STUDIES ON THE LIPID CHEMISTRY OF *ECHINOCOCCUS GRANULOSUS GRANULOSUS* SCOLICES

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HYDATID LIPID CHEMISTRY

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ABSTRACT

The present study dealt mainly with the separation, characterization and biological significance of some of the lipid fractions of Echinococcus granulosus granulosus scolices. The total lipids of E. g. granulosus scolices were extracted and separated into different fractions (Bands) by the use of thin-layer chromatography (TLC). From these fractions, cholesterol, farnesol (esterified), farnesal and squalene were detected while other fractions remained unidentified. Experiments with the larval stages of two other helminths (Trichinella spiralis and Fasciola hepatica) indicated a similarity in the general pattern of their lipid constituents with those of E. g. granulosus scolices.

Using 2-^{14}C-mevalonate, we have shown that scolices did not incorporate the label into cholesterol, farnesol, farnesal and squalene; however, the saponifiable lipids were found to be relatively highly labelled. Cholesterol was obtained from the host in the esterified form and subsequently hydrolyzed into free cholesterol by a possible enzyme system present in the scolices. Cholesterol was also shown to be exogenously supplied by the host in the case of T. spiralis larvae.

The above findings, together with the fact that E. g. granulosus scolices incorporated radioactive mevalonate into lipid fractions which showed relatively strong biological activity, suggested the possibility of finding an endocrine system in the parasite, perhaps similar to that controlling ecdysis in insects.

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INTRODUCTION

1. General review

The work on the lipid chemistry of cestodes has progressed significantly in the last few decades. In his summary on the work of many authors, von Brand (1966) presented the variable amounts of the different classes of lipids present in the larval and adult stages of various cestodes. More recently, the work of Meyer et al. (1966) on both larval and adult Spirometra mansonoides indicated the presence of relatively large amounts of neutral and phospholipids. Ginger and Fairbairn (1966) demonstrated the presence of neutral and polar lipids in adult Hymenolepis diminuta; triglycerides were found to be the major component of the neutral lipids and cholesterol was the only sterol present.

Early studies on the lipids of the hydatid cyst (Echinococcus granulosus) were performed by Cameron and Fitzpatrick (1925) who reported the presence of cholesterol in the scolices, fluid, and cyst wall. Lemaire and Ribère (1935) reported the presence of creatinine and lecithin in the cyst fluid. In 1952, Čmelik found that cholesterol constituted 75% of the unsaponifiable material isolated from hydatid cyst membranes (both laminated cuticle and germinal membrane). More recently, Agosin et al. (1957) reported that the total lipid content of

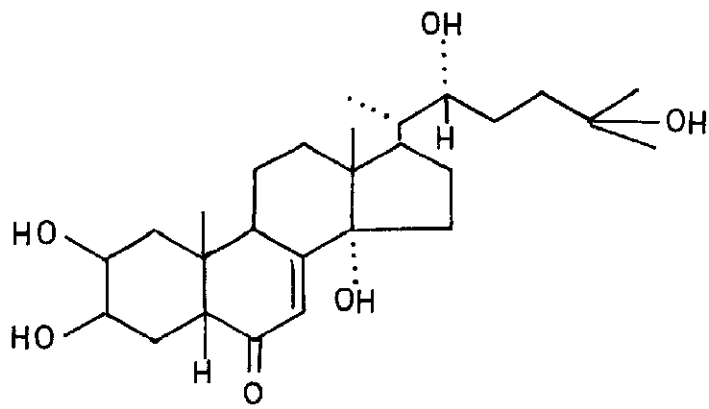
E. granulosus scolices was 13.6% of their dry weight. This was comparable to the lipid content of other helminth parasites. By infrared spectroscopy, Kilejian et al. (1962) reported large amounts of lecithin and a variable amount of cholesterol in crude extracts of E. granulosus scolices and laminated membrane.

Although studies on the lipid (particularly cholesterol) content of several parasitic helminths have been performed, the exact biological significance of the findings has remained obscure. With this in mind, it will be interesting to note the biosynthetic pathway of cholesterol in other classes of animals.

In vertebrates, cholesterol is biosynthesized from acetate or mevalonate (reviewed by Staple, 1963). In many insects, however, radioactive acetate or mevalonate do not incorporate into cholesterol; it has been shown that some insects do not even synthesize squalene, the noncyclic precursor of sterols (Clark and Bloch, 1959a; Robbins et al., 1960). In insects, the biosynthetic pathway of acetate or mevalonate apparently is directed towards the synthesis of specific hormones. To clarify the lines of thought pursued in this study, a brief review of the endocrinological system of insects will be presented, with emphasis on the chemistry of the two important hormones, the juvenile hormone and ecdysone.

Insect development takes place through different molts. Periodic molting appears to be under endocrinological control, the main endocrine centers being the brain and glands associated with it, namely the corpora allata and prothoracic gland. Neurosecretory cells in the brain produce the brain hormone which stimulates the release of the prothoracic gland hormone, ecdysone, which in turn initiates molting. The corpora allata, however, produce the juvenile hormone which is essential for larval molting and whose effects are opposite to that of the molting hormone. The relative concentrations of ecdysone and juvenile hormone control the metamorphosis of insects, from immature stages to the adult.

In 1954, Butenandt and Karlson first isolated 25 mg of ecdysone from 500 Kg of silkworm (Bombyx) pupae. This compound was effective in quantities below 0.0075 μg, when injected into ligated fly abdomens. The hormone was shown to be a steroid (Horn et al., 1966), the correct structural formula of which was presented as



The above formula was recently confirmed by synthesis, independently accomplished in three laboratories (Harrison et al., 1966; Kerb et al., 1966; Furlenmeier et al., 1966). In 1963, Karlson and Hoffmeister demonstrated the incorporation of radioactivity from tritium labelled cholesterol into ecdysone in Calliphora larvae, indicating that cholesterol is a precursor to ecdysone. Clark and Bloch (1959b) and Clayton (1960) showed that 95% of the dietary cholesterol in the beetle Dermestes vulpinus could be replaced by sitosterol but 5% of it remained essential. According to Karlson (1963), it may be assumed that a small part of the cholesterol injected into insects would be converted to ecdysone, the majority still fulfilling cellular functions.

More recently, Hoffmeister (1966) isolated another molting hormone, ecdysterone, from silkworm pupae, which was chromatographically more polar and had stronger biological activity than ecdysone. Horn et al. (1966) isolated crustecdysone from crayfish which was biologically active and proved to be chemically similar to ecdysone, with a hydroxyl group at the C-20 position. Carlisle (1965) demonstrated the biological activity of extracts from crabs, copepods and locusts on the immature shore crab Carcinus maenas, indicating the presence of a common set of interactive ecdysones in crustaceans and insects.

The juvenile hormone which has an effect opposite to that of ecdysone on the molting stages of insects, was first isolated as a yellow oil from the abdomens of Cecropia males by Williams (1956). Its biological activity was tested by applying it on the pupal cuticle and noting the adult cuticle produced. Karlson and Schmialek (1959) injected extracts of the excretions of the beetle Tenebrio molitor into mature larvae and noted the retardation of pupation in 88% of the tested larvae. Later, Gilbert and Schneiderman (1960) developed a bioassay for the activity of the juvenile hormone of insects. In 1961, Schmialek isolated 60 mg of an oil from 80 Kg of Tenebrio feces, which was identified as farnesol and its oxidation product, farnesal. Later, Schmialek (1963) showed that given labelled mevalonic acid, insects can synthesize labelled farnesol and farnesal. Using the farnesol isolated by Schmialek (1961), as well as commercial farnesol, Wigglesworth (1961, 1962) showed that it reproduces all the effects of the juvenile hormone when applied to the surface of the cuticle of the insect Rhodnius. The main effects were retention of larval character and partial reversal of metamorphosis in the molting adult.

Schneiderman and Gilbert (1964) showed that many other compounds such as phytol, farnesyl acetate, farnesinic acid and farnesyl methyl ether also exhibit juvenile hormone activity. Other active compounds have also been

isolated from protozoa and invertebrates (Schneiderman et al., 1960; Fisher and Sanborn, 1962), and even from the balsam fir tree (Bowers et al., 1966). Wigglesworth (in Schmialek, 1961) suggested the possibility of a number of chemicals mimicking the true juvenile hormone. According to Gilbert (1964), it is probable that a few or all of these compounds are precursors or metabolites which could be converted to the true hormone within the animal's body. It seems clear that in insects, the pathway of mevalonate is directed towards the synthesis of specific hormones, whereas in vertebrates, mevalonate biosynthesizes cholesterol. The fate of mevalonate in cestodes, yet another group of organisms, is an intriguing problem.

The work on cholesterol biosynthesis in cestodes is still in its infancy. Ginger and Fairbairn (1966) demonstrated the incorporation of 1-^{14}C-acetate (5.9% of the total neutral lipid incorporation) into the cholesterol fraction of Hymenolepis diminuta. However, Meyer et al. (1966) reported no de novo synthesis of sterols in Spirometra mansonoides, and Frayha (1964) observed that 1-^{14}C-acetate did not incorporate into the cholesterol fraction of E. granulosus scolices, whereas when 26-^{14}C-cholesterol was fed to mice infected with the scolices, the label did appear in the cholesterol isolated from the scolices. The biological activity observed on the nema-

tode Trichinella spiralis larvae by Meerovitch (1965) revealed that farnesol inhibited development of the larvae whereas ecdysone promoted molting.

2. Scope of the current investigation

In this study, the main parasite used was Echinococcus granulosus granulosus (Dailey and Sweatman, 1965). Lipid fractions of E. g. granulosus scolices were isolated and compared with those obtained from a nematode (Trichinella spiralis) and a trematode (Fasciola hepatica). Experiments were also performed to show the chemical state of cholesterol in the cyst fluid of E. g. granulosus, as well as its in vitro transformation in the scolex. Since mevalonate was shown to be a precursor of the juvenile hormone in insects (Schmialek, 1963), an attempt was made to correlate the biological activity of the different lipid fractions of E. g. granulosus scolices with their biosynthesis from mevalonate. The possible existence of a comparable endocrinological system in helminths, particularly E. g. granulosus, was the prevailing objective of the current investigation.

MATERIALS AND METHODS

1. Parasite material

Livers and lungs of sheep and cattle infected with cysts of E. g. granulosus were obtained within a few hours after slaughter of the host. The hydatid fluid and scolices* were withdrawn from the cysts aseptically by a needle and syringe according to the method of Agosin et al. (1957). The scolices were allowed to settle in a conical flask, removed with a Pasteur pipette and washed 3 to 4 times with distilled water. They were then separated by low speed centrifugation, transferred into 125 ml beakers and lyophilized in a Virtis lyophilizer.

For the biosynthetic experiments, the live scolices were washed 3 to 4 times with disodium monohydrogen phosphate and potassium dihydrogen phosphate buffer of pH 7.4 (Clark, 1920), and then separated by decantation.

Hydatid fluid was filtered through Whatman No. 3 filter paper and lyophilized.

2. Extraction of lipids

The total lipids of the scolices were extracted by a procedure similar to that of Folch et al. (1957).

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*The term scolices is used both for the scolices and brood capsules all through the text.

The lyophilized scolices were mixed with 100 ml of chloroform:methanol (2:1, v/v) and agitated on a shaker for 6 hours. The mixture was then centrifuged and the supernatant was filtered through Whatman No. 3 filter paper. This procedure was repeated three times. The combined extracts were evaporated under reduced pressure at 40°C to a volume of 10 ml. This was washed with an equal volume of water (Beames, 1965). After standing for 1 to 2 hours, the chloroform layer was separated and washed with a 0.5 volume of 1% KCl solution. The aqueous layer was mixed with an equal volume of chloroform:methanol (2:1) and the chloroform layer was collected after the clear separation of the interphase. The chloroform extracts were combined and stored overnight at -20°C. They were then filtered in the cold. The filtrate was dried over Na_2SO_4 and evaporated to dryness under reduced pressure at 40°C. The residue was weighed, dissolved in a minimum amount of chloroform and spotted on thin-layer chromatography (TLC) plates to obtain fractionation of the total lipids. The completeness of the extraction was tested by extracting the scolices with chloroform:methanol (2:1, v/v) in a Soxhlet apparatus for 24 hours.

Lipid extraction of the lyophilized cyst fluid was performed in a Soxhlet apparatus for 24 hours. The solvent was evaporated to dryness under reduced pressure at 40°C and the residue was spotted directly on TLC plates.

All separatory funnels were equipped with Teflon stop cocks. All stoppers were covered with aluminum foil. Evaporation of solvents was carried out under an atmosphere of nitrogen from a nitrogen tank. All samples were stored in chloroform at -20°C in an atmosphere of nitrogen. Exposure to light was avoided whenever possible.

3. Thin-layer chromatography (TLC)

Thin layer plates, 0.25 mm thick, were prepared from Silica Gel G (Merck according to Stahl). The plates were spread and subsequently dried at 110°C for 30 minutes. They were reactivated for 10 minutes at 110°C each time before use. The solvent was allowed to run 12 to 13 cm from the point of sample application in preequilibrated chambers away from light. The solvents were renewed after the development of one or two plates. In special cases, the "Chromagram" (Eastman Kodak) plates (0.1 mm thick, type K 301 R 2, Silica Gel) were used.

After development, the plates were air dried, examined under a long wave length lamp (ca 350 mμ), and the spots visualized by either exposing the TLC plates to iodine fumes for a few minutes or by spraying with an appropriate reagent followed by activation at 110°C for 10 minutes.

The solvents used for TLC development were: (1) methylene chloride:acetone (92:8, v/v); (2) methylene

chloride; (3) benzin (50-70°C):ethyl acetate (85:15, v/v); (4) hexane:ethyl acetate (92:8, v/v); (5) hexane; (6) chloroform:acetone (90:10, v/v); (7) chloroform:ethyl acetate (80:20, v/v); (8) chloroform; (9) chloroform: acetone (80:20, v/v); (10) chloroform:methanol (85:15, v/v).

The spray reagents used were Lieberman-Burchard reagent according to Waldi (1965), and Anisaldehyde-sulfuric acid reagent (Waldi, 1965). LB or AS in the results indicates the reagent used as a spray indicator. I_2 indicates exposure to iodine fumes.

Bands and spots were located by spraying each end of a TLC plate with an appropriate reagent; the remainder of the band was scraped off and extracted with chloroform. Bands located by exposure to iodine fumes were extracted with chloroform after the iodine was allowed to sublime in an oven at 100°C.

4. Saponification of esters

Lipid esters were hydrolyzed by refluxing them in 3 to 5 ml of 5% ethanolic KOH for 24 hours under an atmosphere of nitrogen. At the end of the saponification time, 5 ml of distilled water was added to the mixture and the ethanol evaporated by immersing the flask into a water bath at 45°C and bubbling nitrogen into it. The solution was then extracted three times with 5 ml ether portions;

The extracts were dried over Na_2SO_4 and the solvent evaporated under nitrogen. The residue was taken up in a minimal amount of chloroform and directly utilized for the various experiments.

5. Radioactivity measurements

All radioactivity measurements were performed in a Packard tricarb scintillation spectrometer (Model 3003), with an efficiency of 70% and a background count of 8 cpm. The samples were counted in liquid scintillation glass vials (20 ml capacity), with 18 ml of liquid scintillation "cocktail" containing 4 g of 2,5-diphenyloxazol ("PPO") as primary scintillator and 50 mg of dimethyl-1,4-bis /2(5-phenyloxazol)benzene/ ("Dimethyl-POPOP") as secondary scintillator per liter of toluene. Samples which were insoluble in the "cocktail" were first solubilized in 3 ml of dioxane and to them 15 ml of the "cocktail" was added. In the case of samples which were too small to be eluted from the TLC plates, the Silica Gel was scraped off, mixed with the "cocktail" and counted according to the method of Snyder and Stephens (1962).

6. Infrared spectra (IR)

All infrared spectra were recorded with a Perkin-Elmer infrared spectrophotometer (Model 237), in solution form ($CHCl_3$) at a concentration of 10% (w/v).

7. Melting point determinations

All melting points were determined with a Thomas Hoover capillary melting point apparatus.

8. Biological activity tests

The evaluation of the biological activity of the different lipid fractions of E. g. granulosus scolices was performed by Mr. A. Berntzen of the Department of Biology, Portland State College, Portland, Oregon, according to his published procedure, Berntzen and Mueller (1964). Essentially, the method involved the inoculation of the various lipid fractions with scolices obtained from cysticercoids of Hymenolepis diminuta which he dissected from infected Tribolium confusum beetles, according to the method of Rothman (1959). The inocula were maintained at 37°C, and the general appearance, growth and mortality of the worms were observed after a period of 4 to 6 days.

9. Solutions, solvents and chemicals

The antibiotic solution was prepared by diluting with distilled water 200,000 units of penicillin and 200 mg of streptomycin to a final volume of 400 ml. All solvents and substances used as reference standards were reagent grade. Farnesol, farnesal and squalene, produced by Light Co., London, England, were kindly supplied by Dr. Ibrahim Durr and exhibited single peaks in gas-liquid

chromatography. Ecdysone was kindly supplied by Syntex Research Co., Palo Alto, California. 2-^{14}C-DL-mevalolactone and 4-^{14}C-cholesterol were purchased from the Radiochemical Centre, Amersham, England. Radioactive cholesteryl acetate (synthesized from 4-^{14}C-cholesterol) with a specific activity of 1.55×10^4 cpm/mg, was kindly provided by Dr. George Digenis. The ester was shown to be pure by TLC in solvent 2(AS) and subsequent radioautography of the chromatogram.

RESULTS

1. The isolation and characterization of cholesterol, and the detection of fernesol, farnesal and squalene in scolices of E. g. granulosus

When duplicate samples of lyophilized scolices, 0.495 g in weight, were extracted with chloroform:methanol (2:1, v/v), 0.067 g of total lipids were obtained. Thus, the total lipid content of the lyophilized scolices was 13.6% of the dry weight, a figure consistent with that reported by Agosin *et al.* (1957). In order to isolate and identify the lipid constituents of *E. g. granulosus* scolices, thin-layer chromatography (TLC) separation techniques were used. The total lipid residue with preparative TLC in solvent 1 (LB) produced three main zones which were named Bands I (having the same mobility as cholesterol), II (origin and 1 cm above), and III (at the solvent front).

Band I

Elution of Band I with chloroform yielded 0.022 g of a slightly yellowish-white crystalline product, the infrared spectrum of which showed minor differences in the "finger-print" region (Fig. 1) from that of pure cholesterol. Recrystallization of this compound from dilute ethanol yielded 0.015 g of white plates (m.p. 147-148°C), which were identified as cholesterol

following mixed melting point determinations. These results showed that cholesterol amounted to 3.03% of the dry weight of E. g. granulosus scolices (0.21% wet weight, since one gram of lyophilized scolices was equivalent to 14.5 g of wet fresh scolices). A comparison of the total weight residue from Band I (0.022 g) with that of cholesterol (0.015 g) revealed that Band I contained more than one compound. Therefore, a more refined subfractionation of Band I was undertaken with the "Chromagram" (Eastman Kodak) TLC technique, using four solvents 6-9 (I_2), with known cholesterol as reference. At least two additional compounds became recognizable. Subfractionation of a new Band I residue (0.064 g) in solvent 6 (I_2) produced four zones (Fig. 2) which were designated Ia (0.001 g), Ib (0.003 g), Ic (0.038 g) and Id (0.014 g). The Rf values of Band Ic (Table I) proved Ic to be the source of cholesterol previously observed by infrared spectrum and mixed melting point determinations, in the original Band I.

Band Id (Fig. 2) exhibited peaks in its infrared spectrum (Fig. 3) which were similar to, but not identical with, those of farnesal (Fig. 4). Farnesal also appeared at the upper edge of Band I (Fig. 5), when Band I was developed in solvent 1 (I_2). Since farnesal was detected in Band III (see below), it was possible that Band Id contained farnesal as a contaminant from

TABLE I

IDENTIFICATION OF CHOLESTEROL, AND COMPARISON OF Rf VALUES OF BAND I SUBFRACTIONS FROM DIFFERENT SOURCES

Source	Solvent*	Ia	Ib	Ic	Id	Cholesterol
E. g. granulosus scolices	6	0	0.44	0.73	F**	0.74
	7	0	0.43	0.74	F	0.75
	8	0	0.26	0.46	F	0.45
	9	0	0.61	0.80	F	0.80
T. spiralis larvae	6	0	0.48	0.81	F	
	7	0	0.45	0.81	F	
	8	0	0.36	0.57	F	
F. hepatica miracidia	6	0	0.49	0.89	F	
	7	0	0.41	0.80	F	
	8	0	0.35	0.52	F	

*See Materials and Methods for solvent formulation.

**F = Solvent front.

Band III. However, judging from the infrared spectra of farnesal and Band Id, it was also possible that Band Id was a compound chemically similar to farnesal.

Band II

Considering the possibility of ecdysone (a relatively polar compound with a low Rf in low polarity solvents) being a constituent of Band II, it was chromatographed with the residue from Band II in solvent 10 (AS). No ecdysone was detected (Fig. 6). Possibly, Band II contained esterified ecdysone; however, when it was subjected to alkaline hydrolysis and the unsaponifiable fraction isolated, no ecdysone could be detected by TLC in solvent 10 (AS). Therefore, the Band II residue remained unidentified.

Band III

Elution of Band III with chloroform yielded 0.020 g of a viscous liquid, the infrared spectrum of which showed a peak at 1740 cm^{-1}, indicating the presence of esters. When a small amount of the oil was submitted to TLC in solvent 2 (AS), farnesal and cholesterol were identified (Fig. 7). The remaining residue from Band III was subsequently subjected to alkaline hydrolysis and the unsaponifiable matter (0.007 g) was chromatographed with TLC in solvent 2 (AS). Choles-

terol, farnesal and farnesol were detected by comparing the migration of the spots in solvents 2-4 (AS) together with those of pure samples of the above compounds (Figs. 8-10). The spot at the solvent front in Figs. 8-10 was shown to be squalene by TLC using solvent 5 (AS) as in Fig. 11. Table II summarizes the approximate Rf values of the above compounds. With the TLC technique, therefore, cholesterol, farnesol, farnesal and squalene were identified in the scolex lipids.

2. Cholesterol esters in E. g. granulosus cyst fluid

Lipid extraction of 0.500 g of lyophilized fertile cyst fluid yielded 0.110 g of a semisolid residue. In order to determine the chemical state of cholesterol, this residue was dissolved in 0.5 ml of chloroform. An aliquot from it (10-20 λ) was spotted on TLC in solvent 1 (LB), but no cholesterol was observed. Upon saponification of the remaining residue, however, and subsequent TLC of the unsaponifiable matter, cholesterol was detected, indicating that this sterol was found completely esterified in the cyst fluid of E. g. granulosus.

3. Hydrolytic action of homogenates from E. g. granulosus scolices on cholesteryl acetate

To detect the presence of a hydrolytic enzyme

TABLE II

APPROXIMATE Rf VALUES OF COMPOUNDS DETECTED BY TLC FROM THE LIPIDS OF E. G. GRANULOSUS SCOLICES

Compound	Solvent				
	1	2	3	4	5
Cholesterol	0.83	0.28	0.26	0.11	0
Farnesal	0.94	0.72	0.71	0.45	0
Farnesol	F*	0.42	0.34	0.15	0
Squalene	F	F	F	F	0.32

*F = Solvent front.

system in the scolices, one gram of live scolices was homogenized in a Potter-Elvehjem homogenizer. The homogenates were incubated with 1 ml of antibiotic solution, 4 ml of phosphate buffer and 0.006 g of radioactive cholesteryl acetate in a water bath at 37°C for 24 hours with rigorous continuous shaking. As a control, radioactive cholesteryl acetate was incubated under identical conditions with 1 ml of antibiotic solution and 4 ml of phosphate buffer. At the end of the incubation period, the experimental mixture was extracted with chloroform for its lipids. The cholesterol fraction, isolated by developing the lipid extract in solvent 2 (AS) was counted and found to contain 156 cpm. However, no radioactive cholesterol was obtained from the control. The results indicated the hydrolysis of the cholesterol ester into free cholesterol.

4. Lipid extracts of Trichinella spiralis (Nematoda: Trichinellidae) larvae and miracidia of Fasciola hepatica (Trematoda: Fasciolidae)

The TLC technique was used to compare the general lipid patterns of T. spiralis larvae and miracidia of F. hepatica with those of E. g. granulosus scolices. For this study, T. spiralis larvae were obtained by pepsin-HCl digestion of the muscles of two mice infected two months previously. These were then washed and lyophilized to a dry weight of 0.100 g. Miracidia were collected

daily from F. hepatica eggs, released by the adults in tap water at room temperature, over a period of 5 weeks. These were pooled, washed and lyophilized to a dry weight of 0.047 g. Lipid extraction was performed as described for E. g. granulosus scolices. When the total lipids from these species were separated by TLC in solvent 1 (LB), the fractions obtained appeared analogous to Bands I, II and III. Further subfractionation of Band I residues into Bands Ia, Ib, Ic and Id on TLC in solvents 6-8 (I_2) yielded chromatograms for the larvae and miracidia similar to those of the tapeworm (Figs. 12 and 13). Table I shows the comparative similarity of the Rf values of Bands Ia, Ib, Ic and Id from the three sources.

5. Biological activities of the different lipid fractions of E. g. granulosus scolices

In order to determine the effect of scolex lipids as a growth factor, freshly isolated samples of Bands I, II and III, as well as samples of Bands Ia, Ib, Ic, Id, II and III from a second source, were tested for their effect on the development of the unrelated cestode Hymenolepis diminuta by Mr. A. Berntzen. Table III shows that the addition of Bands I and II but not of III, to the culture medium of H. diminuta resulted in shorter worms, suggesting growth supression. Among the subfractions of Band I, Band Ic (in high concentrations)

TABLE III

BIOLOGICAL ACTIVITIES OF VARIOUS LIPID FRACTIONS FROM SCOLICES OF E. G. GRANULOSUS ON CYSTICERCOIDS OF H. DIMINUTA IN IN VITRO CULTURE (EXPT. 1)

Band	Suspected materials	Concentration/ 10 ml medium %	Survivors	Size, mm after 6 days (range)	Appearance
I	Cholesterol Farnesal(?) Other compounds	0.2	6/7	0.5	Vacuolated, motile but abnormal
II	Unknown	0.4	5/7	1.0 (0.5-1.5)	Good, no vacuoles, normal
III	Squalene Farnesol (esterified) Farnesal Cholesterol(?)	0.1	6/7	9.5 (5-11)	Normal
Methanol control	--	--	6/7	10.5 (7-12)	Normal

supressed worm growth, whereas Band Id enhanced it. Bands Ia, Ib, II and III exhibited no marked changes (Table IV). Interestingly enough, the combined extracts of Bands Ia, Ib, Ic and Id, and of Bands Ia, Ib and Id, produced marked supression of growth. The results indicated inconsistent effects on the development of H. diminuta by the different fractions of scolex lipids.

6. Incorporation of 2-^{14}C-mevalolactone into E. g. granulosus scolices

An experiment was performed to trace the pathway of mevalonate in the different lipid fractions obtained from E. g. granulosus scolices. In a 50 ml Erlenmeyer flask, 3.0 g of live scolices were incubated with 4 ml of phosphate buffer, 1 ml of antibiotic solution and 1 ml of 2-^{14}C-mevalolactone (specific activity 3.11×10^5 cpm/ 0.04 μM/ml) in a water bath at 37°C with continuous shaking for 5 hours. At the end of the incubation period, 2 ml of 3N H_2SO_4 was added to the flask. The scolices were separated by low speed centrifugation, washed several times with distilled water, and then transferred to a 125 ml Erlenmeyer flask; their lipids were extracted and fractionated on TLC into Bands I, II, III, Ia, Ib, Ic and Id. To show the presence of ecdysone (undetectable by TLC), Band II was developed in solvent 10 (AS) as previously described, and the adsorbent on the TLC plate

TABLE IV

BIOLOGICAL ACTIVITIES OF VARIOUS LIPID FRACTIONS FROM SCOLICES OF E. G. GRANULOSUS ON CYSTICEROIDS OF H. DIMINUTA IN IN VITRO CULTURE (EXPT. 2)

Band	Tentative identification	Concentration/ 10 ml medium	Mean length* (mm) after 4 days			Mortality %
Ia	Unknown	0.0009 0.009 0.09	1.90	1.93	1.92	5
Ib	Steroid(s)	0.0026 0.026 0.26	1.80	1.85	1.87	50
Ic	Cholesterol Other compounds	0.382 3.82		1.94	0.453	50
Id	Farnesal(?)	0.0140 0.140 1.40	4.18	4.50	4.50	5
II	Unknown	0.0036 0.036 0.36	1.92	1.93	1.92	5
III	Cholesterol Farnesol (esterified) Farnesal Squalene	0.0126 0.126 1.26	1.93	1.91	1.88	5

TABLE IV (continued)

Band	Tentative identification	Concentration/ 10 ml medium	Mean length* (mm) after 4 days	Mortality %
CONTROL	--	--	1.92 1.92 1.91	5-6
I a,b,c,d		a = 0.09 b = 0.26 c = 3.82 d = 1.40	0.196 vs 2.00 (control)	50
I a,b,d		a = 0.09 b = 0.26 d = 1.40	0.191 vs 2.00 (control)	50

*Random measurements on 15 of 50 cultured cysticercoids.

was subdivided into 2.5 cm wide bands which were designated Bands IIa, IIb (ecdysone level), IIc, IId and IIe (Fig. 6); each was scraped off separately and eluted. All the isolated fractions were counted in the scintillation counter (Table V).

The radioactivity in Band IIb (ecdysone level) was negligible; thus, no biosynthesized ecdysone could be detected in Band II.

Band Ic, containing the free cholesterol band, had a specific activity of 30 cpm/mg as calculated from a duplicate sample. To show if the label was incorporated into cholesterol or not, 0.046 g of pure unlabelled cholesterol was added to 0.004 g of Ic, and the whole recrystallized from 95% ethanol. The specific activity of Ic dropped to 7 cpm/mg after the first recrystallization. Two more recrystallizations from acetone:absolute ethanol (1:1, v/v) yielded a final specific activity of 2 cpm/mg. These results indicated that Band Ic, previously identified as cholesterol, contained one or more compounds besides cholesterol, to which the label was incorporated.

Band III was saponified and the different fractions in solvent 2 (AS) were counted. Nearly all the incorporated radioactivity of Band III resided in its saponifiable fraction. Clearly, mevalonate incorporated mostly into the saponifiable fraction of scolex lipids with some

TABLE V

INCORPORATION OF 2-^{14}C-DL-MEVALOLACTONE INTO VARIOUS LIPID FRACTIONS OF E. G. GRANULOSUS SCOLICES

Band		cpm	% Incorporation
I		1096	0.82
	Ia	5	negligible
	Ib	115	0.08
	Ic	148	0.20
	Id	502	0.37
II		171	0.13
	IIa	negligible	negligible
	IIb (Ecdysone level)	8	0.01
	IIc	34	0.02
	IId	93	0.07
	IIe	9	0.01
III		826	0.62
	Origin	81	0.06
Unsaponifiable fraction of Band III	Cholesterol level	4	negligible
	Farnesol level	35	0.02
	Farnesal level	11	0.01
	Squalene level	6	negligible
Saponifiable fraction of Band III		827	0.62

incorporation into traces of unidentified compounds present in Band Ic.

7. The in vivo incorporation of 4-^{14}C-cholesterol into Trichinella spiralis larvae

In this experiment, the transfer of cholesterol from the host to T. spiralis larvae was examined. Two mice, infected with the larvae for four months, were fed for a week on food pellets soaked with 4-^{14}C-cholesterol (3 μc equivalent to 4.66×10^6 cpm). The lipids were extracted from larvae collected from the mice as previously described. Upon isolation and counting of the Band I residue, 310 cpm were found to be incorporated into it. The above residue was saponified, and to the unsaponifiable fraction (0.013 g), carrier cholesterol (0.037 g) was added and the whole mixture recrystallized twice from 95% ethanol and twice from ether:absolute ethanol (1:1, v/v) to a constant specific activity of 38, 31, 31 and 26 cpm/mg. A specific activity of 25 cpm/mg would be expected if all the label resided in the unsaponifiable fraction of the Band I residue. Clearly, cholesterol became incorporated into the larvae by transfer from the host.

DISCUSSION

The total lipid content of E. g. granulosus scolices (13.6% dry weight) was identical with the results obtained by Agosin et al. (1957). McMahon (1961) reported a total lipid content of 6.9% (dry weight) for larval Taenia taeniaeformis, whereas Meyer et al. (1966) reported a value of 16% for larval Spirometra mansonoides. Broad differences are apparent in the lipid content of different species of cestodes.

Cholesterol seems to be the major sterol in helminths. Fairbairn and Jones (1956) showed that 75% of the unsaturated sterols of Ascaris lumbricoides was cholesterol. Thompson and coworkers (1960) reported that 98% and 85% were the respective amounts of cholesterol present in the unsaponifiable matter of adult Taenia taeniaeformis and Moniezia sp. The current study showed that in E. g. granulosus scolices from cysts, free cholesterol constituted 3.03% of the dry weight. The above figure showed an even higher cholesterol content compared to that of Taenia taeniaeformis, for which von Brand and coworkers (1965) reported a value of 1.4% (dry weight).

In the fertile cyst fluid, however, cholesterol was shown to be completely in the esterified form. This observation completed the information obtained from the results of Cameron and Fitzpatrick (1925) whose methods could not

distinguish between free cholesterol and its esters in the cyst fluid.

The detection of farnesal before and after saponification of Band III, proved that it was not an artifact of saponification. Since farnesol was detected only after saponification, it was assumed to be present as esterified. The presence of cholesterol in Band III could be attributed to contamination (due to elution) from Band I.

Although farnesal, farnesol and squalene were detected by TLC, the negligible incorporation of 2-^{14}C-mevalolactone into these compounds and cholesterol suggested that the terpenic pathway was directed towards the biosynthesis of compounds other than cholesterol or its direct precursors. The above suggestion was also supported by the fact that the incorporated radioactivity into Band Ic was found to reside in the compounds accompanying cholesterol. Our results were consistent with those of Frayha (1964) using 1-^{14}C-acetate. Due to the limited availability of the parasitic material, it was not possible to chemically characterize such important Bands as Id, Ic (containing compounds in trace amounts) and the subfractions of Band II. However, further tests are being performed at the present, in an attempt to characterize some of the above bands.

The relatively high incorporation into the saponifiable fraction of Band III (62% of total incorporation) indicated the efficient biosynthesis of glycerides from mevalolactone.

Meyer et al. (1966) reported that the cestode Spirometra mansonoides lacked mechanisms required for the de novo synthesis of sterols, but was able to synthesize triglycerides and sterol esters with exogenously supplied sterols and fatty acids. Our observations seemedto agree with the results of the above workers.

The fact that radioactive cholesterol was isolated from the scolices upon incubation with radioactive cholesteryl acetate, indicated the presence of an enzyme system responsible for the hydrolysis of the cholesterol ester. The above was consistent with the results of Lee and coworkers (1963) who reported the presence of nonspecific esterases in various tapeworms. Frayha (1964) demonstrated the passage of 26-^{14}C-cholesterol from the host (infected mice) to the scolices of E. g. granulosus. Considering the above findings, it seemed reasonable to assume that in E. g. granulosus scolices, cholesterol was obtained from the host esterified, and was subsequently hydrolyzed to free cholesterol by the scolices.

The data concerning the biological activity of the different lipid fractions indicated the greatest activity in Bands I and II (Table III) of which Bands Ic and Id showed striking activities (Table IV). It is interesting to note that Band Ic which was found to contain mainly cholesterol, was active only in high concentrations. The activity could be attributed to either cholesterol acting in relatively

high concentrations with toxic effects, or to the traces of compounds present in this fraction to which 2-^{14}C-mevalonate was incorporated. Since Band Id was suspected to be a compound chemically similar to farnesal (or a contaminant from farnesal), its biological activity was not surprising, in view of the fact that farnesol was previously shown to have juvenile hormone activity on molting Trichinella spiralis larvae (Meerovitch, 1965). It was interesting to note that the overall effect of the combined extracts of Bands Ia, Ib, Ic and Id, and of Bands Ia, Ib and Id was that of striking growth supression. This could be due to the synergistic effect of the bands acting together. However, no conclusions could be drawn on the nature of activity (stimulatory or inhibitory) of the above lipid fractions, due to the lack of additional information concerning the morphology of the developing Hymenolepis diminuta used in the testing system. Moreover, an improvement on the biological activity tests would be to use E. g. granulosus scolices instead of H. diminuta cysticercoids for the testing system. In this way, data resulting from any possible toxic effects on the parasite would be eliminated.

The crude separation of lipid fractions from larval stages of Trichinella spiralis and Fasciola hepatica, and their similarity to those of E. g. granulosus scolices, suggested the presence of a similar pattern of lipids in the three classes of helminths (Cestoda, Nematoda and Trematoda).

The isolation of labelled cholesterol from T. spiralis larvae collected from mice infected for four months and fed on radioactive cholesterol, suggested that the larvae obtained their cholesterol from the host, even at a well encapsulated stage. The above was consistent with the work of Frayha (1964) who isolated 26-^{14}C-cholesterol from E. g. granulosus scolices with similar experiments. However, using 4-^{14}C-cholesterol was advantageous, since loss of radioactivity due to any possible breakage of the side chain would be eliminated.

E. g. granulosus scolices, therefore, rely on an exogenous source of cholesterol (from the host), and incorporate 2-^{14}C-mevalonate into lipid fractions which show relatively strong biological activity. The above suggests the probability of finding an endocrine system in the parasite which could conceivably be similar to that controlling ecdysis in insects.

CONCLUSIONS

1. The total lipid content of E. g. granulosus scolices was 13.6% of their dry weight.

2. Cholesterol proved to be the main constituent of one band derived from the scolices and separated by TLC (designated Band Ic). The amount of cholesterol proved to be 3.03% of the dry weight.

3. Farnesol (esterified), farnesal and squalene were detected by TLC in the lipids of the scolices but ecdysone was not.

4. There was a similarity in the general pattern of lipids of E. g. granulosus scolices, F. hepatica miracidia and T. spiralis larvae.

5. The hydatid cyst fluid was shown to contain cholesterol in the esterified form; this was found to be hydrolyzed to free cholesterol by hydrolytic enzymes in the scolices.

6. E. g. granulosus scolices incorporated radioactive mevalonate mainly into the saponifiable fraction of their lipids, but not into cholesterol, farnesol, farnesal and squalene. The label in the cholesterol fraction (Band Ic) was due to traces of other compound(s) present in addition to cholesterol.

7. T. spiralis larvae were also shown to obtain cholesterol

from their host.

8. The effect of the different lipid fractions from E. g. granulosus scolices on the in vitro growth of H. diminuta revealed a relatively consistent correlation between the fractions exhibiting biological activity and those incorporating radioactivity.

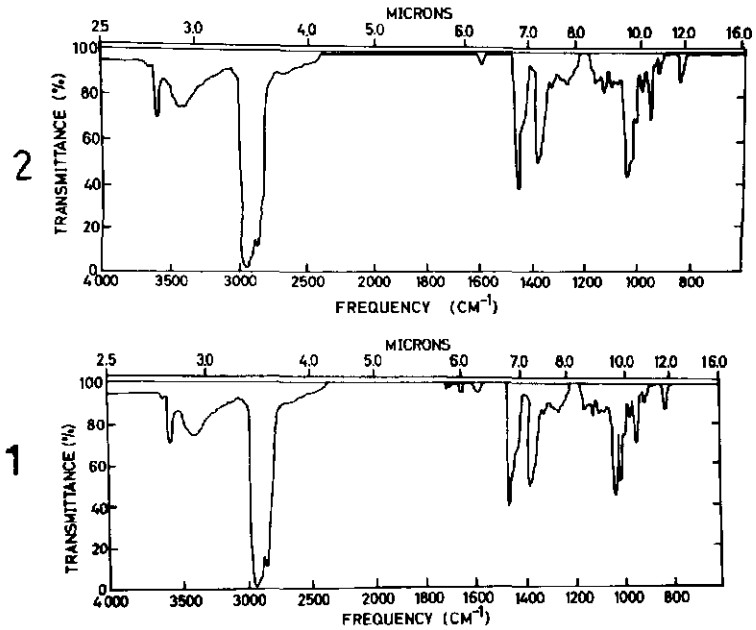

Fig. 1. Comparison of the infrared spectrum (IR) of pure cholesterol (1) with that of Band I (2).

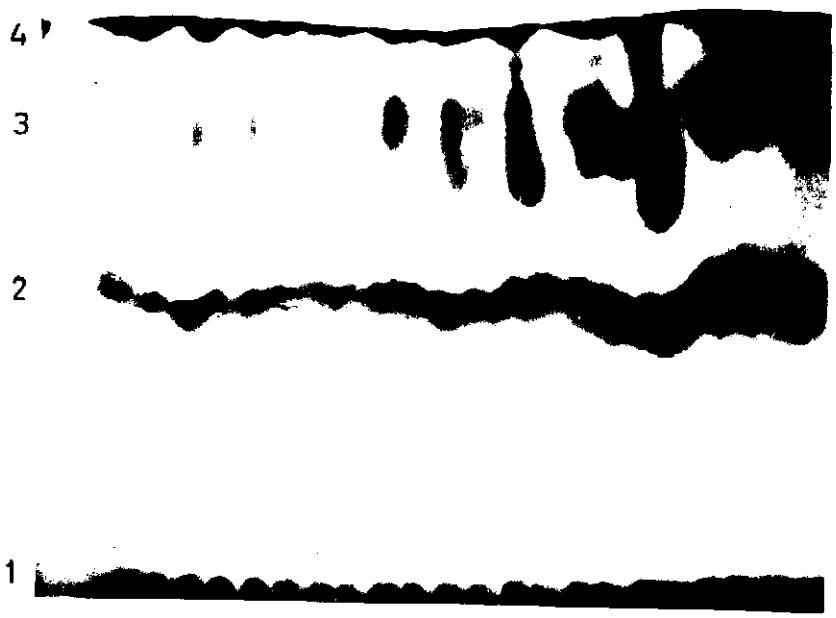

Fig. 2. TLC of Band I in solvent 6 (I_2). 1-Ia; 2-Ib; 3-Ic; 4-Id.

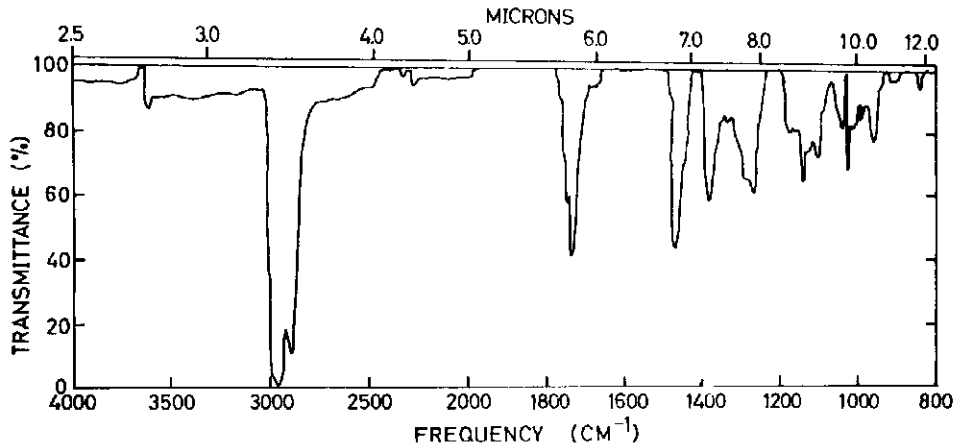

Fig. 3. IR of Band Id.

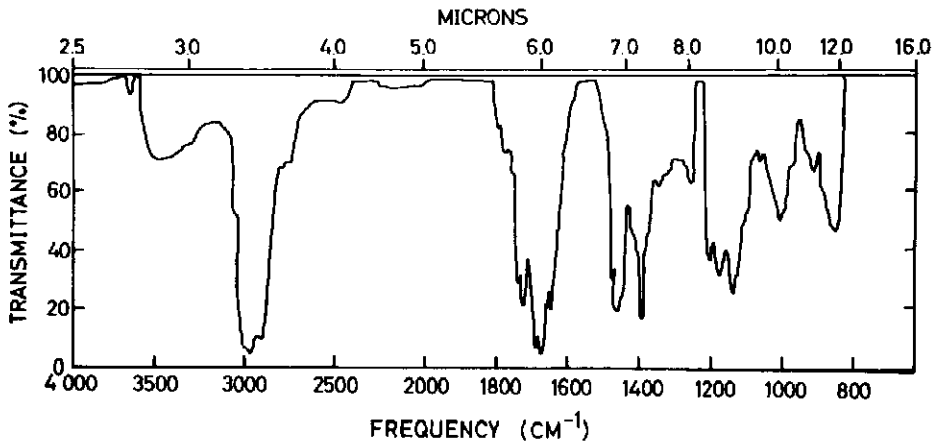

Fig. 4. IR of farnesal.

Fig. 5. TLC of the total lipids of E. g. granulosus scolices in solvent 1 (I_2) with cholesterol and farnesal as reference standards. 1-Cholesterol; 2 and 4-total lipids; 3-farnesal.

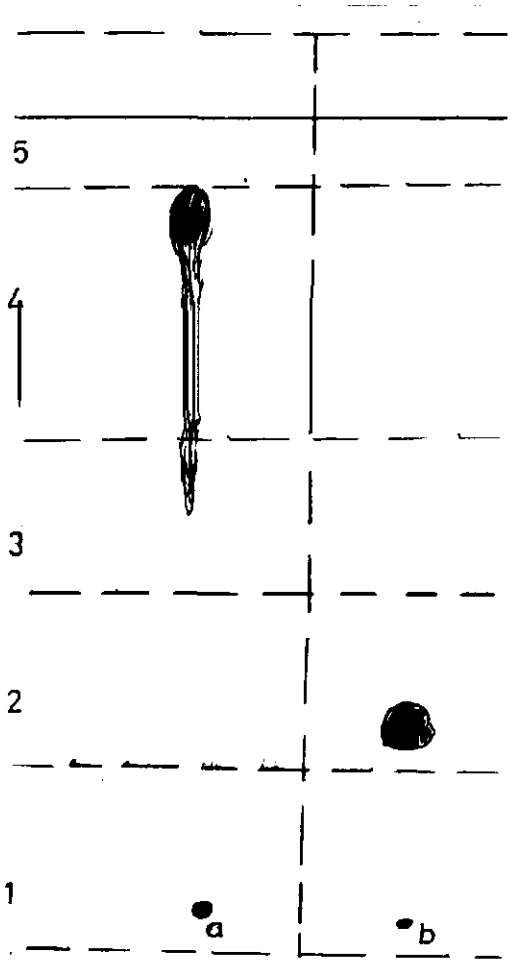

Fig. 6. TLC of Band II and ecdysone in solvent 10 (AS). a-Band II; b-ecdysone; 1-IIa; 2-IIb; 3-IIc; 4-IId; 5-IIe.

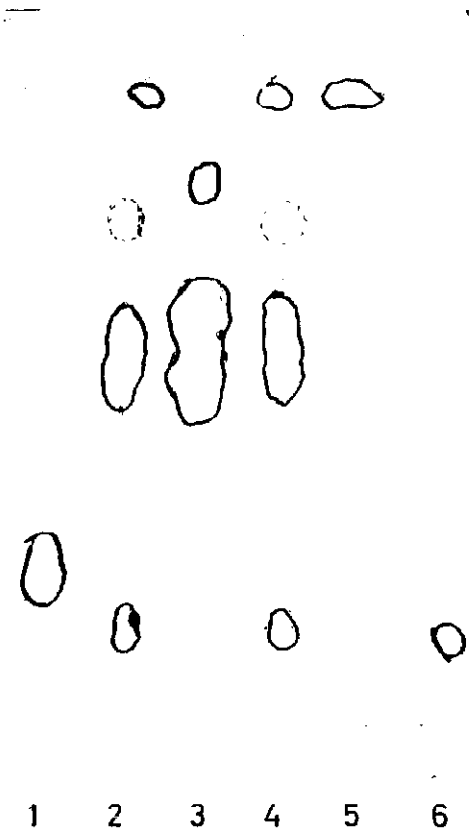

Fig. 7. TLC of Band III (prior to saponification) with reference standards in solvent 2 (AS).
1-farnesol; 2 and 4-Band III; 3-farnesal;
5-squalene; 6-cholesterol.

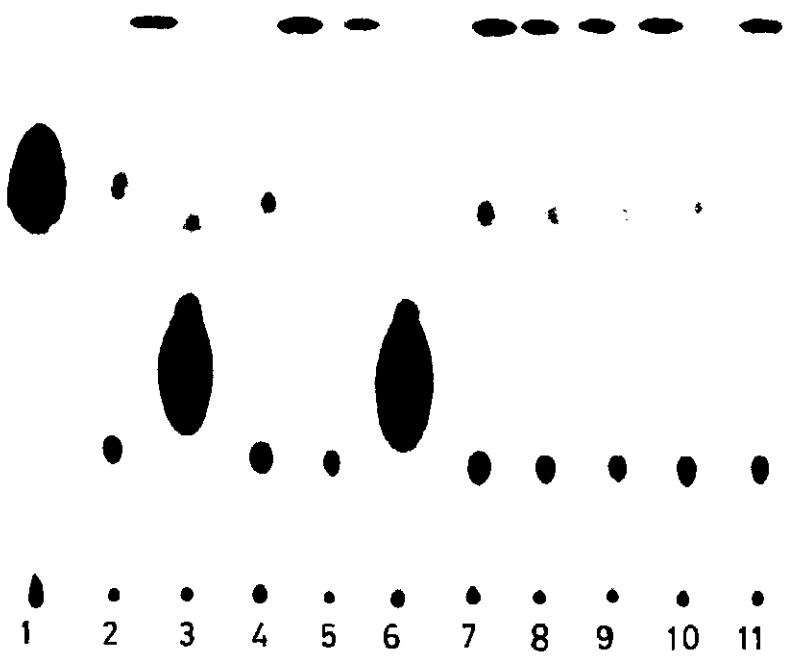

Fig. 8. TLC of the unsaponifiable fraction of Band III with reference standards in solvent 2 (AS). 1-farnesal; 3 and 6-farnesol; 2,4,5,7,8,9,10 and 11-unsaponifiable fraction of Band III. (Farnesol appearing in Band III, although clear on the original plate, is not well defined in the picture.)

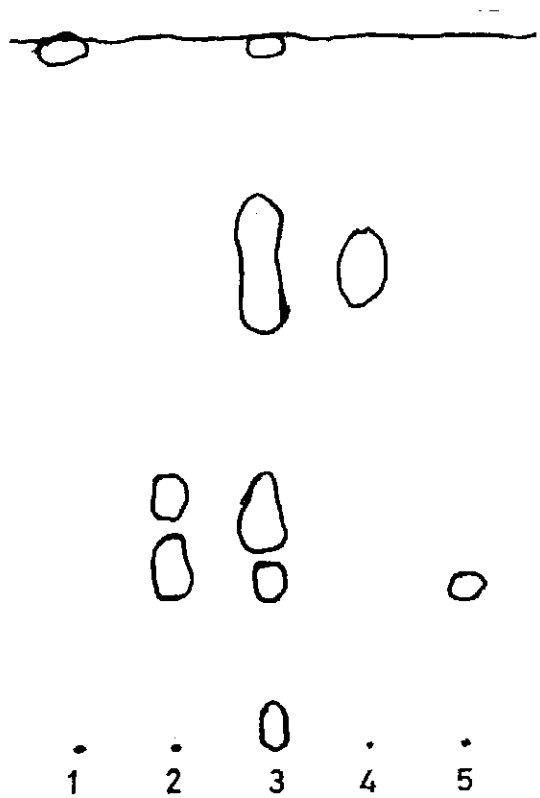

Fig. 9. TLC of the unsaponifiable fraction of Band III with reference standards in solvent 3 (AS). 1-squalene; 2-farnesol; 3-unsaponifiable fraction of Band III; 4-farnesal; 5-cholesterol.

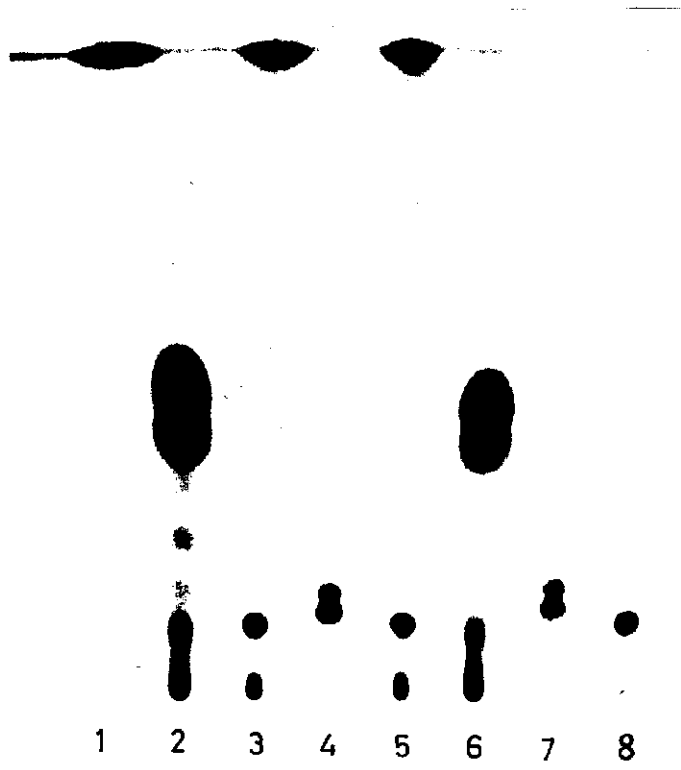

Fig. 10. TLC of the unsaponifiable fraction of Band III with reference standards in solvent 4 (AS). 1-squalene; 2 and 6-farnesal; 3 and 5-unsaponifiable fraction of Band III; 4 and 7-farnesol; 8-cholesterol.

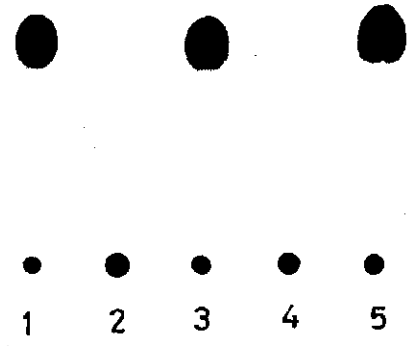

Fig. 11. TLC of the unsaponifiable fraction of Band III with squalene in solvent 5 (AS). 1, 3 and 5-squalene; 2 and 4-unsaponifiable fraction of Band III.

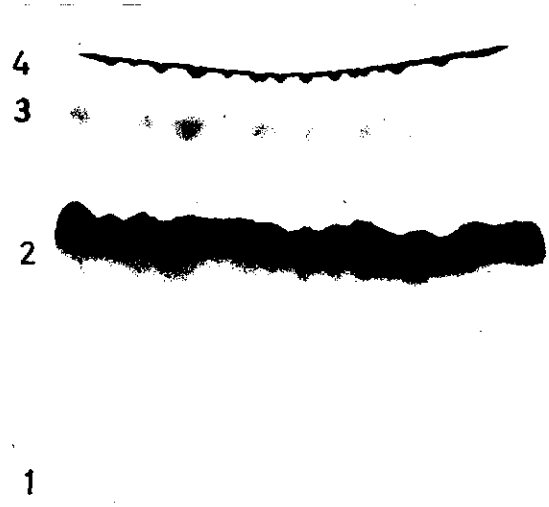

Fig. 12. TLC of the total lipids of T. spiralis larvae in slovent 6 (I_2). 1-Ia; 2-Ib; 3-Ic; 4-Id.

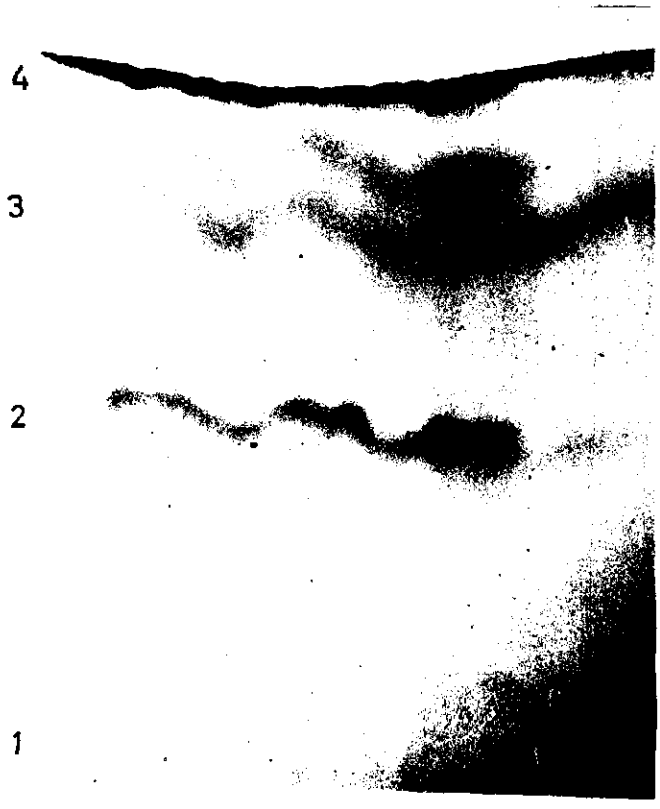

Fig. 13. TLC of the total lipids of F. hepatica miracidia in solvent 6 (I_2). 1-Ia; 2-Ib; 3-Ic; 4-Id.

REFERENCES

Agosin, M., T. von Brand, F. Rivera, and P. McMahon. 1957. Studies on the metabolism of Echinococcus granulosus. 1. General chemical composition and respiratory reactions. Exp. Parasit. 6:37-51.

Beames, C.G., Jr. 1965. Neutral lipids of Ascaris lumbricoides with special reference to the esterified fatty acids. Exp. Parasit. 16:291-299.

Berntzen, A., and J.F. Mueller. 1964. In vitro cultivation of Spirometra mansonoides (Cestoda) from the procercoid to the early adult. J. Parasit. 50:705-711.

Bowers, W.S., H.M. Fales, M.J. Thompson, and E.C. Uebel. 1966. Juvenile hormone: Identification of an active compound from Balsam fir. Science 154:1020-1021.

Brand, T. von 1966. Biochemistry of Parasites. Academic Press, New York. pp. 191-230.

Brand, T. von, Y. Sato, and R. Overton. 1965. Cholesterol and cholesterol esters in Taenia taeniaeformis. J. Parasit. 51:483.

Butenandt, A. von, and P. Karlson. 1954. Über die Isoleirung eines Metamorphose-Hormons der Insekten in kristallisierter Form. Z. Naturforsch. 9b:389-391.

Cameron, G., and A.S. Fitzpatrick. 1925. The hydatid cyst. The microchemical reactions of the hydatid cyst. Am. J. Pathol. 1:227-233.

Carlisle, D.B. 1965. The effects of crustacean and locust ecdysones on molting and proecdysis in juvenile shore crabs, Carcinus maenas. Gen. Comp. Endocrinol. 5: 366-377.

Clark, A.J., and K. Bloch. 1959a. The absence of sterol synthesis in insects. J. Biol. Chem. 234:2578-2582.

Clark, A.J., and K. Bloch. 1959b. Function of sterols in Dermestes vulpinus. J. Biol. Chem. 234:2583-2588.

Clark, W.M. 1920. The Determination of Hydrogen Ions. Williams and Wilkins, Baltimore. p. 114.

Clayton, R.B. 1960. The role of intestinal symbionts in the sterol metabolism of Blatella germanica. J. Biol. Chem. 235:3421-3425.

Čmelik, S. 1952. Zur Kenntnis der Lipoide aus den Cystenmembranen von Taenia echinococcus. Hoppe-Seyler's Z. Physiol. Chem. 289:78-79.

Dailey, M.D., and G.K. Sweatman. 1965. The taxonomy of Echinococcus granulosus in the donkey and dromedary in Lebanon and Syria. Ann. Trop. Med. Parasit. 59:463-477.

Fairbairn, D., and R.N. Jones. 1956. Cholesterol from Ascaris lumbricoides (Nematoda). Can. J. Chem. 34:182-184.

Fisher, F.M., Jr., and R.C. Sanborn. 1962. Production of insect juvenile hormone by the microsporidian parasite Nosema. Nature 194:1193.

Folch, J., M. Lees, and G.H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Frayha, G.J. 1964. Studies on the acetate metabolism of taeniid tapeworms. M.Sc. Thesis, American University of Beirut.

Furlenmeier, A., A. Fürst, A. Longemann, G. Waldvogel, P. Hocks, U. Kerb, and R. Wiechert. 1966. The synthesis of ecdysone. Experientia 22:573.

Gilbert, L.I. 1964. Physiology of growth and development: Endocrine aspects. pp. 149-225. In Rockstein, M. (ed.) The Physiology of Insecta. Vol. I. Academic Press, New York.

Gilbert, L.I., and H.A. Schneiderman. 1960. The development of a bioassay for the juvenile hormone of insects. Tr. Am. Micr. Soc. 79:38-65.

Ginger, C.D., and D. Fairbairn. 1966. Lipid metabolism in helminth parasites. II. The major origins of the lipids of Hymenolepis diminuta (Cestoda). J. Parasit. 52: 1097-1107.

Harrison, L.T., J.B. Siddall, and J.H. Fried. 1966. Steroids CCXCVII. Synthetic studies on insect hormones. Part III. An alternative synthesis of ecdysone and 22-isoecdysone. Tetrahydron Lett. 26:3457-3460.

Hoffmeister, H. 1966. Isolierung eines neuen Insektenhormons aus Seidenspinnerpuppen. Z. Naturforsch. 21:335-336.

Horn, D.H.S., E.J. Middleton, J.A. Wunderlich, and F. Hampshire. 1966. Identity of the molting hormones of insects and crustaceans. Chem. Commun. 11:339-341.

Karlson, P. 1963. Chemistry and biochemistry of insect hormones. Angew. Chem., Internat. edit. 2:175-182.

Karlson, P., and H. Hoffmeister. 1963. Zur Biogenese des Ecdysons. I. Umwandlung von Cholesterin in Ecdyson. Hoppe-Seyler's Z. Physiol. Chem. 331:298-300.

Karlson, P., and H. Schmialek. 1959. Nachweis der Exkretion von Juvenilhormon. Z. Naturforsch. 14b:821.

Kerb, U., P. Hocks, R. Wiechert, A. Furlenmeier, A. Fürst, A. Langemann, and G. Waldvogel. 1966. The synthesis of ecdysone (in Bombyx mori). Tetrahydron Lett. 13:1387-1391.

Kilejian, A., K. Sauer, and C.W. Schwabe. 1962. Host-parasite relationships in Echinococcosis. VIII. Infrared spectra and chemical composition of the hydatid cyst. Exp. Parasit. 12:377-392.

Lee, D.L., A.H. Rothman, and J.B. Senturia. 1963. Esterases in Hymenolepis and Hydatigera. Exp. Parasit. 14:285-295.

Lemaire, G., and R. Ribère. 1935. Sur la composition chimique de la liquide hydatique. Compt. Rend. Seance. Soc. Biol. 118:1578-1579.

McMahon, P.A. 1961. Phospholipids of larval and adult Taenia taeniaeformis. Exp. Parasit. 11:156-160.

Meerovitch, E. 1965. Studies on the in vitro axenic development of Trichinella spiralis. II. Preliminary experiments on the effects of farnesol, cholesterol, and an insect extract. Can. J. Zool. 43:81-85.

Meyer, F., S. Kimura, and J.F. Mueller. 1966. Lipid metabolism in the larval and adult forms of the tapeworm Spirometra mansonoides. J. Biol. Chem. 241:4224-4232.

Robbins, W.E., J.N. Kaplanis, S.J. Louloudes, and R.E. Monroe. 1960. Utilization of 1^{14}C-acetate in lipid synthesis by adult house flies. Ann. Entomol. Soc. Am. 53:128-129.

Rothman, A.H. 1959. Studies on the excystment of tapeworms. Exp. Parasit. 8:336-364.

Schmialek, P. 1961. Die Identifizierung zweier im Tenebriokot und in Hefe vorkommender Substanzen mit Juvenilhormonwirkung. Z. Naturforsch. 16b:461-464.

Schmialek, P. 1963. Über die Bildung von Juvenilhormonen in Wildseidenspinnern. Z. Naturforsch. 18b:462-465.

Schneiderman, H.A., L.I. Gilbert, and M. Weinstein. 1960. Juvenile hormone activity in micro-organisms and plants. Nature 188:1041-1042.

Schneiderman, H.A., and L.I. Gilbert. 1964. Control of growth and development in insects. Science 143:325-333.

Snyder, F., and N. Stephens. 1962. Quantitative carbon-14 and tritium assay of thin-layer chromatography plates. Anal. Biochem. 4:128-131.

Staple, E. 1963. The biosynthesis of sterols. pp. 155-181. In Bernfeld, P. (ed.) Biogenesis of Natural Compounds. Pergamon Press, London.

Thompson, M.J., E. Mosettig, and T. von Brand. 1960. Unsaponifiable lipids of Taenia taeniaeformis and Moniezia sp. Exp. Parasit. 9:127-130.

Waldi, D. 1965. Spray reagents for thin-layer chromatography. pp. 485-502. In Stahl, E. (ed.) Thin Layer Chromatography, a Laboratory Handbook. Springer-Verlag, Berlin.

Wigglesworth, V.B. 1961. Some observations on the juvenile hormone effect of farnesol in Rhodnius prolixus Stål. J. Insect Physiol. 7:73-78.

Wigglesworth, V.B. 1962. Endocrine regulation during development. I. Hormones in relation to metamorphosis. Gen. Comp. Endocrinol., Suppl. 1:316-321.

Williams, C.M. 1956. The juvenile hormone of insects. Nature 178:212-213.