Long-term effects of sterol depletion in *C. elegans*: sterol content of synchronized wild-type and mutant populations

Mark Merris,* Jessica Kraeft,* G. S. Tint,†§ and John Lenard†,*

Department of Physiology and Biophysics,* University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854; Department of Veterans Affairs New Jersey Health Care System,† 385 Tremont Avenue, East Orange, NJ 07018; and Department of Medicine,§ University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07101

Abstract  Three major long-term effects of sterol deprivation in *Caenorhabditis elegans* are described. 1) The life expectancy of sterol-deprived wild-type animals is decreased by more than 40%. Similar decreases are found in animals carrying mutations in the *daf-9*, *daf-12*, *daf-16*, and *clk-1* genes, suggesting that previously described aging pathways involving these genes are not involved in the life-extending effects of sterols. 2) There is a premature loss of motility, measured by response to mild touch. 3) There is a rapid postreproductive onset of sarcopenia (muscle wasting) as measured by total body fluorescence in a *myo3::GFP*-expressing strain. We also report that five sterols (the desmethyl steroids cholesterol, 7-dehydrocholesterol, and lathosterol and the 4α-methyl sterols lophenol and 4α-methyl-cholesta-Δ(14)-en-3β-ol) are found in significant amounts at all stages of development and aging in cholesterol-fed animals. Supplying any one of these as the sole sterol confers similar protection from the long-term effects of sterol deprivation. These findings suggest that sterols are required continuously throughout the animal's life.—Merris, M., J. Kraeft, G. S. Tint, and J. Lenard. *Long-term effects of sterol depletion in C. elegans: sterol content of synchronized wild-type and mutant populations. J. Lipid Res. 2004. 45: 2044–2051.*

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*Caenorhabditis elegans* cannot synthesize sterols and so requires dietary sterol, which is usually supplied in the laboratory as cholesterol (1, 2). Studies of the ability of different sterols to substitute for cholesterol have revealed the presence of at least two independent essential sterol-requiring pathways. One of these pathways can use a highly unusual class of sterols, the C4α-desmethyl sterols (4αMS), and accounts for at least 98% of the total sterol requirement. The other pathway cannot use 4MS, but requires a C4 desmethyl sterol (desMS) (2). 4αMSs were present as major components of the total sterol content in axenically grown unsynchronized (mixed age) populations of *C. elegans* when any of several dietary desMSs were supplied (3). *C. elegans* is unique among presently characterized organisms in possessing the ability to convert dietary desMSs to 4αMSs, while simultaneously lacking the ability to demethylate sterols at C4 (3).

The functions of sterols in *C. elegans* have not been characterized. Sterol deprivation results in decreased fertility in the first generation and developmental arrest with early death in the second. Development to adulthood and timing of reproduction both occur normally in the first generation of sterol deprivation, apparently supported by the utilization of stored sterol (2, 4, 5).

Sterols do not appear to be required for bulk modification of plasma membrane bilayer properties, as indicated by several observations. First, only minute amounts of sterol were required: limited fertility was supported by 10 ng/ml of cholesterol, and normal growth and reproduction by ~100 ng/ml, compared with 5–8 μg/ml present in standard medium (2). Second, sterol-specific staining by filipin showed sterol accumulation largely in five specific cells plus the intestine, rather than uniform distribution in plasma membranes (2). Third, 98% of the *C. elegans* sterol requirement could be satisfied by 4αMSs (2), which are inferior to cholesterol and other desMSs in their ability to modify the bulk properties of membrane bilayers (6). Finally, the enantiomer of cholesterol cannot substitute for natural cholesterol to support *C. elegans*, despite the fact

Abbreviations:  4αMS, C4α-methyl sterol; desMS, C4 desmethyl sterol; GFP, green fluorescent protein; Δ8(14) sterol, 4α-methyl-cholesta-Δ8(14)-en-3β-ol.

1 To whom correspondence should be addressed.
e-mail: lenard@umdnj.edu
that sterol-lipid interactions in model membranes are not generally enantiospecific (5).

Cholesterol itself is not an essential sterol for C. elegans. This was shown when animals supplied with lathosterol or cholesterol as the sole sterol grew and reproduced as well as, and lived as long as, animals grown on cholesterol (Refs. 2, 7, and the present study); sterol analysis of these animals revealed no detectable cholesterol (7). It seems most likely, therefore, that supplied cholesterol is used by C. elegans as starting material for the biosynthesis of other sterols, including 4Ms, which are required for hormonal and/or developmental purposes (2, 8).

This report extends our previous observations (2) of first-generation cholesterol-deprived animals to the postreproductive stage. Effects of sterol deprivation intensified at this stage, resulting in premature loss of motility, sarcopenia (muscle wasting), and death. We also document the presence of the four previously characterized metabolites of cholesterol in C. elegans in significant amounts at all stages of development and senescence, and the ability of each of them to protect against the long-term effects of sterol deprivation. Our findings lend further support to previous suggestions that sterols are needed continuously throughout life (2, 4, 5).

MATERIALS AND METHODS

Reagents

Lophenol (>96% pure) was purchased from Research Plus (Bayonne, NJ). The 4α-methylcholesta-Δ8(14)-en-3β-ol [Δ8(14) sterol] was prepared as previously described (2). All other reagents and sterols were of best available grade.

Animals

Wild-type N2 animals, Bristol type, were used unless other strains are specified. For muscle wasting (sarcopenia) studies, the strain RW1596 myo-3(st386) Ptmyo::GFP rol-6(su1006), which expresses a green fluorescent protein (GFP)-myosin fusion protein, was used (9). For sterol analysis, and to test the relationship between sterol dependence and previously characterized age-related pathways, the following mutant strains were obtained from the Genetic Stock Center, from Garth Patterson (Rutgers University), or from Carl Johnson (Madison, WI): MQL30 dh-1(qm 30), GP8 daf-12(m20), GP555 daf-16(mgDff50), DR2207 daf-9(e1406); daf-12(m20) (double mutant); NS3209 npe-1.

Media

All experiments conducted under sterol-free conditions or with controlled sterol concentrations were performed on animals grown on bacterial (Escherichia coli OP50) lawns on agar plates, using medium containing either-extracted peptone for both nematode and bacterial growth, as previously described (2). Routine propagation of wild-type and mutant animals was performed at 20°C on standard NGM agar plates containing 5 μg/ml cholesterol.

Sterol dependence

To determine the effects of various sterols on longevity and behavior, animals were grown from eggs on experimental media containing no sterol, or containing specified concentrations of different sterols. Adult animals were transferred daily to fresh plates of identical composition during the reproductive period, and then maintained under the same conditions until death. Postreproductive animals were rated by behavior following a light touch on the nose into categories A through D (9), as follows: A) animal moved spontaneously, or following a light touch, in a smooth sinusoidal manner; B) animal moved only when touched, or had obvious irregularities in the movement pattern; C) animal moved only head or tail in small patterns when lightly touched; D) death (no movement when touched, no visible pumping of the pharynx). Animals lost due to dehydration from crawling off the plate were excluded from further analysis. Brood size was determined as described previously (2).

Quantitation of age-dependent muscle wasting (sarcopenia)

Effects of aging on sarcopenia was estimated from changes in mean GFP fluorescence arising from the myosin-GFP fusion protein expressed by the mutant RW1596 myo-3(st386) Ptmyo::GFP rol-6(su1006). Fluorescence was measured in a COPAS BIOSORT analysis and sorting instrument (Union Biometrica, Somerville, MA). Animals were synchronized by allowing eggs to hatch overnight at 20°C in M9 solution. Synchronized populations (~200 animals) of GFP-expressing animals were grown in presence or absence of cholesterol to various ages, and the amount of fluorescence (488 nm excitation, 510 nm emission) emitted by each animal passing through the laser was determined by the BIOSORT. Unlabeled wild-type animals showed negligible fluorescence at identical instrument settings. Separate populations were used for each data point, as animals could not be reused due to low survival and surprisingly low recovery from the sorter.

Sterol analysis

Synchronized worm populations were started by allowing eggs obtained by bleach extraction to hatch overnight in M9 buffer. These animals, assumed to be arrested at 10 h post hatch, were applied to standard plates (OP50 lawns in NGM medium) and harvested at times corresponding to the desired developmental stage (10). Larval stages were confirmed by microscopic observation. The number of animals in each sample was determined by counting the number of animals present in a measured volume (2 μl) on a glass microscope slide.

Sterol determinations were performed as previously described (11). After adding 0.2 μg of coprostanol (5α-cholest-3β-ol) as an internal standard, the pellet (~100 μl) from washed, synchronized worm populations was added to 5 ml ethanol in 25 ml glass centrifuge tubes. Then 0.5 ml of 10 N NaOH was added and thoroughly mixed, then hydrolyzed at 65-70°C for ~1 h, with each tube lightly capped to minimize ethanol loss. Samples were removed from the water bath and were placed in warm tap water (~43°C) for a few minutes to slightly cool the samples. Next, 2.5 ml of deionized H2O was added to each sample, followed by extraction with 3 × 5 ml of hexane. The hexane extract was dried under nitrogen in a 43°C water bath. Samples were treated with 50 μl of Sil-Prep (Alltech, Waukegan, IL) for 30 min at 45°C to form the trimethylsilyl ether derivatives. Following evaporation of the Sil-Prep, the extracts were dissolved in hexane and injected onto a 30 m × 250 μm (inner diameter) HP-5MS having a 0.25 μm 5% phenylmethylsilicone film (Agilent Technologies, Palo Alto, CA) installed in a HP-6890 gas chromatograph interfaced to a HP-5971A mass-sensitive detector. The column was held at 100°C for 2 min and then the temperature was increased to 275°C at a rate of 35°C/min. Helium was used as the carrier gas at a constant flow of 1 ml/min. Cholesterol, 7-dehydrocholesterol, lathosterol, Δ8(14) sterol, and lophenol concentrations were determined by monitoring the ions at m/z 458, 325, 458, 472, and 269, respectively. The areas under the peaks were then compared with the area under the total ion peak of the internal standard coprostanol and the masses were calculated. The system was calibrated by first injecting weighed mixtures of the five sterols together with coprostanol.
RESULTS

Cholesterol dependence of aging and mortality

We have previously shown that animals grown on cholesterol-free medium from hatching develop normally and with normal timing through the larval stages to maturity. They lay fully viable eggs, also with normal timing, but only about half the normal number (2). It was therefore of interest to determine postreproductive effects of cholesterol deprivation. We found that longevity of wild-type worms depended strongly on the presence of cholesterol. Average life expectancy was decreased by more than 40% in cholesterol-free medium compared with cholesterol-replete (1,000 ng/ml) medium (2). A representative experiment is shown in Fig. 1A. A lower concentration of cholesterol (100 ng/ml) gave variable results in repeated experiments (data not shown).

Age-dependent motility was assessed using behavioral criteria established by Herndon et al. (9). They subjected C. elegans to a light nose touch and divided the response into three readily distinguishable categories: A, B, and C (as detailed in Materials and Methods). They showed that each animal is obliged to pass through each stage sequentially during normal aging. As shown in Fig. 1B, the timing of both entry into and exit from stage B occurred much sooner under conditions of cholesterol deprivation than in the presence of 1,000 ng/ml cholesterol. This shows that passage through each of the stages is accelerated by growth in cholesterol-free medium.

Another criterion of aging noted by Herndon et al. (9) was muscle wasting, or sarcopenia. To test this, we used a strain of C. elegans expressing a myosin-GFP fusion molecule behind a myosin promoter. As expected, animals of this strain showed a cholesterol-dependence of life span and aging similar to the wild type (Fig. 2). A significant age-related decrease in GFP-myosin fluorescence was observed in these animals grown on cholesterol-free medium, suggesting pronounced sarcopenia (Fig. 2A). The cholesterol-grown control animals maintained their average GFP-myosin levels to a greater extent through the aging period, but a downward trend and pronounced variability is evident in these animals as well (Fig. 2B). The overall size of animals grown under either condition does not change appreciably during aging (unpublished observations). Thus, a pronounced late-onset sarcopenia results from cholesterol deprivation.

Effects of other sterols

Early analyses of sterol composition revealed that dietary sterols are extensively converted to other sterols by C. elegans (3). A pathway from cholesterol to other major identified sterols was postulated, based solely on these analyses (Fig. 3). Reasoning that this pathway might lead to sterols of functional importance, the ability of each one to substitute for cholesterol in supporting continuous growth and development was investigated. We have previously reported that each of the desMSs (Fig. 3, top row) could sub-

![Fig. 1. Cholesterol dependence of mortality and aging of Caenorhabditis elegans. Animals were grown in presence (filled triangle) or absence (filled diamond) of 1,000 ng/ml cholesterol. A: Mortality. B: Time course of passage through behavioral stage B, as described in Materials and Methods. N = 30.](image-url)
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Fig. 2. Sarcopenia in cholesterol-deprived (A) and cholesterol replete (B) C. elegans animals expressing green fluorescent protein (GFP)-myosin fusion protein. (filled box) Percent surviving animals. (filled diamond) Mean fluorescence (arbitrary units) as measured on COPAS BIOSORT instrument. Control animals not expressing GFP showed negligible fluorescence (data not shown). $N = 50$ for mortality curve; $N = 81$ for each fluorescence data point.

Fig. 3. Biosynthesis of 4MS from cholesterol in C. elegans, as proposed by Chitwood, 1999 (3).

...stitute completely for cholesterol in the maintenance and propagation of C. elegans, starting with cholesterol-depleted eggs. The 4MSs (Fig. 3, bottom row) could substitute partially; each one alone allowed some growth, but the attainment of reproductive maturity required the addition of 1–2% cholesterol or other desMS. This implies the presence of at least two essential sterol pathways, of which one, accounting for >98% of the sterol requirement, can use 4MS, whereas the other requires a small quantity of desMS (2).

These prior findings raised the question of whether the sterols shown in Fig. 3 could confer life-extending effects similar to cholesterol in the first generation of cholesterol depletion. To answer this question, animals hatched from normally grown animals were grown on each of these sterols alone, and mortality rates were observed. As shown in Fig. 4, each of the sterols alone was able to suppress the two major effects of sterol deprivation: reduced egg-laying (Fig. 4A) and decreased life span (Fig. 4B). Greater variability of mortality was encountered in repeat experiments with 7-dehydrocholesterol, lophenol, or $\Delta 8(14)$ sterol than with cholesterol or lathosterol [note, for example, the reduced life expectancy on $\Delta 8(14)$ sterol in the experiment shown in Fig. 4], suggesting that the former sterols may be less efficiently taken up or utilized by the animals.
Sterol analysis of synchronized worm populations

Previous sterol analyses of *C. elegans* were performed on mixed-age populations of worms grown under axenic conditions in liquid culture supplemented with very high concentrations (25 µg/ml) of various sterols (3). We wanted to learn whether similar sterols would be recovered from worms grown on bacterial lawns under standard conditions, and also how they might vary as a function of developmental stage and age.

Representative results are shown in Table 1. Several facts are evident. First, 4MSs are prominently present under normal growth conditions as well as in axenic liquid culture. They cannot be synthesized by *E. coli* (data not shown). Second, 4MSs are present in significant amounts continuously throughout the normal life cycle, from embryonic and early larval stages to senescence. Third, we encountered an unexpected variability in amounts of sterols present in different populations grown at the same time, as well as in supposedly identical populations grown at different times. The most likely explanation for this is that the animals have a large capacity to store sterols (2, 4, 5), and that small differences in growth conditions, especially in the state of the bacterial lawn, can result in disproportionate effects on sterol availability and uptake. Bacteria con-

Table 1. Sterol content of mutant synchronized *C. elegans*

| Strain | Stage | N   | Chol | 2-DHC | Lath | LoΦ | Δ8(14) | 7-DHC/Chol | 4MS/Lath |
|--------|-------|-----|------|-------|------|-----|--------|------------|----------|
| GP8    | L1    | 8,300 | 11.4 | 1.49  | 0.73 | 0.50 | 0.68   | 0.13       | 1.64     |
|        | L2    | 6,860 | 17.3 | 1.34  | 0.66 | 0.43 | 0.42   | 0.08       | 1.28     |
|        | L3    | 6,025 | 24.4 | 4.34  | 1.77 | 0.94 | 0.76   | 0.18       | 3.79     |
|        | L4    | 5,625 | 20.5 | 7.23  | 1.13 | 0.80 | 3.30   | 0.35       | 3.63     |
| DR2207 | L1    | 17,175| 6.19 | 0.57  | 0.15 | 0.60 | 0.09   | 0.69       | 1.74     |
|        | L2    | 10,000| 11.3 | 1.06  | 0.45 | 0.45 | 0.66   | 0.94       | 2.47     |
|        | L3    | 35,100| 17.4 | 7.40  | 0.40 | 0.10 | 1.40   | 0.43       | 3.75     |
|        | L4    | 4,875 | 19.5 | 2.72  | 0.98 | 0.27 | 2.63   | 0.14       | 2.96     |
|        | YA    | 7,400 | 20.3 | 47.8  | 1.66 | 0.68 | 1.90   | 2.35       | 1.55     |

* Growth conditions and abbreviations as in Table 1.
Fig. 5. Cholesterol dependence of mortality of *C. elegans* strains carrying the indicated mutations in age-related pathways. A: *daf-9*(e1406), *daf-12*(m20). B: *clk-1*(qm30). C: *daf-16*(mgDf50). D: *daf-12*(m20), and Animals were grown in the absence (filled diamond) or presence (filled triangle) of 1,000 ng/ml cholesterol. *N* = 30.
centrate cholesterol from the medium passively into their membranes (12, 13), and the dietary bacteria probably constitute the major sterol source for C. elegans (2). This variability implies that much of the total sterol content may be nonfunctional; perhaps it is passively adsorbed in varying amounts on the developing cuticle, or trapped in the intestinal lumen. It appears, therefore, that no conclusions can be drawn from total composition data, at least of bacterially grown worms, regarding functionally important changes in sterol availability during development or senescence.

Table 1 shows representative sterol contents for staged animals mutated in the daf-9, daf-12, and npe-1 genes. Strains were chosen that are most likely to be functional nulls. Npe-1 encodes a transporter of hydrophobic molecules, and is homologous to the human gene mutated in the most common form of Niemann-Pick disease, a disease of cholesterol accumulation (14). Deletion of npe-1 in C. elegans results in hypersensitivity to cholesterol deprivation. The daf-9 and daf-12 mutants were of interest because the genes encode homologues of a vertebrate steroidogenic cytochrome P450 and a vertebrate steroid hormone type nuclear receptor, respectively (15, 16). The double mutant was used to test effects of daf-12 mutation, because the single mutation induces a dauer-constitutive phenotype but the double mutant is dauer-defective. Within the limitations of sample variability discussed above, none of these mutations appeared to grossly affect the sterol distribution at any stage of development.

**Relationship to known age-related pathways**

To address the question of how sterol deprivation affected longevity mutants, several mutant strains were studied. Mutations in daf-9, daf-12, daf-16, and clk-1 were tested, encompassing the major documented age-related genetic pathways (17). As shown in Fig. 5, effects of sterol deprivation on life span were proportionally similar to those found in wild-type worms (Fig. 1) in all the mutants tested. Similar effects on the rate of motility decrease were also seen (not shown). The life-span-increasing actions of sterols are thus unaffected by the actions of any of these age-related genes, including daf-9 and daf-12, which are thought to be related to steroid hormone biosynthesis and function.

**DISCUSSION**

We have previously shown that sterols are required throughout the development of C. elegans. In the first generation of sterol deprivation, animals reach reproductive maturity, presumably by utilizing maternally derived sterols. Sterol depletion is first evidenced in these animals at the reproductive stage, by cessation of growth and subnormal brood size. Development is dramatically affected in the second generation of sterol deprivation; growth is arrested at larval stages L1 to L3, depending on the sterol status of the mother (2, 4, 5).

This report extends observations of first-generation cholesterol-deprived animals to the post-reproductive stage, i.e., past day 7 post hatching. Effects of cholesterol deprivation intensify at this stage, resulting in a significantly decreased life span (Fig. 1A). There is also a more rapid loss of motility (Fig. 1B), accompanied by accelerated sarcopenia (Fig. 2). These observations suggest that under cholesterol-replete conditions sufficient sterol is stored in the fertilized egg to support development to reproductive maturity, at which point usable sterols are exhausted and increasingly severe consequences ensue.

From these observations, it seems that sterols are required continuously throughout life. The presence of all the major sterol metabolites at every stage of life (Table 2) is consistent with this conclusion, and provides a further indication that sterol metabolites, not the added cholesterol itself, are the functional molecules.

The life-extending effects of sterols are most likely not mediated by the known age-related genes daf-16 (which is downstream from, and mediates, the age-related effects of daf-2), daf-9, daf-12, or clk-1, as evidenced by the observation that mutations in any of these genes failed to mitigate the effects of sterol deprivation (Fig. 5). We conclude that sterols act through as yet uncharacterized pathways, which may control specific biochemical processes, and might also help to prevent the occurrence of nonspecific stochastic disintegration and loss of function.

We have previously shown that 4MSs can provide >98% of required sterol for animals grown from the eggs of cholesterol-deprived mothers, but that the addition of 1–2% of any desMS is required for complete developmental func-

**TABLE 2. Sterol content of synchronized wild-type (N) Caenorhabditis elegansa**

| Stage          | N | Chol  | 7-DHC | Lath | Loph | Δ8(14) | 7-DHC/Chol | Σ4MS/Lath | Ratios   |
|----------------|----|-------|-------|------|------|--------|------------|-----------|----------|
| Eggs/Embryo    | 31,000 | 3.30  | 4.3   | 1.04 | 0.34 | 1.47   | 1.30       | 1.74      |          |
| L1             | 23,000 | 26.5  | 0.30  | 0.30 | 0.26 | 0.26   | 0.11       | 1.67      |          |
| L2             | 36,570 | 5.29  | 1.8   | 0.36 | 0.10 | 0.92   | 0.35       | 2.85      |          |
| L3             | 11,950 | 11.3  | 5.0   | 0.65 | 0.45 | 1.24   | 0.44       | 2.60      |          |
| L4             | 32,000 | 5.90  | 3.8   | 0.50 | 0.20 | 1.10   | 0.64       | 2.60      |          |
| YA             | 3,070  | 33.7  | 3.22  | 1.67 | 0.50 | 0.76   | 0.10       | 0.75      |          |
| 7–10 days      | 2,500  | 40    | 139   | 48   | 15   | 56.5   | 3.50       | 1.50      |          |
| 15–18 days     | 2,200  | 634   | 1,076 | 67.7 | 38.4 | 81.5   | 1.80       | 2.10      |          |
| Dauer          | 195,000 | 5.82  | 40    | 3.27 | 0.20 | 6.13   | 6.90       | 2.00      |          |

Chol, cholesterol; 7-DHC, 7-dehydrocholesterol; Lath, lathosterol; Loph, lophenol; YA, young adult; Δ8(14), 4α-methylcholesta-8(14)-en-3β-ol [Δ8(14) sterol]; Σ4MS, lophenol + Δ8(14) sterol.

a Grown on OP50 lawns on NGM agar plates.
tion (2). However, any of the 4MSs or desMSs could provide similar fertility-maintaining and age-retarding effects as cholesterol in animals that have never been sterol deprived (Fig. 4), even though 4MSs cannot be demethylated at C4 by *C. elegans* (7). This appears to be another example of what has been termed “cholesterol sparing” (18, 19); an adequate external supply of either of the 4MSs facilitates the conservation of the small amount of desMS required for full function. An alternative possibility is that the long-term effects described here are mediated exclusively by a 4MS pathway.

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