CHEMOATTRACTION OF *ECHINOSTOMA TRIVOLVIS* (TREMATODA) REDIAE TO LIPOPHILIC EXCRETORY-SECRETORY PRODUCTS AND THIN LAYER-CHROMATOGRAPHIC ANALYSIS OF REDIAL LIPIDS

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Summary :

High performance thin-layer chromatography (HPTLC) was used to analyze the lipophilic excretory-secretory (ES) products of *Echinostoma trivolvis rediae*. These products were free sterols at a concentration of 0.83 ± 0.03 µg/100 ml and a lesser amount of free fatty acid that was not quantified. Preparative layer chromatography (PLC) was used to obtain the free sterol and free fatty acid fractions of redial ES products, and silica gel squares containing these lipids were tested against single rediae in a Petri dish bioassay. Rediae were significantly attracted to squares containing either free sterol or free fatty acid. HPTLC was also used to analyze the major lipid fractions in the redial bodies, which were free sterols, phosphatidylcholine (PC), and phosphatidylethanolamine (PE). The major wet weight percentage ± SE (n = 3) of free sterols was 2.59 ± 0.16 and for PC and PE was 0.011 ± 0.002 and 0.010 ± 0.002, respectively.

KEY WORDS : *Echinostoma trivolvis*, Trematoda, redia, chemical attraction, thin-layer chromatography, neutral lipids, phospholipids.

MOTS CLÉS : Echinostoma trivolvis, *trématode, rédie, chémoattraction, chromatographie en couche mince, lipides neutres, phospholipides.*

Résumé : PRODUITS D'EXCRÉTION-SÉCRÉTION LIPIDIQUES DE RÉDIES *D'ECHINOSTOMA TRIVOLVIS* ET ANALYSE PAR CHROMATOGRAPHIE EN COUCHE MINCE

*La chromatographie sur couche mince de haute performance (HPTLC) a été utilisée pour analyser les produits d'excrétion-sécrétion (ES) lipidiques de rédies d'*Echinostoma trivolvis. *Ces produits sont d'une part des stérols libres à la concentration de 0,83 ± 0,03 g/100 ml, et d'autre part des acides gras libres dont la concentration n'a pas été déterminée. La chromatographie préparative (PTLC) a été employée pour séparer les fractions de stérols et d'acides gras libres à partir de produits ES, et des carrés de gels de silice contenant ces lipides ont été examinés contre des rédies isolées dans une boîte de Pétri selon la technique déjà employée. Les rédies sont significativement attirées par les carrés contenant soit des stérols libres, soit des acides gras libres. La HTPLC a été aussi utilisée pour analyser les fractions lipidiques majeures des rédies elles-mêmes. Les composés trouvés sont des stérols libres de la phosphatidylcholine (PC) et la phosphatidyléthanolamine (PE). Les pourcentages (en % du poids humide) de ces composés ± SE (*n *= 3) sont respectivement de 2,59 ± 0,16 pour les stérols, de 0,011 ± 0,002 pour la PC et de 0,010 ± 0,002 pour la PE.*

INTRODUCTION

Relatively little work has been done on the lipid composition of the redial stages of digenetic trematodes (see references in Frayha & Smith, 1983; Fried & Sherma, 1990), and most of the studies are based on the analyses of lipids in rediae contained within the digestive gland gonad complex (DGG) of the snail host (Fried *et al.*, 1990; Beers *et al.*, 1995). A recent study in our laboratory using daughter rediae of the ubiquitous North American digenetic trematode *Echinostoma trivolvis* (see review in Huffman & Fried, 1990) showed that these rediae are attracted to each other *in vitro* and that lipids released from them are chemoattractants (Reddy & Fried, 1996). The purpose of this study was to identify the lipid classes released by rediae of *E. trivolvis* into a bioassay medium and to determine which lipids are chemoattractive. Additionally, analyses were done on the lipids in the redial bodies of this echinostome.

MATERIALS AND METHODS

OBTAINING REDIAE AND SAMPLE PREPARATION

Helisoma trivolvis snails naturally infected with daughter rediae of *Echinostoma trivolvis* were obtained from a farm pond in Northampton County, PA, USA. Rediae were dissected from the DGG of snails and rinsed in several changes of Locke's solution prior to use. The morphology of the redia of this species was described by Fried & Awatramani (1992).

To obtain redial excretory-secretory (ES) products, 500 rediae were placed in 2 ml of Locke's 1:1 solution in a 10 × 75 mm disposable glass culture tube (Becton-Dickinson Co., Rutherford, NJ, USA) at 22 °C for 1 h. The redial-free incubate (2 ml) was removed by pipet and extracted in 3 ml of chloroform-methanol (2:1).

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The extract was then filtered through glass wool and treated with the Folch wash (0.88 % KCl) to obtain a hydrophilic (top) and a lipophilic (bottom) phase. The hydrophilic layer was removed and discarded, and the lipophilic layer was used for the behavior and thin-layer chromatography (TLC) studies described herein. A total of 2,500 rediae were used in five trials to obtain the ES products needed to complete these studies.

TLC analysis of redial bodies was done by extracting 500 rediae (about 60 mg wet weight) in 3 ml of chloroform-methanol (2:1) and then preparing the lipid extract essentially as described for the ES products. Five trials, each with 500 rediae, were used for the HPTLC analyses of redial bodies.

Identification of lipids in ES products and redial bodies was done by high performance thin-layer chromatography (HPTLC) as described in the last section of the « Materials and methods ». After it was determined that the redial ES products were free sterols and free fatty acids, preparative layer chromatography (PLC) was used to isolate these fractions from the silica gel plates. To do PLC, from 100 to 200 µl of the ES lipophilic extract was streaked on the origin of a 20 × 20 cm silica gel sheet (Bakerflex IB2, J.T. Baker Chemical Co., Phillipsburg, NJ, USA) in a narrow, 10 cm band, 5 cm from each edge as described in Fried *et al.* (1980). Ten µl of sample and 5 µl of neutral lipid standard (see last section of the « Materials and methods ») were spotted on the origin within 5 cm of each edge. The sheet was developed in petroleum ether-diethyl ether-acetic acid (80:20:1), and each 5 × 20 cm edge was cut and removed from the center 10 × 20 cm piece. The edges were sprayed with 5 % ethanolic phosphomolybdic acid (PMA) and heated at 115 °C to produce blue lipid zones on a light yellow background, and then matched against the center piece of the plate (see Fig. 12.2 on page 192 of Fried & Sherma, 1986). PLC showed two neutral lipid bands in the ES products, a free sterol band at R_f= 0.27-0.32, and a free fatty acid band at R_f= 0.50-0.55. These bands (about 1 cm wide) were cut from the center portion of the silica gel and prepared as 0.5 cm² squares for use in the behavior studies (see next section) along with a lipid-free band at R_f= 0.75-0.80.

Chemoattraction of rediae to silica gel squares

To observe redial chemoattraction toward the free sterol and free fatty acid zones removed from the silica gel sheets, attraction of a single redia to a silica gel square was tested in a 3.5 cm Petri dish bioassay containing an agar substratum and a Locke's overlay (see Reddy & Fried, 1996). The dish was divided into three approximately equal areas of about 4.2 mm². One area (A) was designated attractive and received a piece of 0.5 cm² silica gel at its edge containing either the free sterol or free fatty acid fraction. A blank piece of 0.5 cm² silica gel was placed at the opposite end of the dish, designated the non-attractive area (C). The central area (B) was considered neutral, and a single redia was placed in it equidistant between the two silica gel squares. Squares were placed in the dish 10 min prior to inoculating a single redia into area B. Rediae that moved into area A were scored as being attracted. Redial attraction to the free sterol square was tested 12 times, with observations made at 0, 0.25, 0.50, 1.0, 1.25, 1.5, 2.0, and 4.0 h. A similar protocol was used to test the free fatty acid squares. Chemoattraction was defined as the percent of rediae in the attractive area at each time.

HPTLC analysis of redial excretory/secretory (ES) products and redial bodies

To determine neutral lipids in the ES products and in redial bodies, HPTLC was performed as described by Masterson *et al.* (1993) using Whatman (Clifton, NJ, USA) LHP-KDF high performance 10 × 20 cm channeled, preadsorbent silica gel plates. The standard used was the neutral lipid standard 18-4A (Matreya, Pleasant Gap, PA, USA), which contained 0.20 µg/µl each of cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol. The standard and reconstituted sample solutions (the lipophilic extract of 500 rediae was evaporated to dryness and reconstituted with 100 µl of chloroform-methanol, 2:1) were spotted in 2.0, 4.0, 8.0, and 16.0 µl aliquots on the same plate using a 25 µl Drummond (Broomall, PA, USA) digital microdispenser. Plates were developed with petroleum ether-diethyl ether-acetic acid (80:20:2) to a distance of 7.5 cm past the preadsorbent-silica gel interface in a glass, paper-lined Camag (Wilmington, NC, USA) twin-trough HPTLC chamber. The plates were dried using a hair dryer, and lipids were detected by spraying with PMA. Sample and standard zones of free sterols were scanned using a Shimadzu CS-930 densitometer operated in the single beam, single wavelength mode at 700 nm. Calculation of sterol percentages based on the scan areas of the four standards and the single sample zone with an intensity closest to the two middle standards was performed as described by Higgs *et al.* (1990).

Phospholipid analysis was done as described in Perez *et al.* (1994). The standard used was Matreya polar lipid mix No. 1127, containing 0.25 mg/ml each of cholesterol, phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine. The silica gel HPTLC plates were spotted and developed with chloroform-methanol-water (65:25:4) as described above for neutral lipids. Phospholipids were detected as black spots

on a white background by spraying the dried plate with a 10 % solution of cupric sulfate in 8 % phosphoric acid, and heating in an oven at 160°C for 10 min Phospholipid zones were scanned at 370 nm.

RESULTS

Daughter rediae of *Echinostoma trivolvis* were significantly attracted to silica gel squares impregnated with the neutral lipid standard.

LIPIDS IN ES PRODUCTS AND REDIAL BODIES

Daughter rediae of *E. trivolvis* released free sterols (R_f = 0.23) and free fatty acids (R_f = 0.31) but not phospholipids into the medium. Natural pigments, *i.e.*, carotenoids, which occur in the redial bodies (see Fried *et al.*, 1993), were not released into the medium. The free sterols released into the medium were quantified in three separate trials against the cholesterol standard based on the fact that greater than 95 % of *E. trivolvis* free sterol is cholesterol (see Chitwood *et al.*, 1985) and were found to be present at a concentration of 0.83 ± 0.03 µg/100 ml. The free fatty acids released into the medium were detected but not quantified. The free fatty acids were detected as trace amounts but could not be quantified because the scan area of the 16.0 µl sample free fatty acid zone was below the scan area of the 2.0 µl standard zone, *i.e.*, no sample zone was bracketed between the standards using the 500 redial sample.

The most abundant neutral lipid fraction in the redial bodies was free sterol (R_f = 0.23), along with lesser amounts of triacylglycerols (R_f = 0.65), free fatty acids (R_f = 0.31) and sterol esters (R_f = 0.89). The mean wet weight percentage ± SE of free sterols in the redial bodies (n = 3) was 2.59 ± 0.16. The other neutral lipids were not quantified because their zones could not be clearly distinguished from pigment bands that reacted with PMA after the plates were heated.

Phospholipids detected in the redial bodies were phosphatidylcholine (PC; R_f = 0.36) and phosphatidylethanolamine (PE; R_f = 0.54). The mean wet weight percentage (n = 3) of PC and PE was 0.011 ± 0.002 and 0.010 ± 0.002, respectively.

ATTRACTION OF REDIAE TO FREE STEROLS AND FREE FATTY ACIDS ON THE SILICA GEL SQUARES

Preliminary studies showed that single rediae did not migrate out of area B in the absence of a stimulus. Therefore, if areas A and C contained blank silica gel squares, the percent attraction was always zero. In the presence of silica gel squares with either free sterol or free fatty acid fractions, rediae either migrated into area A or remained in area B, but never moved into area C. The results of the chemoattraction studies are summarized in Table I and show that attraction became greater as a function of time when rediae were tested against both free sterols and free fatty acids. Student's *t*-test ($P < 0.05$ being considered significant) showed that redial attraction was significantly greater to the free fatty acid fraction versus the free sterol fraction at 15, 30, 45, and 60 min. Beyond that time there was no significant difference in the fatty acid versus free sterol fraction.

Table I. — Chemoattraction of single rediae of *E. trivolvis* to silica gel squares containing free fatty acid or free sterol excretory-secretory redial products.

	Percentage of rediae ± SE in area A*	
Time in min	Free fatty acids	Free Sterols
0	0.0 + 0	0.0 + 0
15	17 ± 1	8.3 ± 0.8
30	33 ± 2	25 ± 2
45	67 ± 4	42 ± 3
60	75 ± 6	42 ± 3
90	83 ± 4	75 ± 5
120	83 ± 4	75 ± 5
240	92 ± 6	83 ± 5

Rediae not in area A were in area B; rediae never migrated into area C.

The experimental set up of the Petri dish bioassay was as follows: area A was designated attractive and received a 0.5 cm^2 piece of silica gel containing either the free sterol or fatty acid fraction; area C was at the opposite end of the dish and was designated the non-attractive area and received a blank piece of 0.5 cm^2 silica gel; the central area (B) was considered neutral, and a single redia was placed in it equidistant between the two silica gel squares.

DISCUSSION

Reddy and Fried (1996) showed that lipophilic, but not hydrophilic, ES products were involved in chemoattraction of echinostome rediae *in vitro*. The chemical classes of lipids were not identified in that study. The present study has determined that the lipophilic ES products of *E. trivolvis* rediae are free sterols and free fatty acids, and that both of these lipid classes can produce significant intraspecific redial attraction *in vitro*. Free sterols have been implicated as chemical attractants for both hermaphroditic and dioecious adult trematodes (see reviews in Fried, 1986 and Haseeb & Fried, 1988) and free fatty acids in vertebrate skin as chemoattractants for avian and mammalian schistosome cercariae (Haas, 1992).

Quantitative analysis of lipids in echinostome rediae devoid of host DGG are not available, and this study provides such information for the first time for free sterols and phosphatidylcholine and phosphatidyletha-

nolamine. Knowledge of the amounts of free sterols and phospholipids in the redial stages of trematodes may be useful for a better understanding of the physiology and biochemistry of this poorly understood larval stage.

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