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Ecdysteroids in Axenically Propagated *Caenorhabditis elegans* and Culture Medium[1]

D. J. CHITWOOD[2] AND M. F. FELDLAUFER[3]

Abstract: Ecdysteroids (insect molting hormones) from *Caenorhabditis elegans* were chromatographically purified and quantified by radioimmunoassay. Nematodes from semidefined medium contained the immunoreactive equivalent of 460 pg ecdysone per gram dry weight. Culture medium, however, contained the immunoreactive equivalent of 68 times the quantity within the nematodes. In a defined medium lacking immunoreactivity, *C. elegans* contained 520 pg ecdysone equivalents per gram dry weight but reproduced slowly. Reproduction of *C. elegans* in defined medium was enhanced by formulation in agar. Propagation of *C. elegans* in either agar-based or aqueous defined medium supplemented with [^{14}C]cholesterol of high specific activity failed to result in production of radiolabeled free ecdysteroids or polar or apolar ecdysteroid conjugates. Failure to demonstrate ecdysteroid biosynthesis in *C. elegans* raises questions about the ecdysteroids identified previously in nematodes being products of endogenous biosynthesis, a necessary condition for these compounds to be nematode hormones.

Key words: Caenorhabditis elegans, culture, ecdysteroid, hormone, steroid.

Molting and certain other developmental processes in insects are regulated by the hormones termed ecdysteroids (16), which are polyhydroxylated steroids with a keto group at C-6 and a double bond at C-7. Because nematodes also molt, it has long been speculated that ecdysteroids function similarly in nematodes and insects. The two general approaches involved in investigation of the biochemical and physiological roles of these compounds within nematodes have been reviewed thoroughly (1,4,8,12,21,24,25). In the first approach, nematodes are cultured in, or treated with, solutions containing specific ecdysteroids. These experiments have yielded interesting biological responses, including stimulation of molting (2,11) and reinitiation of oocyte meiosis (1). Although such experiments have demonstrated that exogenous ecdysteroids can affect nematode development, many factors can obscure the interpretation of these experiments (4).

The second approach is direct analysis of nematodes for endogenous ecdysteroids. The identification of ecdysteroids from nematodes has been reported in at least 13 species, predominantly animal parasites (12). Analysis of nematode ecdysteroids usually has relied upon radioimmunoassay (RIA), a relatively nonspecific technique often accompanied by cross-reactivity problems. The combination of high performance liquid chromatography (HPLC) with radioimmunoassay and gas chromatography-mass spectrometry (GC-MS) in the selected ion monitoring (SIM) mode has demonstrated clearly that ecdysteroids occur in *Anisakis simplex, Ascaris suum, Caenorhabditis elegans,* and *Dirofilaria immitis* (6,9,20,23). Because ecdysteroids are common constituents of higher plants (13), one must consider the possibility that ecdysteroids isolated from nematodes originated from the diet of the nematodes or their hosts. Such contributions have been evaluated infrequently, however.

Because they are easily propagated in artificial media, free-living or microbivorous nematodes would seem to be the most suitable nematodes for experiments to determine whether nematodes are capable of ecdysteroid biosynthesis. The purpose of this investigation was to isolate ecdysteroids from *Caenorhabditis elegans* and de-

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[2] Zoologist, Nematology Laboratory, and [3]Research Entomologist, Insect Hormone Laboratory, USDA ARS, Building 467, Beltsville Agricultural Research Center, Beltsville, MD 20705.

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termine if they were the products of endogenous biosynthesis.

Materials and Methods

Culture methods: Caenorhabditis elegans (Maupas, 1900) Dougherty, 1953 var. Bristol strain N2 was propagated initially at 22 C in a semidefined aqueous medium containing yeast extract (cat. no. 0127-01, Difco Laboratories, Detroit, MI; 30 mg/ml), soy peptone (cat. no. P-0521, Sigma Chemical Co., St. Louis, MO; 30 mg/ml), glucose (cat. no. G-5000, Sigma; 10 mg/ml), hemoglobin (cat. no. H-2500, Sigma; 0.5 mg/ml), cholesterol (> 99% purity by GC; 10 μg/ml), Tween 80 (cat. no. P-1754, Sigma; 0.5 mg/ml), and silicone Antifoam C emulsion (cat. no. A-8011, Sigma; 0.2 μl/ml). The yeast extract and glucose were extracted with chloroform : methanol 2:1 (v: v) and the hemoglobin with dichloromethane to remove endogenous sterol contaminants. Subsequent experiments involved a commercially available defined medium, *Caenorhabditis briggsae* maintenance medium (CbMM) (30), or CbMM formulated in the authors' laboratory and lacking pyridoxamine, pantetheine, calcium folinate, and niacinamide. Defined media were also supplemented with hemoglobin, cholesterol, and Tween 80 in identical concentrations as in the semidefined medium. In general, 3-liter cultures in 4-liter side-arm flasks were employed, with air being pumped through a filter unit (0.22-μm-pore d) and then through a stainless steel HPLC pump inlet filter (5-μm-pore d) to vigorously aerate the cultures. Nematodes from late logarithmic phase cultures were isolated by centrifugation and flotation on 30% sucrose, were rinsed five times with distilled water, and were immediately lyophilized.

Because reproduction in either of the defined media was inferior to that in semidefined medium, in some experiments the defined medium was solidified by addition of 1.5% agar (Bacto agar, Difco) previously extracted with methanol : water 3:1, methanol, and chloroform to remove any contaminating sterols and ecdysteroids. No antifoaming agent was added to solid medium. Two liters of solid medium were poured into 10-cm-d culture dishes, and nematodes were subsequently rinsed from the upper surface of the agar (where they accumulated in great numbers) and were cleaned as described in the previous paragraph.

In some experiments with semidefined and liquid and solidified defined media, cultures were supplemented with [^{14}C]cholesterol of high specific activity (see "radiochemicals"). Only 1 μg of the sterol was added per milliliter of medium in order to maximize the percentage of exogenous sterol taken up by nematodes.

Analysis of free ecdysteroids: Lyophilized nematodes were extracted twice with methanol : water 7:3 and then twice with methanol to produce a crude ecdysteroid extract. This extract was partitioned between countersaturated hexane and 70% aqueous methanol; the dried methanolic residue was then partitioned between countersaturated *n*-butanol and water (5). The butanolic residue was fractionated by passage through a 5.0-g, 1.0-cm-i.d. column of silica gel 60 (70–230 mesh, E. Merck, Darmstadt); compounds were eluted with 50-ml portions of chloroform; 5, 10, 25, and 40% ethanol (95%) in chloroform; and methanol. The dried 25% ethanolic fraction, containing putative free ecdysteroids, was applied in 5 ml 10% methanol in water to a C_{18} SepPak (Waters Associates, Milford, MA) previously conditioned with 5-ml volumes of methanol, water, methanol, water, and 10% methanol. Five milliliters 30% methanol, 6 ml 60% methanol, and 5 ml methanol were applied to the SepPak. Because free ecdysteroids elute in the 60% methanol fraction (31), this fraction is subsequently referred to as the putative free ecdysteroid fraction. Free ecdysteroids were then quantified by RIA (10). All results are expressed as ecdysone equivalents, i.e., units of immunoreactivity equal to that of a unit quantity of ecdysone. As a precautionary step to safeguard against unexpected chromatographic behavior of free ecdysteroids from nematodes, the 40% ethanolic eluate from

the silica column was also fractionated on a C_{18} SepPak, and all C_{18} SepPak fractions were analyzed by RIA.

In some cases, ecdysteroid fractions were analyzed by reversed phase HPLC with a Spectra-Physics model SP8700XR solvent delivery system pumping methanol : water 38:62 at 1.0 ml/minute through a Shandon C_{18} column (ODS-Hypersil, 25 cm × 4.6 mm i.d., 5 μm particles) at 30 C. Absorbance at 254 nm was monitored with a Waters model 441 detector. In radiotracer studies, 1.0-ml aliquots of the eluate were added to 5 ml Hydrofluor (National Diagnostics, Manville, NJ) and analyzed by liquid scintillation counting (LSC) in a Beckman model LS 5801 instrument with quench determinations by the external H-number method.

In a few radiotracer experiments, some C_{18} SepPak fractions were further purified by normal phase HPLC on a Rainin silica column (15 cm × 4.6 mm i.d., 5 μm particles) with a solvent system of dichloromethane : isopropanol : water 125:30:2 at 30 C. The effluent was collected in 1-ml fractions and 5 ml scintillation cocktail (0.5% PPO and 0.03% dimethyl POPOP in toluene) was added. After analysis by LSC, solvents were removed with a rotoevaporator. The residues were transferred with three 5-ml volumes of chloroform to a silica SepPak previously conditioned with 5 ml chloroform. The compounds of interest were separated from the scintillants by elution of the SepPak with an additional 10 ml chloroform, 7-ml aliquots of 10, 25, and 40% ethanol in chloroform, and 7 ml methanol. The 25% ethanol fractions were then analyzed by C_{18} HPLC as described.

Some chromatographic separations were monitored by thin-layer chromatography (TLC) on high performance silica gel 60 chromatoplates (E. Merck, Darmstadt, Federal Republic of Germany) developed with a solvent system of chloroform : 95% ethanol 70:30. In radiotracer experiments, areas from developed chromatoplates were scraped into scintillation vials, 100 μl water and 5 ml Hydrofluor were added, and the vials were analyzed by LSC.

Analysis of free ecdysteroids from media: Six grams of each component of the semidefined medium was dissolved in 60 ml water in a 500-ml separatory funnel, and 140 ml methanol and 200 ml hexane were added to create a hexane–70% methanol partition. Partitions were performed with unextracted medium components, except for yeast extract, in which case the chloroform : methanol-extracted material was used. After five transfers across three funnels, the methanolic phases were combined, and the dried residue was partitioned between *n*-butanol and water, chromatographed on silica columns and C_{18} SepPaks, and analyzed by RIA as described in the previous section. In addition to the usual medium components, we similarly analyzed Difco Bacto-peptone, tryptose, and brain–heart infusion. For defined media and preparations of the entire semidefined medium, the hexane–70% methanol partitions were achieved after initial concentration of 200–1,000 ml medium into 60 ml with a rotoevaporator; the remaining partitions and chromatographic purifications were as described in the previous section.

The C_{18} SepPak fraction containing putative free ecdysteroids from the Bacto-peptone was treated with a protease (from *Streptomyces griseus,* 6 units/mg, cat. no. P-5147, Sigma) in order to determine the specificity of the immunoreactivity. Reaction mixtures contained the peptone substrate and 1.0 mg enzyme in 1.0 ml 0.1 M Tris buffer, pH 7.5, and were incubated at 40 C for 5 hours with vortexing every 30 minutes, at which time the reaction mixtures were frozen in order to stop proteolytic hydrolysis. Controls consisted of enzyme alone or enzyme plus 2.0 ng ecdysone or enzyme plus peptone substrate plus 2 ng ecdysone. The products were purified via C_{18} SepPak chromatography and analyzed by RIA as described.

Analysis of polar ecdysteroid conjugates: The aqueous phase from the butanol-water partition of nematode extracts, which would contain polar ecdysteroid conjugates (29), was applied to a C_{18} SepPak (conditioned

as described in the section on analysis of free ecdysteroids) in 5 ml water and fractionated with 10 ml 10% methanol in water, 10 ml 30% methanol, and 5-ml aliquots of 40, 60, and 100% methanol. To the 30 and 40% methanol fractions were added 6.0 ml 0.2 M NaCl and 6.0 ml 0.2 M sodium acetate buffer, pH 5.0, containing 3.5 mg *Helix pomatia* β-glucuronidase (cat. no. G-0751, Sigma; 407 units/mg), 3.5 mg *Patella vulgata* β-glucuronidase (cat. no. G-8132, Sigma; 1,500 units/mg), 2.0 mg almond β-glucosidase (cat. no. G-8625, Sigma; 5.5 units/mg), and 2.5 mg yeast α-glucosidase (cat. no. G-5003, Sigma; 5.6 units/mg) (29). After incubation for 48 hours at 30 C with occasional vortexing, the solution was partitioned across equal volumes (12 ml) of countersaturated *n*-butanol and water, with five transfers of the upper, butanolic phase across three centrifuge tubes containing butanol-saturated water (29). The dried butanolic residue was repartitioned across equal volumes of countersaturated hexane and 70% methanol by identical procedures. Putative free ecdysteroids released from conjugates by enzymic hydrolysis were purified by chromatography on a C_{18} SepPak and C_{18} HPLC as described for free ecdysteroids.

Analysis of acyl esters of ecdysteroids: Ecdysteroid acyl esters occur in the methanolic eluates from the C_{18} SepPaks to which are applied the 25 and 40% ethanolic eluates from the silica column (26). The dried methanolic residues from *C. elegans* were dissolved in 0.5 ml 0.1 M potassium borate buffer (pH 8.0), and 2.3 mg (five units) *Pseudomonas fluorescens* cholesterol esterase (cat. no. C-9281, Sigma) and 6.3 μl (10 units) pork liver esterase (cat. no. E-31281, Sigma) were added to each sample. After incubation for 24 hours at 37 C, 2 ml 95% ethanol was added to denature the enzymes, and the mixtures were extracted after addition of 4 ml methanol, vortexing, and centrifugation. Extraction with methanol was repeated three times. The combined methanolic extracts from each sample were dried and applied to C_{18} SepPaks as described for free ecdysteroids.

Spent media: Liquid media in which *C. elegans* had been cultured previously was analyzed in order to detect possible secretion or excretion of free ecdysteroids. After centrifugation had removed most of the nematodes from the spent medium, remaining nematodes and eggs were removed by passing the medium through a sintered glass funnel. The culture flask and all glassware subsequently in contact with nematode suspensions before lyophilization were rinsed with methanol, which was filtered and added to the spent medium in order to guard against the unlikely adsorption of released ecdysteroids to the glass surfaces. The equivalent of 700 ml of spent medium was concentrated to 200 ml with a rotoevaporator and extracted four times with 200 ml *n*-butanol saturated with water. The combined butanolic layers were then repartitioned between countersaturated butanol and water (15-ml layers, five transfers across three centrifuge tubes). The butanolic phase was chromatographed as described for free ecdysteroids.

Radiochemicals: The 4-[^{14}C]cholesterol (Amersham, Arlington Heights, IL) was purified by C_{18} HPLC with a solvent of 2% water in methanol flowing at 1.0 ml/minute and was used at a specific activity of 61 mCi/mmol. Following purification, no impurities were detected by HPLC or GLC. The 4-[^{14}C]22,25-dideoxyecdysone was prepared as previously described (28) and was determined to be 97.7% pure by thin-layer chromatography (TLC). It was used at a specific activity of 0.40 mCi/mmol and was added to aqueous defined medium at 2.94 μg/ml. The 23,24-[^{3}H]ecdysone, a gift of Zoecon Corporation (Palo Alto, CA), was purified by reversed phase HPLC and was used at a specific activity of 55–60 Ci/mMole. To ensure that ecdysteroids were not being destroyed during analysis nor migrating unexpectedly as a result of lipids contained within nematode extracts, 70,000 dpm (490–530 pg) tritiated ecdysone was added to the tissue homogenizer during the initial extraction of one harvest of nematodes. Radioactivity was measured in all fractions generated during the usual

partitions and chromatographic fractionations, including HPLC separations.

Results

Semidefined medium: In semidefined medium, the free ecdysteroid fraction from *C. elegans* contained the immunoreactive equivalent of 0.46 ng ecdysone per gram nematode dry weight. Analysis of medium, however, revealed that 31.2 ng ecdysone equivalents occurred in the 544-ml volume of medium necessary to produce 1 g dry weight of *C. elegans.* When individual medium components were similarly analyzed, the soy peptone and yeast extract contained 2.03 and 0.14 ng ecdysone equivalents per gram, respectively, whereas glucose, hemoglobin, cholesterol, Tween 80, and agar were not RIA-positive. In attempts to discover proteinaceous medium components devoid of RIA activity, 1.82, 1.31, and 0.82 ng ecdysone equivalents per gram of tryptose, Bacto-peptone and brain-heart infusion, respectively, were detected by RIA of the appropriate C_{18} SepPak fractions. Protease treatment of the Bacto-peptone fraction containing putative free ecdysteroids did not reduce its immunoreactivity, nor did such treatment reduce the immunoreactivity of ecdysone.

When a solvent blank was identically evaporated, chromatographed, and analyzed by RIA, no immunoreactivity was detected. When the tritiated ecdysone was added to the nematodes during extraction, its subsequent chromatographic behavior was the same as that of pure ecdysone. The final recovery of radiolabel following the final silica HPLC analysis was 84.9%.

Defined media: Because of the large amounts of immunoreactivity in the semidefined medium, defined media were examined for immunoreactivity. A 200-ml volume of commercially available CbMM contained 1.86 ng ecdysone equivalents by RIA. An equivalent batch of modified CbMM formulated in our laboratory contained 2.49 ng ecdysone equivalents, 1.73 ng of which had the same retention time as ecdysone during C_{18} HPLC. When the modified CbMM was prepared from newly purchased, reagent grade chemicals, however, the putative free ecdysteroid fraction was devoid of immunoreactivity, with a threshold of detection of 0.10 ng ecdysone equivalents per liter, approximately the volume (1,040 ml) from which 1 g dry weight of *C. elegans* was readily obtained. One gram of *C. elegans* from this medium contained the immunoreactive equivalent of 0.52 ng ecdysone in its C_{18} SepPak fraction; although reversed phase HPLC fractions of this material were RIA-negative.

Caenorhabditis elegans reproduced very rapidly on defined medium when it was solidified with agar; 1 g dry weight of *C. elegans* was readily obtained from 564 ml of the solid medium and possessed 0.59 ng ecdysone equivalents in the appropriate C_{18} SepPak fraction. Activity equivalent to 0.53 ng ecdysone occurred in the corresponding fraction obtained from hydrolyzed ecdysteroid polar conjugates from 1 g *C. elegans.* The analogous fractions from enzyme blanks were devoid of immunoreactivity.

Media supplemented with [^{14}C]cholesterol: Caenorhabditis elegans from 400 ml semidefined medium supplemented with [^{14}C]cholesterol contained 21.6×10^6 dpm. The 60% methanolic eluate from the C_{18} SepPak contained 5,700 dpm, but neither TLC/LSC nor C_{18} HPLC detected any material comigrating with ecdysone or 20-hydroxyecdysone or other ecdysteroids. Similarly, when the putative polar ecdysteroid conjugates were hydrolyzed, the corresponding eluate from the C_{18} SepPak contained 440 dpm, but none of the radioactivity migrated during TLC or C_{18} HPLC like free ecdysteroids. The free ecdysteroid fraction prepared from spent medium did not contain free ecdysteroids when analyzed by C_{18} HPLC.

Nematodes from 700 ml liquid defined medium supplemented with radiolabeled cholesterol contained 54.8×10^6 dpm. The 60% methanolic C_{18} SepPak fraction contained 441,000 dpm, but none of the radiolabeled components behaved similarly to free ecdysteroids during TLC or C_{18} HPLC. No radioactivity was present in the analogous C_{18} SepPak fraction obtained

from hydrolyzates of the putative polar ecdysteroid conjugates from nematodes propagated in this medium. When the spent medium used for propagating these nematodes was analyzed, 13,900 dpm occurred in the C_{18} SepPak fraction possibly containing free ecdysteroids. Of this radioactivity, 3,300 dpm comigrated with ecdysone or 20-hydroxyecdysone during TLC; but C_{18} HPLC indicated that free ecdysteroids were absent; all of the injected radioactivity emerged with the solvent front or was highly apolar.

Extracts from *C. elegans* propagated in 2 liters solidified defined medium contained 232.6×10^6 dpm, of which 71,700 dpm emerged in the putative ecdysteroid-containing eluate from the C_{18} SepPak. When this material was purified by normal phase HPLC, fractions with retention times corresponding to ecdysone and 20-hydroxyecdysone contained 272 and 140 dpm, respectively. After the radiolabeled components were separated from the scintillants by silica SepPak chromatography and were analyzed by C_{18} HPLC, no radioactivity emerged in fractions corresponding to ecdysone or 20-hydroxyecdysone. Identical analysis of several silica HPLC fractions bordering ecdysone and 20-hydroxyecdysone also failed to produce any radiolabeled peaks during C_{18} HPLC; the radiolabeled components emerged either near the solvent front or were eluted only when a greater percentage of methanol was pumped through the column. When the putative ecdysteroid polar conjugates were combined and hydrolyzed, 16,100 dpm occurred in the subsequent C_{18} SepPak fraction that would have contained liberated free ecdysteroids. Analysis of this fraction by C_{18} HPLC revealed that all of its radioactivity was associated with material much more apolar than ecdysone. Similarly, when the putative ecdysteroid acyl esters were hydrolyzed, no radioactive free ecdysteroids were detected by C_{18} HPLC.

22,25-Dideoxyecdysone: When *C. elegans* propagated in aqueous defined medium supplemented with 100 μg/ml 22,25-dideoxyecdysone, nematodes reproduced much slower than in similar medium containing 0, 1.0, or 10 μg/ml of the compound. No inhibition was apparent at 10 μg/ml. When nematodes were propagated in semidefined medium supplemented with 1.29×10^6 dpm [^{14}C]22,25-dideoxyecdysone (2.94 μg/ml), their combined methanolic extracts contained 359,000 dpm. The C_{18} SepPak fraction that would have contained free ecdysteroids was devoid of radioactivity. Analysis of all fractions generated during ecdysteroid purification by TLC/LSC did not reveal substantial amounts of radiolabeled compounds other than the original 22,25-dideoxyecdysone. Similarly, spent medium did not contain free ecdysteroids or substantial quantities of other compounds produced by nematodes from the supplemented 22,25-dideoxyecdysone.

Discussion

Caenorhabditis elegans was cultured initially in a semidefined medium because it is ideal for rapid, inexpensive axenic propagation of *C. elegans* in a manner suitable for investigation of nematode biochemistry. *Caenorhabditis elegans* from semidefined medium contained the immunoreactive equivalent of 0.46 ng ecdysone, a quantity similar to the 0.47 ng reported in *C. elegans* propagated on *Escherichia coli*; the bacterium contained 0.59 ng per gram dry weight (23). Because nematode-free semidefined medium contained 68 times the immunoreactivity found in the nematodes produced in it, this medium was judged inappropriate for further comparison of ecdysteroids from nematodes and medium. Analysis of individual medium components revealed that substantial immunoreactivity occurred in the yeast extract and soy peptone. In an attempt to replace the yeast extract and soy peptone with ecdysteroid-free materials, tryptose, mammalian peptone, and brain–heart infusion were analyzed, but each contained substantial immunoreactivity.

Identification of the immunoreactive components within the medium ingredients was beyond the scope of our investi-

gation. Protease treatment of the material purified from the mammalian peptone did not decrease its immunoreactivity and thereby failed to show that the binding during RIA was nonspecific. Most mammalian tissues have not been examined for existence of ecdysteroids. Recent interest in the possible diagnosis of parasitic infections in humans by quantification of possible ecdysteroids released by nematodes into mammalian sera or urine has resulted in detection of ecdysteroids in sera of uninfected mammals (1).

When radiolabeled cholesterol of high specific activity was added to the semidefined medium, no radiolabeled free ecdysteroids or ecdysteroids hydrolyzed from polar conjugates were detected in *C. elegans*. This was not definitive evidence that *C. elegans* is incapable of ecdysteroid biosynthesis because the potentially high ecdysteroid concentrations in the medium could have caused product inhibition of the biosynthetic pathway.

Failure to develop a satisfactory semidefined medium resulted in investigation of the suitability of defined media for investigation of nematode ecdysteroid biochemistry. A modified CbMM formulated in our laboratory contained the immunoreactive equivalent of less than 0.1 ng ecdysone per liter. The putative free ecdysteroid fraction from *C. elegans* contained the same level of immunoreactivity as occurred in the homologous fraction from nematodes grown in semidefined medium. When identical experiments were performed with cultures supplemented with radiolabeled cholesterol, however, no radioactive free ecdysteroids or ecdysteroids liberated by hydrolysis of polar ecdysteroid conjugates were detected. Failure to detect radiolabeled ecdysteroids in these experiments was not conclusive evidence for lack of ecdysteroid biosynthesis because reproduction of *C. elegans* in this medium was much slower than in semidefined medium.

Previously, we had been investigating various axenic culture systems for the evaluation of inhibitors of steroid metabolism on *C. elegans*. Historically, *C. elegans* has been propagated monoxenically on *E. coli* (3) or axenically in aqueous media. One disadvantage of aqueous media is that free-living nematodes move very rapidly in it and are difficult to observe and count. When semidefined medium containing 3% yeast extract, 3% soy peptone, 1% glucose, 0.05% hemoglobin, 10 μg/ml sterol, and 0.5 mg/ml Tween 80 is solidified by the addition of agar, the result is a medium that supports high nematode reproduction (Chitwood, unpubl.). As in monoxenic culture on lawns of *E. coli*, *C. elegans* moves slow enough to be closely observed, is readily manipulated, and accumulates on the upper surface of the agar (Chitwood, unpubl.). Because this culture system is simple and inexpensive and does not require maintenance of separate bacterial cultures, it would seem to be a useful substitute for monoxenic cultures in many cases.

Caenorhabditis elegans reproduced very rapidly when the defined medium was solidified by agar that had been extracted to remove any possible contaminating ecdysteroids and was RIA-negative. The free ecdysteroid fraction from nematodes from this medium contained approximately the same level of ecdysone equivalents as found in similar fractions from *C. elegans* from semidefined or liquid defined medium. In addition, polar conjugates appeared to contain about the same amount of free ecdysteroid when hydrolyzed. When radiolabeled cholesterol was used in this medium, however, no radiolabeled free ecdysteroids or ecdysteroids hydrolyzed from polar conjugates were isolated from the nematodes.

The nature of the immunoreactive material in *C. elegans* was not further characterized. Possible explanations include the concentration by *C. elegans* of trace quantities of ecdysteroids occurring in the medium at levels below the threshold of detection, the hydrolysis and subsequent uptake by *C. elegans* of polar or apolar ecdysteroid conjugates in the medium, and a possible nonspecific nature of the immunoreactivity. The material was not pro-

duced artifactually because solvent blanks chromatographically fractionated identical to nematodes were devoid of immunoreactivity. Regardless, our experiments that failed to demonstrate biosynthesis of ecdysone or 20-hydroxyecdysone from radiolabeled cholesterol clearly indicate that *C. elegans* does not biosynthesize ecdysone or 20-hydroxyecdysone in quantities sufficient to account for the amounts reported to occur in this and many other species of nematodes (12). Similarly, our radiotracer experiments in *C. elegans* failed to demonstrate endogenous biosynthesis of polar or apolar ecdysteroid conjugates. Of course, *C. elegans* may biosynthesize ecdysteroids of such greatly different polarity from that of ecdysone or 20-hydroxyecdysone that they would not have been detected by the HPLC systems employed. The possibility of the existence of specific pools of cholesterol for ecdysteroid biosynthesis in *C. elegans* was considered; for this reason, the nematodes used as inoculum in experiments with radiolabeled cholesterol were cultured in identical medium for at least three generations. Still, *C. elegans* may biosynthesize ecdysteroids, but in quantities lower than the 0.02 ng/g dry weight threshold of detection in the experiments with [^{14}C]cholesterol. The lowest level of ecdysteroids reported previously in nematodes is 0.37 ng/g dry weight in dauer juveniles of *C. elegans* (23). Another possibility is that animal-parasitic nematodes synthesize greater quantities of hormones than microbivorous nematodes because development of the animal parasites may depend more upon external stimuli and may be under greater hormonal control.

Although unlikely, a nutritional requirement for ecdysteroids in nematodes is possible; failure to detect cytochrome P-450 in *Dirofilaria immitis* adults led Comley (7) to question whether the cytochrome P-450-mediated hydroxylations involved in insect ecdysteroid biosynthesis occurred in *D. immitis* adults. Interestingly, Mercer et al. (23) did not detect hydroxylation of radiolabeled ecdysone in monoxenically propagated *C. elegans.*

Radiolabeled 22,25-dideoxyecdysone was added to nematode cultures to determine if *C. elegans* can hydroxylate steroids at C-22 and C-25, two metabolic steps required for ecdysone biosynthesis. The resultant failure to isolate radiolabeled ecdysone or other hydroxylated metabolites from *C. elegans* could be regarded as further evidence of lack of ecdysteroid biosynthesis in nematodes; but one must recognize that some insects do not significantly convert 22,25-dideoxyecdysone to ecdysone, although substantial hydroxylation occurs (14,15,28).

Preliminary analysis of ecdysteroids by RIA of sera or urine from nematode-infected mammals indicated that ecdysteroids occur in higher concentrations in these fluids than in corresponding fluids from uninfected animals (17–19) and possess diagnostic value (17,18). It has been speculated that higher ecdysteroid concentrations in sera or urine of infected mammals could result from ecdysteroid excretion or secretion by nematodes (17,18). Although initially promising, the present outlook for diagnosis of mammalian helminth infections by ecdysteroid analysis of sera or urine is less encouraging because of the abundance of falsely negative results in such assays (27) and questions about the source of the elevated ecdysteroid levels (1). Direct evidence for secretion or excretion of ecdysteroids endogenously biosynthesized by nematodes is lacking. Indirect evidence occurs in *Onchocerca gibsoni*-infected cattle, where nodule tissue immediately next to the nematodes contains much greater levels of free ecdysteroids than peripheral nodule tissues or the parasites (22). Our radiotracer experiments failed to demonstrate that *C. elegans* releases free ecdysteroids or apolar conjugates into its culture medium.

In summary, *C. elegans* does not biosynthesize ecdysteroids in the quantities reported previously in this and other species of nematodes. Because demonstration of endogenous biosynthesis is crucial for establishment of a classic hormonal role for ecdysteroids in nematodes, our results in-

dicate that widely held assumptions about the likely hormonal role for these compounds in nematodes should be accompanied by a modest amount of skepticism. Regardless, the often powerful biological effects of exogenous ecdysteroids on nematodes warrant continued investigation of nematode ecdysteroid biochemistry.

Literature Cited

1. Barker, G. C., D. J. Chitwood, and H. H. Rees. 1990. Ecdysteroids in helminths and annelids. Invertebrate Reproduction and Development 17:1–11.

2. Barker, G. C., J. G. Mercer, H. H. Rees, and R. E. Howells. 1990. Functional roles of ecdysteroids in the filarial nematodes *Dirofilaria immitis* and *Brugia pahangi*. Invertebrate Reproduction and Development 17:103–104 (Abstr.).

3. Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. Genetics 77:71–94.

4. Chitwood, D. J. 1987. Inhibition of steroid or hormone metabolism or action in nematodes. Pp. 122–130 *in* J. A. Veech and D. W. Dickson, eds. Vistas on nematology. Society of Nematologists.

5. Chitwood, D. J., M. A. McClure, M. F. Feldlaufer, W. R. Lusby, and J. E. Oliver. 1987. Sterol composition and ecdysteroid content of eggs of the root-knot nematodes *Meloidogyne incognita* and *M. arenaria*. Journal of Nematology 19:352–360.

6. Cleator, M., C. J. Delves, R. E. Howells, and H. H. Rees. 1987. Identity and tissue localization of free and conjugated ecdysteroids in adults of *Dirofilaria immitis* and *Ascaris suum*. Molecular and Biochemical Parasitology 25:93–105.

7. Comley, J. C. W. 1985. Isoprenoid biosynthesis in filariae. Tropical Medicine and Parasitology 36 (supplement):10–14.

8. Davey, K. G. 1988. Endocrinology of nematodes. Pp. 63–86 *in* H. Laufer, ed. Endocrinology of selected invertebrate types. New York: Alan R. Liss.

9. Evershed, R. P., J. G. Mercer, and H. H. Rees. 1987. Capillary gas chromatography–mass spectrometry of ecdysteroids. Journal of Chromatography 390: 357–369.

10. Feldlaufer, M. F., W. R. Lusby, J. A. Svoboda, and M. J. Thompson. 1984. Identification of 20-hydroxyecdysone from the pupa of the gypsy moth *Lymantria dispar*. Archives of Insect Biochemistry and Physiology 1:323–330.

11. Fleming, M. W. 1985. *Ascaris suum*: Role of ecdysteroids in molting. Experimental Parasitology 60:207–210.

12. Franke, S., and G. Käuser. 1989. Occurrence and hormonal role of ecdysteroids in non-arthropods. Pp. 296–307 *in* J. Koolman, ed. Ecdysone. Stuttgart: Georg Thieme Verlag.

13. Horn, D. H. S., and R. Bergamasco. 1985. Chemistry of ecdysteroids. Pp. 185–248 *in* G. A. Kerkut and L. I. Gilbert, eds. Comprehensive insect physiology, biochemistry, and pharmacology, vol. 7. Oxford: Pergamon Press.

14. Kaplanis, J. N., S. R. Dutky, W. E. Robbins, and M. J. Thompson. 1974. The metabolism of the synthetic ecdysone analog, 1α-^{3}H-22,25-bisdeoxyecdysone in relation to certain of its biological effects in insects. Pp. 161–175 *in* W. J. Burdette, ed. Invertebrate endocrinology and hormonal heterophylly. New York: Springer-Verlag.

15. Kaplanis, J. N., M. J. Thompson, S. R. Dutky, W. E. Robbins, and E. L. Lindquist. 1972. The metabolism of 4-^{14}C-22,25-bisdeoxyecdysone during larval development in the tobacco hornworm, *Manduca sexta* (L.). Steroids 20:105–120.

16. Koolman, J. 1989. Ecdysone. Stuttgart: Georg Thieme Verlag.

17. Koolman, J., and H. Moeller. 1986. Diagnosis of major helminth infections by RIA detection of ecdysteroids in urine and serum. Insect Biochemistry 16:287–291.

18. Koolman, J., J. Walter, and H. Zahner. 1984. Ecdysteroids in helminths. Pp. 323–330 *in* J. Hoffmann and M. Porchet, eds. Biosynthesis, metabolism and mode of action of invertebrate hormones. Berlin: Springer-Verlag.

19. Lansoud-Soukate, J., A. Dupont, M. L. De Reggi, G. E. Roelants, and A. Capron. 1989. Hypogonadism and ecdysteroid production in *Loa loa* and *Mansonella perstans* filariasis. Acta Tropica 46:249–256.

20. Mendis, A. H. W., M. E. Rose, H. H. Rees, and T. W. Goodwin. 1983. Ecdysteroids in adults of the nematode, *Dirofilaria immitis*. Molecular and Biochemical Parasitology 9:209–226.

21. Mercer, J. G. 1985. Developmental hormones in parasitic helminths. Parasitology Today 1:96–100.

22. Mercer, J. G., G. C. Barker, R. E. Howells, and H. H. Rees. 1989. Analysis of ecdysteroids in *Onchocerca gibsoni* and *Onchocerca volvulus* and nodule tissues. Tropical Medicine and Parasitology 40:434–439.

23. Mercer, J. G., A. E. Munn, and H. H. Rees. 1988. *Caenorhabditis elegans*: Occurrence and metabolism of ecdysteroids in adults and dauer larvae. Comparative Biochemistry and Physiology 90B:261–267.

24. Rees, H. H., and A. H. W. Mendis. 1984. The occurrence and possible significance of ecdysteroids during nematode and cestode development. Pp. 338–345 *in* J. Hoffmann and M. Porchet, eds. Biosynthesis, metabolism and mode of action of invertebrate hormones. Berlin: Springer-Verlag.

25. Rees, H. H., and J. G. Mercer. 1986. Occurrence and fate of parasitic helminth ecdysteroids. Pp. 173–186 *in* M. Porchet, J.-C. Andries, and A. Dhainaut, eds. Advances in invertebrate reproduction, vol. 4. Amsterdam: Elsevier Science Publishers.

26. Russell, G. B., and D. R. Greenwood. 1989. Methods of isolation of ecdysteroids. Pp. 97–105 *in* J. Koolman, ed. Ecdysone. Stuttgart: Georg Thieme Verlag.

27. Simon, P., and J. Koolman. 1989. Ecdysteroids in vertebrates: Pharmacological aspects. Pp. 254–259 *in* J. Koolman, ed. Ecdysone. Stuttgart: Georg Thieme Verlag.

28. Thompson, M. J., E. P. Marks, W. E. Robbins, S. R. Dutky, P. A. Filipi, and H. Finegold. 1978. Isolation and identification of the metabolites of 22,25-dideoxyecdysone from cockroach fat body cultures. Lipids 13:783–790.

29. Thompson, M. J., J. A. Svoboda, and G. F. Weirich. 1984. Ecdysteroids in developing ovaries and eggs of the tobacco hornworm. Steroids 43:333–341.

30. Vanfleteren, J. R. 1980. Nematodes as nutritional models. Pp. 47–79 *in* B. M. Zuckerman, ed. Nematodes as biological models, vol. 2. New York: Academic Press.

31. Weirich, G. F. 1985. Ecdysone 20-monooxygenase. Pp. 454–458 *in* J. H. Law and H. C. Rilling, eds. Methods in enzymology, vol. 111. Steroids and isoprenoids Part B. Orlando: Academic Press.