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Reviewed work(s):

Source: *The Journal of Parasitology*, Vol. 66, No. 4 (Aug., 1980), pp. 585-590

Published by: [The American Society of Parasitologists](#)

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STEROLS IN THE TROPHOSOMES OF THE MERMITHID NEMATODES *NEOMESOMERMIS FLUMENALIS* AND *ROMANOMERMIS CULICIVORAX* RELATIVE TO STEROLS IN THE HOST HEMOLYMPH

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ABSTRACT: The sterols in the trophosomes of the mermithid nematodes *Romanomermis culicivorax* and *Neomesomermis fluminalis* were identified tentatively and compared with the sterols in the hemolymph of their larval insect hosts, *Aedes aegypti* and *Simulium venustum*, respectively. The C₂₆ sterol 22-*trans*-24-norcholesta-5, 22-dien-3 β -ol was predominant in the free sterol and sterol ester fractions of both nematodes. 22-dehydrocholesterol was a major component of esterified and nonesterified sterols in the trophosomes of *R. culicivorax* and of free sterols in the trophosomes of *N. fluminalis*. Cholesterol was not a major trophosomal component in either of the nematodes. The host hemolymph contained cholesterol and β -sitosterol as the principal free sterols in *A. aegypti* and as the only detectable free sterols in *S. venustum*. The evidence suggests that the mermithids have some capacity to interconvert sterols supplied by the hosts.

Mermithid nematodes parasitize the hemocoel of insects during a portion of the larval development of the insects. Nutrients available in the host's hemolymph are transported across the cuticle of the nematode (Rutherford et al., 1977), then stored in a structure known as a trophosome for subsequent utilization by nonfeeding, free-living stages. Lipids, apparently in the form of a lipid-protein complex, constitute the predominant storage product of *Agamermis decaudata* (Chitwood and Jacobs, 1938) and *Mermis nigrescens* (Denner, 1968), mermithid parasites of grasshoppers. In *Romanomermis culicivorax* and *Neomesomermis fluminalis*, mermithid parasites of larval mosquitoes and blackflies, respectively, trophosomes contain substantial amounts of lipids (Ittycheriah et al., 1977; Gordon et al., 1979); in decreasing order of prevalence, these are in the form of triacylglycerols, phospholipids, sterol esters, and free sterols (Gordon et al., 1979). The fatty acid composition of the triacylglycerol and sterol ester fractions was investigated and related to metabolites available in the host hemolymph (Gordon et al., 1979), but no information was presented concerning the sterol components of the trophosomes and their derivation from the host hemolymph.

Nematodes thus far investigated require sterols as essential dietary components (Dut-

ky et al., 1967b; Hieb and Rothstein, 1968; Cole and Dutky, 1969; Lu et al., 1977). Thus, as part of our continuing investigation for nutritional information pertinent to the in vitro culture of *R. culicivorax* and *N. fluminalis*, we examined the sterol composition of their trophosomes and precursors available in the host hemolymph. Nematodes studied thus far are unable to biosynthesize sterols de novo (Rothstein, 1968; Cole and Krusberg, 1968; Barrett et al., 1970; Willett and Downey, 1974). Thus, it is probable that the mermithids rely upon sterols in the host hemolymph for dietary sterol requirements. Although the sterol composition of whole insects has been investigated extensively (Thompson et al., 1972; Svoboda et al., 1975), there is a dearth of information concerning hemolymph sterols—no studies have been done on the sterol composition of culicid or simuliid hemolymph. Accordingly, the hemolymph sterol composition of larval *Simulium venustum*, a host in Newfoundland for *N. fluminalis*, and of larval *Aedes aegypti*, laboratory host for *R. culicivorax*, was examined to provide basic information on the availability of sterols to the mermithids.

MATERIALS AND METHODS

Biological material

Newly hatched *A. aegypti* larvae were infected with controlled doses of *R. culicivorax* and maintained at 27 C (Bailey and Gordon, 1973). Mermithids were dissected from the hosts' hemocoel 6 days

Received for publication 31 July 1979.

postinfection, intact trophosomes were removed, pooled, and stored at -20°C until analysis (Ittycheriah et al., 1977). Pooled hemolymph samples were taken separately from uninfected hosts under a dissecting microscope by puncturing their cuticle in the prothoracic region and drawing up the exuded blood into a micropipet. A few crystals of phenylthiourea were added to these samples which were frozen at -20°C until analysis.

Advanced parasitic stages of *N. flumenalis* were dissected from larval blackflies (*S. venustum*) collected locally from previously surveyed streams (Condon, 1975). Trophosomes were removed and treated as above. Pooled hemolymph samples were collected from the respective, uninfected, simuliid hosts, phenylthiourea added, then frozen (Gordon and Bailey, 1976).

To undertake the entire study, it is estimated that blood was withdrawn from approximately 8,000 mosquito and 2,000 blackfly larvae. Dissections were done on approximately 1,200 *R. culicivora* larvae and 500 *N. flumenalis* larvae.

Extraction and separation of sterols

Trophosomes (300—*R. culicivora*; 120—*N. flumenalis*) were homogenized in a tissue grinder with 300 μl phosphate-buffered Dulbecco's saline (Grand Island Biological Co., Grand Island, New York). The homogenate was transferred to microtubes and 100 μl chloroform:methanol (2:1) (Folch et al., 1957) added to extract lipids. After 15 min, 100 μl 9% sodium chloride solution was added. After centrifugation (15,000 rpm; 30 sec), the lower, lipid-containing layer was removed and spotted on heat-activated, silica-gel-coated (Alumina GF) thin-layer chromatography (tlc) plates. Residual lipids were reextracted from the upper layer by the same method and spotted on top of the initial extract on the tlc plate. Lipids were extracted from hemolymph samples (200 μl —*A. aegypti*; 150 μl —*S. venustum*) and spotted on tlc plates in a similar fashion, except that the saline/chloroform:methanol was added directly to the blood without homogenizing. The double solvent system of Skipski et al. (1968) was used to separate lipid fractions. Lipid fractions separated in this way that were not destined to be processed further through gas liquid chromatography (glc) were stained with iodine vapor to allow location and identification of the major hemolymph and trophosomal lipid fractions. Following tlc of both insect hemolymph and nematode trophosomes, areas corresponding in R_f values to the stained (unbound) sterol fractions were scraped from unstained plates; sterol esters were recovered only from trophosomal lipids separated by tlc. To insure that the sterols obtained by tlc were uncontaminated, the tlc scrapings were incubated first (40°C ; 50 min) with 4 ml alcoholic KOH (6 ml 33% KOH:94 ml absolute ethanol). Nonsaponifiable lipids then were removed by extracting with petroleum ether (2 ml), 10% chloroform in petroleum ether (2 ml), and finally chloroform (2 ml). The combined ether/chloroform extracts were washed with distilled water, evaporated to dryness with nitrogen, then stored (4°C) in a desiccator.

Gas liquid chromatography

Sterol (esterified and nonesterified) fractions recovered by tlc were further analyzed by glc. The trimethylsilyl (TMS) ether derivatives were prepared (Idler et al., 1978) by adding to the extracted sterols 10 μl of a solution consisting of hexamethyldisilazane (1 ml)/trimethylchlorosilane (20 μl)/pyridine (1 ml). After 30 min, the sterol TMS ethers could be separated on a column (length 1.8 m; internal diameter 0.4 cm) of 3% OV-17 (Applied Science Laboratories, Bellefonte, Pennsylvania). The gas chromatograph used was a Packard Series 7400 equipped with a hydrogen-flame-ionization detector. The inert carrier gas was nitrogen with a flow rate of 80 ml/min; the detector gas flow rates were 400 ml/min for air and 60 ml/min for hydrogen. The temperatures of the column, detector and injection were 212, 248, and 245 $^{\circ}\text{C}$, respectively.

Identification (tentative) of individual sterols was based on their retention times (R_c) relative to cholestane (Barrett et al., 1970). Sterol standards were supplied by Dr. D. R. Idler, Marine Sciences Research Laboratory, Memorial University of Newfoundland, Canada.

Systematic nomenclature

22-dehydrocholesterol = cholesta-5, 22-dien-3 β -ol; cholesterol = cholest-5-en-3 β -ol; cholestane = 5 α cholestane; β -sitosterol = (24R)-24-ethylcholest-5-en-3 β -ol.

RESULTS

The tlc separations (Fig. 1) confirmed our earlier conclusions (Gordon et al., 1979) that the trophosomes of both nematodes contained triacylglycerols, phospholipids, sterol esters, and sterols in decreasing order of prevalence. In *S. venustum* hemolymph, triacylglycerols constituted the most abundant lipid type, followed by free fatty acids and phospholipids. Sterols were present in both esterified and nonesterified forms. The lipid composition of *A. aegypti* hemolymph differed from that of *S. venustum* in that monoacylglycerols were present (along with triacylglycerols) as a major lipid fraction. Also, sterols were present in *A. aegypti* hemolymph only in nonesterified form.

Glc analyses showed that the trophosomes of *N. flumenalis* contained four free sterols (Fig. 2a), three of which could be tentatively identified on the basis of R_c values as 22-*trans*-24-norcholesta-5, 22-dien-3 β -ol (a C26 sterol), *cis* 22-dehydrocholesterol, and cholesterol. Compared to the other sterols identified, cholesterol was present in relatively small amounts. Bound sterols were present in the trophosomes of *N. flumenalis* only as es-

T.L.C. OF TROPHOSOME AND HOST BLOOD

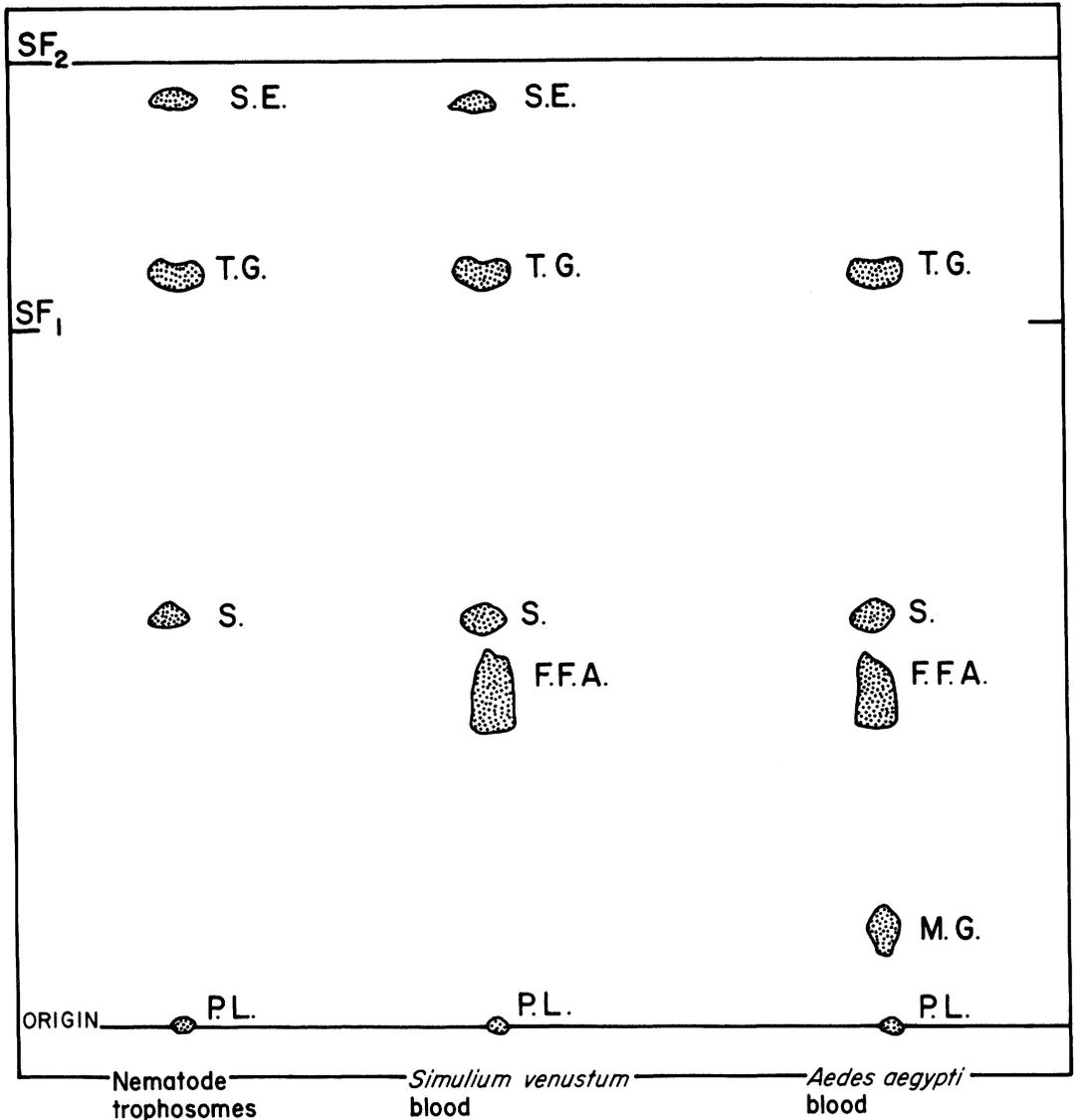


FIGURE 1. Thin-layer chromatography separation of lipid classes in the trophosomes of *R. culicivorax* and *N. flumenalis* and in the hemolymph of their hosts, *A. aegypti* and *S. venustum*, respectively. SF₁, first solvent front; SF₂, second solvent front; FFA, free fatty acids; MG, monoacylglycerols; PL, phospholipids; S, sterols; SE, sterolesters; TG, triacylglycerols.

ters of the C26 sterol—no cholesterol was detectable in this fraction (Fig. 2b). The hemolymph of the simuliid host *S. venustum* contained only two free sterols—cholesterol and β -sitosterol (Fig. 2c).

The trophosomes of *R. culicivorax* contained the same free sterols as *N. flumenalis*, except that the unidentified sterol (peak 4) was not present in the mermithid from mos-

quitoes (Fig. 3a). Cholesterol appeared to be the most abundant of the free sterols identified. As with *N. flumenalis*, the sterol ester fraction of *R. culicivorax* trophosomes did not contain cholesterol; nonesterified sterols were present as esters of the C26 sterol and *cis* 22-dehydrocholesterol (Fig. 3b). The hemolymph of the culicid host *A. aegypti* contained three sterols—the C26 sterol, choles-

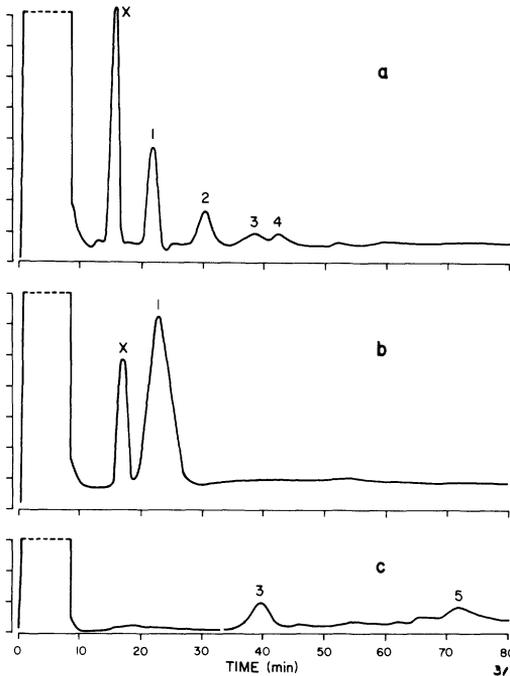


FIGURE 2. Gas-liquid chromatograms of sterols (trimethylsilyl ether derivatives), nonesterified (Fig. 2a) and esterified (Fig. 2b) in the trophosomes of *N. flumenalis* and in the hemolymph of its host, *S. venustum* (Fig. 2c). Retention times of reference standards relative to cholestane (R_c) are given in brackets for each peak below. x. Unknown (1.00). 1. 22-*trans*-norcholesta-5, 22-dien-3 β -ol (1.46). 2. 22-*cis*-cholesta-5, 22-dien-3 β -ol (2.13). 3. Cholesterol (2.66). 4. Unknown (2.87). 5. β -sitosterol (4.69).

terol, and β -sitosterol (Fig. 3c). A peak (X) with retention time identical to cholestane was apparent in all samples except for the simuliid hemolymph. It seems unlikely that the peak resulted from cholestane, because the cholesterol nucleus rarely has been identified from living tissues. In view of its short retention time, the peak is probably of nonsterol origin.

DISCUSSION

The principal lipids in hemolymph were found to be triacylglycerols in *S. venustum* and triacylglycerols/monoacylglycerols in *A. aegypti*. This contrasts with the situation which exists in most other insects, in which diacylglycerols predominate (Downer and Matthews, 1976). The fact that sterols and sterol esters were detected in the hemolymph of *S. venustum*, whereas only free sterols

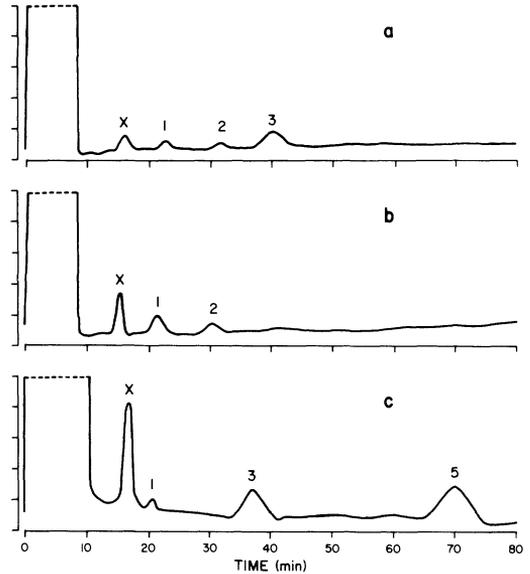


FIGURE 3. Gas-liquid chromatograms of sterols (trimethylsilyl ether derivatives), nonesterified (Fig. 3a) and esterified (Fig. 3b) in the trophosomes of *R. culicivorax* and in the hemolymph of its host, *A. aegypti* (Fig. 3c). Enumerating of peaks and R_c values as in Figure 2.

were found in *A. aegypti* hemolymph accords with available information that some insects transport sterols in free form only (Wang and Patton, 1969), whereas others transport them in esterified form as well (Thomas, 1974). The presence of cholesterol and β -sitosterol as the two major hemolymph sterols in both insects is consistent with knowledge gained from studies on homogenates of whole insects. Both phytosterols and cholesterol are of widespread occurrence in insects and pathways exist for converting phytosterols to cholesterol (Thompson et al., 1972; Svoboda et al., 1975).

The mermithids, *R. culicivorax* and *N. flumenalis*, contained a mixture of sterols in their trophosomes both as free sterols and as sterol esters. The sterol composition differs from that found in tissues (whole organism homogenates) of other free-living and parasitic nematodes in several ways.

Cholesterol, the predominant sterol of most other nematodes investigated (Cole and Krusberg, 1967, 1968; Dutky et al., 1967a; Barrett et al., 1970; Tulk and Shorb, 1971), was present in relatively small amounts in the mermithids. In contrast to ascarids (Barrett et al., 1970; Tarr and Fairbairn, 1973; Fulck and

Shorb, 1971) and the plant parasitic nematode *Globodera solanacearum* (Orcutt et al., 1978), the mermithids did not appear to contain phytosterols—at least, in their trophosomes. This was somewhat surprising in view of the fact that the hemolymph of both host species contained β -sitosterol as a major sterol. The insect parasitic nematode *Neoaplectana carpopap-sae* DD136 similarly lacks phytosterols (Dutky et al., 1967a), but can use several phytosterols (including β -sitosterol) as a dietary replacement for cholesterol when supplied in culture media (Dutky et al., 1967b). Moreover, it has been demonstrated that the free-living nematode *Turbatrix aceti* can dealkylate β -sitosterol and fucosterol to cholesterol (Cole and Krusberg, 1968). Thus, it is possible that trace amounts of β -sitosterol in the trophosomes were undetected by our procedures and the possibility remains that the mermithids could utilize β -sitosterol available in the host hemolymph.

22-dehydrocholesterol was a major sterol component in the trophosomes of both mermithids; this sterol has not been recorded from other nematodes. Because this sterol was not found to be a hemolymph constituent of either host species, the mermithids must be capable of synthesizing it from other sterols (probably cholesterol). Based on relative retention times, the mermithids appear to contain the C26 sterol 22-*trans*-24-norcholesta-5,22-dien 3 β -ol, a sterol which first was identified from scallops (Idler et al., 1970). C26 sterols have been identified from several groups of invertebrates (Alcaide et al., 1971; Yasuda, 1974; Idler et al., 1978; Ballantine et al., 1979) but not, thus far, from nematodes. Although this sterol is available to *R. culicivora*x as a minor component of the host hemolymph, it was not found in *S. venustum* hemolymph. Presumably, the blackfly parasite *N. flumenalis* must synthesize it from other sterols.

ACKNOWLEDGMENTS

We thank Dr. J. J. Petersen of the U.S. Department of Agriculture at Lake Charles, Louisiana, for supplying *R. culicivora*x. We also thank Dr. D. R. Idler, Marine Sciences Research Laboratory, Memorial University of Newfoundland, for his many helpful suggestions. This investigation was supported by a grant from the Natural Sciences and Engi-

neering Research Council of Canada (Operating Grant A6679).

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