Developmental Nutrition of Nematodes: the Biochemical Role of Sterols, Heme Compounds, and Lysosomal Enzymes[1]

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Abstract: Attempts to develop defined *in vitro* culture systems for the growth, reproduction and development of free-living nematodes have yielded much basic information about their nutritional requirements and biochemistry. Requirements for sterol and heme have been identified suggesting that some nematodes lack *de novo* synthesis of these molecules. Possible pathways of metabolism of these nutritional requirements can be derived from *in vitro* experiments that use a variety of sterol and heme sources as supplements to the culture medium. These pathways are reviewed as well as the possible role of sterol and heme in the biology of free-living and parasitic nematodes. Since these molecules must be acquired dietarily, the possible involvement of lysosomal enzymes in digestion is discussed. Also considered is the possibility that lysosomal enzymes change when nematodes are fed on a heme protein source. *Key Words:* Hemoglobin, cholesterol, cathepsin D, acid protease, free-living, plant and animal parasitic nematodes.

Studies on the developmental biochemistry of nematodes have historically lagged behind biochemical studies in other areas because of the small size of most nematodes, and the inability to rear large synchronous populations of nematodes, especially many of the parasitic ones. This has made it difficult to obtain enough material for biochemical studies. However, the recent interest in free-living nematodes as a model system for aging studies has produced much information on nematode biochemistry, physiology, genetics, and nutrition. This information has led to the development of defined *in vitro* culture systems. These systems are now being modified for the culture of parasitic nematode species. During the design of culture media for *in vitro* growth of some nematodes, specific nutritional requirements for heme and sterol have been identified. This has led to several studies on the metabolism of these molecules by nematodes. Because these requirements must be obtained dietarily, the studies have led to

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a consideration of the role of lysosomal enzymes in nematode digestion.

This paper considers the observations we have made during our attempts to develop a defined medium for *in vitro* culture of the free-living and early parasitic stages of the rat parasitic nematode, *Nippostrongylus brasiliensis*. Our findings here are related to the work of other investigators in an attempt to familiarize the reader with the current knowledge of nematode sterol and heme metabolism during development and growth.

A) *Nematode sterol synthesis:* In mammalian systems, sterols are synthesized by condensation of acetyl CoA to mevalonate. Six 5-carbon units from mevalonate residues are then condensed to squalene which is converted to the 2,3 oxide. This molecule undergoes an anaerobic cyclization, catalyzed by squalene oxide cyclase, to lanosterol. The lanosterol is converted to desmosterol and then to cholesterol. 7-Dehydrocholesterol can also serve as an immediate precursor of cholesterol (54).

Cholesterol is the major structural lipid of biomembranes and is a direct precursor for the synthesis of steroid hormones. In mammalian systems cholesterol is catabolized to the various bile acids, such as cholic and chenodeoxycholic acid, to 7-dehydrocholesterol, a precursor of vitamin D and to the fecal products coprostanol, coprostanone and cholestanol. These last compounds are readily available to the free-living stages of animal-parasitic nematodes that develop in feces.

The sterol composition of several nematodes has been characterized. In all cases the nematodes have been found to contain cholesterol, cholesterol esters, and in some species precursors of sterol biosynthesis (4, 19, 20, 50). It has been reported that the sterol composition of nematodes may be affected at least in part by the host diet (46).

The possibility that bacteriophagous and plant-parasitic nematodes are incapable of *de novo* sterol biosynthesis was discovered through attempts to maintain them in a synthetic medium (28, 35, 37, 41). It has been further demonstrated that these nematodes are unable to synthesize sterols from the simple precursors acetate and mevalonate. Thus *Turbatrix aceti, Panagrellus redivivus,* and *Caenorhabditis briggsae* are unable to incorporate ^{14}C acetate or mevalonate into cholesterol (20, 40, 41).

Nippostrongylus brasiliensis has also been shown to be incapable of *de novo* sterol synthesis (7). Free-living stages of *N. brasiliensis* were grown on a medium of formalin-killed *Escherichia coli,* supplemented with either precursors to sterol synthesis, sterols of plant and animal origin or end products of sterol metabolism (7). Acetate and mevalonate supported development only to second-stage larvae (L_2), which were small and degenerate. Squalene at 100 μg/ml, however, supported about 9% development to normal-appearing third-stage infective larvae (L_3) within the time required for 80% development to L_3 in cholesterol-supplemented control cultures. Lanosterol, on the other hand, supported about 40% development to normal L_3. 7-Dehydrocholesterol was somewhat better in supporting development to L_3 than was cholesterol. Farnesol failed to support any development and was lethal. Ninety percent of the first-stage larvae from farnesol-supplemented cultures were dead after 5 days of incubation. The plant sterols ergosterol and β-sitosterol could be substituted for cholesterol, but the metabolic end products of cholesterol metabolism, coprostanol and coprostanone, which are present in the natural environment in which these larvae develop, did not support development (7).

Similar results have been reported by Lu *et al.* (35). Those workers observed that *C. briggsae* and *C. elegans* completed their life cycles on a medium supplemented with 200 μg/ml lanosterol; *T. aceti* completed its life cycle with lower concentrations. *P. redivivus* makes cholesterol from lanosterol. The lanosterol is synthesized by the cyclization of squalene-2,3-oxide (51, Willett, personal communication). Squalene at 400 μg/ml also supported the growth, development, and reproduction of *C. briggsae* and *C. elegans* (35). Since we (7) did not test the effect of squalene concentrations at the high levels used by Lu *et al.* (35), a greater developmental response of *N. brasiliensis* might have occurred at these levels.

β-Sitosterol has been reported to support the growth and reproduction of *T. aceti, C. briggsae, C. elegans* (35), and *N. brasiliensis*

(7). Barrett (4) was unable, however, to demonstrate the conversion of β-sitosterol to cholesterol in adult Ascaris. This would suggest that the nematodes capable of using β-sitosterol either do so by substituting the molecule unmodified into their metabolic pathways, or they convert β-sitosterol to cholesterol. This point needs further investigation.

Results of *in vitro* experiments with free-living nematodes and the free-living stages of parasitic nematodes suggest that either, through evolution, nematodes have lost enzymes necessary for the conversion of simple precursors of cholesterol biosynthesis to squalene or that these enzymes are repressed by negative feedback of the high concentration of sterols readily available in the nematode diet. It is apparent, however, that these nematodes retain to various degrees the enzymes necessary for the conversion of squalene to cholesterol. Thus the data from *in vitro* studies suggest that the enzymes squalene epioxidase and squalene oxide cyclase are present and active in the free-living nematodes. Although these enzymes may be present in *N. brasiliensis* they are present at low concentration, have low specific activity, or are partially inhibited by end-product feedback. Enzymes for the conversion of farnesol to squalene are apparently lacking or inactive in the nematodes investigated. Enzymes required for the conversion of lanosterol to cholesterol are apparently present since lanosterol will support the growth and development of both free-living nematodes and free-living stages of parasitic nematodes (7, 35).

Although these observations give much insight into the problem of nematode sterol biosynthesis and metabolism, conclusive proof of the presence of the various enzymes will depend upon their isolation and characterization.

B) *Functional role of sterols:* Cholesterol and cholesterol esters have been shown to be major components of, and contribute to the structural integrity of, all biological membranes. Hence these molecules probably have a similar role in nematodes. Involvement of dietary cholesterol would, therefore, be extremely important during molting (11). The hypodermal changes that occur during molting require synthesis of rough endoplasmic reticulum and Golgi (11). These membranous cellular organelles, therefore, require sterols. Only the parasitic nematode *Nematospiroides dubius* has been shown to develop in sterol-free media; apparently there is sufficient endogenous cholesterol in the egg to supply the needs for subsequent development (36). Sterol synthesis has not been demonstrated in this nematode (36).

Several workers (5, 24, 25, 30) have recently proposed that nematode molting is hormonally regulated in a manner similar to that of insects and that ecdysone or ecdysonelike steroids are involved. Hitcho and Thorson (30) have isolated two lipid fractions from the larvae of *Trichinella spiralis.* One fraction appears to have juvenile hormonelike activity, and the other ecdysonelike activity. These compounds inhibit and stimulate molting and growth in a manner similar to that observed in insects. Similar ecdysonelike steroids have been identified by radioimmune assay in extracts of *P. redivivus, Aphelenchus avenae* and *Haemonchus contortus* (24). Thus, since cholesterol serves as a direct precursor for steroid hormone biosynthesis, it is likely that it is involved in the synthesis of nematode "molting-regulatory hormones" or of any other steroid hormonelike compounds which may function in nematodes. Additionally, it has been suggested that nematode sterols are involved in chemical attraction (39, 45).

C) *Heme metabolism in nematodes:* Several species of nematodes have been shown to contain hemoglobin in perienteric fluid and myoglobin in tissues (8, 33, 46). Of the nematodes investigated to date, only three species of parasitic nematodes, all members of the order Oxyuroidea, lack hemoglobin (46). Additionally, hemoglobin has been isolated from two species of free-living nematodes (2, 14). The physical and physiological characteristics of hemoglobins of parasitic nematodes have been reviewed by Lee and Smith (33) and by Smith (43). They pointed out that it is unlikely that hemoglobins function mainly as an oxygen transport mechanism or, in the case of the perienteric fluid hemoglobin, as an oxygen storage source in animal-parasitic nematodes.

Animal-host and parasitic-nematode

hemoglobins differ in their oxygen affinities, deoxygenation kinetics, absorbance spectra, isoelectric points and molecular weight (27, 33). These differences apparently reside in the globin rather than the heme moiety. This suggests that the parasitic nematodes either synthesize their hemoglobin *de novo*, or ingest and digest host hemoglobin, which they then convert into nematode hemoglobin by replacing the host globin moiety with a globin molecule of nematode origin.

Heme, in some form, has been shown to be necessary for *in vitro* growth, reproduction, and development of many nematodes (8, 29, 37, 41, 46). Furthermore, it has been shown that the hemoglobin content of adult Ascaris is dependent on heme concentration in the host diet (17). The *in vitro* requirement for hemoglobin has been supplied by addition to culture medium of such compounds as hemoglobin, hematin, blood cell lysate, or tissue extracts (8, 12, 29, 48). To determine the nature of this requirement Hieb *et al.* (29) and Vanfleteren (48) reported that *C. briggsae* would grow and reproduce on a medium containing hemin chloride or several heme proteins (heated liver extract, myoglobin, hemoglobin, cytochrome-C). *In vitro* growth, development and reproduction did not occur, however, on the same medium containing nonheme proteins. These experiments demonstrated that the heme moiety was the required portion of the molecule for support of *in vitro* culture of *C. briggsae*.

D) *Hemoglobin synthesis:* Hemoglobin synthesis in mammals is initiated by the synthesis of aminolevulinic acid (ALA) from pyridoxal phosphate and succinyl CoA. This reaction, which is product-feedback regulated, is catalyzed by the enzyme ALA synthetase. Thus the rate of heme synthesis is also regulated by this rate, controlling enzyme. The various porphyrin precursors are synthesized from porphobilinogen by condensation into the typical tetrapyrrole ring. The appropriate acetate, propionate, methyl, and vinyl side chains are added to make protoporphyrin. Iron is inserted into protoporphyrin to form heme by the enzyme ferrochelatase.

We tested the ability of several known intermediates in mammalian and bacterial heme synthesis and bilirubin and biliverdin (the end products of hemoglobin metabolism) to support the *in vitro* development of *N. brasiliensis* larvae from L_1 to L_3 (8). Coproporphyrin I resulted in slightly enhanced development of the larvae to third stage and a significant increase in the number of viable larvae recovered after 5 days. Porphobilinogen, and uroporphyrin supplements, while not resulting in increased percent development, had a positive effect on worm size. Coproporphyrin I, which is abundant in rat feces (52), was found to be the best growth supplement, suggesting that the larvae can utilize this molecule to synthesize heme. This seems to imply that the nematode retains the enzyme coproporphyrinogen oxidase needed to convert coproporphyrin I to protoporphyrin-IX, the direct precursor of heme. That protoporphyrin-IX added as a supplement had a slight enhancing effect on larva survival suggests that the larvae maintain the enzyme ferrochelatase. Furthermore, since ALA and porphobilinogen supplements had little, if any, effect on worm growth and development, it might be suggested that the enzymes ALA synthetase and ALA dehydratase are either absent or repressed in the worm.

E) *Heme metabolism in N. brasiliensis:* Heme metabolism by *N. brasiliensis* larvae was studied by maintaining the larvae in a medium containing U ^{14}C rat-blood hematin for 6 days until L_3 larvae had developed. The ^{14}C porphyrins were identified in these larvae. In addition to protoporphyrin IX, which could have arisen by removal of iron from heme during the extraction procedure, radioactive coproporphyrin was present. This observation suggests that the worm is capable of converting ingested protoporphyrin and/or hematin to coproporphyrin. It was also observed that L_3 recovered from feces cultures contained protoporphyrin, coproporphyrin, and uroporphyrin (3.1, 5.5, and 2.6 nmoles/mg dw, respectively), and that adult worms recovered from the rat 11 days postinfection showed an increased concentration of these porphyrins (14.0, 9.8, and 10.0 nmoles/mg dw, respectively). The possibility that porphyrin ratios changed in these larvae in response to feeding on host hemoglobin was tested *in vitro* by maintaining exsheathed L_3 filariform larvae in "parasitic" culture in a medium of rat hemoglobin, medium 199

and vitamin B_{12} in roller bottles at 37 C in an atmosphere of 92% air, 8% CO_2. After 2 days in this culture the worms showed morphological changes comparable to early lung-stage larvae (50). When the porphyrins were isolated from these developing hemoglobin-fed larvae and quantitated, there was a 3-fold increase in protoporphyrin, a slight increase in coproporphyrin and a 2-fold increase in uroporphyrin. This suggests that prior to attaching the globin moiety, the nematode must restructure the host molecule and resynthesize a heme porphyrin which can be used in nematode hemoglobin. This breakdown, which may be only a rearrangement of the side chains of heme, is not a novel suggestion since Lee and Smith (33) proposed that nematodes metabolized host hemoglobin to bile pigments and then resynthesized heme. Cain and Welshman (17) have cited evidence that no breakdown of host heme to bile pigments occurs. No conversion of bile pigments to heme apparently occurs in *N. brasiliensis* larvae, since the bile pigments bilirubin and biliverdin had no enhancing effect on the development of this worm from egg to third-stage larvae (8). Additionally, these molecules did not replace rat-blood lysate as a growth factor in "parasitic" culture used for the development of *N. brasiliensis* from infective third-stage larvae to L_4 parasitic lung-stage larvae (Bolla and Weinstein, unpublished observations).

Although the experiments with *N. brasiliensis* suggest the presence of the enzyme coproporphyrinogen oxidase in the worm, specific assays for the presence of this enzyme were not done. It was originally thought that the conversion of coproporphyrin to protoporphyrin had an absolute requirement for molecular oxygen. If this were true it would be difficult to explain how a conversion of coproporphyrin to heme could occur in adults of those intestinal parasitic nematodes, which are in a micro aerobic environment (32). Perhaps the bacterial pathway for anaerobic conversion of corporphyrin to heme (26) is functional in some nematodes. Cain and Bassow (16) reported the presence of coproporphyrin-III and protoporphyrin-IX in the perienteric fluid of Ascaris but they could not identify the compounds in muscle. These workers were unable to identify uroporphyrin in Ascaris perienteric fluid or muscle although this porphyrin was found in whole-body extracts of *N. brasiliensis* (8). This difference might simply indicate a rapid conversion of uroporphyrin to coproporphyrin in Ascaris, or it might suggest a metabolic difference between Ascaris and *N. brasiliensis*. Cain and Bassow (16) further proposed that the perienteric concentration of porphyrins in Ascaris was dependent on the nematode diet. Nematodes which had darkly pigmented guts had approximately 8 times as much protoporphyrin and 10 times as much coproporphyrin as did nematodes with lightly pigmented guts. The proto/copro ratio, however, was the same in both nematode types. Protoheme was the major porphyrin present in Ascaris perienteric fluid. The heme to protoporphyrin ratio is about 5000 (16). That observation supports the earlier hypothesis of Cain and Welshman (17) that protoheme from the host diet is directly incorporated without modification into nematode perienteric fluid hemoglobin. This suggested ability to utilize host heme directly could explain the inability of nematodes to synthesize hemoglobin *de novo* from ALA since heme present in high concentrations in the perienteric fluid could act as a repressor to the enzymes ALA synthetase and ALA dehydratase which initiate *de novo* hemoglobin synthesis.

It is of interest, however, that Ascaris retains the ability to convert coproporphyrin to protoporphyrin. Cain (15) reported coproporphyrinogen oxidase activity in adult Ascaris gut, and in muscle mitochondria, and in 6-day embryonated eggs. This enzyme was low in activity compared with rat liver, but that might be accounted for by feedback inhibition by heme on the rate-limiting enzymes of prophyrin biosynthesis.

Besides the possible incorporation of dietary porphyrins, the origin of various porphyrins in nematodes remains somewhat obscure. It is apparent that nematodes are capable of utilizing exogenous supplies of heme to synthesize nematode hemoglobin. Although it can be suggested that nematodes do not directly utilize exogenous heme but must first degrade it and resynthesize a "nematode heme," this would require an as-yet-undescribed pathway. It is more

likely, therefore, that the nematode is capable of taking any available porphyrin and converting it to "nematode heme" by slightly altering the side chains or of utilizing heme directly from the host without having modified it. Based on results with *N. brasiliensis* (8), the possibility of breakdown and resynthesis cannot be ruled out.

F) *Functional role of heme:* In addition to its role in oxygen storage and transport as hemoglobin, heme is involved in the (1) synthesis, structure and activity of cytochrome molecules, (2) synthesis of vitamin B_{12}, (3) regulation of protein synthesis, and (4) synthesis of enzymes, such as catalase and peroxidase. Nematodes have never been shown to synthesize vitamin B_{12} and in many instances have been shown to require that vitamin as a nutritional supplement (34, 50). Thus it is unlikely that exogenous heme is required for synthesis of this vitamin by the nematode. Within the last decade, heme has been shown to have a major functional role in the regulation of globin protein synthesis (1, 22). This regulation involves the hemin-dependent cAMP binding to the regulatory subunit of cAMP-dependent protein kinase (23). Met-tRNA-binding factor eIF_2, when phosphorylated, is inhibited from binding to the ribosome. It has been demonstrated by DeHaro *et al.* (23) that hemin blocks the activation of cAMP-dependent protein kinase responsible for phosphorylation of eIF-2. When insufficient heme is present, cAMP is activated to phosphorylate, a protein kinase which then phosphorylates eIF-2, resulting in inhibition of binding of eIF-2-GTP-met $tRNA_f$ to the 40s ribosomal subunit, thus inhibiting protein synthesis. The presence of heme blocks the initial step in this regulatory pathway and allows protein synthesis initiation (23, 44).

The regulation of protein synthesis by hemin could be of great importance to nematodes, particularly during early embroyogenesis and molting. Lee and Smith (33) and Smith (47) have suggested that hemoglobin in adult female *Ascaris* serves as a source of hemin for egg production and for early embryogenesis. The heme moiety is possibly used for the synthesis of cytochromes needed in large amounts by the developing embryos and larvae (15). The function of hemoglobin in male *Ascaris* is not known. It is also possible that the heme present in eggs and embryos (33) and in larvae and adult forms of many nematodes serves a regulatory function in protein synthesis. Regulation of protein synthesis by heme may explain the 58% increase in protein synthesis reported by Cain and Welshman (17) in *Ascaris* from pigs fed a diet rich in hemin. Such regulation may have a key role in the developmental changes that occur when parasitic nematodes switch from a free-living existence to a parasitic mode of life. Many infective larvae are nonfeeding (32), and therefore may be deficient in heme. Such a condition could result in repressed protein synthesis. Induction of protein synthesis could then occur with infection of the host, when the larvae begin feeding in a hemin-rich environment. This mechanism could also apply to nonfeeding stages of infective larvae of plant-parasitic nematodes which reinitiate development once they begin feeding on plant tissues. The mechanism may be particularly true in species infecting leguminous plants which synthesize leghemoglobin.

The hemoglobin requirement demonstrated for growth, development, and reproduction of nematodes may have a practical application to the legume-nematode relationship that exists. Hussey and Barker (31) reported that nodulation was inhibited in soybean plants infected with *Belonolaimus longicaudatus* and *Heterodera glycines,* and was stimulated by *Meloidogyne hapla* and *Pratylenchus penetrans.* This stimulation was, however, of no advantage to the plant, since all four species of nematode inhibited nitrogen fixation. The observation may be important, however, since a link has been demonstrated between nodulation, leghemoglobin synthesis, and nitrogen fixation (21). The synthesis of leghemoglobin is apparently a cooperative effort between the nodule bacteroids and the plant, since it has been demonstrated that the heme moiety is synthesized by the bacteroid and the globin moiety by the plant (21). Furthermore, globin synthesis by the plant is regulated by the presence of heme synthesized by the bacteroid, again stressing the regulation of protein synthesis by heme. After the plant is infected, the parasitic nematodes may use

enough heme from the plant to repress globin synthesis. That would then interfere with leghemoglobin synthesis and prevent nitrogen fixation. On the other hand, it might be of advantage for some nematodes to induce increased nodulation of the infected plant as a mechanism of increasing their supply of heme. Sharma and Sethi (42) measured the leghemoglobin content of cowpea nodules infected with *M. incognita* and *H. cajani*. Forty-five days postinfection, *M. incognita* had caused a 52% decrease in leghemoglobin concentration, and *H. cajani* had caused a 37% decrease. Mixed infection with both nematodes resulted in a 44% decrease. This work represents the first report of a direct effect of plant-parasitic nematodes on the leghemoglobin content of a nodulated plant. It is not, however, clear from that work whether the parasite affects the leghemoglobin by feeding directly on the heme protein or by influencing the bacteroid which causes nodulation and synthesis of the heme moiety. The reported decrease in leghemoglobin content, however, is large enough to result in decreased nitrogen fixation by the plant. Such a relation between infected plants and the parasitic nematodes infecting the plant might explain observations of Hussey and Barker (31) and surely poses a problem worth investigating further.

An exogenous source of heme may also be required by nematodes for synthesis of the heme-containing enzymes of H_2O_2 metabolism. Aueron and Rothstein (3) have demonstrated the presence of catalase and peroxidase in *T. aceti*. Thus heme proteins in nematodes have many and varied functions totally different from the major mammalian role in the storage and transport of oxygen.

G) *Lysosomal enzymes:* Because the nutritional requirements discussed above must be derived from the nematode diet (7, 8, 13, 29, 48) it is of interest to consider aspects of protein digestion by nematodes. In a few nematodes protein digestion has been suggested to be extracellular and to involve lysosomal enzymes (18, 50).

Weinstein (49) reported that before *N. brasiliensis* feeds as a parasite on a heme protein source, its gut cells contain a large number of colorless, small inclusions having many characteristics of lysosomes (49). However, after feeding in vitro on lysed red blood cells, or after recovery from host lung, the intestinal cell inclusions become pigmented a golden brown, and lose their birefringence in polarized light. These inclusions thus resemble residule bodies. It is interesting to consider whether these morphological changes are related to the change in diet that occurs with the transition from free-living forms to parasitic forms. It is also interesting to consider whether biochemical changes in lysosomal enzymes accompany the morphological changes. Major nutritional changes occur when free-living larvae, feeding on bacteria and detritus, switch to parasitic larvae feeding on blood and cellular products.

Toward determining the role of lysosomes in the transition from free-living to parasitic life styles, we have investigated developmental changes in two major lysosomal enzymes—acid phosphatase and acid protease of the cathepsin type (9, 10). We have demonstrated that acid phosphatase activity changes coincidental with nematode development and age postinfection (9). The specific activity of this enzyme was highest in infective L_3 larvae, decreased in lung-stage L_4, then increased again coincident with differentiation to adult worms. The increase in activity of the enzyme was greatest during differentiation from L_2 to L_3 larvae. By 40 hours postinfection, the time at which the intestinal cell inclusions differentiate to residual bodies (49), the specific activity of the enzyme decreased about 14-fold. This change in specific activity of acid phosphatase from L_3 to L_4 larvae corresponded to a loss in the histochemical reaction of the gut cell inclusions for this enzyme (49). Although Weinstein (49) equated this loss of histochemical reaction when the worm differentiated to a parasitic form, to a loss of lysosomal activity, it may rather represent a change in the major lysosomal enzyme present.

These observations have led us to suggest that synthesis of a protease capable of digesting hemoglobin is required when *N. brasiliensis* begins feeding on host blood. Changes in acid protease activity in *N. brasiliensis* coincident with a shift in the diet from that of free-living larvae to that of parasitic larvae and adults were therefore

investigated (10). Assay conditions were such that protease activity, in the pH range of 3.2 to 3.8, hydrolyzed hemoglobin but not bovine serum albumin or other nonheme proteins. This is characteristic of a cathepsin-D-like enzyme (53). The activity of such a protease in *N. brasiliensis* was high in L_1 and L_2 larvae, declined in L_3 larvae, and began to increase following infection of the host, reaching a maximal level in fourth-stage larvae 4 days postinfection (10). The patterns of inhibition by heavy metals and a variety of protease inhibitors and the stimulation by sulfhydral compounds of this activity were similar to those reported for cathepsin-D (53). This suggests that acid protease in *N. brasiliensis* is a cathepsin-D-like enzyme(s). The high levels of acid protease activity in L_1 and L_2 larvae, followed by a decline in third-stage larvae, may be related to the extensive feeding that occurs in the early larval forms but not in nonfeeding ensheathed L_3. The increase in enzyme activity coincident with feeding on a tissue or heme protein substrate supports the hypothesis that lysosomal acid protease activity increases when the nematode switches to a parasitic mode (10).

This hypothesis was further substantiated by *in vitro* "parasitic" culture studies (10). Acid protease activity increased in exsheathed L_3 larvae maintained on parasitic culture medium containing a protein or peptide source. It did not increase in the basal medium [Difco Laboratories (Detroit, Michigan) Medium 199 + vitamin B_{12}] which contains only amino acids (Table 1). The results are consistent with those of Pinnock *et al.* (38). They reported the protein growth factor required by *C. briggsae* (12, 13, 47, 48) could be replaced by a peptide source of at least tripeptide length. They also noted free amino acids did not replace the protein requirement.

The increase in acid protease activity coincident with the parasitic phase of the life cycle of *N. brasiliensis* appears to be due to an increase in the concentration of the enzyme (10). Actinomycin D, which had earlier been shown to inhibit RNA synthesis in *N. brasiliensis* in culture (7), was added to parasitic culture medium supplemented with soy peptone or horse serum. After 2 days in "parasitic" culture, the exsheathed larvae were recovered and assayed for acid protease activity. Slight but measurable activity could be detected in these larvae. The levels were, however, below that of larvae used to initiate the cultures and of control larvae maintained without actinomycin-D (10). This suggests protein turnover in the absence of *de novo* synthesis. This observation would also suggest that the increase in acid protease activity above a maintenance level in response to feeding is a result of increased synthesis of the enzyme rather than "activation" of a stored form of the enzyme (10).

TABLE 1. Cathepsin-D-like activity from L_3 larvae of *N. brasiliensis* reared in "parasitic" culture (9) supplemented with either horse serum, rat blood cell lysate plus horse serum, or soy peptone.

Medium	μg tyrosine produced/μg protein/hr in 4 days	μg tyrosine produced/μg protein/hr in 6 days
Basal medium[a]	0.527	0.589
Basal medium + horse serum[b]	1.162	1.417
Basal medium + horse serum + rat red blood cell lysate	1.245	1.117
Basal medium + soy peptone	0.924	1.030

[a]Basal medium: Difco medium 199 + vitamin B_{12}.
[b]The horse serum used contained significant amounts of hemoglobin.

Additional research is needed on the synthesis of specific lysosomal enzymes in response to feeding and on the possible role of heme in the regulation of this response. If this form of regulation can be shown to operate in nematodes, it may help to clarify the reason for the heme requirement for nematode growth, development and reproduction.

H) *Conclusions:* Nematode nutritional biochemistry and the mechanisms of utilization of sterols and heme compounds during development, growth, and reproduction remain unclear. It is apparent, however, that sterols are not synthesized by many nematodes, although a variety of sterols supplied in the diet can be metabolized to forms which can be utilized by the worms. Sterols are an integral part of nematode

membranes; other roles played by these lipids in nematode biochemistry remain obscure. Additional studies are needed to determine whether steroid homones are active in regulating nematode molting. It is also clear that many nematodes are unable to synthesize heme *de novo* but the metabolism of heme proteins ingested by nematodes remains somewhat equivocal. Of greater interest in the area of hemoglobin utilization by nematodes is the host-parasite relationship, particularly between plant-parasitic nematodes and leguminous plants; and the role that heme metabolism may play in the initiation of nematode protein synthesis. Both of these problems certainly merit further investigation.

All of these problems of nematode biochemistry are now within the reach of the researcher interested in basic biochemistry and molecular biology. With the advent, in the last decade, of suitable culture technique for these animals and the several highly sensitive micromethods for biochemical research, we now have the methodology and expertise to pursue problems related to the biochemistry of nematodes and the host-parasite relationship.

It is hoped that this review has outlined some areas of nematode developmental biology and parasitism that will foster research in this exciting and fairly unexplored area of biochemistry and molecular biology.

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