Organic Acids of *Ditylenchus triformis* and *Turbatrix aceti*[1]

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Abstract: Pyruvic acid, lactic acid and several tricarboxylic acid cycle acids were extracted from *Ditylenchus triformis* and *Turbatrix aceti* and identified. Fumaric acid was predominant in both nematodes. Small amounts of malic and α-ketoglutaric acids and intermediate quantities of lactic, citric, succinic, and pyruvic acids occurred in *D. triformis*. In *T. aceti* citric, lactic, and α-ketoglutaric acids were less abundant than succinic, malic and pyruvic acids. Only traces of aconitic and oxalacetic acids occurred in both nematodes. All the organic acids detected accounted for only about one per cent of the dry weight of nematodes of the two species. *Key Words:* Tricarboxylic acid cycle acids.

The tricarboxylic acid cycle (TCA-cycle) has been studied in only a few species of nematodes, mostly animal parasites. Evidence for the presence of this cycle in nematodes has been obtained by various means, such as: detecting enzyme activities in nematode preparations (4, 5, 6, 9, 14), determining TCA-cycle acids in nematodes (3, 22), testing the ability of intact nematodes or nematode homogenates to utilize different TCA-cycle intermediates (5, 12, 21), using inhibitors of specific enzyme systems and respiratory metabolism in nematodes (5, 13, 14), and determining incorporation of ^{14}C from labelled substrates into TCA-cycle intermediates in nematodes (7, 14). Succinic was the major organic acid in *Ascaris lumbricoides* whereas pyruvic, lactic, oxalacetic and α-ketoglutaric acids occurred in small amounts (22).

The purpose of this study was to determine whether organic acids of the TCA cycle and some metabolically-related acids occur in the two nematodes, *Ditylenchus triformis* (Hirschmann and Sasser), a fungus-feeder, and *Turbatrix aceti* (Muller), a free-living obligate aerobe which thrives in fermenting cultures.

Materials and Methods

Ditylenchus triformis originally isolated from soil and maintained in laboratory culture for 10 years was cultured on the fungus *Pyrenochaeta terrestris* growing on potato dextrose agar in 26 C. Four- to six-week-old cultures were harvested by modified Baermann funnel. *Turbatrix aceti* was propagated axenically in liquid medium containing 3% yeast extract, 4% soy peptone, 2% (v/v) glacial acetic acid and 5% (v/v) fresh beef liver extract. Cultures were incubated at 30 C and harvested after 3–4 weeks. Around 4–6 ml of wet packed *D. triformis* per 100 petri plates and 10–20 ml of *T. aceti* per 5–10 flasks (100 ml of medium/flask) were obtained at each harvest. Nematodes were suspended in 5–10 ml of distilled water and disrupted with a cold French pressure cell. The homogenates were immediately frozen at –5 C and lyophilized.

EXTRACTION OF NEMATODES AND PREPARATION OF ESTERS OF ORGANIC ACIDS: Lyophilized homogenate (300 to 800 mg) was rehomogenized in boiling 80% ethanol and the homogenate filtered. The filtrate was evaporated to dryness at 40 C and the residue extracted with chloroform followed by water to obtain the lipids and the water-soluble sub-

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stances, respectively. The water-soluble fraction, containing the organic acids, was concentrated to about one ml at 40 C and then acidified to *p*H 2 with concentrated sulfuric acid (10). The acidified solution was mixed thoroughly with acid-washed silicic acid (0.5 ml/g) and packed into a glass column (30 × 1 cm), from which the organic acids were eluted with about 400 ml of diethyl ether. The eluate was evaporated to dryness at 25 C and the residue was subsequently dissolved in 1–2 ml of diethyl ether containing 10% methanol. The acids were methylated with diazomethane prepared from N-methyl-N-nitroso-*p*-toluene sulfonamide (20). The solution containing the esters was concentrated with a stream of dry nitrogen and dried over anhydrous sodium sulfate. Methyl esters of commercial acids were also prepared in this way. Samples were chromatographed immediately or stored at –5 C.

GAS CHROMATOGRAPHY (GLC) OF ESTERS: Methyl esters of acids were analyzed using a Chromalab Model A-110 Gas Chromatograph with an argon β-ionization detector. Silanized glass columns (183 cm × 3.4 mm) were packed with Chromosorb W (80–100 mesh) coated with 15% diethylene glycol succinate (DEGS). Argon carrier gas was maintained at 30 psig and a flow rate of 27 ml/min.

Samples were chromatographed isothermally at 135 C to separate the low-boiling esters, and 185 C to separate the high-boiling esters (19). Temperature programming, when used, was from 70 C to 185 C at the rate of 5 C/min. The relative retention times on GLC of the unknown methyl esters of nematode acids were compared to retention times of known esters, both individually and in mixtures. Methyl esters were quantitated by triangulation of peaks on chromatograms. Individual esters were trapped from the gas chromatograph and analyzed by infrared spectrophotometry.

EXTRACTION OF KETO ACIDS, PREPARATION OF 2,4-DINITROPHENYLHYDRAZONES AND TLC OF HYDRAZONES: Keto acids were extracted from lyophilized nematode tissues with 1–5 ml of 5% perchloric acid. One to two ml of 0.5% 2,4-dinitrophenylhydrazine in 2N HCl was added to the filtrate and the hydrazones formed were extracted with ethyl acetate and aqueous 10% sodium carbonate (11).

Hydrazones of keto acids were dissolved in ethyl acetate and spotted on TLC plates coated with cellulose powder 250 μ thick. Chromatograms were developed with *n*-butanol/ethanol/0.5M NH_4OH (7:1:2, v/v/v) at 25 C. R_f values of hydrazones of nematode keto acids were compared with those of known keto acids. Spraying developed plates with 2% NaOH in 90% ethanol caused the hydrazones to change from a yellow to red-brown confirming their identities (1). Cellulose containing individual hydrazones was scraped from unsprayed plates and the hydrazones were eluted with 0.1M potassium phosphate buffer at *p*H 7.4 for quantitation. Optical densities of solutions were measured at 365 nm (11). Standard concentration curves were prepared with chromatographed authentic acid hydrazones and were used to quantitate the nematode acid hydrazones.

Hydrazones were recrystallized from cold ethyl acetate solution by addition of hexane, and crystals were then utilized for melting point determinations and for infrared spectral analyses.

RESULTS

Organic acids identified from both *D. triformis* and *T aceti* were pyruvic, succinic, lactic, fumaric, malic, α-ketoglutaric, aconitic, citric and oxalacetic acids.

Hydrazones of pyruvic, α-ketoglutaric and oxalacetic acids were separated and identified by TLC. Two spots were obtained for

TABLE 1. Organic acids isolated from *Ditylenchus triformis*, in μmoles/g dry wt. of nematodes.

Acid	Experiment Number				Mean
	1	2	3	4	
Lactic	4.7	3.6	4.4	2.9	3.9
Fumaric	33.7	37.7	33.1	34.8	34.8
Succinic	7.8	6.5	6.6	7.9	7.2
Malic	1.2	0.9	0.9	1.3	1.1
Citric	3.7	3.0	3.7	3.9	3.6
Pyruvic	9.3	11.0	8.4		9.4
α-Ketoglutaric	0.5	0.7	0.6		0.6

pyruvic acid and were designated as pyruvate hydrazone-1 (R_f 0.78) and pyruvate hydrazone-2 (R_f 0.57). α-Ketoglutarate hydrazone had an R_f of 0.13 and oxalacetate hydrazone an R_f of 0.17. Melting points and infrared spectra of the keto acid hydrazones from nematodes agreed with those for the corresponding authentic keto acid hydrazones.

Fumaric acid was the predominant organic acid (57% of the total organic acids) in *D. triformis* (Table 1). Small quantities of malic and α-ketoglutaric acids and intermediate quantities of lactic, succinic, citric and pyruvic acids were also detected. In *T. aceti* fumaric acid was also the predominant organic acid (65% of the total organic acids) (Table 2), occurring in almost twice the concentration found in *D. triformis*. Citric, lactic and α-ketoglutaric acids were least abundant, and succinic, malic and pyruvic acids were intermediate in abundance.

TABLE 2. Organic acids isolated from *Turbatrix aceti*, in μmoles/g dry wt. of nematodes.

Acid	Experiment Number				Mean
	1	2	3	4	
Lactic	1.0	0.8	0.2	0.2	0.6
Fumaric	68.2	77.6	71.0	54.6	67.9
Succinic	8.5	9.2	11.4	24.7	13.4
Malic	8.3	22.2	9.9	7.1	11.9
Citric	3.0	1.4	3.5	3.0	2.7
Pyruvic	8.5	7.4	5.7		7.2
α-Ketoglutaric	0.6	0.4	0.3		0.4

Aconitic and oxalacetic acids occurred in amounts too small to measure in the quantities of nematodes available although the former was tentatively identified by GLC in the methylated acids and the latter by its hydrazone on TLC. Isocitric acid was not detected in nematode preparations.

DISCUSSION

The total organic acids recovered constituted about 0.8% of the dry wt. of *D. triformis* and 1.2% of *T. aceti*. These findings are consistent with the general observation that organic acids occur in low quantities in animal tissues (8).

The presence of these acids supports the evidence based on studies of enzymes from *D. triformis* (9) and *T. aceti* (5) that the TCA cycle is operative in these nematodes. Ells and Read (5), however, suggested that a typical TCA cycle does not occur in *T. aceti* based (i) on the limited ability of some nematode preparations to carry out reactions of this cycle, (ii) on the failure of some intermediates to stimulate respiration in homogenates and in intact nematodes, and (iii) on the failure to detect cytochrome oxidase. In contrast, *D. triformis* preparations possessed several active TCA-cycle enzymes as well as active cytochrome oxidase (9).

Studies with the animal parasite, *Ascaris lumbricoides*, have shown succinic acid to be the major acidic component of the perienteric fluid (3, 22) and muscles (22). Pyruvic, lactic, oxalacetic and α-ketoglutaric acids were detected in small amounts, and several other organic acids occurred only in traces. Although an operative TCA cycle was indicated in the muscles of *Ascaris* (14), the cycle must be only of minor importance since its metabolism is essentially anaerobic. Carbon dioxide fixation and subsequent dismutation reactions between carboxylic acids at different

oxidation levels may explain the reason for the kind of oxidative metabolism occurring in *Ascaris* (14). Bueding *et al.* (2) suggested that a reversal of the succinoxidase reaction in *Ascaris* may be an anaerobic adaptation and may account for the accumulation of succinic acid. Ells and Read (5) suggested that the same reaction may occur in *T. aceti.*

The accumulation of fumarate in *T. aceti* and *D. triformis* may be due to inhibition of fumarate reductase under the aerobic conditions of the nematode cultures. Oxygen has been found to inhibit this enzyme in certain animal parasites (15). If anaerobic incubation of nematodes caused a decrease in fumarate and an increase in succinate the presence of a fumarate reductase resembling that in animal parasites would be suggested.

Isocitrate lyase and malate synthetase, two enzymes of the glyoxylate pathway, were detected in *Caenorhabditis briggsae* (16), and in *T. aceti, Panagrellus redivivus* and *Rhabditis anomala* (17, 18). Glyoxylic acid was not detected in *T. aceti* or *D. triformis* in this study, although the pathway may be functioning in these nematodes. The glyoxylate pathway is probably operative in *T. aceti,* since this nematode thrives in fermenting media containing relatively high levels of acetic acid, a precursor of the two-carbon compounds metabolized in this pathway (17, 18). The glyoxylate pathway has not been investigated in *D. triformis.*

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