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Polyamine Synthesis by the Mermithid Nematode *Romanomermis culicivorax*[1]

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Abstract: The polyamine and amino acid composition of the mermithid nematode, *Romanomermis culicivorax*, and its host, *Aedes aegypti*, was determined. Putrescine, spermidine, spermine, cadaverine and two acetylated spermidine derivatives were present in parasitic juveniles, newly-emerged postparasites, and eggs of *R. culicivorax*. Whole insect homogenates of fourth-instar *A. aegypti* contained the same array of polyamines, except that the putrescine : spermidine ratio was the inverse of that in parasitic *R. culicivorax*. Polyamines and amino acids were in greater concentrations in the nematode eggs than in other developmental stages investigated. Both the host and nematode possess the biosynthetic capacity for polyamine biosynthesis, as evidenced by measurable activities of ornithine decarboxylase in the host's tissues and the nematode's free-living stages.

Key words: Aedes aegypti, amino acid, mermithid, mosquito parasite, ornithine decarboxylase, polyamine, *Romanomermis culicivorax*.

Polyamines are important constituents for all living cells. There is substantial evidence to indicate that they enhance protein synthesis, stabilize the molecular structure of nucleic acids and phospholipids, and, in specific instances, constitute essential growth factors (17).

Despite the importance of polyamines in physiological processes, little is known about these compounds in nematodes and insects. Among nematodes, the capacity for cadaverine synthesis has been demonstrated in *Trichinella spiralis* (13) and *Ascaris lumbricoides* (3,13,19) and for putrescine synthesis, in *A. lumbricoides* (3). From the limited information available, it appears that polyamines fulfill an important role in embryogenesis and subsequent metamorphosis of insects, such as *Drosophila melanogaster* (6) and *Calliphora erythrocephala* (20), and constitute dietary growth factors for certain grain pests, reared on synthetic diets (4,5).

To enhance our knowledge of polyamines in nematodes (and insects) and to assess the importance of these compounds as a dietary constituent for *R. culicivorax*, we investigated the synthesis of polyamines by the mermithid nematode, *Romanomermis culicivorax* Ross and Smith, and its laboratory host, the larval culicid *Aedes aegypti* (L.).

Materials and Methods

A laboratory colony of *R. culicivorax* was maintained by propagating the mermithid through newly hatched *A. aegypti* larvae (1). For physiological assays, parasitic juveniles were dissected out of *A. aegypti* fourth-instar larvae that had been experimentally infected with equal numbers of preparasites 7 days previously, so that 1–3 nematodes developed in infected hosts (16). Postparasitic juveniles, adults and eggs were obtained from nematodes maintained at 27 C (11).

Amino acids and polyamines were determined for uninfected hosts (fourth-instar) and for nematodes (ca. 2 ♀:1 ♂) of four developmental stages: parasitic, newly emerged (0–2 days), spent (5 weeks postemergence), eggs (0–5 days old). To establish whether the nematode was itself able to synthesize polyamines distinct from the host, enzyme assays were conducted on newly emerged postparasites (ca. 2 ♀:1 ♂), nematode eggs, and early and late fourth-instar uninfected *A. aegypti* larvae.

Host and nematode samples were prepared for free amino acid and polyamine analyses by washing them three or four

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times with sterile distilled water. Nematodes (n = 90–240), eggs (75–100 μl packed volume), or *A. aegypti* larvae (n = 30) were homogenized in 1.5 ml distilled water in a ground glass homogenizer with a motor-driven pestle. To prevent melanization, a few crystals of phenylthiourea were added to distilled water used to homogenize host larvae. To effect complete disruption of the egg shells, egg homogenates were sonicated (85 watts; 20 kHz; 30 seconds). Samples were analyzed with a Beckman Model 121MB amino acid analyzer for free amino acids (11) and polyamines. The procedure for polyamine analysis was modified from that of Hall et al. (12). A fluorescence detection reagent, o-phthaldehyde (OPA), was used to improve sensitivity. Sulfonated styrene copolymer resin with 7.5% cross-linkage, bead diameter 10 ± 1 μm, was converted to the sodium form with 0.2 M NaOH containing 0.01% EDTA and packed to a height of 6 cm in a 0.6-cm-d glass column. The column was then washed with 116.7 mM trisodium citrate (pH 5.4), containing 4.0 M NaCl, to obtain maximum shrinkage of resin. Deproteinized, buffered (pH 2.2; 0.2 M sodium citrate buffer) extracts (250 μl) were applied to the column and polyamines were eluted at 65 C with buffer A (0.2 M trisodium citrate, pH 6.4, and 1.0 M NaCl) for 20 minutes, followed by buffer B (116.7 mM trisodium citrate, pH 5.4, and 2.0 M NaCl) for 24 minutes, then buffer C (116.7 mM trisodium citrate, pH 5.4, and 4.0 M NaCl) for 10 minutes. Buffer and OPA flow rates were 35 ml/hour and 17.5 ml/hour, respectively. The column was regenerated with 0.2 M NaOH (4 minutes) and equilibrated with buffer A (16 minutes) after each sample. The lower limit of detection for polyamines was 0.001 or 0.002 nanomoles/milligram dry weight of tissue, depending upon the sample tested; lower limits of detection for ninhydrin positive substances ranged from 0.01 (host) to 0.05 (eggs) nanomoles/milligram dry weight of tissue.

To determine ornithine decarboxylase (ODC; EC 4.1.1.17) activities, postparasites (n = 30–60) or eggs (20–70 μl) of *R. culicivorax* were homogenized in 1 ml buffer D (0.25 M sucrose, 0.002 M EDTA, 0.005 M dithiothreitol, 0.002 M HEPES, pH 7.4). Egg homogenates were sonicated. Replicates of host larvae for ODC analysis were prepared by homogenizing 50 insects in 5 ml buffer D containing a few crystals of phenylthiourea. Homogenates were centrifuged (20,000 *g* at 4 C for 20 minutes), and the ODC activity in the supernatants was determined by measuring the release of $^{14}CO_2$ from {1-^{14}C}ornithine (15). L-ornithine was present in the incubation mixture at a final concentration of 0.4 mM and specific radioactivity of 0.5 μCi/μmole. Assays were incubated for 30 minutes at 28 C. Activity was linear with respect to protein concentration and time under the conditions used. The enzyme was assayed within 1 hour of sample preparation, since its activity was quite unstable on freezing.

Dry weight measurements were obtained, using a parallel set of host and nematode samples, of equivalent size to those used for the physiological determinations. To obtain dry weights, samples were heated (60 C; ca. 30 minutes) and weighed to constant weight on an electrobalance.

Data obtained for the various developmental stages of *R. culicivorax* were compared by means of a single classification analysis of variance and means were separated with Duncan's multiple-range test (18).

Results and Discussion

Putrescine, spermidine, and spermine, which are polyamines of common occurrence in animal tissues, were present in *R. culicivorax* at all stages of development examined (Table 1). In addition, the mermithid contained cadaverine, a polyamine only occasionally reported from eukaryotes, and relatively low concentrations of two acetylated derivatives of spermidine. The relative proportions of the two most prevalent polyamines, putrescine and spermidine, varied with developmental stage. In the eggs and spent postparasites, from

TABLE 1. Polyamine composition of *Romanomermis culicivorax* and its host, *Aedes aegypti.*

Polyamine	Eggs	Parasitic juveniles	Newly emerged postparasites	Spent postparasites	Fourth-instar larvae
Putrescine	15.53 ± 4.43 a	2.74 ± 0.74 b	2.25 ± 0.40 b	4.32 ± 0.56 b	12.26 ± 2.19
Cadaverine	0.50 ± 0.06 a	0.03 ± 0.00 b	0.01 ± 0.01 b	0.07 ± 0.00 b	0.13 ± 0.04
Spermidine	8.00 ± 1.55 a	6.90 ± 0.71 a	3.00 ± 0.08 b	1.92 ± 0.04 b	4.12 ± 0.78
Spermine	0.10 ± 0.03 a	0.12 ± 0.07 a	0.30 ± 0.01 a	0.18 ± 0.03 a	0.37 ± 0.04
N^1-acetyl spermidine	0.11 ± 0.04 a	0.03 ± 0.01 b	0.01 ± 0.00 b	0.01 ± 0.00 b	0.02 ± 0.00
N^8-acetyl spermidine	0.08 ± 0.02 a	0.02 ± 0.01 a	0.01 ± 0.00 a	0.03 ± 0.00 a	0.15 ± 0.08

Data are mean values ± SE of four separately prepared nematode or insect host homogenates and are expressed as nanomoles per milligram dry weight of designated polyamine.

Means followed by different letters in a horizontal row were significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

which the eggs were derived, the putrescine : spermidine ratio was about 2:1; in parasitic juveniles and newly emerged postparasites, spermidine was the predominant polyamine.

The egg stage contained the highest concentration of polyamines; the putrescine content of the eggs was more than three times greater than that of spent postparasites and six times that of parasitic juveniles and newly emerged postparasites. Spermidine levels in parasitic juveniles approximated those in the egg stage. Except for spermidine, the polyamine composition of stages other than the eggs was similar. Host tissues contained the same array of polyamines as the nematode, but the putrescine : spermidine ratio (ca. 3:1) was the reverse of that observed in the mermithid's parasitic juvenile stage (ca. 1:3).

As was the case for polyamines, the egg stage of the mermithid contained the greatest concentrations of free amino acids, compared with the other developmental stages investigated (Table 2). The composition of the eggs was significantly different ($P \leq 0.05$) from other developmental stages for almost all of the ninhydrin positive substances detected; however, stages of development, other than the eggs, were similar to one another. In particular, concentrations of methionine and ornithine, the primary precursors of putrescine, spermidine and spermine (17), as well as of lysine, the precursor of cadaverine (8), were much higher in eggs of *R. culicivorax* than in other developmental stages. Values obtained for the amino acid content of the eggs agreed closely with those obtained in a previous study (11). The high levels of polyamines and amino acid precursors in the eggs of *R. culicivorax*, relative to other developmental stages, agree with observations made on insects (6,20) concerning the essential role of polyamines in embryogenesis. The concentrations of amino acids determined in this study for *R. culicivorax* cannot be directly compared with those obtained for this nematode by Giblin and Platzer (9), since the units of measurement and the majority of the stages examined were different. However, the predominance of aspartic acid–asparagine and glutamic acid–glutamine, evident within the free amino acid pool in parasitic and postparasitic stages, accords with observations made on preparasites and newly emerged postparasites (9). Relative to these amino compounds, other amino acids constituted a lower proportion of the total amino acid fraction in nematodes that we analyzed, in contrast with the results of Giblin and Platzer (9). Such differences may be accounted for, at least in part, by the fact that Giblin and Platzer used a different mosquito species, *Culex pipiens,* as host. Thus, while the hemolymph of *C. pipiens* contained serine and histidine as the two predominant amino acids (9,25), such was not the case in the tissues of *A. aegypti.*

Notwithstanding the difficulty of comparing data obtained from whole insects with those obtained from the hemolymph, the effect of host species, and consequent nutritional milieu, upon the physiology of the mermithid deserves attention.

TABLE 2. Ninhydrin positive substances in *Romanomermis culicivorax* and its host, *Aedes aegypti.*

Substances tested	Eggs	Parasitic juveniles	Newly emerged postparasites	Spent postparasites	Fourth-instar larvae
α amino acids					
Aspartic acid	31.50 a	3.14 b	1.77 b	2.21 b	12.61
Hydroxyproline	BLD	BLD	BLD	BLD	BLD
Threonine	18.87 a	0.68 b	0.38 b	0.56 b	22.79
Serine	12.48 a	1.58 b	1.48 b	1.90 b	28.12
Asparagine	25.98 a	1.05 b	3.20 b	1.71 b	12.59
Glutamic acid	62.05 a	2.92 b	3.98 b	2.61 b	33.92
Glutamine	12.36 a	0.23 b	1.10 b	0.32 b	28.60
α-aminoadipic acid	5.86 a	BLD b	0.18 b	0.07 b	0.15
Proline	11.38 a	0.31 b	0.42 b	0.07 b	26.04
Glycine	15.29 a	1.41 b	0.45 b	1.26 b	22.29
Alanine	35.71 a	1.81 b	2.17 b	1.63 b	56.13
Citruline	0.15 a	BLD a	BLD a	BLD a	0.52
α-amino-n-butyric acid	0.10 a	BLD a	BLD a	BLD a	1.78
Valine	10.36 a	0.62 b	0.45 b	0.51 b	19.40
Cystine	0.24 a	0.14 a	0.13 a	0.13 a	6.92
Methionine	9.39 a	0.29 b	0.18 b	0.43 b	5.93
Isoleucine	8.20 a	0.29 b	0.27 b	0.45 b	12.85
Leucine	19.13 a	0.45 b	0.56 b	0.72 b	21.12
Tyrosine	7.02 a	0.40 b	0.25 b	0.48 b	81.22
Phenylalanine	12.37 a	0.77 b	0.53 b	0.97 b	13.87
Tryptophan	2.45 a	0.20 b	0.34 b	0.53 b	5.17
Ornithine	2.73 a	0.49 b	0.46 b	1.52 a	0.76
Lysine	27.19 a	1.42 b	2.16 b	3.65 b	22.17
Histidine	14.08 a	0.21 b	0.42 b	0.36 b	24.89
Arginine	18.08 a	2.18 b	0.52 b	4.07 b	56.11
Other substances					
Taurine	19.60 a	4.97 b	0.16 b	1.25 b	16.13
Phosphoethanolamine	2.59 a	0.77 b	BLD b	0.10 b	2.24
Cystathionine	7.30 a	2.83 b	0.39 b	0.24 b	1.91
γ-aminobutyric acid	3.03 a	0.06 b	0.14 b	0.25 b	0.83
Ethanolamine	44.67 a	3.31 b	8.27 b	8.65 b	2.17

Data are mean values of three or four separately prepared nematode or insect host homogenates and are expressed as nanomoles of the ninhydrin positive substance per milligram dry weight of tissue. Standard errors have been omitted for clarity. Means followed by different letters in a horizontal row were significantly different ($P \leq 0.05$). BLD = below the limits of detection.

The finding that the putrescine : spermidine ratio of parasitic juveniles of *R. culicivorax* was the inverse of that prevailing in the host tissues suggests that the nematode may be able to synthesize polyamines. Confirmation of such a biosynthetic capability was obtained from assays of ODC, considered in mammalian tissues to be the rate-controlling enzyme in the biosynthesis of putrescine, spermidine and spermine (17). Measureable levels of ODC activity were recorded from homogenates of the larval host and newly emerged postparasites and eggs of *R. culicivorax* (Table 3).

TABLE 3. Ornithine decarboxylase activities in *Romanomermis culicivorax* and its host, *Aedes aegypti.*

Organism	Developmental stage	Enzyme specific activity
R. culicivorax	postparasites	256 ± 14
	eggs	417 ± 80
A. aegypti	early fourth instar	1,960 ± 146
	late fourth instar	282 ± 59

Specific activity is expressed as pmoles $^{14}CO_2$ produced per 30 minutes per mg soluble protein. Enzyme was assayed at 28 C. All values are means ± SE of 4–6 separately prepared nematode or insect host homogenates.

The higher ODC activity of early fourth-instar *A. aegypti* larvae, compared with late fourth instars, may be related to changes in polyamine levels associated with somatic development, as reported in *D. melanogaster* (6). Consistent with the high polyamine content of the nematode eggs, ODC activ-

ity appeared to be greater at this stage than in newly emerged postparasites. Differences in ODC activity were not significant, however, due to the high variability among replicates of egg homogenates. The rate of polyamine synthesis, and associated ODC activity, varies during embryonic development in a wide range of metazoa (14). Since in this study enzyme extracts were prepared from eggs of mixed, but variable, age (0–5 days postemergence), major differences in ODC activity among replicates are to be expected. The correlation between polyamine synthesis and stage of embryogenesis warrants closer attention; however, the egg stage of *R. culicivorax* is capable of synthesizing polyamines and does not depend directly on the parent female for these metabolites.

Effective commercialization of *Romanomermis culicivorax* as a mosquito biocontrol agent (21–23) can only be accomplished when an in vitro method has been devised for mass cultivating the nematode's infective stage (7,22). Numerous attempts to culture the nematode in artificial media (2,7,10) have been unsuccessful. Researchers have attempted to improve conditions for development by adding hormones and defined undefined nutrients (2) to the media or by modifying the amino acid (24) and salt content (10). Apparently from this study, in vitro culture conditions should be conducive to polyamine synthesis by the developing nematode. Given the mermithid's biosynthetic capacity for polyamines, however, the polyamine content of the culture medium should not be a critical factor governing development.

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