the differences between paired samples was determined using the Student's *t*-test. To

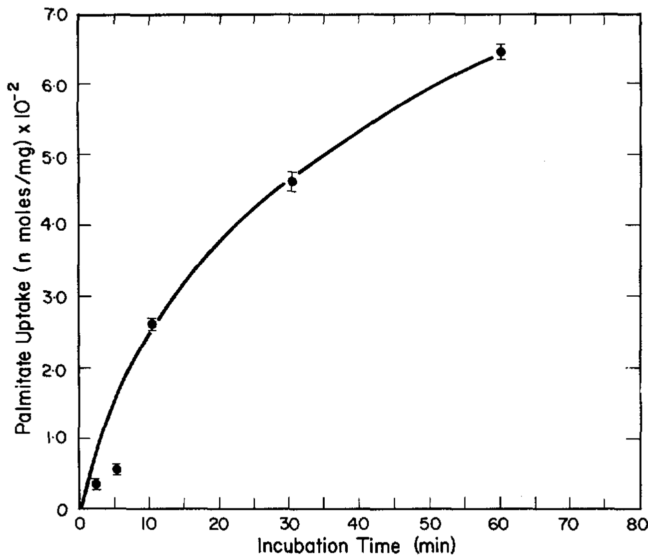

FIG. 1. Relationship between uptake of palmitic acid by *Romanomermis culicivorax* parasitic juveniles and incubation time. Data are expressed as nmoles palmitic acid absorbed/mg nematode dry weight. Each point represents the mean ± SE of three determinations.

analyze the data from experiments which deployed varying concentrations of palmitic acid in the presence of a fixed concentration of another fatty acid, it was necessary to conduct a Lineweaver and Burk (12) linear transformation of the Michaelis-Menten equation (a double reciprocal plot of $1/v$ versus $1/s$, where v is nmoles uptake/mg/5 minutes and s is μM substrate concentration). The least squares linear regression lines for such double reciprocal plots were drawn. The Eadie-Hofstee (5,10) linear transformation of the Michaelis-Menten equation (a plot of v versus v/s) was used to confirm conclusions drawn from the Lineweaver and Burk (12) analysis.

RESULTS

The rate of absorption of palmitic acid by the mermithid juveniles was logarithmic (Fig. 1).

Separation of nonpolar lipids, extracted by TLC from whole nematodes, showed triacylglycerols to be the most abundant lipid category. Also present, in decreasing order of prevalence, were free fatty acids, sterol esters, mono and diacylglycerols, and free sterols (Fig. 2).

Specific activity determinations of these lipid fractions after the nematodes had been incubated with the radiolabelled palmitic acid for 5 minutes showed that 65% of the isotope absorbed by the mermithid was still in the form of free fatty acid(s). Less than 20% had been incorporated into triacylglycerols, while incorporation into other lipid fractions collectively accounted for only 17.8% of the absorbed palmitic acid (Fig. 3). With 1-hour incubation, however, this situation was reversed; 60% of absorbed palmitate had been converted to triacylglycerols, while only 21.6% remained in free fatty acid form. Other lipid fractions accounted for 18.9% of the absorbed isotope, similar to the amount after 5-minute incubation. The fact that after 5 minutes most of the absorbed isotope was in the free fatty acid form, while after 1-hour incubation the triacylglycerol fraction of the nematode contained the highest level of radioactivity, suggests that during the shorter time period, transcuticular uptake was the principal process being measured, while the longer incubation period permitted substantial storage product synthesis to occur. It is possible, though outside the scope of this study, that a proportion of the absorbed palmitic acid may, particularly in the longer term incubation experiments, have been oxidized by the nematode for energy metabolism.

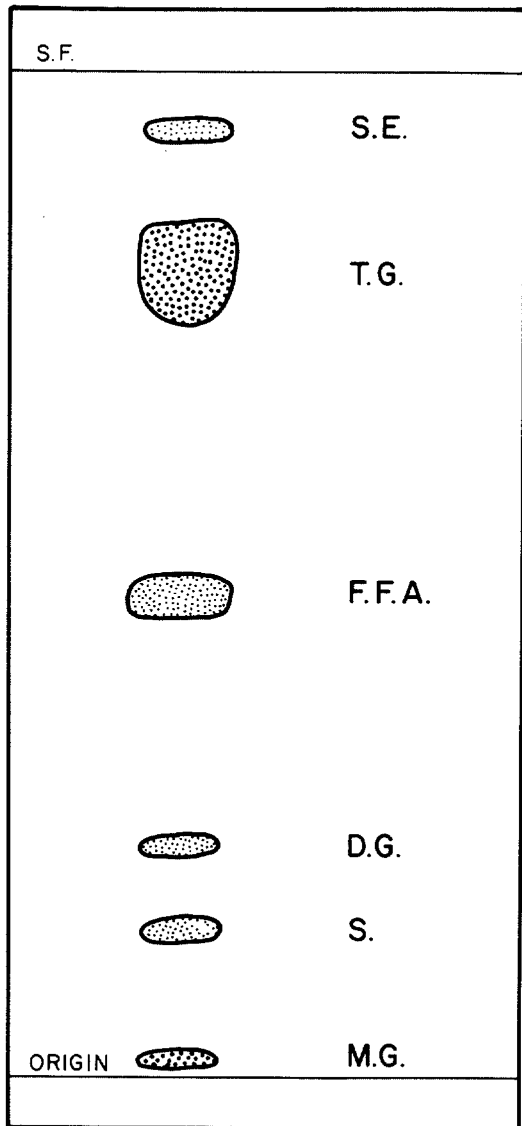

FIG. 2. Thin layer chromatography separation of nonpolar lipids in parasitic juveniles of *Romanomermis culicivorax*, 7 days postinfection. D.G. = diacylglycerols. F.F.A. = free fatty acids. M.G. = monoacylglycerols. S. = sterols. S.E. = sterol esters. S.F. = solvent front. T.G. = triacylglycerols.

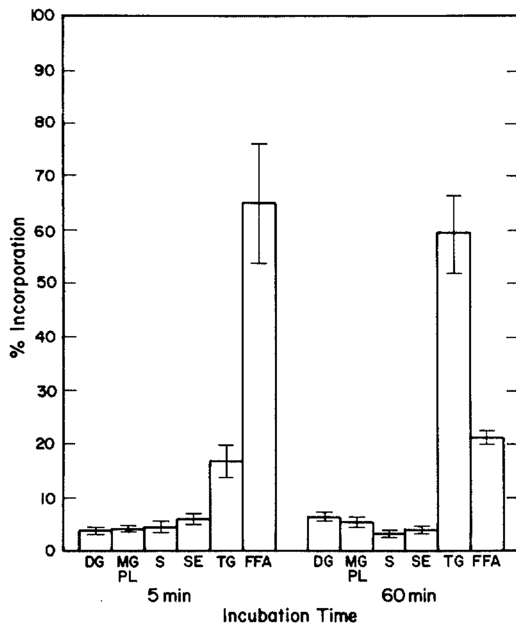

FIG. 3. Incorporation of palmitic acid into various lipid fractions of *Romanomermis culicivorax* parasitic juveniles after 5-minute and 60-minute incubation. Each value represents the mean (SE of five determinations) percentage of the isotope absorbed by the nematode that had been incorporated into the lipid fraction indicated. DG = diacylglycerols. FFA = free fatty acids. MG = monoacylglycerols. PL = phospholipids. S = sterols. SE = sterol esters. TG = triacylglycerols. Since monoacylglycerols separated out close to the base-line, they were pooled with phospholipids for specific activity determinations.

Over the range of concentrations tested, uptake of palmitic acid by the living nematodes was nonlinear (Fig. 4). The linear regression coefficient for the uptake of palmitic acid by osmotically killed nematodes was significant ($P < 0.025$). When the diffusion component was subtracted from the total uptake curve of the living nematodes, the mediated transport system that was resolved displayed saturation kinetics (Fig. 4).

The metabolic inhibitors KCN and DNP reduced the capacity of the nematode to take up palmitic acid during 5-minute (DNP—$P < 0.01$; KCN—$P < 0.001$) and 1-hour ($P < 0.001$ for both DNP and KCN) incubation periods (Fig. 5). For both time periods, absorption of palmitic acid was approximately two-thirds of control values when the nematodes were incubated in the presence of DNP and slightly less than half of control values when in the presence of KCN.

The sodium pump inhibitor ouabain reduced ($P < 0.001$) uptake of palmitic acid by the nematode to approximately one-third of control levels during both 5-minute and 1-hour incubation periods (Fig. 6).

Increasing concentrations of either stea-

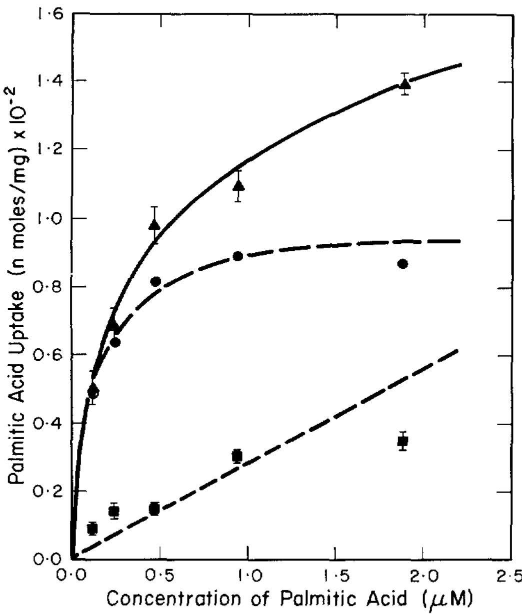

FIG. 4. Net uptake of palmitic acid by parasitic juveniles of *Romanomermis culicivorax* as a function of substrate concentration. Data are expressed as nmoles palmitic acid absorbed/mg nematode dry wt. For living (▲——▲; total uptake) and osmotically-killed (■---■; diffusion only) nematodes, each point represents the mean ± SE of six and four determinations, respectively. The linear regression coefficient, *r*, for the diffusion line is 0.883. The curve (●---●) depicting the mediated transport system was obtained by subtracting the diffusion component from the total uptake curve.

ric acid or myristic acid inhibited uptake of palmitic acid from a 0.5-μM solution in an apparently competitive manner, as shown by the saturation of inhibition with increasing concentration of the fatty acid inhibitor (Fig. 7). Concentrations of stearic and myristic acids in excess of 5 μM maximally inhibited uptake of the palmitic acid by approximately 85% and 65%, respectively. Data from experiments in which the concentration of palmitic acid was varied in the presence of a constant (1 μM) level of stearic or myristic acid confirmed that the unlabelled fatty acids competitively inhibited uptake of the labelled palmitic acid. Regression lines drawn from double reciprocal plots ($1/v$ versus $1/s$) for uptake of palmitate (corrected for diffusion) in the absence of, and in the presence of, another fatty acid intersected at a common intercept on the $1/v$ axis regardless of whether the fatty acid inhibitor was myristic (Fig.

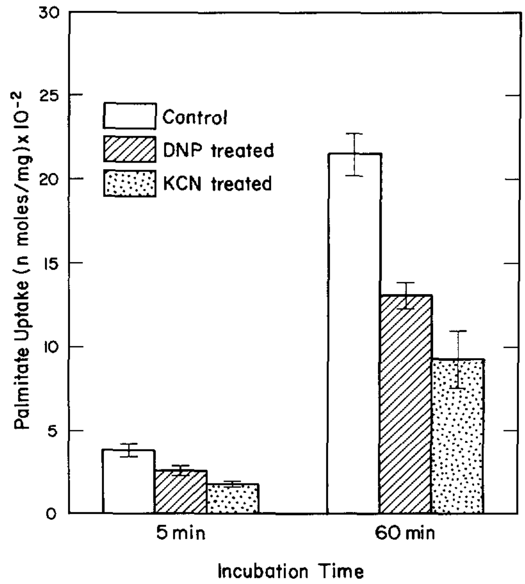

FIG. 5. Effect of a 1.0 mM solution of the metabolic inhibitors 2,4-dinitrophenol (DNP) and potassium cyanide (KCN) on uptake of palmitic acid by parasitic juveniles of *Romanomermis culicivorax* over 5-minute and 60-minute time intervals. Data are expressed as nmoles palmitic acid absorbed/mg nematode dry weight. Each value represents the mean ± SE of six determinations.

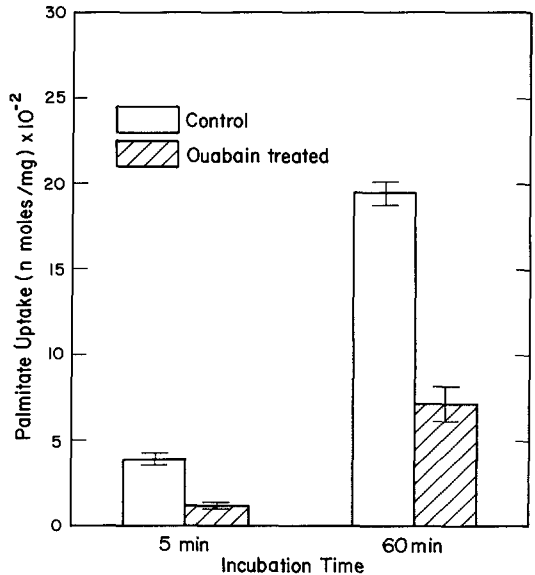

FIG. 6. Effect of the sodium pump inhibitor ouabain (1.0 mM) on uptake of palmitic acid by *Romanomermis culicivorax* parasitic juveniles over 5-minute and 60-minute time intervals. Data are expressed as nmoles palmitic acid absorbed/mg nematode dry weight. Each value represents the mean ± SE of six determinations.

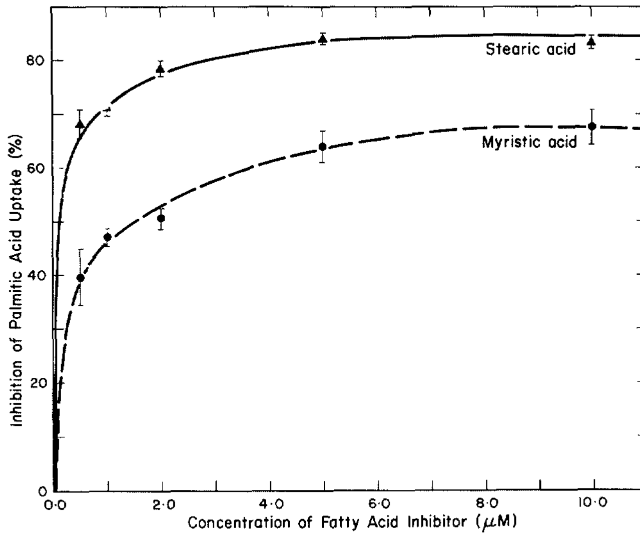

Fig. 7. Percentage inhibition of uptake of palmitic acid by *Romanomermis culicivorax* parasitic juveniles from 0.5-μM substrate by the presence of increasing concentrations of myristic acid (●——●) or stearic acid (▲——▲) in the media. Each value shown for myristic acid and stearic acid inhibition represents the mean ± SE for five and four determinations, respectively.

8) or stearic acid (Fig. 9). This situation is characteristic of competitive inhibition and implies that while the maximum uptake velocity (V_{max}) for palmitic acid was not affected, the apparent Michaelis' constant (K_m) was increased by the presence of stearic or myristic acid. It should be further noted that these findings were corroborated when regression analysis was done on Eadie-Hofstee plots of the same data.

Discussion

The data reported in this study suggest that parasitic juveniles of *R. culicivorax* transport palmitic acid across their tegument by a combination of nonmediated and mediated processes. Uptake of palmitic acid was nonlinear with respect to concentration, but saturation kinetics was not displayed. Such a relationship, characteristic of a wide variety of membrane transport systems in cestodes (15), is highly suggestive of the presence of concurrent mediated and nonmediated systems. The diffusion component, resolved and measured in osmotically killed nematodes, accounted for a quantitatively significant proportion of the overall fatty acid uptake. After the diffusion process had been taken into consideration, the mediated system that was re-

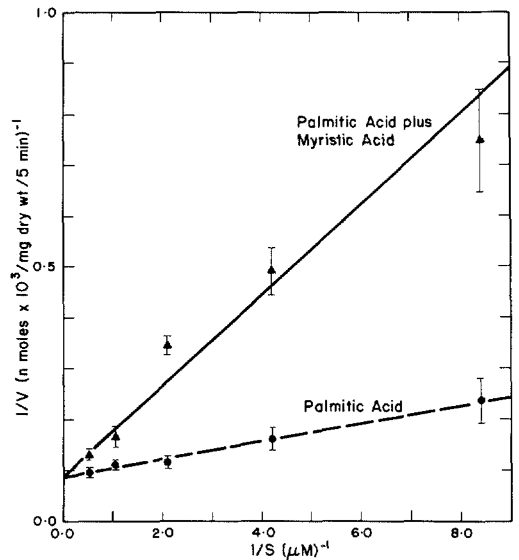

Fig. 8. Double reciprocal plots for the uptake of palmitic acid by parasitic juveniles of *Romanomermis culicivorax* with (▲——▲) and without (●——●) 1.0 μM myristic acid in the medium. Uptake of palmitic acid was expressed as nmoles palmitic acid absorbed/mg dry weight over a 5-minute incubation period, and the ordinate reflects the reciprocal of such values. Each value has been corrected for the diffusion component and represents the mean ± SE of five determinations. Linear regression coefficients: r_1, with myristic acid = 0.980; r_2, without myristic acid = 0.995.

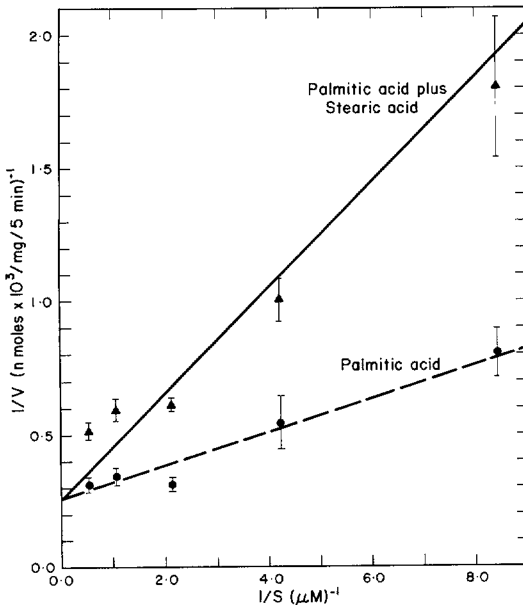

FIG. 9. Double reciprocal plots for the uptake of palmitic acid by parasitic juveniles of *Romanomermis culicivorax* with (▲——▲) and without (●---●) 1.0 μM stearic acid in the medium. Uptake of palmitic acid was expressed as nmoles palmitic acid absorbed/mg dry weight over a 5-minute incubation period, and the ordinate reflects the reciprocal of such values. Each value has been corrected for the diffusion component and represents the mean ± SE. of five determinations. Linear regression coefficients: r_1, with stearic acid = 0.990; r_2, without stearic acid = 0.970.

solved displayed, as expected, saturation kinetics. The stereospecific loci for palmitic acid uptake are also implicated in transport of myristic and stearic acids, as each of these fatty acids competitively inhibited palmitic acid uptake.

The inhibitory effects of DNP (an uncoupling agent which prevents phosphorylation of ADP to ATP) and KCN (an inhibitor of electron transport) on palmitate uptake during 5-minute incubation periods indicate that the mediated transport system is energy dependent. In the absence of ATP, storage product synthesis would also be inhibited in addition to the transport system itself. The principal category of trophosomal lipids in *R. culicivorax* are triacylglycerols (8). To what degree suppression of triacylglycerol synthesis would affect the free fatty acid pool within the tissues of the nematode, thereby disrupting transcuticular diffusion gradients and consequent diffusion processes, is not known. Thus, effects of the metabolic inhibitors on net palmitic acid uptake during the 1-hour incubation period may be attributed, with high probability, to direct impairment of the carrier system and, with less certainty, to indirect retardation of the diffusion process caused by disruption of triacylglycerol synthesis. The uptake of isotope which did occur in the presence of DNP and KCN likely resulted from a combination of diffusion, limited provision of ATP through energy reservoirs present within the nematode's tissues, and/or routes of ATP synthesis other than via oxidative phosphorylation.

The inhibitory effect of ouabain on uptake of palmitate by the parasitic juveniles suggests that the carrier-mediated system for the fatty acid is linked to a Na^+K^+ ATPase pump. Such a Na^+ and energy dependent transport mechanism is characteristic of sugar and amino acid, but not of free fatty acid, transport by the epithelial cells of vertebrates (20). While the precise mechanism for transport of free fatty acids by vertebrate cells has not been elucidated, it is known to be energy independent (14). The precise location of the membrane transport loci for fatty acids is not known. The cuticle of the parasitic stage(s) of this mermithid is traversed by pores (18), so it is possible that the functional absorptive surface is between the cuticle and the trophosome, possibly the hypodermis.

No parallel studies have been done on uptake of lipids by other nematode species that possess a transtegumentary mode of nutrition. Among nematodes, lipid absorption has been studied only for *Ascaris lumbricoides,* which transports palmitate across the mucosal cells of its gut and into the pseudocoelom by diffusion and an energy dependent transport mechanism, applied in sequence (2,21). Information on the nature of uptake mechanisms used by mermithids for nonlipid nutriment is available for *Mermis nigrescens,* parasitic in locusts. The transport mechanisms used by *M. nigrescens* for glucose and amino acids, however, appear to be quite different from that used by *R. culicivorax* for fatty acid uptake, being of the facilitated diffusion variety and not coupled to co-transport of sodium ions (19). Among helminths, only cestodes have been examined in detail with respect to mechanisms used for fatty acid

transport. Cestodes resemble mermithid nematodes in feeding exclusively by transtegumentary absorptive processes. Like *R. culicivorax*, a cestode such as *Hymenolepis diminuta* absorbs fatty acids across its tegument by a combination of diffusion and mediated systems. The sensitivity of the cestode mediated transport system(s) for fatty acids to sodium and metabolic pump inhibitors is unknown, however. Preliminary findings that uptake of palmitic acid in *H. diminuta* was unaffected by exposure of the cestode's surface membrane to such inhibitors (4) must be interpreted in light of the subsequent discovery that the transport loci are in the inner tegumentary membrane (17).

Studies are underway in our laboratory to elucidate the nature of the transport mechanisms used by *R. culicivorax* for absorbing other categories of lipids, especially mono and triacylglycerols, across its tegument.

Literature Cited

1. Bailey, C. H., and R. Gordon. 1973. Histopathology of *Aedes aegypti* (Diptera: Culicidae) larvae parasitized by *Reesimermis nielseni* (Nematoda: Mermithidae). J. Invertebr. Pathol. 22:435–441.

2. Beames, C. G., Jr., and G. A. King. 1972. Factors influencing the movement of materials across the intestine of *Ascaris*. Pp. 275–282 *in* H. van den Bossche, ed. Comparative biochemistry of parasites. New York: Academic Press.

3. Castillo, J. M., P. Chin, and D. W. Roberts. 1982. Growth and development of *Romanomermis culicivorax* in vitro. J. Nematol. 14:476–485.

4. Chappell, L. H., C. Arme, and C. P. Read. 1969. Studies on membrane transport. V. Transport of long chain fatty acids in *Hymenolepis diminuta* (Cestoda). Biol. Bull. 136:313–326.

5. Eadie, G. S. 1942. The inhibition of cholinesterase by physostigmine and prostigmine. J. Biol. Chem. 146:85–93.

6. Finney, J. R. 1981. Mermithid nematodes: In vitro culture attempts. J. Nematol. 13:275–280.

7. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–509.

8. Gordon, R., J. R. Finney, W. J. Condon, and T. N. Rusted. 1979. Lipids in the storage organs of three mermithid nematodes and in the hemolymph of their hosts. Comp. Biochem. Physiol. 64B:369–374.

9. Gordon, R., I. R. Burford, and T. L. Young. 1982. Uptake of lipids by the entomophilic nematode *Romanomermis culicivorax*. J. Nematol. 14:492–495.

10. Hofstee, B. H. J. 1952. Specificity of esterases. I. Identification of two pancreatic aliesterases. J. Biol. Chem. 199:357–364.

11. Ittycheriah, P. I., R. Gordon, and W. J. Condon. 1977. Storage material of the nematode *Romanomermis culicivorax*, a mermithid parasite of larval mosquitoes. Nematologica 23:165–171.

12. Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56:658–666.

13. Mangold, H. K. 1969. Aliphatic lipids. Pp. 363–421 *in* E. Stahl, ed. Thin layer chromatography. New York: Springer-Verlag.

14. Nikkila, E. A. 1971. Transport of free fatty acids. Progr. Biochem. Pharmacol. 6:102–129.

15. Pappas, P. W., and C. P. Read. 1975. Membrane transport in helminth parasites: A review. Exp. Parasitol. 37:469–530.

16. Platzer, E. G. 1981. Biological control of mosquitoes with mermithids. J. Nematol. 13:257–262.

17. Podesta, R. B., W. S. Evans, and H. E. Stallard. 1977. *Hymenolepis diminuta* and *Hymenolepis microstoma*: Effect of ouabain on active nonelectrolyte uptake across the "epithelial" syncytium. Exp. Parasitol. 43: 25–38.

18. Poinar, G. O., Jr., and R. Hess. 1977. *Romanomermis culicivorax*: Morphological evidence of transcuticular uptake. Exp. Parasitol. 42:27–33.

19. Rutherford, T. A., J. M. Webster, and J. S. Barlow. 1977. Physiology of nutrient uptake by the entomophilic nematode *Mermis nigrescens* (Mermithidae). Can. J. Zool. 55:1773–1781.

20. Schultz, S. G., and P. F. Curran. 1970. Coupled transport of sodium and organic solutes. Physiol. Rev. 50:637–718.

21. Van den Bossche, H., and S. de Nollin. 1973. Effects of mebendazole on the absorption of low molecular weight nutrients by *Ascaris suum*. Int. J. Parasitol. 3:401–407.