Sterol effects and sites of sterol accumulation in *Caenorhabditis elegans*: developmental requirement for 4α-methyl sterols

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Abstract ***Caenorhabditis elegans* requires sterol, usually supplied as cholesterol, but this is enzymatically modified, and different sterols can substitute. Sterol deprivation decreased brood size and adult growth in the first generation, and completely, reversibly, arrested growth as larvae in the second. After one generation of sterol deprivation, 10 ng/ml cholesterol allowed delayed laying of a few eggs, but full growth required 300 ng/ml. *C. elegans* synthesizes two unusual 4α-methyl sterols (4MSs), but each 4MS supported only limited growth as the sole sterol. However, addition of only 10 ng of cholesterol to 1,000 ng of 4MS restored full growth and egg-laying, suggesting that both a 4MS and an unmethylated sterol are required for development. Filipin stained sterols in only a few specific cells: the excretory gland cell, two amphid socket cells, two phasmid socket cells and, in males, spicule socket cells. Sterols were also present in the pharynx and in the intestine of feeding animals in a proximal-to-distal gradient. This non-random sterol distribution, the low concentration requirements, and the effects of 4MSs argues that sterols are unlikely to be used for bulk structural modification of cell membranes, but may be required as hormone precursors and/or developmental effectors.**—Merris, M., W. G. Wadsworth, U. Khamrai, R. Bittman, D. J. Chitwood, and J. Lenard. **Sterol effects and sites of sterol accumulation in *Caenorhabditis elegans*: developmental requirement for 4α-methyl sterols. *J. Lipid Res.* 2003.** 44: **172–181.**

Supplementary key words cholesterol • filipin • amphid socket cell • phasmid socket cell • excretory gland cell

Dietary sterol is required by *Caenorhabditis elegans* (1, 2) because, like insects, *C. elegans* is incapable of synthesizing the four-ring sterol nucleus, but its functions remain largely unknown. The existence of sterol-based hormones in *C. elegans* has recently been suggested, but no hormone has yet been identified (3, 4). Cholesterol is known to be extensively metabolized by *C. elegans* to form several other sterols, including two unusual 4α-methyl sterols (4MSs), which are present in substantial amounts (5–8, 9). These sterols might thus be functional, instead of or in addition to cholesterol itself.

Insects resemble these nematodes in requiring sterols but being unable to synthesize them. Two functions for cholesterol are known in insects: as the metabolic precursor of the molting hormone ecdysone (10), and as the moiety required for activation by covalent attachment to the morphogen protein hedgehog (11). Insect cells, unlike vertebrate cells, grow normally under sterol-free conditions, and thus do not need cholesterol in their plasma membranes (12–14).

We have now characterized the sterol requirements of *C. elegans* in some detail. Conditions for stringent sterol deprivation were developed, and the consequences are described. The use of these conditions allowed us to investigate minimum cholesterol requirements, and the ability of other sterols to substitute. Partial and synergistic effects were found, suggesting that different sterols have diverse effects mediated by several pathways. The accumulation of sterol in the intestinal tract and in a few specific cells in *C. elegans* was also demonstrated by filipin staining, which stains all 3β-hydroxy sterols.

These observations provide a basis for a comprehensive study of sterol functions in *C. elegans*.

MATERIALS AND METHODS

Reagents

Sterols and steroid hormones were obtained from Steraloids Inc. (Newport, RI) or from Sigma. Lophenol was purchased

Manuscript received 13 August 2002 and in revised form 15 October 2002.

Published, JLR Papers in Press, November 4, 2002.
DOI 10.1194/jlr.M200323-JLR200

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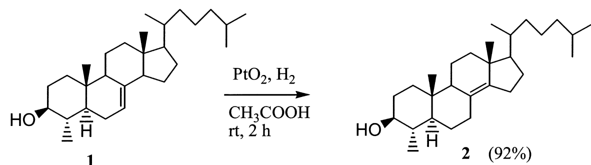
from Research Plus (Bayonne, NJ) and determined to be >96% pure. Filipin III was obtained from Sigma. Electrophoresis grade agarose of several brands proved equally acceptable. All other reagents were of best available grade.

Synthesis of 4-α-methyl-5-α-cholest-8(14)-en-3β-ol

The key step in the synthesis is the PtO_2-catalyzed isomerization of the 7(8)-double bond of lophenol (**Scheme 1**, part 1) to the more stable 8(14) isomer (Scheme 1, part 2) (22).



Scheme 1. Formation of $\Delta^{8(14)}$ Sterol from Lophenol.

NMR spectra were recorded on a 400 MHz (for ^{1}H) and 100 MHz (for ^{13}C) Bruker spectrometer using $CDCl_3$ as the solvent. The δ values are referenced to residual $CHCl_3$ or TMS. Merck silica gel $60F_{254}$ coated (0.25 mm thick) on aluminum sheets were used for TLC. The melting point (mp) was measured on a Hoover capillary apparatus and is uncorrected. The optical rotation was measured in a 1.0-dm cell on a JASCO DIP-140 digital polarimeter.

Prereduced PtO_2 (10 mg) and $\Delta^{7(8)}$-lophenol (Scheme 1, part 1) (17.0 mg, 42.0 μmol) was dissolved in 1 ml of CH_3CO_2H in a small reaction vial (2.5 ml). A tiny magnetic bar was introduced and the solution was degassed and stirred at room temperature under H_2 for 2 h. The solution was poured into ether (3 ml) and neutralized slowly with $NaHCO_3$. After the mixture was filtered through a Celite pad, the ether layer was withdrawn and the aqueous phase was extracted with ether (2 × 3 ml). The combined organic phases were washed with saline (4 ml) and dried ($MgSO_4$). Evaporation of the solvent afforded 16 mg (94%) of the product (Scheme 1, part 2) as a white solid, mp 142–144°C; $[\alpha]^{25}_{D}$ +20.42° (*c* 0.24, CH_2Cl_2). The solid showed a single alkaline permanganate active spot (R_f 0.15, hexane:EtOAc 10:1). Analysis by GC/MS confirmed an overall purity of 92+%, with minor contamination by lophenol, a diene, and a 4,4 dimethylsterol. The disappearance of the doublet at δ 5.2 ppm in the ^{1}H-NMR spectrum indicated the essentially complete conversion of lophenol to the $\Delta^{8(14)}$ isomer. The vinylic carbons of $\Delta^{8(14)}$ sterol (Scheme 1, part 2) in the ^{13}C-NMR spectrum appeared at δ 126.0 and 142.2 ppm, whereas those for lophenol (Scheme 1, part 1) appeared at δ 117.4 and 139.2 ppm. The disappearance of the peaks at δ 126.0 and 142.2 ppm in a ^{13}C DEPT 135 experiment indicates the quaternary nature (15) of the vinylic carbons of $\Delta^{8(14)}$ sterol (Scheme 1, part 2). ^{13}C-NMR ($CDCl_3$) peaks were found at δ 13.9, 15.3, 18.2, 19.1, 20.0, 22.6, 22.8, 23.7, 24.9, 25.7, 27.1, 28.0, 29.7, 31.2, 34.5, 36.0, 36.3, 37.4, 37.5, 39.6, 39.7, 42.7, 49.4, 50.7, 56.9, 126.0, 142.2 ppm.

Media

Bacterial and *C. elegans* media contained 3.5 mM Tris.Cl, 2 mM Tris, 34 mM NaCl, and 3.1 g/l of ether-extracted peptone. Ether extracted peptone was prepared in a large beaker in a fume hood. The peptone powder was mixed with an excess volume of ether, allowed to settle, decanted, and the process was repeated twice more. The extracted peptone was allowed to dry overnight in the hood to remove the remaining ether. The extracted peptone contained ~0.1 μg cholesterol/g by gas chromatographic-mass spectrometric analysis or <0.4 ng/ml cholesterol in the growth media. *Escherichia coli* strain OP50 was grown directly in this sterol-free medium. For the nematode medium, agarose (1.6%), Tween 80 (.005%), and the indicated amounts of various sterols were added. Stock sterol solutions were 1 mg/ml in absolute ethanol. Plates in each experiment were balanced so as to contain identical quantities of ethanol.

C. elegans growth

Wild-type N2, Bristol variety *C. elegans* was routinely propagated at 20°C on standard NGM plates containing 5 μg/ml cholesterol.

Cholesterol-depleted animals (CF1) were grown from the eggs of gravid animals that had crawled away from the bacterial lawn on standard plates, thus minimizing cholesterol transfer through adherent bacteria. These animals were placed in M9 buffer and transected at the mid-line of the gonad. Released eggs were transferred onto a plate containing cholesterol-free medium (as described above) using a flame-drawn micropipette in a hematology mouth pipetter. Except for **Fig. 1A** eggs from CF1, animals were used for all experiments in which animal or brood size was measured.

In order to initiate an experiment, 3–4 eggs from CF1 animals were transferred to each well of a 12-well plate (well diameter, 22 mm) containing the experimental sterol condition. When eggs from CF1 animals were placed on sterol-free plates, the resulting animals are referred to as "CF2". No more than two animals/well were retained after one day of growth.

Microscopy, imaging, and data analysis

In order to measure growth rate, images of each animal were captured daily through a dissecting microscope fitted with either a CoolSnap or a Diagnostic Instruments, Inc. HereSpot 3.2.4 CCD digital camera. Images were stored and analyzed using Media Cybernetics Image-Pro version 4.1 software. This software traced the outline of each animal semi-automatically, yielding values for perimeter and area, which were then calibrated and transferred to spreadsheets for further analysis. No significant differences were found between growth rates based on perimeter or area, so only perimeter values are shown.

Determination of brood size

Each well in a 12-well plate, containing one or two animals, was examined daily for the presence of eggs or hatched larvae. These were counted and removed using a flame drawn Pasteur pipette connected to a vacuum source.

Filipin staining

One to three 60 mm plates containing unsynchronized nematode populations were washed into a 15 ml conical centrifuge tube and washed 3× with ice-cold distilled water. After aspiration of the last wash, the volume was adjusted to ~200 μl, and an equal volume of ice-cold 2X RFB (16), plus 25 μl of 37% formaldehyde solution, 2.5 μl of β-mercaptoethanol, and 1 μl of phenoxypropanol were added. The sample was freeze-thawed three times in an ethanol-dry ice bath and 25 μl of freshly prepared filipin III (10 mg/ml in dimethyl sulfoxide) was added. The sample was flushed with nitrogen, capped, and incubated for 1–4 h in an ice-water bath in the dark. It was then washed three times with PBS. After aspiration, 5 μl of liquid from the bottom of the conical tube containing the highest concentration of animals, were transferred to a glass slide using a plastic pipet tip that had been cut with a razor to enlarge the diameter of the aperture to ~1 mm. Five microliters of Vectashield (Vector Laboratories, Inc.) was added, and the mixture was viewed under a 22 × 22 mm cover slip. Maximum absorption (fluorescence excitation) of filipin is at 338 nm, emission at 480 nm. Filipin-stained animals were viewed using the same filter set used for DAPI stain.

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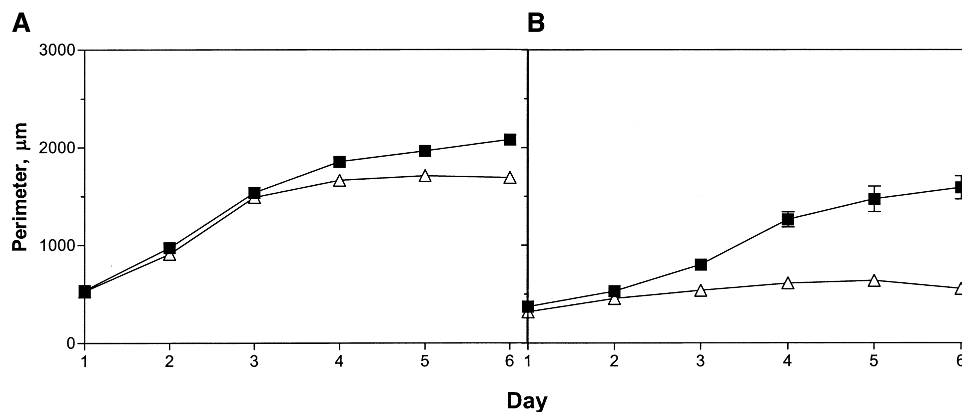

Fig. 1. Effects of cholesterol deprivation on growth of *C. elegans*. A: CF1 generation. Eggs from animals grown under routine maintenance conditions were applied on day 0 to plates containing (square) or lacking (triangle) 1 µg/ml cholesterol as described in Materials and Methods. B: CF2 generation. Eggs laid by CF1 animals were applied at day 0 to plates containing (square) or lacking (triangle) 1 µg/ml cholesterol. Worm perimeters were measured daily as described in Materials and Methods. N = 22–24 animals each curve. Error bars indicate SD.

RESULTS

Conditions for complete cholesterol deprivation

Simply omitting cholesterol, generally added at 5–8 µg/ml, from the standard NGM agar mix gave inconsistent results. Growth under these conditions was clearly sub-optimal, but some animals were able to mature and lay eggs, continuing on sometimes for several generations (cf. 17). The consistent effects of complete cholesterol deprivation, as reported below, were obtained by modifying standard procedures in several ways: *1*) Agarose was used in place of agar. *2*) Peptone was extracted with ether. *3*) Seed bacteria were grown in the same cholesterol-free medium, minus agarose, because bacteria concentrate sterols in their membrane bilayers (18, 19). *4*) No more than two animals were grown in each 22 mm well.

Effects of complete cholesterol deprivation

Growth curves for animals grown for one or two generations under cholesterol-free conditions are shown in Fig. 1. In the first generation of cholesterol-free growth (CF1), the daily increase in size was not significantly affected for the first 3 days, but growth of CF1 animals essentially ceased by day 4 (Fig. 1A).

CF1 animals matured and laid viable eggs at normal times, but brood size was decreased by about half (**Fig. 2A**). CF1 animals possessed normal numbers of oocytes and sperm throughout the egg-laying period as estimated by DAPI staining of 3–6-day-old animals (not shown). The rate of fertilization was slowed, however, as indicated by the smaller number of fertilized eggs present in the uterus each day (Fig. 2B). Unfertilized eggs were also sometimes found in the uterus (**Fig. 3D**), perhaps corresponding to the endomytotic oocytes reported by Shim et al. (17). No evidence was seen for premature exit from pachytene meiosis, as has been reported to occur in response to RNAi of the caveolin-1 gene or brief treatment with the cholesterol scavenger β-methyl cyclodextrin (20). DIC microscopy showed extensive necrotic cell death and disruption of the gonads in the CF1 animals by day 7, when egg-laying was essentially complete (not shown).

When eggs from CF1 animals were transferred to fresh +cholesterol plates, the hatched animals grew well after a lag and developed to maturity, eventually achieving the

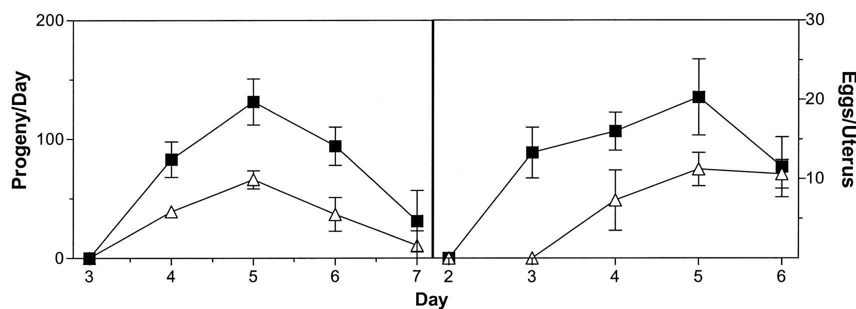

Fig. 2. Effects of cholesterol deprivation on reproduction. A: Eggs from animals grown under routine maintenance conditions were applied on day 0 to plates containing (square) or lacking (triangle; CFI) 1 µg/ml cholesterol. Eggs were counted and removed daily as described in Materials and Methods. B: Average number of fertilized eggs present each day in the uteruses of CF1 and control animals from Fig. 2A. N = 6 animals each curve. Error bars indicate SD.

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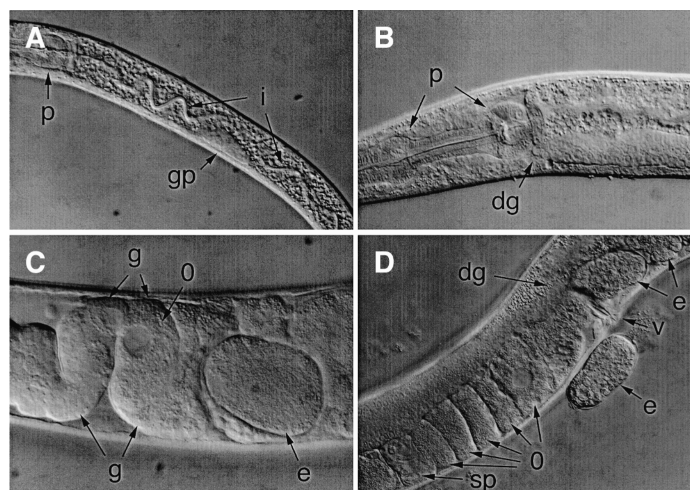
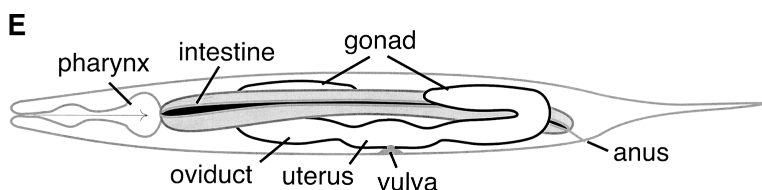


Fig. 3. DIC micrographs of cholesterol deprived animals. A: An early CF2 larva with an intestine (i) showing pronounced sinusoid morphology. B: A late CF2 larva where the distal tip of a gonad arm (dg) has developed ventrally into the head. C: An adult grown on 4α-methyl sterol with a 180 degree twist in a gonad arm. D: A CFI adult with unfertilized oocytes (o) in the uterus of the anterior arm of the gonad. Developing eggs are present in the uterus of the posterior arm and one egg has been laid. egg; gp, gonadal primordium; p, pharynx; sp, spermatheca; v, vulva. E: Schematic diagram of an adult hermaphrodite showing the normal arrangement of the intestine and gonad arms.



same size as those that had never been cholesterol deprived (top curve, Fig. 1B). In contrast, eggs from CF1 animals placed on −cholesterol plates hatched, becoming CF2 animals, but grew only slowly. CF2 animals never reached maturity, generally arresting in the L2 or L3 stage (bottom curve, Fig. 1B). DIC microscopy showed random necrotic cell deaths at later times, correlating with a tendency to become uncoordinated and with increased mortality (not shown). The intestine showed a much more pronounced sinuosity in cholesterol-deprived CF2 animals than in control animals (Fig. 3A), perhaps as a consequence of uneven growth arrest. Notably, somatic gonad development tended to be abnormal, with inhibition of the ventral-to-dorsal migration (Fig. 3B). Cholesterol deprivation did not in our hands result in any noticeable difficulty in cuticle shedding (in contrast to a previous report) (21). Dauer formation (an alternate developmental pathway from L3, initiated by crowding and starving), which has recently been reported to occur in 6% of cholesterol-deprived wild-type animals (3), was not observed, perhaps because the animals were grown in isolation.

During our early attempts to define standard cholesterol-free growth conditions, CF2 animals were grown in large groups, and occasional breakthrough to maturity and egg-laying occurred, probably due to scavenging of sterols from dead animals. When the resulting eggs were subjected to a third generation of growth on −cholesterol plates (CF3 animals), growth was even more severely inhibited than in the CF2 generation, resulting in arrest at an even smaller size, and at an earlier stage of development at the L1 or L2 larval stage (**Fig. 4**). It appears, therefore, that growth and development stalled when the animals' sterol was exhausted, rather than arresting at any discrete point.

Effects of cholesterol deprivation were reversible. CF2 ani-

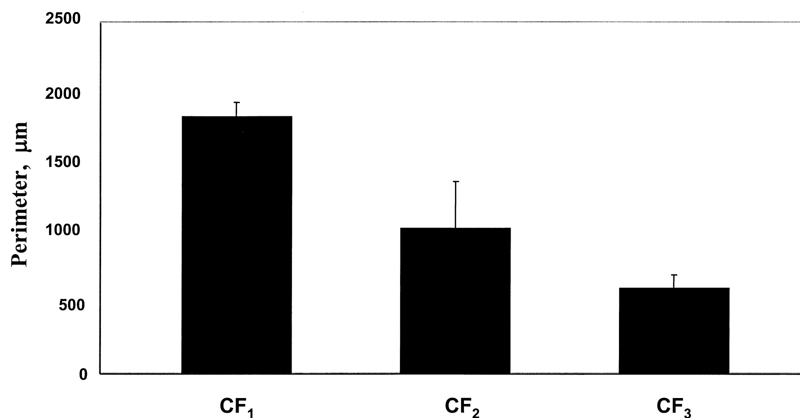


Fig. 4. Size of *C. elegans* grown under cholesterol-free conditions for 1, 2, or 3 generations (CF1, CF2, and CF3, respectively), measured 4 days after hatching. N = 22–24 animals each generation. Error bars indicate SD.

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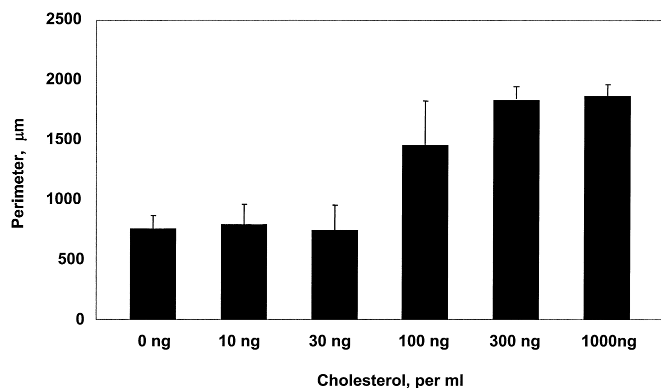

Fig. 5. Cholesterol dependence of *C. elegans* size. Eggs laid by CF1 animals were placed on plates containing the indicated concentrations of cholesterol and measured 4 days after hatching. N = 18–24 animals each concentration. Error bars indicate SD.

mals transferred to +cholesterol plates after 4–5 days progressed to maturity with normal appearance within a few days (not shown). Thus, growth and development were arrested by cholesterol depletion, not irreversibly distorted.

Quantitation of the cholesterol requirement

Eggs from CF1 animals were placed on plates containing various amounts of cholesterol in order to assess minimum requirements. Perimeter measurements of animals after 4 days of growth are shown in **Fig. 5**. Animals grown on 10 ng/ml and 30 ng/ml cholesterol were hardly larger than those grown in the complete absence of cholesterol, while those grown at 100 ng/ml were substantially larger. At 300 ng/ml and above, size at 4 days was independent of cholesterol concentration.

A different cholesterol dependence was found for egg-laying (**Fig. 6**). Viable eggs were laid at only 10 ng/ml, although in sub-normal numbers and with a delay of several days. Normal numbers of eggs were laid at 30 ng/ml, but with a 1-day delay (Fig. 6). Above 100 ng/ml, both the brood size and the timing of egg-laying was independent of cholesterol concentration (Fig. 6). Egg-laying thus occurred at cholesterol levels that profoundly retarded overall growth, suggesting that these properties are independently regulated by sterols.

Substitution of cholesterol by other steroids and sterols

4MSs are found as significant metabolites in *C. elegans* (5, 6), in contrast to most animals and yeast. Although 4MSs have been found in substantial quantities in some plants (22), they have no known function in any organism.

The metabolic pathway shown in **Fig. 7** has been proposed for 4MS formation in *C. elegans* (9). In order to assess the possible functional significance of 4MSs, and thus of this pathway, the growth and egg-laying properties of animals grown on each of the sterols shown in Fig. 7 was determined. The sterol requirement for *C. elegans* could be met equally well by any of the sterols shown in the top row of Fig. 7. Other unmethylated sterols that fully supported *C. elegans* growth and development included allocholesterol (Δ^4), the unmethylated form of $\Delta^{8(14)}$ sterol and the fully saturated sterol cholestanol (not shown). These differ from the compounds shown in the top row of Fig. 7 only in the position (or presence) of a double bond, suggesting that *C. elegans* has a broad ability to modify double bonds in order to produce its required sterol(s).

In contrast, either of the two 4MSs shown in Fig. 7 (bottom line) was only able to support partial growth, but not maturation to the reproductive stage when used as the sole sterol source (**Fig. 8**). Both sterols were cytotoxic when used alone, resulting in extensive necrotic cell death, gross tissue degradation including twisted and ruptured gonads (Fig. 3C), and a high incidence of mortality due to exploding vulva. These effects were significantly more pronounced for lophenol than for $\Delta^{8(14)}$ sterol. Thus, 4MSs alone provide a limited, but incomplete, ability to support development.

Remarkably, however, nearly complete support of growth and egg-laying was provided if a small amount of cholesterol was added to the 4MSs. The addition of 10 ng/ml of cholesterol to 1 µg/ml of either 4MS restored essentially complete growth (Fig. 8A, B) and egg-laying (Fig. 8C, D). There was no synergistic effect when the other 4MS (e.g., lophenol with $\Delta^{8(14)}$ sterol or vice versa) was used in place of cholesterol (not shown). Because *C. elegans* can methylate sterols at C-4 but cannot demethylate them (8), the simplest interpretation of these results is that two different sterols are required for development, a 4MS and an unmethylated sterol, acting through separate pathways. It is noteworthy that each 4MS

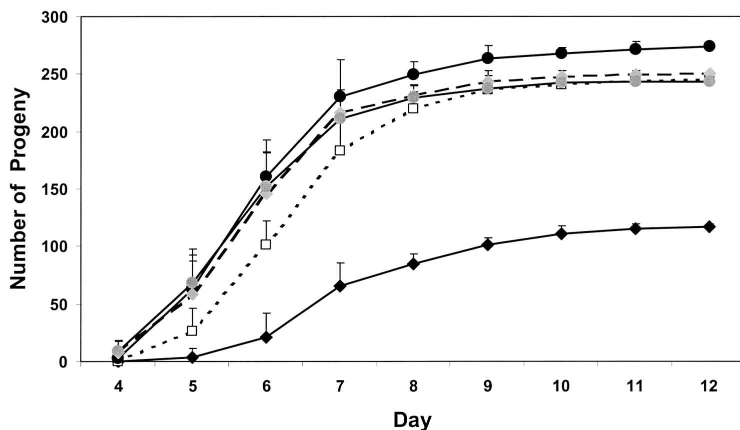

Fig. 6. Cholesterol dependence of egg-laying and brood size. Eggs laid by CF1 animals were placed on plates containing the following concentrations of cholesterol (in ng/ml): black diamond, 10; square, 30; black circle, 100; gray diamond, 300; gray circle, 1000. Eggs and newly hatched larvae were counted and removed daily. N = 22–24 animals each concentration. Error bars indicate SD.

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Fig. 7. The biosynthesis of 4MSs from cholesterol, as proposed by Chitwood (9).

can serve quantitatively as the major, but not as the sole, required sterol.

Animal or insect steroid hormones were unable to substitute for cholesterol. Growth from eggs of CF1 animals on plates containing α-ecdysone or the vertebrate hormones aldosterone, cortisol, cortisone, dexamethasone, estradiol, progesterone, or testosterone (at 3 μg/ml) was not significantly better than growth in the absence of cholesterol. In agreement with these findings, neither pregnenolone nor ecdysone were able to ameliorate certain *daf-9* phenotypes, while addition of excess cholesterol or 7-dehydrocholesterol was able to do so (4).

Other sterols and related substances were tested for their ability to promote growth and maturation of eggs laid by CF1 animals. As expected, squalene could not substitute for cholesterol, confirming the inability of *C. elegans* to synthesize the sterol ring from an acyclic precursor (2). The 4,4-dimethyl sterol lanosterol, the first sterol in the cholesterol biosynthetic pathway, was also inactive, providing an additional indication that *C. elegans* cannot demethylate sterols at C-4 (8). 3β-methoxycholesterol was also unable to substitute for cholesterol, suggesting that a free hydroxyl group at C-3 is required.

Distribution of sterols

In order to determine the distribution of sterols within *C. elegans*, filipin staining was employed. Filipin is a fluorescent polyene antibiotic that forms tight, specific com-

Fig. 8. Growth and reproduction on 4MSs. A, B: Growth of *C. elegans* the 4MSs (A) lophenol or (B) $\Delta^{8(14)}$ sterol in the presence or absence of cholesterol. Diamond, no sterol; square, cholesterol alone, 10 ng/ml; white triangle/white circle, the indicated 4MS alone, 1 μg/ml; black triangle/black circle, the indicated 4MS, 1 μg/ml + cholesterol, 10 ng/ml. C, D: Egg-laying by *C. elegans* grown on the 4MSs lophenol (C) or $\Delta^{8(14)}$ sterol (D) in the presence or absence of cholesterol. Symbols are as in A and B. N = 9–12 animals each sterol. Error bars indicate SD.

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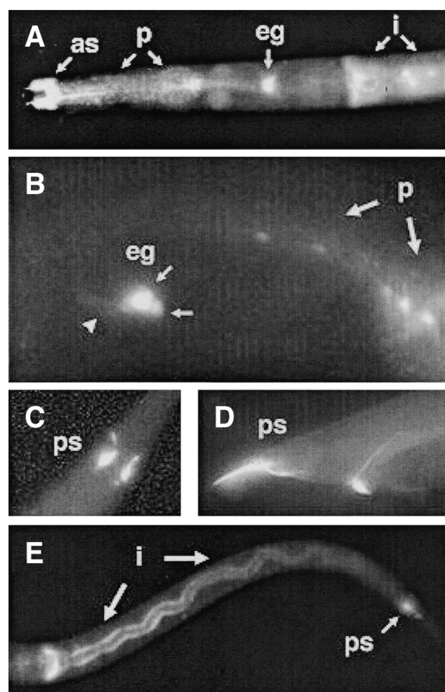

Fig. 9. Fluorescence micrographs of filipin-stained animals. A: In the head region, cholesterol accumulates in the amphid socket cells (as), diffusely in the pharynx (p), in the excretory gland cell (eg), and in the intestine (i). Ventral aspect. B: The bilobed excretory gland cell (arrows) is connected (arrowhead) to the large excretory cell, which sends two arms that extend along most of the length of the animal. Lateral aspect. C: In the tail of the hermaphrodite, the phasmid socket cells (ps) accumulate sterol. Ventral aspect. D: In the male tail, the phasmid socket cells and cells associated with the spicules stain. Lateral aspect. Two phasmid socket cells and one spicule socket cell are visible. E: The intensity of staining decreases posteriorly along the intestine.

plexes with 3β-hydroxy sterols (23–26). These are detectable using either fluorescence or freeze-fracture electron microscopy (e.g., 12).

Filipin consistently stained five discrete cells plus the pharynx and the intestine in hermaphrodites of all ages (Fig. 9). The five cells have been tentatively identified as two amphid socket cells (Fig. 9A), the excretory gland cell (Fig. 9A, B), and two phasmid socket cells, which have different morphologies in hermaphrodites (Fig. 9C) and in males (Fig. 9D). In males, filipin staining of the spicule socket cells is also prominent (Fig. 9D). Costaining experiments (not shown) showed that filipin has a different, nonoverlapping staining pattern from fluorescein, a dye that stains cells of the amphid sensillae that are open to the environment (27). CF1 animals were not stained under identical staining conditions (data not shown), showing that staining occurred only in the presence of sterols.

The pharynx and the intestinal epithelium were also heavily labeled in a gradient that decreased distally from the intestinal valve to the anus (Fig. 9E). This most likely marks the route of ingestion of cholesterol by the animal. Most of the ingested cholesterol was probably incorporated into the membranes of the dietary bacteria (18, 19) and subsequently absorbed during the digestive process. A similar gradient of sterol concentration down the intestine was observed for dehydroergosterol (DHE), a fluorescent sterol that can satisfy the cholesterol requirement in *C. elegans* (28).

The observation that sterols accumulate in only a few cells, rather than in the plasma membranes of many or all cells, is consistent with observations on another free-living nematode, *Turbatrix aceti.* A freeze-fracture study of filipin-stained *T. aceti* revealed that all the detectable sterol in that animal was concentrated in the internal vacuoles of a single cell, which was not identified further (12).

DISCUSSION

We consider the results reported here in the context of some well-established biological functions of cholesterol: as a structural component of biological membranes, as a precursor for steroid hormone biosynthesis, and as a covalent activator of developmentally important molecules such as hedgehog. It is likely that other uses remain to be discovered.

Membrane structure

Our findings suggest that sterols are not used by *C. elegans* as a structural component of cell membranes. Cholesterol intercalates readily into phospholipid bilayers, decreasing cooperativity between phospholipid molecules and creating, at mole ratios exceeding ~15%, the so-called "thermal buffer" effect (29). Discrete cholesterol-rich lipid phases or "membrane rafts" in the plasma membranes of vertebrate cells are thought to form important loci for cellular signaling (30, 31). One or both of these effects are probably essential for vertebrate cells, since they do not survive without high levels of cholesterol (13, 32, 33).

In contrast, many lines of insect cells can be maintained in the complete absence of any sterol (12–14), showing that the membrane-modifying properties of cholesterol are not obligatory for cellular function in eukaryotes.

The concentration of sterol in a few specific cells in *C. elegans* (**Fig. 9**) suggests a non-uniform distribution, in contrast to the prediction if all plasma membranes contained comparable amounts of cholesterol. In another free living nematode, *T. aceti,* sterol was present in internal vacuoles of the single sterol accumulating cell, but not in its plasma membrane (12).

The small amount of cholesterol needed for growth and egg-laying in *C. elegans* provides a further argument against a membrane role. As little as 10 ng/ml of cholesterol allowed limited maturation and reproduction (Fig. 6). Although the extent of concentration of cholesterol from the medium by the bacteria and by the worms is not known, it seems unlikely that quantities sufficient to significantly modify membrane structures could be extracted from such deficient medium.

Results obtained with 4MSs also argue against a major role of sterols in membrane structure. These are less effective modifiers of membrane structure than is cholesterol

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because of their three-dimensional structure (34), yet *C. elegans* synthesizes them from cholesterol, and they can supply the bulk of the sterol requirement (Fig. 8). Taken together, these arguments suggest that the essential functions of sterols in *C. elegans* do not include significant modification of the physical properties of membrane bilayers.

A recent report showed that the RNAi-induced inhibition of expression of caveolin-1, a cholesterol-binding protein associated with membrane rafts and caveolae in mammalian cells, caused premature exit from pachytene meiosis in oocytes of *C. elegans*. Furthermore, brief treatment of live animals with β-cyclodextrin, a cholesterol-scavenging reagent, resulted in a similar effect (20), implying a role for cholesterol. We observed no such effects in our experiments, however. In any case, it is hard to see how this rather subtle non-lethal effect could reflect an essential function for cholesterol.

Sterol hormones and sterol distribution

A much stronger case can be made for sterols as precursors of sterol-based hormones, i.e., metabolites that regulate transcription through nuclear receptors. The *C. elegans* genome possesses ∼270 nuclear receptors, compared with 21 in *Drosophila* and ∼50 in humans (35). One of these, DAF-12, mediates important developmental decisions during L3, including dauer formation and the ventral-to-dorsal migration of the distal tip cell. Genetic experiments place *daf-*9, a gene encoding a cytochrome P450 related to steroidogenic mammalian enzymes, upstream of *daf-*12, and sterol deprivation phenocopies certain mutant phenotypes of both genes (3, 4). The steroid hormone thought to be synthesized by DAF-9 and to activate DAF-12 has not yet been identified, however. It should be noted that this putative hormone must be present at very low levels, because no possible products of cytochrome P450 oxidation (i.e., possessing additional hydroxyl or keto groups) were detected in the exhaustive characterization of sterol metabolites (9). The DAF-12 ligand is thus unlikely to account for the bulk of the *C. elegans* sterol requirement.

The invariant presence of substantial amounts of the unusual 4MSs in *C. elegans* suggests these might have important functions. The attachment of a 4α-methyl group onto unmethylated sterols by *C. elegans* (Fig. 7) is astonishing because it represents a precise reversal of reactions that occur in other organisms. In animals, yeast and many plants 4MSs arise only as transient intermediates during the demethylation of sterol precursors, and are thus usually present in minute amounts. These organisms possess enzymes that remove methyl groups from C-4, but they cannot re-attach them at this position. *C. elegans* in contrast can methylate but not demethylate at C-4 (9) while lacking a functional sterol biosynthetic pathway.

Likely locations for the synthesis or storage of sterol-based hormones are the cells identified by filipin staining (Fig. 9). Of these, the excretory gland cell has a particularly suitable morphology. It possesses abundant secretory granules, and makes connections with both the pharyngeal nerve ring and the excretory canal, which extends through much of the length of the worm, a suitable location for neuroendocrine communication (36). Much less is known about the other sites of accumulation, the amphid, phasmid, and spicule socket cells. It should be noted that filipin stains by complexing with any 3β-hydroxysterol; it is not specific for cholesterol. Thus, the cells stained by filipin (Fig. 9) need not contain cholesterol, but could alternately contain any of the metabolites shown in Fig. 7. Filipin cannot stain any of the known animal or insect steroid hormones, however.

A comparison of our results with those of Matyash et al. (28) suggests that the amphid and phasmid socket cells are accumulating a sterol metabolite rather than unmodified, exogenously added cholesterol itself. Matyash et al. (28) used the intrinsically fluorescent sterol dehydroergosterol (DHE) as the sole supplied sterol, and observed its distribution in vivo without staining. DHE accumulated in only one of the five cells identified by filipin staining (Fig. 9), the excretory gland cell. However, DHE fluorescence arises from the arrangement of three conjugated double bonds within its ring system. Isomerization or elimination of any of these eliminates fluorescence. As *C. elegans* possesses abundant ability to rearrange sterol double bonds, and as this activity is probably functionally important, fluorescence observations using DHE will detect only the earliest stages of sterol distribution, before any metabolic changes. The excretory gland cell may thus accumulate the unmodified ingested sterol, while the amphid and phasmid socket cells accumulate later metabolites.

Covalent attachment to proteins

Cholesterol activates the *Drosophila* morphogen protein hedgehog by forming an ester bond with a newly generated carboxyl terminus, and similar proteins have been described in vertebrates (11). No close relative of hedgehog was found in the *C. elegans* genome, but two extensive families of more distantly related proteins are present (37). Although no direct evidence for sterol attachment to these proteins has yet been reported, this could be an important function for sterols. Such covalent attachment need not be limited to cholesterol. Specific requirements for other sterols could also exist.

Other uses

It is intriguing that certain 4,4-dimethyl sterols have been found to activate meiosis in mouse oocytes (38), as treatment of *C. elegans* with RNAi against the gene encoding caveolin-1 or with the sterol scavenger β-methylcyclodextrin was found by others to accelerate meiotic progression (20). It is likely that presently unknown developmental roles of sterols remain to be discovered, as suggested by the variety of developmental defects displayed by human patients with genetic disorders of cholesterol metabolism. The Smith-Lemli-Opitz syndrome, for example, arises from defects in the gene encoding 7-dehydrocholesterol reductase, which catalyzes the final step in cholesterol biosynthesis (the reverse of the first reaction shown in Fig. 7). Abnormal morphogenesis is evident in most affected patients, and patients with other lesions in the cholesterol pathway also show multiple developmental defects (39), many of which cannot be accounted for by the known uses of cholesterol. It may be

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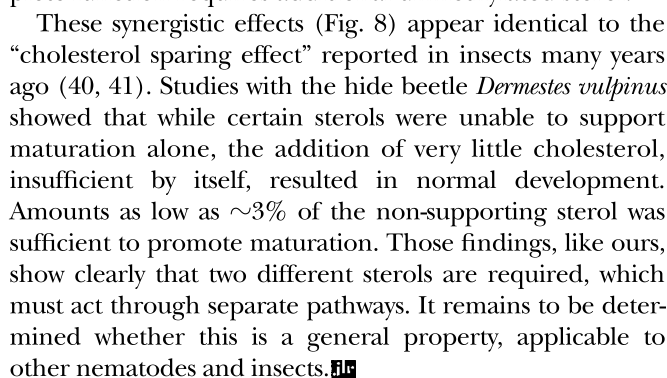
speculated that novel sterol-mediated developmental pathways might be more easily revealed in *C. elegans* than in vertebrate models, because it lacks the high cholesterol background from plasma membranes.

Multiple effects of multiple sterols in *C. elegans*

We have provided evidence here for at least two essential roles for sterols during development, controlled by at least two different sterols. A general indication of multiple roles for sterols comes from the observation (Fig. 4) that cholesterol-deprived animals apparently do not arrest at any particular point in development, but whenever they run out of sterol. Two independent roles are indicated from a comparison of the very different cholesterol dependences of animal size (Fig. 5) and brood size (Fig. 6).

Experiments with the 4MSs (Fig. 8) provide further evidence. The very limited growth promoting properties of these sterols alone, and the dramatic synergism with minute amounts of cholesterol (Fig. 8), suggests that the 4MSs can satisfy the bulk of the sterol requirement, but provide only part of the growth requirement, while complete function requires additional unmethylated sterol.



These synergistic effects (Fig. 8) appear identical to the "cholesterol sparing effect" reported in insects many years ago (40, 41). Studies with the hide beetle *Dermestes vulpinus* showed that while certain sterols were unable to support maturation alone, the addition of very little cholesterol, insufficient by itself, resulted in normal development. Amounts as low as ~3% of the non-supporting sterol was sufficient to promote maturation. Those findings, like ours, show clearly that two different sterols are required, which must act through separate pathways. It remains to be determined whether this is a general property, applicable to other nematodes and insects.

The authors thank David Hall for assistance in identification of the filipin-stained cells, David Axelrod for assistance in an early measuring experiment, and G. S. Tint for sterol analysis. This work was supported in part by Grants from the National Institutes of Health to R.B., J.L., and W.G.W.

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