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Glyoxylate Pathway in the Free-Living Stages of the Entomophilic Nematode *Romanomermis culicivorax*

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Abstract: Isocitrate lyase and malate synthetase, key enzymes of the glyoxylate cycle, were present in postparasites of the mermithid nematode *Romanomermis culicivorax*. Specific activities of enzymes were higher in adult postparasites than in newly emerged juveniles. Isocitrate lyase had a well-defined pH optimum (7.5), whereas malate synthetase functioned optimally over a broad range of alkaline pH (7.5–9.0). Substrate affinities of the two enzymes were measured.

Key words: glyoxylate pathway, isocitrate lyase, malate synthetase, physiology, postparasite, *Romanomermis culicivorax*.

The mermithid nematode *Romanomermis culicivorax* Ross and Smith is a promising biocontrol agent of larval mosquitoes (18). Some of the juvenile stages of this nematode develop within the hemocoel of larval culicids, abstracting and storing metabolites from the insect's hemolymph for growth and development. Upon completion of parasite development, the mermithid exits the host, piercing its intersegmental membranes or passing through natural openings, to commence the free-living phase of its life cycle. Molting of the free-living juveniles (postparasites) to adults, mating, and oviposition occur in the substratum of the host's fresh water habitat. Eggs hatch to release infective juveniles (preparasites) that infect mosquito larvae by penetration of their exoskeleton.

Free-living stages of mermithids are presumed to be nonfeeding (18,23), relying for metabolism upon nutrients stored in the trophosome during the parasitic stages. In *R. culcivorax*, lipids, especially triacylglycerols, constitute the predominant storage metabolite (4,9) and the energy metabolism of postparasites is accomplished initially by β-oxidation of such lipid reserves (5). It is improbable, however, that oxidative catabolism could occur within the pond sediment, a microhabitat of minimal oxygen content (6). Accordingly, *R. culicivorax* is a facultative anaerobe (6) that relies upon carbohydrate metabolism via the fumarate reductase pathway (7) once it has burrowed into the substratum of the larval mosquito's habitat. Maintained under aerobic conditions, postparasites were found to increase their reserves of glycogen at the expense of lipids during the early portion of free-living development, indicating the presence of a glyoxylate pathway (8).

The glyoxylate cycle is common in micro-organisms, germinating seeds of higher plants and spores of ferns, but of scattered occurrence among metazoa (14). Enzymes of the cycle have been demonstrated for several species of free-living nematodes (15–17,21,22). Among parasitic helminths, however, enzymes of the glyoxylate pathway have been shown only for the adult trematode *Fasciola hepatica* (19), the acanthocephalan *Moniliformis dubius* (11), embryonating eggs of the parasitic nematodes *Ascaris lumbricoides* (2) and *Ascaris suum* (16,17), and postembryonic stages of *Strongyloides ratti* (10).

My purpose was to ascertain whether postparasitic juveniles and adults of *R. culicivorax* possess isocitrate lyase and malate synthetase, key enzymes of the glyoxylate cycle, and to gain insight into the role of the cycle by monitoring enzyme activities at varying stages of postparasitic development.

MATERIALS AND METHODS

A laboratory colony of *R. culicivorax* was maintained by propagating the mermithid through newly hatched *Aedes aegypti* (L.)

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larvae (1). Postparasites were obtained from experimentally infected *A. aegypti* larvae maintained at 27 C. Under these conditions, postparasitic nematodes emerged from hosts 7–9 days after infection. All biochemicals used in this study were obtained from Sigma Chemical Co., St. Louis, Missouri, and were the purest available commercially.

All enzyme assays were carried out using postparasites (sex ratio ca. 2 males : 1 female) of known age that had been reared (27 C) in distilled water in petri dishes (5). To prepare enzyme extracts, nematodes were washed twice in buffer (pH 7.5) by centrifugation (250 *g*; 5 minutes). The buffer used to wash the postparasites had the same composition as that used subsequently to homogenize them. For malate synthetase, a 50 mM Trizma (Sigma Chemical Co., St. Louis, Missouri) buffer was used, whereas isocitrate lyase assays were done using a 0.1 M morpholinopropanesulfonic acid (MOPS) buffer containing 1 mM ethylenediamine tetraacetic acid, disodium salt (EDTA), 5 mM MgCl, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 20% (v/v) glycerol (16,17). The nematodes (0.2–0.3 ml packed volume after centrifugation) were homogenized in 1–1.5 ml buffer using a motor-driven ground glass homogenizer; the extract was subjected for 30 seconds to sonic treatment (85 watts; 20 kHz) using a Braun-Sonic 2000 sonicator (Canlab Laboratory Equipment, Dartmouth, N.S., Canada). Extracts were clarified by centrifugation at 34,000 *g* for 20 minutes at 4 C. The supernatant was removed and assayed immediately for isocitrate lyase or malate synthetase activity.

Isocitrate lyase (E.C. 4.1.3.1) was assayed colorimetrically (20). The reaction mixture consisted of 3.45 ml assay buffer (homogenization buffer minus glycerol) and 0.1 ml of 0.04 M sodium-DL-isocitrate, warmed (10 minutes) to 30 C. The reaction was started by the addition of 0.05 ml enzyme extract and incubations carried out at 30 C for 30 minutes. Under these incubation conditions, the yield of glyoxylate was determined to be linear with respect to time over a 1-hour incubation period. The reaction was stopped by the addition of 0.4 ml of 1 M oxalic acid, followed by 0.1 ml of 5% phenylhydrazine HCl. Glyoxylate in the sample was then determined (13) and the specific activity expressed as nmoles of glyoxylate formed·minute^{-1}·mg protein^{-1}.

Malate synthetase (E.C. 4.1.3.2) was assayed at 20 C (3). The reaction mixture comprised 4.0 ml Trizma buffer (50 mM), 0.15 ml of 0.1 M MgCl, and 0.1 ml of 0.002 M acetyl-CoA. Of this solution, 0.6 ml was pipetted into each of two silica cells, followed, in the test cell, by 20 µl of enzyme extract. The reaction was started by the addition of 20 µl of 0.02 M sodium glyoxylate and the consequent change in extinction of the reaction mixture at 232 nm followed by use of a Shimadzu UV-260 recording spectrophotometer (Shimadzu Corportion, Kyoto, Japan). Specific activity was expressed as nmoles acetyl-CoA cleaved·minute^{-1}·mg protein^{-1}.

The protein content of the nematodes was determined colorimetrically (12) using bovine serum albumin as a protein standard.

Apparent Michaelis-Menton constants for substrates were determined from double reciprocal plots. Regression lines were plotted by the least mean square method.

Results and Discussion

Postparasitic juveniles and adults of *R. culicivorax* were found to possess both isocitrate lyase and malate synthetase, key enzymes of the glyoxylate pathway (Table 1). This pathway consists of a cyclic set of reactions involving five enzymes. The other three enzymes involved in the pathway, malate dehydrogenase, citrate synthetase, and aconitase, are also involved in the tricarboxylic acid cycle; their presence may be inferred from the observation that nematodes produced $^{14}CO_2$ consequential to β-oxidation of ^{14}C-palmitic acid (5). Malate dehydrogenase has been assayed and characterized from postparasites of this mermithid (7). The finding that glycogen

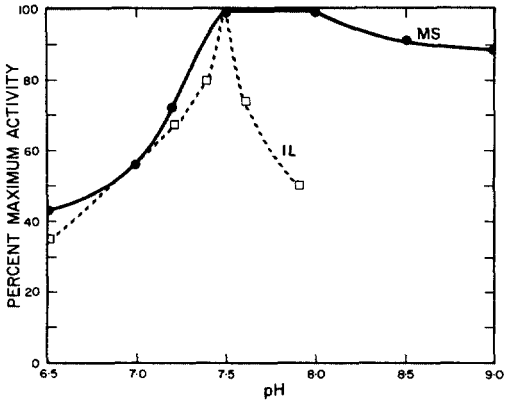

FIG. 1. Isocitrate lyase (IL, □- - -□) and malate synthetase (MS, ●——●) from *Romanomermis culicivorax* (4 weeks after emergence from host): pH–enzyme activity curves. Activities expressed as percent of maximum obtained at optimum pH. Each point is the mean of 3–5 determinations.

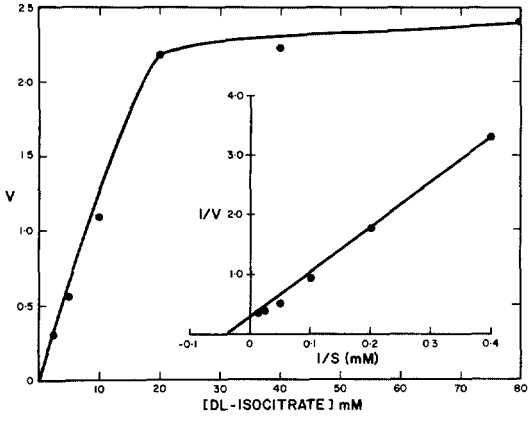

FIG. 2. Substrate–activity curve for isocitrate lyase in extracts of *R. culicivorax* postparasites (7 days after emergence). Specific activities (V) are expressed as nmoles glyoxylate produced·minute^{-1}·mg protein^{-1}. Each point is the mean of three determinations. Inset is a double reciprocal plot of the substrate–activity curve, 1/V being expressed as (nmoles glyoxylate produced·minute^{-1}·mg protein^{-1})$^{-1}$.

reserves increased relative to lipids during the first 10 days of postparasitic development (8) indicates that the glyoxylate pathway is functional in this mermithid. Although isocitrate lyase and malate synthetase have been found in several species of free-living nematodes (21,22), in the animal-parasitic nematodes *S. ratti* (10) and *A. suum* (16,17), as well as in isolated species within other helminth groups (11,19), a functional glyoxylate pathway has been established to date only for the eggs of *A. lumbricoides* (2) and the postembryonic stages of *Caenorhabditis elegans* (14,17).

Isocitrate lyase had a narrow pH optimum of 7.5, whereas malate synthetase functioned at maximum, or near-maximum, activity over a broad range of tested pH conditions from 7.5 to 9.0 (Fig. 1). In the eggs of *A. lumbricoides,* these enzymes also function most effectively at alkaline pH, but the pH optima for both isocitrate lyase (7.7) and malate synthetase (9.0) are separate and distinct (2).

Substrate concentration–reaction velocity curves were determined for isocitrate lyase (Fig. 2) and malate synthetase (Fig. 3), and hyperbolic saturation curves were obtained. The enzymes followed Michaelis-Menten kinetics, and double reciprocal plots (inset of figures) were used to determine apparent K_ms and V_{max} (Table 2). Malate synthetase had more than four times greater affinity for acetyl-CoA (0.71 mM) than for glyoxylate (3.2 mM). The isocitrate lyase of *R. culicivorax* had a considerably lower substrate affinity than did the comparable enzyme extracted from *C. elegans* and *A. suum* (17).

Several studies done on isocitrate lyase and malate synthetase in helminths involved measurements of enzyme activities in units other than those used herein. However, the specific activities of both enzymes in *R. culicivorax* fall within the range of

TABLE 1. Enzyme activities in *Romanomermis culicivorax* postparasites.

Days post-emergence†	Specific activity‡	
	Isocitrate lyase	Malate synthetase
0–2	0.25 ± 0.08	4.35 ± 0.62
9	1.49 ± 0.17	15.23 ± 1.00
15	1.53 ± 0.17	12.74 ± 0.82
30	1.40 ± 0.07	20.51 ± 1.18

† Days postemergence from the host: 9 days postemergence, nematodes are newly molted adults; 15 days after emergence represents a stage of development when female nematodes are ovipositing; by 30 days postemergence, female nematodes have completed oviposition.

‡ Specific activity expressed as nmoles glyoxylate produced·minute^{-1}·mg protein^{-1} (isocitrate lyase) or nmoles acetyl-CoA cleaved·minute^{-1}·mg protein^{-1} (malate synthetase). Isocitrate lyase was assayed at 30 C, malate synthetase at 20 C. All values are means ± SE of 4–7 determinations.

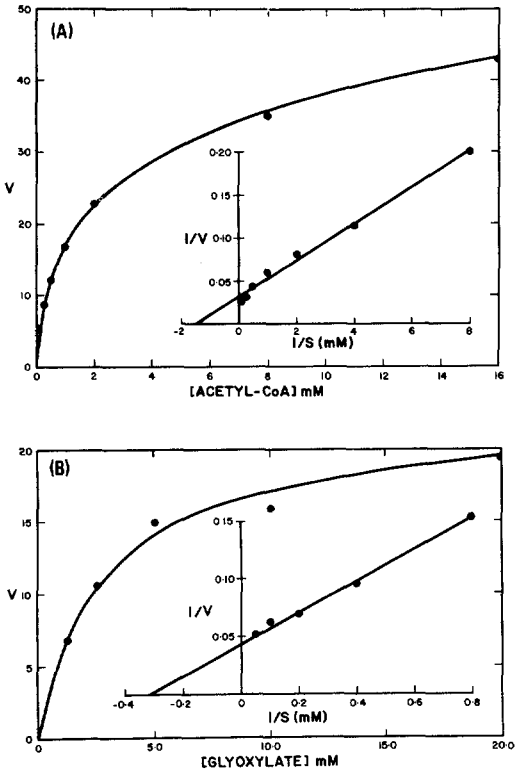

FIG. 3. Substrate–activity curves for malate synthetase in extracts of *R. culicivorax* postparasites (7 days after emergence), the substrates being acetyl-CoA (Fig. 3A) and glyoxylate (Fig. 3B). Each point is the mean of three determinations. Specific activities are expressed as nmoles acetyl-CoA cleaved·minute^{-1}·mg protein^{-1}. Inset are double reciprocal plots: (A) acetyl-CoA affinity; (B) glyoxylate affinity.

values obtained for *S. ratti* (10), *M. dubius* (11), *A. lumbricoides* (2), and *A. suum* and *C. elegans* (17). Isocitrate lyase activity recorded for *F. hepatica* (19) was several orders of magnitude higher than for other species examined.

Specific activities of isocitrate lyase and malate synthetase were higher in newly molted and sexually mature adults than in newly emerged juveniles (Table 1), indicating that the function of the glyoxylate pathway is associated with free-living development within the substratum of the larval mosquito's habitat, rather than with the parasitic portion of the life cycle.

In other organisms studied, the glyoxylate pathway permits gluconeogenesis from lipid sources. In circumventing the decarboxylation steps of the tricarboxylic acid cycle and causing the production of succinate and malate from isocitrate (and acetyl-CoA), the glyoxylate cycle replenishes intermediates of the tricarboxylic acid cycle, conserving carbon that would otherwise be oxidized. It has been suggested that the conversion of lipid reserves to glycogen in *R. culicivorax* is necessary because the pond substratum, in which adult nematodes develop, is essentially anaerobic (6,8). *R. culicivorax* would appear to possess all of the ingredients necessary for gluconeogenesis: a β-oxidation pathway (5) to generate acetyl-CoA, a glyoxylate cycle, and phosphoenolpyruvate carboxykinase (7) to bypass pyruvate kinase, which catalyzes an essentially irreversible transformation. The evidence indicates that energy metabolism of postparasites developing in the substratum of the larval mosquito's habitat is accomplished by a fumarate reductase pathway (7), provision for which is furnished, in part, by the glyoxylate cycle.

TABLE 2. Kinetic parameters of isocitrate lyase and malate synthetase in *Romanomermis culicivorax* postparasites.

Enzyme	Km (mM)		Vmax†
Isocitrate lyase	DL-isocitrate	26.32	3.33
Malate synthetase	glyoxylate	3.20	23.81
	acetyl-CoA	0.71	33.33

† Expressed as nmoles of product formed (isocitrate lyase) or substrate utilized (malate synthetase)·minute^{-1}·mg protein^{-1}. Enzyme extracts were prepared from postparasites 7 days after they had emerged from the host.

LITERATURE CITED

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

2. Barrett, J., C. W. Ward, and D. Fairbairn. 1970. The glyoxylate cycle and the conversion of triglycerides to carbohydrates in developing eggs of *Ascaris lumbricoides*. Comparative Biochemistry and Physiology 35:577–586.

3. Dixon, G. H., and H. L. Kornberg. 1962. Malate synthetase from baker's yeast. Pp. 633–637 *in* S. P. Colowick and N. O. Kaplan, eds. Methods in enzymology, vol. 5. New York and London: Academic Press.

4. Gordon, R., J. R. Finney, W. J. Condon, and T.

A. Rusted. 1979. Lipids in the storage organs of three mermithid nematodes and in the hemolymph of their hosts. Comparative Biochemistry and Physiology 64B:369–374.

5. Gordon, R., D. J. Walsh, and I. R. Burford. 1981. Beta-oxidation in the free-living stages of the entomophilic nematode *Romanomermis culicivorax*. Parasitology 83:451–457.

6. Imbriani, J. L., and E. G. Platzer. 1981. Gaseous requirements for postparasitic development of *Romanomermis culicivorax*. Journal of Nematology 13: 470–476.

7. Imbriani, J. L., and E. G. Platzer. 1982. Characterization of lactate dehydrogenase, malate dehydrogenase, pyruvate kinase, and phosphoenolpyruvate carboxykinase in *Romanomermis culicivorax* post-parasitic larvae and adults. Comparative Biochemistry and Physiology 72B:21–29.

8. Imbriani, J. L., E. G. Platzer, and S. N. Thompson. 1982. The effect of oxygen on the metabolism of postparasites of the facultative anaerobe *Romanomermis culicivorax* (Nematoda: Mermithidae). Comparative Biochemistry and Physiology 72B:13–19.

9. Ittycheriah, P. I., R. Gordon, and W. J. Condon. 1977. Storage material of the nematode *Romanomermis culicivorax*, a mermithid parasite of larval mosquitoes. Nematologica 23:165–271.

10. Körting, W., and D. Fairbairn. 1971. Changes in beta-oxidation and related enzymes during the life cycle of *Strongyloides ratti* (Nematoda). Journal of Parasitology 57:1153–1158.

11. Körting, W., and D. Fairbairn. 1972. Anaerobic energy metabolism in *Moniliformis dubius* (Acanthocephala). Journal of Parasitology 58:45–50.

12. Lowry, O. H., N. J. Rosebrough, A. L.Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. Journal of Biological Chemistry 193:265–275.

13. McFadden, B. A., and W. V. Howes. 1960. The determination of glyoxylic acid in biological systems. Analytical Biochemistry 1:240–248.

14. Patel, T. R. 1983. Spruce budworm and energy metabolism. Pp. 25–31 *in* Forest defoliator-host interactions: A comparison between gypsy moth and spruce budworm. General Technical Report NE-85, U.S. Department of Agriculture, Forest Service, Northeastern Station.

15. Patel, T. R. 1977. Particulate isocitrate lyase and malate synthetase in *Caenorhabditis elegans*. Archives of Biochemistry and Biophysics 183:24–30.

16. Patel, T. R., and B. A. McFadden. 1978. *Caenorhabditis elegans* and *Ascaris suum*: Fragmentation of isocitrate lyase in crude extracts. Experimental Parasitology 44:72–81.

17. Patel, T. R., and B. A. McFadden. 1978. *Caenorhabditis elegans* and *Ascaris suum*: Inhibition of isocitrate lyase by itaconate. Experimental Parasitology 44:262–268.

18. Petersen, J. J. 1984. Nematode parasites of mosquitoes. Pp. 797–820 *in* W. R. Nickle, ed. Plant and insect nematodes. New York and Basel: Marcel Dekker.

19. Prichard, R.K., and P. J. Schofield. 1969. The glyoxylate cycle, fructose-1-6-diphosphatase and gluconeogenesis in *Fasciola hepatica*. Comparative Biochemistry and Physiology 29:581–590.

20. Roche, T. E., J. O. Williams, and B. A. McFadden. 1970. Effect of pH and buffer upon K_m and inhibition by phosphoenolpyruvate of isocitrate lyase from *Pseudomonas indigofera*. Biochimica Biophysica Acta 206:193–195.

21. Rothstein, M., and H. Mayoh. 1965. Nematode biochemistry—7. Presence of isocitrate lyase in *Panagrellus redivivus, Turbatrix aceti,* and *Rhabditis anomala*. Comparative Biochemistry and Physiology 16: 361–365.

22. Rothstein, M., and H. Mayoh. 1966. Nematode biochemistry—8. Malate synthetase. Comparative Biochemistry and Physiology 17:1181–1188.

23. Rutherford, T. A., and J. M. Webster. 1976. Effects of the nematode *Mermis nigrescens* on some chemical components of the insect host's hemolymph. Proceedings of the 1st International Colloquium on Invertebrate Pathology. Queen's University, Kingston, Canada, pp. 272–275.