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Recent Developments in Nematode Steroid Biochemistry

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Abstract: Current knowledge of steroid nutrition, metabolism, and function in free-living, plant-parasitic and animal-parasitic nematodes is reviewed, with emphasis upon recent investigation of *Caenorhabditis elegans*. A number of 4-desmethylsterols with a *trans*-A/B ring configuration can satisfy the steroid nutritional requirement in *C. elegans*, but sterols with a *cis*-A/B ring configuration or *trans*-A/B sterols with a 4-methyl group cannot. *C. elegans* removes methyl or ethyl substituents at C-24 of the plant sterols sitosterol, campesterol, stigmasterol, stigmastanol, and 24-methylenecholesterol to produce various sterols with structures partially dependent upon that of the dietary sterol. Additional metabolic steps in *C. elegans* include reduction of Δ^{22}- and Δ^{5}-bonds, C-7 dehydrogenation, isomerization of a Δ^{7}-bond to a $\Delta^{8(14)}$-bond, and 4α-methylation. An azasteroid and several long-chain alkyl amines interfere with the dealkylation pathway in *C. elegans* by inhibiting the Δ^{24}-sterol reductase; these compounds also inhibit growth and reproduction in various plant-parasitic and animal-parasitic nematodes. A possible hormonal role for various steroids identified in nematodes is discussed.

Key words: biochemistry, *Caenorhabditis elegans*, ecdysteroid, hormone, steroid, sterol.

Although investigation of nematode steroids began 30 years ago (18), knowledge of nematode steroid biochemistry has advanced slowly for several reasons. These include the microscopic size of most nematodes, their low steroid content, and the consequent difficulty of propagating sufficient quantities of nematodes, especially parasitic species, for biochemical analysis. Nonetheless, the existence of key differences in steroid metabolism between parasitic nematodes and their hosts and the potential benefit of selective inhibition of nematode steroid metabolism have caused researchers to intensify their efforts in this important area of nematode biochemistry.

NOMENCLATURE

A seemingly complex system of trivial and systematic names for steroids understandably has alienated many biologists from steroid research papers. Initially, steroids may appear to be complex, but they differ from each other in only a few ways: the presence of a side chain on the tetracyclic ring system (or steroid nucleus) and the presence and location of double bonds and methyl, ethyl, keto, or hydroxyl substituents. Systematic nomenclature of steroids is based on the structure of cholestane (Fig. 1). Steroids with a complete side chain and a single hydroxyl group are called sterols; they function primarily as integral structural components of cell membranes. Most sterols contain double bonds, usually at C-5 (a Δ^{5}-bond); however, some sterols do not contain a double bond, and they are referred to as stanols (e.g., cholestanol, Fig. 1). Plant sterols differ from animal sterols in that the former usually possess an alkyl (i.e., methyl or ethyl) substituent at C-24; indeed, the terms "phytosterol" and "24-alkylsterol" are often regarded as synonymous, although cholesterol does occur in a number of plant species at very low concentrations. The plant sterol sitosterol (Fig. 1) is 24α-ethylcholesterol; its systematic name is 24α-ethylcholest-5-en-3β-ol, where "α" and "β" denote specific stereochemical orientation. Alternatively, sitosterol can be defined in relation to stigmastane (= 24α-ethylcholestane) as stigmast-5-en-3β-ol. Because all of the sterols described in this review are 3β-ols, we shall omit the "3β" descriptor.

Sterols also serve as metabolic precursors to steroid hormones, which in vertebrates typically lack a side chain (e.g., testosterone, estrone; Fig. 1). Ecdysteroids (e.g., ecdysone, Fig. 1) are polyhydroxylated Δ^{7}-6-ketosteroids with complete side chains and are involved in hormonal regulation of molting and other developmental processes in insects. The reader is

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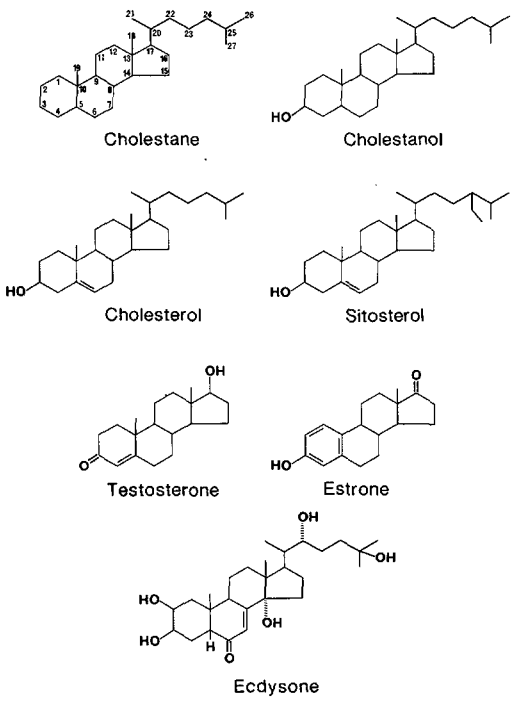

FIG. 1. Structures of some common steroids.

referred to the excellent book of Nes and McKean (31) for further discussion of steroid nomenclature and function.

ANALYTICAL METHODS

Recent advances in analytical methodology and instrumentation have facilitated the identification of minute quantities of nematode steroids. These techniques include capillary gas-liquid chromatography (GLC), with resolution superior to conventional packed-column GLC; capillary GLC-mass spectrometry (GC-MS), which provides information on the molecular weight and structure of unknown compounds; high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC), with better resolution and sensitivity than their early predecessors; and radioimmunoassay (RIA), employed for the detection of subnanogram quantities of nematode ecdysteroids.

One should be cognizant of the limitations of the above methods. For example, TLC, even HPTLC, provides a nondefinitive indication of the identity of a compound, as many classes of compounds have approximately the same polarity as sterols and thus migrate similarly on TLC in many solvent systems. In our own work, putative sterol fractions homogeneous by TLC have contained occasionally less than 5% sterol. Although HPLC and GLC provide better separation than TLC, comigration of two or more sterols as well as nonsteroidal contaminants can occur; precise structural determinations can be made only when chromatographic data is supplemented by MS or other spectroscopic data. Even MS cannot differentiate between many stereoisomers. Many researchers lack access to a mass spectrometer; for these, erroneous identification can be minimized by subsequent analytical steps such as acetylation, argentation column chromatography, and GLC on two or more liquid phases.

NUTRITIONAL REQUIREMENT FOR STEROL IN NEMATODES

Interest in nematode sterol metabolism was stimulated by the discovery that *Steinernema feltiae* DD-136 and *Caenorhabditis briggsae* require sterols for growth and reproduction (17,24). Detailed historical reviews of investigation of the structural specificity for the essential sterol as well as the lack of de novo sterol biosynthesis in nematodes can be found (3,7). Nutritional experiments should be evaluated cautiously because of the possible occurrence of sterol contaminants in media ingredients or in the supplemented sterol. For example, we have found a commercial hemin preparation to contain 0.2% cholesterol and a commercial sitosterol sample to contain 44% sterols other than sitosterol. In addition, one should be aware of the possible occurrence of a "sparing" process, well documented in insects (26), in which many sterols can substitute for cholesterol in a structural role in membranes and thus make available small quantities of endogenous cholesterol for use as a specific precursor to steroid hormones or other metabolites. Fortunately, several investigations with nematodes have been performed with satisfactory methodology, including GLC analyses. The results of our recent investigations with *Caenorhabditis elegans* (7) agree with similar findings in other nematodes in that a large number of structurally diverse sterols can satisfy the nutritional requirement. Sterols that did not satisfy the requirement in *C. elegans* include 4α-methyl- or 4,4-dimethylsterols

TABLE 1. Relative percentages of sterols in some plant-parasitic nematodes and their respective hosts.

	Heterodera zeae	Corn roots	*Globodera solanacearum*	*Rotylenchulus reniformis*	Cotton roots
Cholesterol	8.9	0.6	11.5	22.6	0.6
Cholestanol	0.4	Trace		0.7	
Lathosterol	0.3	0.1			
24-methylcholesta-5,22-dienol	0.6	0.1			
24-methylenecholesterol	0.2	0.1			
Campesterol	15.2	20.4	9.2	4.2	5.1
Campestanol	1.7	1.8	43.1	0.7	Trace
Stigmasterol	12.4	57.0		14.6	43.4
Stigmast-22-enol	2.8	1.7	19.8		
24Z-ethylidenecholesterol	0.8	0.4	7.7		
Sitosterol	51.3	15.5	(combined)	49.1	48.5
Stigmastanol	4.6	1.2	8.7	2.7	Trace
Stigmast-7-enol	0.5	0.4			
24Z-ethylidenecholest-7-enol	0.3	0.6			
Unknown				5.5	2.4
Literature citation	(6)	(6)	(32)	(37)	(37)

or sterols with a *cis*-A/B ring configuration (as opposed to the *trans*-configuration of cholesterol).

STEROLS OF PARASITIC NEMATODES

Because of the lack of suitable techniques for axenic mass propagation, investigation of sterol metabolism in parasitic nematodes principally has been limited to comparison of the sterol compositions of host and parasite. Cholesterol is the major sterol of the few vertebrate-parasitic nematodes analyzed thus far (1,5,12,23). Significant amounts of sitosterol, campesterol (24α-methylcholesterol), cholestanol, stigmastanol (24α-ethylcholestanol) and campestanol occur in *Ascaridia galli* (23) and *Ascaris suum* (1,12). Presence of 24-alkylsterols in these digestive tract parasites is probably due to occurrence in the host diet. Movement of cholesterol and sitosterol across the intestinal wall of *A. suum* has been demonstrated (2), but passage of cholesterol through the body wall is the primary route of short-term cholesterol absorption in this nematode (22).

Among phytoparasitic nematodes, the only sterols detected in *Ditylenchus triformis* and *D. dipsaci* were cholesterol and lathosterol (cholest-7-enol), except for traces of phytosterols in the latter species (11). Their hosts contained only 24-alkylsterols. Curiously, the sedentary plant parasites examined to date (Table 1) contain greater relative proportions of phytosterols than the migratory *Ditylenchus* spp. It is not known whether the more highly evolved parasitism of the sedentary plant parasites has included an adaptation of these organisms to utilize or store substantial amounts of plant sterols. The relative lack of stanols in *Heterodera zeae* (6) compared to *Globodera solanacearum* (32) (Table 1) is another interesting difference among plant-parasitic nematodes. The possibility that the two major genera of cyst nematodes have characteristically different sterol compositions has interesting phylogenetic ramifications and requires further investigation.

Because plant-parasitic nematodes contain higher relative percentages of cholesterol than their hosts and because many phytophagous insects (35), as well as subsequently described free-living nematodes, convert plant sterols to cholesterol by a C-24 dealkylation process, it has been speculated that similar dealkylation pathways exist in phytoparasitic nematodes (6,11,37). However, the presence in some insects of selective uptake mechanisms (36,39) dictates that experiments involving radiolabeled 24-alkylsterols should be performed. Interestingly, *A. suum* did not metabolize injected [^{14}C]sitosterol to any other sterol (1).

STEROL METABOLISM IN FREE-LIVING NEMATODES

C-24 dealkylation: The most specific information about nematode sterol metab-

TABLE 2. Relative percentages of sterols from *Caenorhabditis elegans* propagated with different dietary sterols.

Recovered sterol	Supplemented sterol				
	Sitosterol*	Stigmasterol	Campesterol	24-methylene-cholesterol	Stigmastanol
Cholesterol	8.1	9.1	4.0	9.4	
7-dehydrocholesterol	56.4	50.6	26.3	48.2	
Lathosterol	5.5	4.8	3.4	4.3	65.4
Cholesta-5,7,9(11)-trienol	2.0	5.2	1.5	2.2	
Cholest-8(14)-enol					3.9
Cholestanol					3.9
Cholesta-5,7,24-trienol				0.1	
Campesterol	0.6		38.9		
24-methylenecholesterol			3.2	27.6	
24-methylcholesta-5,7-dienol			13.9		
24-methylcholest-7-enol			0.7		
24-methylenecholesta-5,7-dienol			1.1	3.4	
24-methylcholesta-5,7,9(11)-trienol			0.9		
Sitosterol	18.2				
Stigmasterol		20.8			
Stigmastanol					15.4
Fucosterol	0.1				
4α-methylcholest-8(14)-enol	8.6	9.2	4.6	4.3	10.6
4α-methylcholest-7-enol	0.5	0.3	0.2	0.3	0.8
4α,24-dimethylcholest-8(14)-enol			0.8		
4α,24-dimethylcholestanol			0.5		

* Contained 1.5% campesterol.

olism has been obtained through investigations of axenically propagated free-living nematodes. Cole and Krusberg (13) conclusively demonstrated the existence of a C-24 dealkylation capability in nematodes via conversion of [^{3}H]sitosterol to [^{3}H]cholesterol and [^{3}H]7-dehydrocholesterol (cholesta-5,7-dienol) by *Turbatrix aceti*.

More recently we have performed a comprehensive investigation of sterol metabolism in *C. elegans* (Table 2) axenically propagated in a liquid medium containing one of five different 24-alkylsterols and chloroform/methanol-extracted basal ingredients (9,10,27–29). In these experiments, *C. elegans* removed the 24-ethyl group of [^{14}C]sitosterol and produced several different [^{14}C]24-desalkylsterol metabolites of approximately the same specific activity as the dietary sitosterol. In addition to converting [^{14}C]sitosterol to [^{14}C]cholesterol, *C. elegans* introduced a double bond at C-7 to produce 7-dehydrocholesterol as its major sterol and, to a lesser extent, reduce the Δ^5-bond of 7-dehydrocholesterol to form smaller quantities of lathosterol. The sterol composition of *C. elegans* propagated in medium supplemented with stigmasterol (stigmasta-5,22-dienol) was similar to that of sitosterol-fed nematodes; thus, the presence of a Δ^{22}-bond did not prevent dealkylation. Similarly, *C. elegans* removed 24α-methyl or 24-methylene substituents in experiments with campesterol-supplemented or 24-methylenecholesterol-supplemented medium (Table 2). Unlike sitosterol, the nucleus of campesterol was directly modified to a substantial extent without dealkylation occurring, as substantial quantities of campesta-5,7-dienol (i.e., 24α-methylcholesta-5,7-dienol) and campest-7-enol were produced. It is not known whether the direct nuclear modification of campesterol results from the existence of separate enzyme systems for nuclear modification of 24-methylsterols or from the ability of sterols with a less bulky 24-methyl group to bind to the same enzymes involved in the nuclear modification of 24-desalkylsterols.

Phytosterol dealkylation in *C. elegans* is not dependent on existence of unsaturation in the sterol nucleus, as *C. elegans* metabolized stigmastanol to produce mainly lathosterol (Table 2). Because cholesterol,

7-dehydrocholesterol, or any other sterols with a Δ^5-bond were not detected, *C. elegans* could lack a C-5 dehydrogenase.

Nuclear methylation: In animals with de novo sterol biosynthetic capabilities, six five-carbon isoprenoid units are linked together and cyclized to form lanosterol (4,4,14α-trimethylcholesta-8,24-dienol) as the first cyclized product; in the formation of cholesterol, the three methyl groups are sequentially removed. In our experiments with *C. elegans* (Table 2), we unexpectedly detected substantial quantities of a few 4α-methylsterols, the most abundant one having the unusual $\Delta^{8(14)}$-bond. The steryl ester fraction of *C. elegans* was especially rich in these compounds (9,28). In experiments with radiolabeled dietary desmosterol (cholesta-5,24-dienol) or sitosterol, the 4α-methylsterols from *C. elegans* contained approximately the same specific activity as the dietary sterol and hence were produced by a direct nuclear methylation pathway. Concurrent detection of 4α-methylcholest-7-enol and lathosterol indicates that the latter compound could be a tentative precursor for 4α-methylsterol synthesis.

We have discovered a similar nuclear methylation pathway in *T. aceti.* Surprisingly, 4α-methylcholestanol comprised approximately one-third of the 4α-methylsterols from this species (8). The function of nematode 4-methylsterols is obscure; we have speculated about a possible specific hormonal, pheromonal or other physiologic role for 4α-methylsterols or their metabolites (10). The failure of previous investigators to detect 4-methylsterols in nematodes could have resulted from comigration of these compounds with fatty alcohols rather than 4-desmethylsterols during TLC. Alternatively, many species may not contain 4-methylsterols; indeed, we could not detect such compounds in *H. zeae* cysts (6), although other life stages of this or other parasitic nematodes could contain such compounds. Additionally, it is possible that 4α-methylsterols are metabolic products of a similarly unique biotransformation of the dietary sterol by some microorganism, even an endosymbiont, contaminating our *C. elegans* culture. Although it is empirically impossible to rule out such a possibility, we have been unable to isolate such an organism, inhibit 4-methylsterol production by addition of antibiotics to the medium, or detect 4-methylsterols in incubated, nematode-free medium.

Inhibitors of sterol metabolism: Like nematodes, insects lack the ability to biosynthesize sterols de novo. As a result of extensive chemical synthesis and subsequent structure–activity investigations, other investigators at our laboratory discovered that many azasteroids and related nonsteroidal alkylamines and alkylamides inhibit growth, development, and steroid metabolism in several insects (38). Collaborative investigations with other researchers revealed that many of these compounds at concentrations as low as 1.0 μg/ml are toxic to several diverse genera of nematodes, including *Panagrellus redivivus* (19), *Meloidogyne incognita* (19), *Ostertagia ostertagi* (16), *Nematospiroides dubius* (4), and *Nippostrongylus brasiliensis* (4). Specific biological effects of these compounds in some of these experiments include decreased motility, paralysis, inhibition of reproduction, blocked or incompleted molting, and similar morphological abnormalities as observed in nematodes cultured in sterol-deficient medium.

The most highly specific effects of these inhibitors in nematodes were obtained through our recent investigations of *C. elegans* propagated in inhibitor-supplemented media (10,27,29). When *C. elegans* was cultured in medium supplemented with sitosterol and the azasteroid 25-azacoprostane hydrochloride, 96% of the isolated sterols (excluding dietary sitosterol) were Δ^{24}- or $\Delta^{24(28)}$-sterols, which are usually present in no more than trace quantities (Table 3); e.g., desmosterol (cholesta-5,24-dienol), cholesta-5,7,24-trienol, cholesta-7,24-dienol, and fucosterol (24E-ethylidenecholesterol). The azasteroid-induced accumulation of these four sterols indicates that they are key intermediates in the pathway for sitosterol dealkylation and subsequent metabolism in *C. elegans* (Fig. 2) and that their production results from inhibition of the Δ^{24}-sterol reductase enzyme system in *C. elegans* by the azasteroid. Also, because Δ^7- and $\Delta^{7,24}$-4α-methylsterols occurred in inhibited *C. elegans* in greater proportions than their $\Delta^{8(14)}$- and $\Delta^{8(14),24}$-

TABLE 3. Relative percentages of total sterols from *Caenorhabditis elegans* propagated with 25 μg/ml sitosterol and various metabolic inhibitors. Dietary sitosterol contained 1.5% campesterol.

Recovered sterol	None	25-azacoprostane HCl (5 μg/ml)	C_{12} amine* (25 μg/ml)	C_{14} amine† (25 μg/ml)	C_{16} amine‡ (25 mg/ml)	C_{12} amide§ (35 μg/ml)	Branched C_{12} amine‖ (25 μg/ml)
Cholesterol	8.1	0.4	9.0	9.3	8.5	9.0	2.6
7-dehydrocholesterol	56.4	1.2	23.1	33.7	29.0	47.0	3.7
Lathosterol	5.5	0.2	7.7	6.1	7.2	6.0	2.0
Cholesta-5,7,9(11)-trienol	2.0	0.1	0.7	3.8	11.0	6.4	0.5
Desmosterol		9.0	4.7		0.8		11.8
Cholesta-5,7,24-trienol		44.5	14.8	9.7	2.4		7.4
Cholesta-7,24-dienol		2.1	1.2	1.2	1.1		6.9
Cholesta-5,7,9(11),24-tetraenol		1.2	1.4	1.6			
Cholesta-8,24-dienol		0.1	0.1				3.3
Campesterol	0.6	0.9	1.5	1.3	2.2	1.0	3.6
Fucosterol	0.1	2.3	0.4	2.7	1.7	1.1	3.8
Sitosterol	18.2	29.8	19.6	16.8	20.0	20.1	35.7
4α-methylcholest-8(14)-enol	8.6	0.2	4.1	5.3	11.4	6.7	2.8
4α-methylcholest-7-enol	0.5	0.5	2.9	2.6	2.9	0.7	2.1
4α-methylcholesta-8(14),24-dienol		2.5	2.2	1.0	1.4		9.1
4α-methylcholesta-7,24-dienol		3.1	1.7	0.9	0.4		4.5
Others		1.9	4.9	4.0		2.0	0.2
Total Δ^{24}-sterols	0.1	64.8	26.5	17.1	7.8	1.1	46.8
Total 24-alkylsterols	18.9	33.0	21.5	20.8	23.9	22.2	43.1

* N,N-dimethyldodecanamine.
† N,N-dimethyltetradecanamine.
‡ N,N-dimethylhexadecanamine.
§ N,N-dimethyldodecanamide.
‖ N,N,3,7,11-pentamethyldodecanamine.

analogs, 25-azacoprostane very likely inhibits a second metabolic site; i.e., the isomerase that converts Δ^{7}- to $\Delta^{8(14)}$-4α-methylsterols.

The *C. elegans* Δ^{24}-sterol reductase is inhibited by four nonsteroidal alkylamines: N,N,3,7,11-pentamethyldodecanamine, N,N-dimethyldodecanamine, N,N-dimethyltetradecanamine, and N,N-dimethylhexadecanamine, in decreasing order of Δ^{24}-sterol reductase inhibition (Table 3). The first of these compounds, however, is the least toxic to *C. elegans* (27). Conversely, even though N,N-dimethyldodecanamide inhibited growth and reproduction in *C. elegans* at the concentrations employed, it did not inhibit Δ^{24}-sterol reductase activity (Table 3). These inhibitors could affect some other site of steroid metabolism, as several azasteroids and alkylamines alter ecdysteroid metabolism in a number of insects (35). However, the paralytic or convulsive effects seen immediately upon addition of *C. elegans* to medium containing alkylamines or alkylamides (but not azacoprostane) indicates that the amines and amides may also possess an initial, direct neurotoxicity or neurochemical action.

Experiments with dietary campesterol and stigmasterol further demonstrated the utility of N,N-dimethyldodecanamine as a tool for probing metabolism of other phytosterols by *C. elegans* (29). The compound inhibited the Δ^{24}-sterol reductase in nematodes fed campesterol less than in sitosterol-fed *C. elegans*, but the observed production of several Δ^{24}-sterols indicates that the major pathway for campesterol metabolism occurs as depicted in Figure 2. In stigmasterol-supplemented cultures, N,N-dimethyldodecanamine induced accumulation of desmosterol, cholesta-5,7,24-trienol, cholesta-5,22,24-trienol, cholesta-5,7,22,24-tetraenol, and stigmasta-5,22E,24(28)-trienol. Lack of detection of sitosterol, fucosterol, and any $\Delta^{5,22}$-, $\Delta^{5,7,22}$- or $\Delta^{7,22}$-dealkylated sterols indicates that reduction of the Δ^{22}-bond occurs after dealkylation but before Δ^{24}-reduction (Fig. 3).

STEROID HORMONE BIOSYNTHESIS

Sterols have at least two major roles in animals. First, sterols function as integral structural components of membranes, and sterols have been detected in intracellular membranes of *T. aceti* (34). Second, sterols

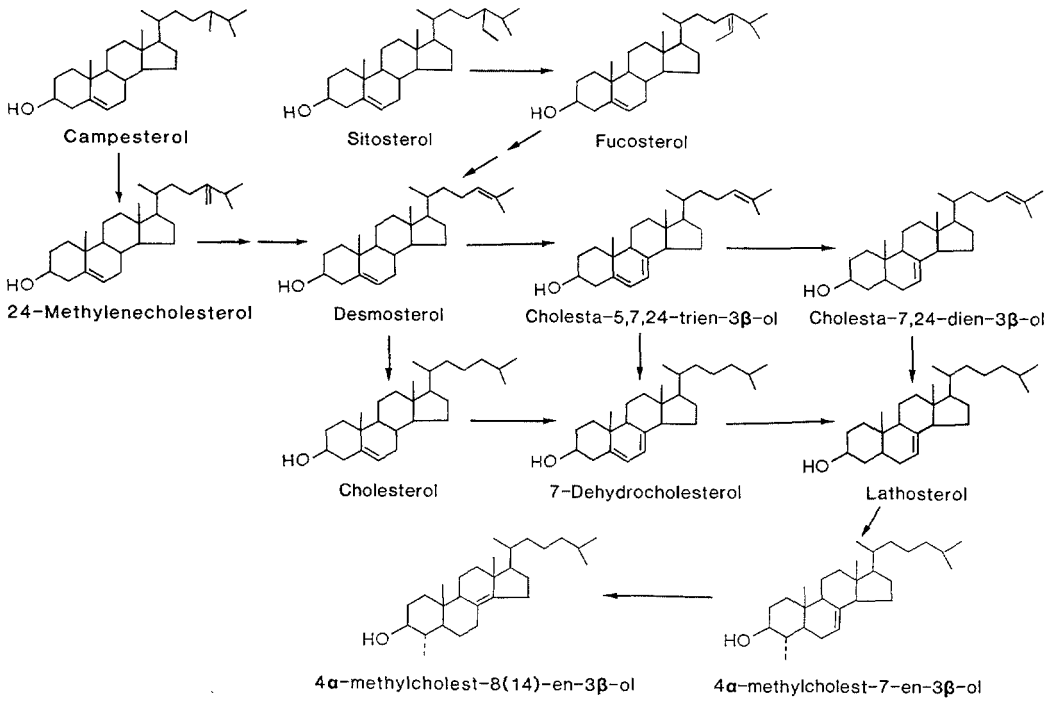

FIG. 2. Major pathways for metabolism of sitosterol and campesterol by *Caenorhabditis elegans.*

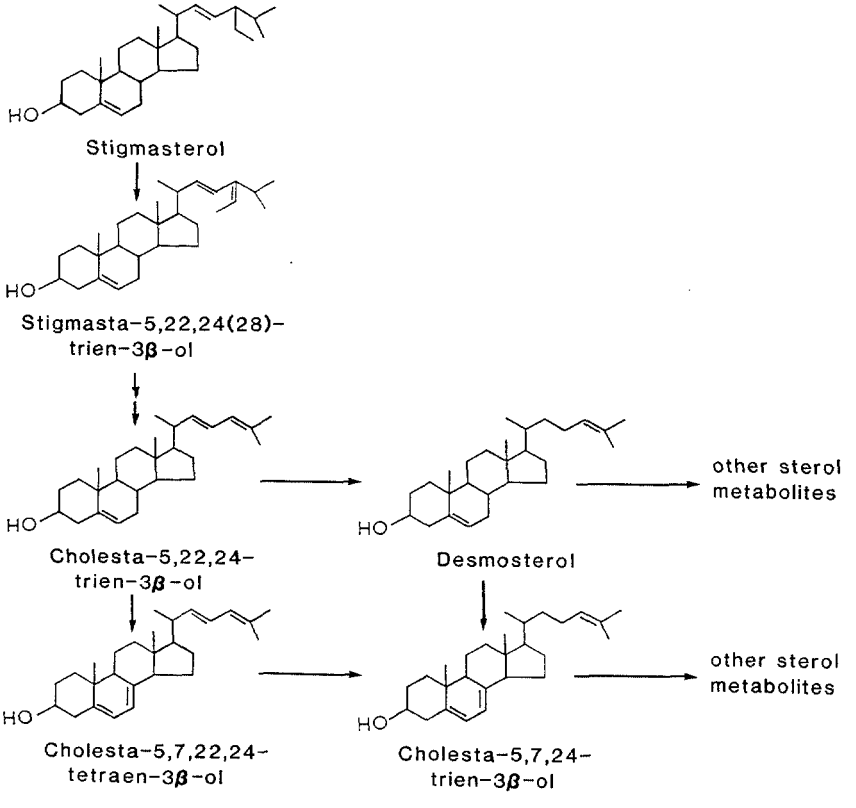

FIG. 3. Major pathways for metabolism of stigmasterol by *Caenorhabditis elegans.*

are metabolic precursors to a variety of other compounds, most notably steroid hormones. Unfortunately, the biosynthesis of steroid hormones from a sterol precursor by nematodes has not yet been demonstrated.

Knowledge of hormonal control of nematode development has progressed slowly. Evidence for the existence of hormonal regulation of exsheathment or ecdysis in a few animal-parasitic nematodes has been excellently reviewed (14,40). The identity of any such hormone is unknown; analogs of insect juvenile hormones (epoxyfarnesoic acid methyl ester derivatives) have received greater attention than steroids.

Because both insects and nematodes molt periodically and because ecdysteroids hormonally regulate insect molting, many investigators have attempted to isolate and identify such compounds from nematodes. The search for ecdysteroids in nematodes, recently reviewed (33), has progressed slowly because of the much smaller concentrations of these compounds in nematodes than in insects. In an excellent combination of classic with modern analytical methodology, Mendis et al. (30) identified ecdysone and 20-hydroxyecdysone as the major ecdysteroids of the dog heartworm by GC-MS, HPLC, and RIA. A similarly conclusive identification of a typical vertebrate steroid hormone in a nematode has not been presented, although Willett et al. (40) preliminarily described the detection of progesterone in *P. redivivus.*

The presence in nematodes of steroids with hormonal function in other phyla does not imply that such compounds have similar roles in nematodes. A detailed description of the biological effects in nematodes of exogenously applied ecdysteroids is beyond the scope of this review, but the stimulation of molting or growth of various species by low concentrations of various ecdysteroids (15,20,21,25) indicates that these compounds are strongly bioactive within nematodes, if not hormones in the classic sense. Conclusive proof of hormonal function of a nematode steroid would involve identification of the site of synthesis or release of the compound and a subsequent combination of an organ-specific bioassay system with analytical chemistry.

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