Growth Inhibition of *Caenorhabditis elegans* and *Panagrellus redivivus* by Selected Mammalian and Insect Hormones

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Abstract: *Caenorhabditis elegans* and *Panagrellus redivivus* were cultured in axenic medium in microwells. The addition of selected steroids and terpenoids to the medium caused quantitative inhibition of numbers of offspring produced per well. Three out of 14 vertebrate sex hormones and analogs, and seven out of 10 insect juvenile hormones and analogs inhibited growth at 25 or 50 micrograms per ml. In addition, two insecticide synergists which mimic juvenile hormones, propyl 2-propynyl phenyl phosphonate and piperonyl butoxide, inhibited growth at 7 μg/ml. Total lipids from *Panagrellus* and from *Nematospiroides dubius* were inhibitory. Separation by silicic acid column chromatography yielded active and inactive portions. We concluded that the inhibition observed was non-specific. *Key Words:* Microwells, Steroids, Sesqui-terpenoids.

Recent data on arthropod endocrines have shown insect hormones and analogues to be valuable in insect control. The discovery that certain plants contain compounds with molecular structure and activity resembling insect hormones suggests that search for parallel systems against nematode parasites might be fruitful (13).

Very little is known about growth and differentiation in nematodes. Nerve cells of certain nematodes exhibit a secretory cycle correlated with molting (3). Hypodermal and gonadal growth abnormalities were induced by exposure of sugarbeet nematode larvae to an exogenous steroid androgen and to insect juvenile hormone mimics (8). Although there is no critical proof of direct effects of mammalian host hormones on animal-parasitic nematodes, there is an ill-defined relation between the host's reproductive cycle and the growth of nematode parasites (4).

The purpose of the studies reported here was to measure the effect of selected mammalian sex hormones, insect hormones, and lipid extracts from nematodes on the growth of free-living nematodes. We report inhibition of growth of two species of free-living nematodes in axenic culture by a number of mammalian sex hormones, insect hormones, and related compounds. In addition, we examined the effects of lipid extracts from two species of nematodes and from other sources.

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MATERIALS AND METHODS

Second-stage larvae of *Panagrellus redivivus* (Linn) T. Goodey or *Caenorhabditis elegans* (Maupas) Dougherty were cultured in groups of two or three, respectively, at 23 C. The medium consisted of 3% soy-peptone, 3% yeast extract, with 10% crude yeast supplement (9). Stock cultures were maintained in 2 ml of medium in 25 × 100-mm screw-cap test tubes.

Microtiter plates of flexible vinyl plastic were sterilized for 12 hr in sealed Mason jars by chlorine gas generated from NaOCl and HCl. Each well received 0.0625 ml of medium. The microtiter plates were subsequently enclosed in sterile plastic dishes sealed with wide rubber bands to reduce evaporation.

Second-stage larvae of *P. redivivus* and of *C. elegans* were collected for assay by filtration of mixed populations through sand (11). Filters were prepared from acid washed sand sieved through a 60-mesh, and caught on an

80-mesh sieve. A 1.8-cm diam glass centrifuge tube (12-ml capacity), drawn into a cone with a 4-mm opening at the bottom, was loosely packed with about 0.5 cm of fiberglass topped with a 2-cm column of dry, sieved sand. The assembly was fitted into a conical screw-capped centrifuge tube and sterilized by autoclaving. Nematode suspensions from axenic cultures were transferred onto the sand and washed into the centrifuge tube with 10 ml of sterile Fenwick's solution (5). When the small larvae which emerged settled to the bottom of the tube, they were transferred by pipette to a well of a microtiter plate. Individual larvae were aseptically dispensed into the microwells by micropipette under 12× magnification in a laminar flow hood.

In each experiment, the effects of three test substances or three concentrations of a single substance were compared with one or two controls on unamended medium. Each treatment and control medium was dispensed to 21 replicate microwells. In the *P. redivivus* series, larval counts were recorded directly; in the *C. elegans* series, a scoring system permitted more rapid collection of data. Wells with no larvae were scored −1; with 1–10 larvae, +1; with 11–20 larvae, +2; and with more than 20 larvae, +3.

Lipids to be tested were dissolved in appropriate solvents (ether, acetone, or ethanol) and added to the medium by one of several methods: (i) addition to the lyophilized soy-peptone-yeast portion; (ii) addition to lyophilized crude yeast supplement; and (iii) addition to liquid soy-peptone-yeast portion. The medium was reconstituted or completed after evaporation of the solvent at 40 C (ether and acetone). Ethanol solvent was removed by desiccation in a lyophilizer. Method (iii) was used for most experiments since it proved to be as effective as the other methods and much simpler.

Vertebrate sex hormones and analogs were donated by J. Fried, Syntex Corp., and insect hormones and analogs by G. B. Staal, Zoecon Corp., both at Palo Alto, California.

Results

Lipid Extract: Unmated female *P. redivivus* contained relatively few oocytes in the uterus, whereas mated females produced eggs which were fertilized as they enter the uterus. Six days after isolation of a pair of larvae, the uteri of mated females contained 50 ± 5 (SE) eggs and embryos compared with 7 ± 1 oocytes in uteri of unmated females. To test whether lipid extracts might stimulate an increase in oocyte production of unmated females, chloroform-methanol and ether extracts of *P. redivivus* were added to culture media containing unmated females. No effect was detected.

Lipid extracts of both *P. redivivus* and *Nematospiroides dubius*, an intestinal parasite of mice, inhibited reproduction of *P. redivivus*. Reproduction failed completely in cultures exposed to 5.76 mg/ml of lipids from *P. redivivus*. An ether extract of *N. dubius* completely blocked reproduction of *P. redivivus* at 0.7 mg/ml and reduced larval production to approximately half that of the controls at a concentration of 0.07 mg/ml. The extracts were not lethal to the assay organisms. Table 1 shows the total scores in a dilution series of lipid extracts assayed on *C. elegans*. Results are comparable to those with *P. redivivus*.

The medium used for cultivation of *P. redivivus* mass cultures was a mixture of oatmeal, yeast and bacteria. Total lipid extracted from this mixture incubated in the absence of nematodes, and lipid from yeast alone were inhibitory to both species of nematodes. We might conclude that lipids in general are toxic to these nematodes. But total lipids extracted from mouse liver were inactive at 0.25 to 0.063 mg/ml. Further,

TABLE 1. Effect of lipid extracts from nematodes and yeast on reproduction of *Caenorhabditis elegans*

Source of extract	Extract conc. (μg/ml)	Total reproduction score[a]	Replicate micro-wells
Panagrellus redivivus	530	−3	28
	265	39	
	132	76	
	0	72	
Nematospiroides dubius	350	−11	21
	70	7	
	35	19	
	0	19	
	0	15	
Yeast	700	−21	21
	350	−18	
	70	−13	
	0	35	
	0	11	

[a] Reproduction scoring system: 0 larvae/well = −1; 1–10 larvae = +1; 11–20 larvae = +2; 21 or more larvae = +3.

some chromatography fractions of *Panagrellus* lipids separated on silicic acid columns were highly active, but other fractions caused no depression of reproduction in bioassays with both species.

MAMMALIAN SEX HORMONES: Table 2 summarizes results of bioassays with *C. elegans* of 14 mammalian sex hormones and analogues. Compound-4 (17 α-acetoxy-6-chloro-pregna-1,4,6-triene-3,20-dione) was the most inhibitory steroid tested. Progesterone inhibited early reproduction, but the nematodes recovered reproductive capacity. Inhibitory properties of some steroids were inversely correlated with the nutritional levels of the medium. The culture fluid consisted of soy-peptone, yeast extract medium to which 10% v/v of crude yeast supplement was added (9). Each batch of crude yeast supplement differed in its ability to support

TABLE 2. Influence of mammalian sex hormones and analogues on growth of *Caenorhabditis elegans.*

Cpd. No.	Compound name	Activity and dosage (μg/ml)	No. of tests[a]
1.	Testosterone	Inactive at 25, 50, 100, 250	2
2.	Progesterone	Inhibitory at 25, 50, 100, 200, 250	2
3.	17α-acetoxy-6-chloro-pregna-4,6-diene-3,20-dione	Inconsistent results	2
4.	17α-acetoxy-6-chloro-pregna-1,4,6-triene-3,20-dione	Inhibitory at 25, 50, 100, 200, 250	2
5.	11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione	Inhibitory at 50, 100; inactive at 25 and 250	1
6.	Estradiol	Inactive at 50, 100, 200, 250	2
7.	2α-methyldihydrotestosterone-17-tetrahydropyranyl ether	Inconsistent results, see Table 3.	2
8.	6α-fluoro-11β,16α-17α-21-tetrahydroxy-pregna-1,4-diene-3,20-dione-16,17 acetonide-21-acetate	Inconsistent results	2
9.	17α-methyl-19-nor-testosterone	Inactive at 50, 100, 200, 250	1
10.	17α-methyl-β-nor-testosterone	Inactive at 50, 100, 200	1
11.	17α-acetoxy-progesterone	Inactive at 25, 50, 100	1
12.	6-fluoro-6-dehydro-17α-acetoxy-progesterone	Inactive at 25, 50, 100	2
13.	6-fluoro-6α,7α-difluoromethylene-17α-acetoxy-progesterone	Inactive at 25, 50, 100	1
14.	6-fluoro-6α,7α-difluoromethylene-16-methylene-17α-acetoxy progesterone	Inactive at 25, 50, 100	1

[a] Each test was a separate experiment with 21 replications per treatment.

TABLE 3. Nutrient level effect on the inhibition of growth of *Caenorhabditis elegans* by mammalian sex hormones and analogues.

Compound[a] No.	Nutrient level[b] and hormone dosage (μg/ml)						
	High nutrient				Low nutrient		
	250	100	50	25	200	100	50
2		–[c]		–	–	–	–
4	–	–	–	–	—	—	—
7	–	0	0	0	—	—	—

[a] Compound-2 = progesterone; Compound-4 = 17α-acetoxy-6-chloro-pregna-1,4,6-triene-3,20-dione; Compound-7 = 2α-methyldihydrotestosterone 17-tetrahydro pyranyl ether.

[b] High nutrient = culture medium in which F_1 larvae developed in 120 hr or less after transfer of 2nd stage larvae. Low nutrient = culture medium in which F_1 larvae did not develop until more than 120 hr after transfer.

[c] – = moderate inhibition; — = strong inhibition; 0 = no effect.

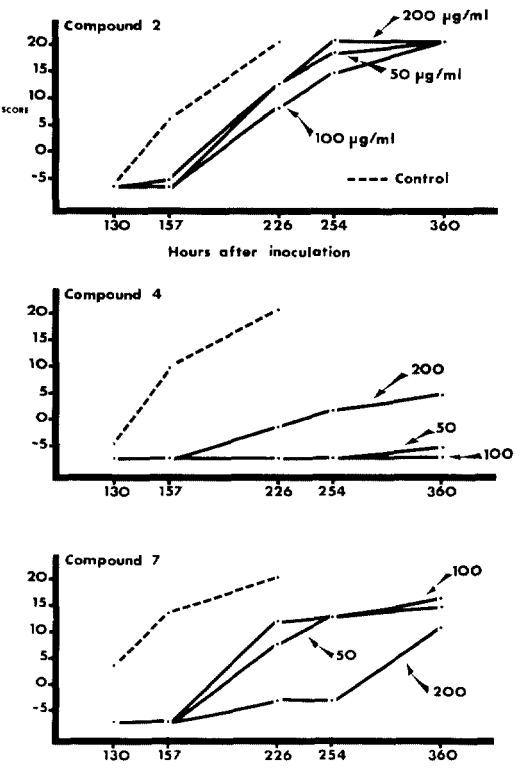

FIG. 1. Inhibition of production of F_1 larvae by *Caenorhabditis elegans* grown in nutrient medium containing vertebrate sex hormones. Compound-2 = progesterone; Compound-4 = 17α-acetoxy-6-chloro-pregna-1,4,6-triene-3,20-dione; Compound-7 = 2α-methyldihydrotestosterone-17-tetrahydropyranyl ether. (Reproduction scoring system: 0 larvae/microwell = –1; 1–10 larvae = +1; 11–20 larvae = +2; >20 larvae = +3.)

nematode growth. F_1 larvae were present in microwells with the best crude yeast supplement at about 90 hr after inoculation of 2nd stage larvae. Such media are classed as high nutrient media. If development to F_1 larvae required longer than 120 hr, the media were classed as low nutrient media. Table 3 shows that Compound-2 was moderately inhibitory at both high and low nutrient levels, but Compounds 4 and 7 were strongly inhibitory at low nutrient levels and much less inhibitory at high levels. Two compounds (3 and 8) were moderately inhibitory in one experiment at 25, 50 and 100 μg/ml but inactive at 25, 100 and 250 in another. Nutrient levels for both assays were high.

Fig. 1 presents plots of larval production in typical bioassays at low nutrient levels. The strongly inhibitory compounds (4 and 7) continued to depress reproduction for more than 7 days after the controls had reached maximum scores of 21 per set of seven wells. The moderately active Compound-2 delayed reproduction about 1 day.

INSECT HORMONES, ANALOGUES AND MIMICS: A mixture of synthetic sesquiterpenoid derivatives which showed a broad spectrum of activity in insect hormone assays ("Juvenile Hormone, synthetic, B-grade," Calbiochemical Corp.) inhibited growth of *C. elegans* at 63 to 250 μg/ml. We therefore examined the inhibitory properties of a series of insect juvenile hormones and analogues. Table 4 summarizes results of this series.

In general, the inhibition by sesquiterpenoids was greater than by the steroids. Three compounds (Nos. 19, 20 and 22) were strongly inhibitory at 50 μg/ml. Fig. 2A shows larval production by *C. elegans* in microwells with nutrient medium containing various concentrations of Compound-22. At 25 μg/ml, reproduction was delayed, but

eventually F_2 larvae were produced. At 50 and 100 μg/ml, no F_2 larvae appeared, and at 100 μg/ml, the production of F_1 larvae was severely curtailed. Nematodes remained smaller than their counterparts in medium without the compound. Larvae isolated by sand filtration measured 400 ± 17 μm in length. Lengths (μm) of *C. elegans* after 10 days in medium with Compound-22 were as follows: unamended medium, 1245 ± 15; 50 μg/ml, 1017 ± 35; 100 μg/ml, 721 ± 32; 200 μg/ml, 607 ± 25. At the highest concentration the nematodes grew very little. With decreasing concentration, nematode growth approached the normal rate.

The three most inhibitory compounds are compared in Fig. 2B with one of slight activity. Reproductive scores were recorded when the control series first reached maximum. The assay was sensitive enough to distinguish between 25 and 50 μg/ml of an inhibitory compound.

Five sesquiterpenoid compounds were tested at both high and low nutrient levels; nutrient level did not affect the degree of activity.

A number of insecticide synergists have been reported to mimic the action of juvenile

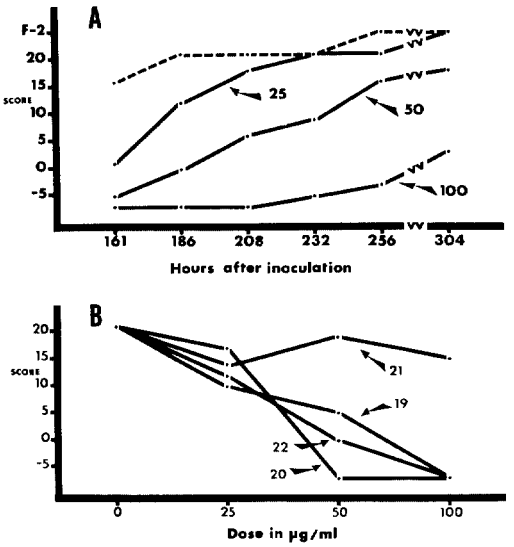

FIG. 2. Inhibition of production of F_1 larvae by *Caenorhabditis elegans* growing in nutrient medium amended with: **A.** An insect juvenile hormone mimic (Compound-22), 3′-methyl-7′-ethylnona-2′, 6′-dienyl 3,4-methylenedioxyphenyl ether at 25, 50, and 100 μg/ml compared with untreated check (dashed line), or **B.** Synthetic insect juvenile hormone (Compound-19) and three mimics rated when control score was maximum. (Reproduction scoring system: 0 larvae/microwell = −1; 1–10 larvae = +1; 11–20 larvae = +2; >20 larvae = +3.)

TABLE 4. Influence of insect juvenile hormones and analogues on growth of *Caenorhabditis elegans*.

Cpd. No.	Compound name	Dosage (μg/ml) and relative activity	Number of tests
15.	2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane (Sesamex)	Inactive at 25–200	2
16.	Methyl 4-[1′,5′-dimethyl-3′-oxo] hexyl cyclohex-1-enoate (Juvabione)	Inactive at 25–200	2
17.	Ethyl 7,11-dichloro-3,7,11-trimethyldodec-2-(*trans*)-enoate	Inhibitory at 50–200, inactive at 25	2
18.	*Trans, trans, cis* methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate (Cecropia JH-I)	Inactive at 25–200	2
19.	*Trans, trans, cis* methyl 10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate (Cecropia JH-II)	Inhibitory at 25–100, inactive at 0.008-5	2
20.	6′,7′-epoxygeranyl 3,4-methylene-dioxyphenyl ether	Inhibitory at 25–200	2
21.	3′-methyl-7′-ethylnona-2′,6′-dienyl 4-nitrophenyl ether	Inhibitory at 25–200	2
22.	3′-methyl-7′-ethylnona-2′,6′-dienyl 3,4-methylene-dioxyphenyl ether	Inhibitory at 25–200	2
23.	*Trans* ethyl 11-chloro-3,7,11-trimethyldodec-2-enoate	Inhibitory at 50–200	1
24.	*Trans* ethyl 3,7,7,11,11-pentamethyl-9-oxo-dodec-2-enoate	Inhibitory at 50–200, inactive at 25	2

hormones in insects (1). We tested three such compounds (obtained from J. H. Fales, Pesticide Chemicals, U. S. Department of Agriculture). Two of the three insecticide synergists with juvenile hormone mimetic activity were inhibitory in the *P. redivivus* assay. Both propyl 2-propynyl phenyl phosphonate and piperonyl butoxide prevented reproduction at 7 μg/ml. The 2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane prevented reproduction at 670 μg/ml but had no effect at 67. Piperonyl butoxide strongly inhibited elongation and reproduction of *C. elegans* at 5 μg/ml, but was ineffective at 1 μg/ml. Nematodes exposed to this chemical in the nutrient medium for 7 days averaged 85 $\pm$ 3.3 μm in length while the controls averaged 605 $\pm$ 20.4 μm.

Four phytoecdysones and one insect steroid were examined. Ponasterone A, cyasterone, ecdysone, and inokosterone (obtained from T. Takemoto, Tohuku Univ., Japan) and β-ecdysone were all inactive at 200 to 0.01 μg/ml.

Discussion

The compounds tested were selected on the basis of their activity as hormones or mimics of hormonal action in vertebrates and insects. Effective concentrations in our assays were in the range of 10^{-4} M or higher. The most active compound, piperonyl butoxide, was effective at 10^{-5} M. We concluded that the growth inhibition in assays on free-living nematodes was non-specific. This contrasted with Johnson and Viglierchio's finding that specific hypertrophy of the gonad of *Heterodera schachtii* resulted from exposure of larvae to farnesyl derivatives (8). We observed no aberrations other than retarded growth and development. Our findings also differ from those of Shanta and Meerovitch's report that low concentrations of farnesyl methyl ether inhibited development of male copulatory appendages (12).

We found no correlation between molecular structure and growth inhibition. Many of the steroids tested were inactive but much more work will be required to define the particular configurations toxic to the test nematodes. The inhibitory activity of insect juvenile hormones and mimics did not parallel their activity as insect hormones. Compound-19 is generally less active in insects than Compound-18, but the reverse was true in our assays. Some of the other inhibitory compounds are active as juvenile hormones on *Pyrrhocoris* bugs but not on other insects; however, Compound-22 (see Fig. 2) was ineffective in *Pyrrhocoris* but effective in other insects (personal communication of G. B. Staal).

That a variety of mammalian sex hormones and analogues, and insect juvenile hormones, analogues and mimics were non-specifically inhibitory to nematode reproduction suggested that further search would be useful. Differences between our results and those of other workers indicated that various species of nematodes react differently to such compounds.

The search for specific compounds in plants that act as growth regulators for parasites has hardly begun. Exogenous sterols are important for reproduction in fungi (6) and are also required for the growth of some nematodes (2, 7). The occurrence in plants of terpenoid regulators of insect growth (13) makes it probable that specific growth regulators of other pathogens also exist in plants. It will therefore be useful to study plant resistance to nematodes from this point of view.

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