

Development of the Reproductive System of *Caenorhabditis elegans*

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A morphological study of the growth and the development of the reproductive system of the nematode *Caenorhabditis elegans* has been carried out. When the first stage larva hatches from the egg it contains four primordial gonadial cells. These cells proliferate and form the entire adult reproductive system, consisting of approximately 2500 nuclei, in 45 hr at 25°C. Several distinctive morphological features of gonadogenesis and early embryogenesis that are recognizable in the compound microscope can be used to chart the development of the nematode. The mature gonad presents a linear developmental axis both temporally and morphologically of the formation of oocytes, fertilization, and the early stages of embryogenesis. The structure of the adult ovary indicates that the cytoplasm of each newly formed oocyte is derived from a common core of cytoplasm within the multinuclear ovary.

INTRODUCTION

A major problem in biology today is to understand the patterns of control that genetic functions exert during the development of a higher organism. One approach to this problem is to probe specific stages of development with mutational analysis using an organism in which distinct morphological characterization can be done.

It was apparent to cytologists as long as 75 years ago that nematodes were valuable for cytological analysis of development. Van Beneden in 1883 discovered from his cytological studies on nematode chromosomes that equal amounts of nuclear material were contributed by egg and sperm. In 1899, Boveri published his now classical studies on the early cleavage patterns of *Ascaris* eggs, and from these studies emerged the idea of cell lineages and the demonstration that the fate of cells in nematode eggs was determined at a very early cleavage. Further, he was able to show that the primordial germ cell was set aside from the somatic cells early in embryonic cleavages.

We have chosen the small nematode *Caenorhabditis elegans* for our studies because it has a simple morphology with a relatively small number of cells, its reproduction has been extensively studied by

Honda (1925) and Nigon (1965), it is easy to culture in the laboratory, its genetics has recently been elucidated in a series of elegant studies by Brenner (1974), and mutants are easily obtained. Our approach has been to isolate a set of temperature-sensitive mutants blocked in various stages of development and to begin characterization of the phenotypes by morphological studies.

It was first necessary to understand the chronology and morphology of development in the wild-type strain of *C. elegans*. These studies serve as a basis of comparison for phenotypes observed in the mutants and for the timing of major events during the development of the mutants. Our anatomical studies focus on the reproductive system of *C. elegans* because it undergoes a dramatic development during the growth of the nematode and because many of the temperature-sensitive mutants appear to have their primary lesions in gonadogenesis or in the function of the adult reproductive system. This paper describes the anatomical studies on the wild-type strain of *C. elegans*.

MATERIALS AND METHODS

Nematode strain. The *C. elegans* var. Bristol used in these studies is a free-liv-

ing soil nematode. The wild-type strain, designated N2, came from the stock maintained by Brenner (1974) in Cambridge, England. Honda (1925) and Nigon (1965), in their descriptive work on the reproduction and embryology of *C. elegans*, used the strain originally isolated by Maupas in 1900. Whereas their descriptions indicate that their strain is very similar to the *C. elegans* used in our studies, it is not the identical worm, as evidenced by its different temperature responses. For example, the Bristol strain that we use grows and reproduces at 25°C, but the French strain must be maintained below 24°C.

Media. NG agar and M9 buffer have been described by Brenner (1974). M9 medium used for growth of *E. coli* OP50 is M9 buffer containing 4 g glucose, 1.25 mg vitamin B₁, and 4 mg uracil per liter.

C. elegans Ringer's solution is 8.21 g anhydrous sodium acetate, 0.182 g sodium chloride, 1.46 potassium chloride, 1.03 g calcium chloride hexahydrate, 0.80 g magnesium chloride hexahydrate, 2.06 g sucrose, and 1.19 g HEPES per liter and adjusted to pH 7.55 using sodium hydroxide.

Laboratory culture methods. The basic culture methods are those of Brenner (1974). *C. elegans* is routinely cultured in the laboratory on petri plates filled with NG agar which contain a lawn of *E. coli* strain OP50. Worms are transferred *en masse* with M9 buffer or with paper strips; individuals are transferred with sharpened applicator sticks. Stocks are stored by freezing slowly in M9 buffer containing 15% DMSO or in a 15% solution of glycerol in S buffer, and stored in liquid nitrogen.

Cultures of N2 worms are normally maintained at 20°C, but since our work involves the use of the temperature-sensitive mutants, the worms are grown at 16 or 25°C, the permissive and restrictive temperatures used for the mutants (Hirsh and Vanderslice, 1976).

Synchronous cultures of *C. elegans* are prepared by allowing gravid worms to lay

fertilized eggs on petri plates seeded with *E. coli* OP50. The worms are gently rinsed off the plates with M9 buffer. Any worms remaining after the rinse are removed with a small glass tube connected to a vacuum line. The eggs stick to the agar surface. After 2 hr of hatching, the first stage larvae (L1's) are collected by rinsing the plate again. They are then transferred to fresh bacteria seeded NG plates, which have been equilibrated at the desired growth temperature.

Microscopy. General observations of worms are done with a Wild M5 dissecting microscope equipped with a KG-1 heat filter. Detailed light microscopic observations are done with a Zeiss Universal Photomicroscope.

The structure of the adult gonad of wild-type worms has been studied using both whole worms and dissected gonads. The gonad can be dissected out of the worm by cutting the worm immediately below the pharynx with irridectomy scissors or a scalpel. The internal pressure usually forces the gonad from the worm, but often an eyelash glued to an applicator stick can be used to move the gonad away from the worm. Dissections are done in *C. elegans* Ringer's solution. Live, whole worms are observed in the Ringer's solution or M9 buffer supplemented with 0.5% phenoxypropanol (Eastman, P6448) or 0.125% Tricaine as anesthesia. Bright-field and Zeiss-Nomarski differential interference contrast optics are used to observe whole worms and dissected gonads. Bright-field microscopy is also performed on whole worms and dissected gonads fixed in Carnoy's solution, mounted on slides with Mayer's albumin, and stained by the Feulgen reaction (Humason, 1967). Transmission electron microscopy is performed on thin sections of whole N2 worms and dissected gonads. Material is fixed in a solution containing 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and postfixes with 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.4. After

extensive washing with water, the specimen is stained with 1% uranyl acetate, then dehydrated with ethanol, and embedded with Epon. The sections are stained with uranyl acetate and lead citrate.

Observation of early embryogenesis is done with Nomarski interference and phase-contrast optics on the *in utero* zygotes in anesthetized worms. In several instances, for example, in order to follow cleavages prior to the 30 blastomere stage, the fertilized eggs are released from the parent by gently tapping the coverslip. The zygotes are very sensitive to anoxia. Therefore, the gravid parents were placed in an M9 buffer solution which also contains *Euglena* to supply oxygen. This permits complete embryogenesis to occur under a sealed coverslip.

MORPHOLOGY OF *C. elegans*

Adult anatomy. Adult *C. elegans* is 1.2 mm long and 70 μm in diameter and contains approximately 600 somatic cells (Fig. 1a). It is encased in a tough elastic cuticle probably similar to the *Ascaris* cuticle, which is composed chiefly of a specialized cross-linked collagen surrounded by a proteinaceous cortical layer (Josse and Harrington, 1964; Bird and Deutsch, 1957). Because the worm is transparent, major internal structures can be seen with the light microscope without sectioning. Large hypodermal cells lie beneath the cuticle and probably secrete cuticular components. EM examination of these cells shows that they contain a large amount of rough endoplasmic reticulum. The worm feeds through an oral cavity, and a bilobed muscular pharynx pumps bacteria into the intestine, which is composed of 32 epithelioid cells. The intestine exists at the anus. Four longitudinal rows of muscle cells extend down the body of the animal. This musculature, as well as the pharyngeal muscles, has recently been studied by Epstein *et al.* (1974) and Waterston *et al.* (1974). *C. elegans* contains a simple nervous system made up of approximately 200

to 300 nerve cells. Most of the sensory receptors are organized into sensilla located on the front of the animal. These include sensilla assumed to be mechanoreceptive or thermosensitive and amphids, that are most likely chemoreceptive. The worm exhibits chemotaxis (Ward, 1973; Dusenbery, 1974), and the complete sensory neuroanatomy has recently been elucidated (Ward *et al.*, 1975; Ware *et al.*, 1975).

C. elegans is a self-fertilizing hermaphrodite containing an extensive reproductive system that makes both sperm and eggs. It has a 4-day generation time at 16°C. A sample of 25 worms produced 280 ± 97 progeny per parent. Two worms produced no progeny; therefore, the average for fecund worms is 304 ± 52 progeny per parent. Much of our effort has been devoted to a morphological description of the reproductive system and will be given in detail below. Hermaphrodites have five pairs of autosomal linkage groups and are XX.

Males occur spontaneously in *C. elegans* with a frequency of about one per 700 worms (Hodgkin, 1974). Males are XO and are morphologically distinguishable from hermaphrodites by the copulatory bursa on their tails. Stocks of males are maintained by crossing the spontaneously appearing males with hermaphrodites. Males are used for the transfer of genetic markers, mapping mutations, and complementation studies (see Brenner, 1974).

Larval growth. A growth curve for N2 worms at 16, 20, and 25°C is shown in Fig. 2. When the worm hatches from the egg shell, it is a 240- μm first-stage larva, or L1, which is sexually immature. As it grows to adulthood, it undergoes four larval molts, in which the old cuticle is shed and replaced by a new underlying cuticle. Each intermolt period is designated as a separate larval stage, L1 through L4, followed by adulthood. Worms stop pharyngeal pumping and become very lethargic while molting. Extensive proliferation of

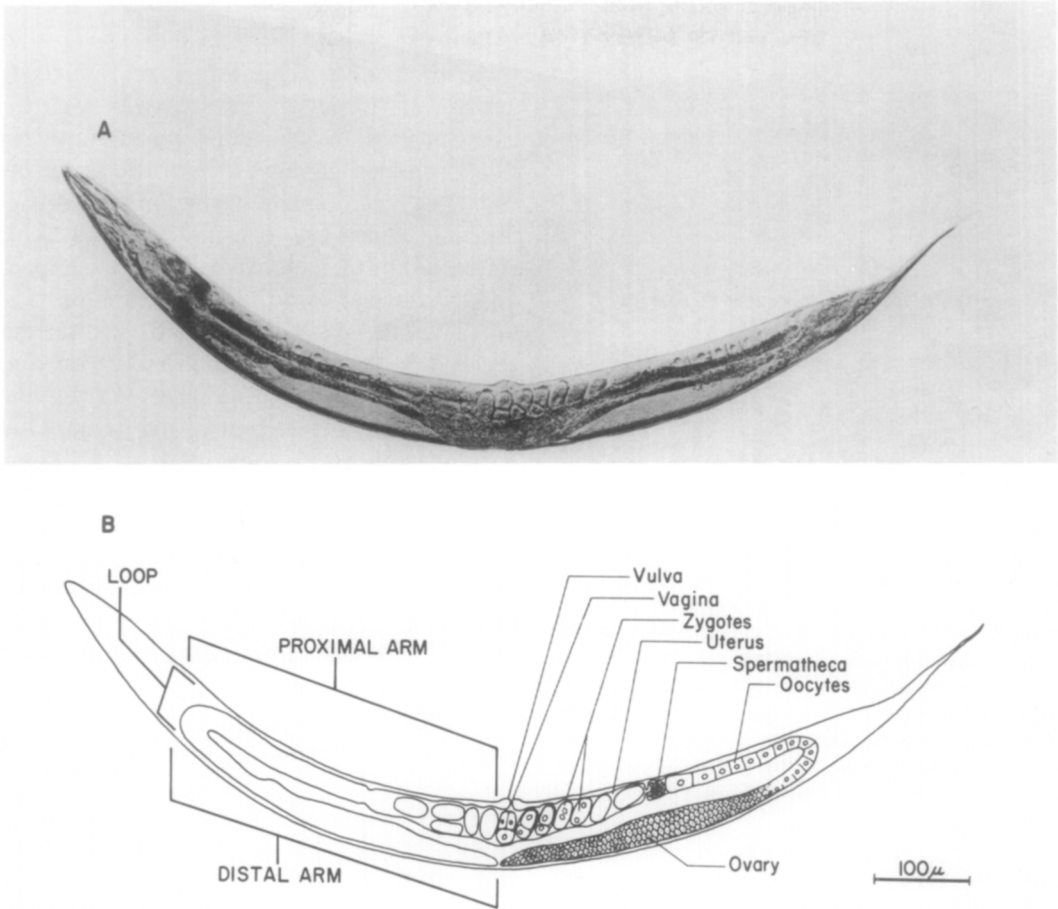


FIG. 1. Wild-type (N2) *C. elegans* hermaphrodite. (A) Photomicrograph taken with Zeiss-Nomarski differential interference contrast optics. (B) Diagram showing the reproductive system.

the germ line occurs during the growth from L1 to adulthood. This proliferation is described in greater detail below.

The growth rate shown in Fig. 2 at 25°C is 2.1 times that at 16°C; the growth rate at 20°C is 1.3 times that at 16°C. At 25°C, the worms begin laying fertilized eggs 45 hr after hatching, continue for 3 days, and then lay oocytes for 2 days. At 16°C, laying of fertilized eggs begins 94 hr after hatching, continues for 4 days, then unfertilized eggs are laid for 3 days. An L1 hatches from the fertilized egg approximately 12 hr after it is laid. The limited number of fertilized eggs is laid because the worms become depleted of sperm. Some of these unfertilized eggs must be viable, since older

hermaphrodites mated with males produce offspring, half of which are males.

The number of progeny produced by a worm is a function of the growth temperature. This relationship as well as the growth rate as a function of temperature is shown in Fig. 3. The mean life span of *C. elegans* at 16°C is 23 ± 3 days, at 20°C is 14.5 ± 2 days, and at 25°C is 8.9 ± 1.1 days. If the food source is depleted in a mixed population of worms, the L2 worms undergo a molt to become Dauer larvae, which are thinner than any normal larval stage and can live for several weeks without food (Bird, 1971). Upon refeeding, Dauer larvae molt and become L4 worms thus entering the normal developmental

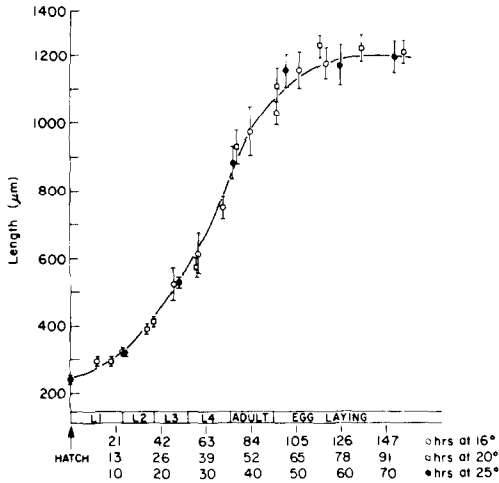


FIG. 2. Growth curves of wild-type *C. elegans* hermaphrodites. A synchronous population of worms (see Methods) was prepared so that all worms had hatched within a 2-hr period. They were immediately distributed to plates at the three temperatures. Zero time is taken as 1 hr before the distribution. At various times, worms were anesthetized and photographs taken. Lengths were measured from photographs using a map reader. Each point represents measurements from 20 worms, and \pm SD is indicated by the vertical bars. The larval stages, which are separated by molting, are indicated as L1 through L4.

pathway and growing to be sexually mature adults.

The most extensive morphological changes during the growth of a worm from L1 to adult is its increase in size and the development of its reproductive system. We have studied the morphology of the adult reproductive system and its development. As a result a distinct set of developmental features during gonadogenesis have been identified which can be used to chart the time of development of a wild-type worm. In addition, a remarkable anatomical simplicity exists in the adult reproductive system. These morphological landmarks in development also serve as phenotypic markers for development of the temperature sensitive mutants (Hirsh and Vanderslice, 1976). Similarly, distinct anatomical features of the adult reproductive system also serve as phenotypic markers for certain mutants.

STRUCTURE OF THE REPRODUCTIVE SYSTEM

Adult gonad. The adult reproductive system of *C. elegans* is shown schematically in Fig. 1b. An adult worm contains two U-shaped gonads, one in the anterior half, the other in the posterior half of the animal. These are in mirror symmetrical relationship to each other. Each U-shaped gonad consists of an ovary that is on the dorsal side of the animal connected through a 180° bend to the oviduct on the ventral side of the animal. The ovary buds off oocytes in the region of the bend. The oocytes enlarge as they move in a linear array down the oviduct and toward the spermatheca. The spermatheca is a hollow receptacle in which the sperm is stored. As oocytes pass through the spermatheca, they are fertilized and then enter the uterus. The two uteri join at a common

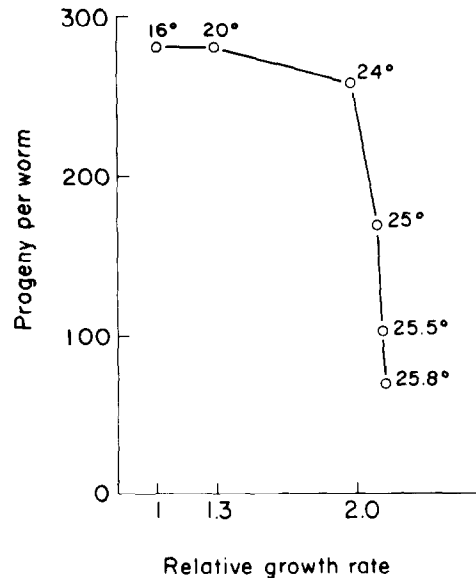


FIG. 3. Progeny production and relative growth rates as a function of temperature. Relative growth rates were determined from curves as in Fig. 2. Progeny per worm was determined by plating individual worms 2 hr after hatching at the indicated temperatures and transferring them daily to fresh plates after egg laying began. The number of progeny worms was subsequently counted. Each point represents the average number of progeny from 25 individuals.

vagina. The vagina connects to the vulva, an opening located midway along the ventral surface of the animal. Fertilized eggs exit through the vulva to the exterior. Normally, a young gravid adult contains four or five fertilized eggs advancing through embryogenesis as they pass down the anterior uterus toward the vulva, and four of five similar eggs moving anteriorly down the posterior uterus.

Therefore, each gonad can be divided conveniently into an arm that is distal from the vulva, an arm proximal to the vulva, and a loop connecting the two arms. The arms are parallel and the loop refers to the segment that curves through 180° and links the two arms. The length of each arm is about $400\ \mu\text{m}$. The cross section of this tubular structure is circular everywhere; however, its diameter is not constant throughout its length. At the distal tip, the diameter increases rapidly to $17\ \mu\text{m}$ at about $9\ \mu\text{m}$ from the tip. The diameter then increases slowly to about midway down the distal arm where it reaches a diameter of $50\ \mu\text{m}$ and then begins decreasing again. At the loop, the diameter decreases to $28\ \mu\text{m}$. The diameter of the proximal arm increases gradually down its length from 28 to $40\ \mu\text{m}$. The entire gonad is surrounded by a tubular layer of extremely thin, flat cells.

Distal arm. The general organization of the distal arm is that of a central, nucleate, undivided core of cytoplasm, surrounded by a cylindrically disposed layer of 1300 nuclei. This is particularly apparent when the dissected gonad is observed with interference contrast optics at a surface and a middle focal plane (Fig. 4). Serial cross sections observed both in the light and electron microscopes also reveal this organization. This pattern of organization is constant from the tip to the loop with two exceptions. Sections taken within the first micrometer of the tip show four cells that are contiguous without a central core, but 2 or $3\ \mu\text{m}$ from the tip, the core is present. Also, some gonads show a discontinuity in

the central core. That is, about $35\ \mu\text{m}$ from the distal tip, the core is of smaller diameter and is surrounded by two circumferential layers of nuclei. At $60\ \mu\text{m}$ from the tip, the core reappears for the remaining length of the distal arm. The basic structure of the distal arm in cross section is of 8 to 12 nuclei arranged as a single layer circumferentially around the central cytoplasmic core (Fig. 5). Each nucleus is surrounded by cytoplasm and a plasma membrane. The plasma membrane is, however, incomplete. The core of cytoplasm is continuous with the cytoplasm directly surrounding the nuclei, communicating through large gaps in the plasma membranes. In cross sections, the plasma membranes appear complete at the periphery of the gonad and appear to extend radially inward between adjacent nuclei. The discontinuity in membranes is also evident at the edges of a gonad viewed in mid focal plane with interference contrast optics (Fig. 6). Occasionally, gaps in the membrane between adjacent nuclei are seen in the EM sections.

All cytoplasm of the distal arm is filled with ribosomes that are, for the most part, not membrane-bound, although occasional lengths of rough endoplasmic reticulum are seen. Numerous mitochondria, often surrounded by a ring of rough endoplasmic reticulum can be observed. Very few microtubules are present. Toward the end of the distal arm closest to the loop region, the central core contains mitochondria, lipid drops, yolk bodies, and other cytoplasmic components characteristic of oocyte cytoplasm. The observations strongly suggest that the core cytoplasm becomes oocyte cytoplasm as it moves toward the loop.

The nuclei of the distal arm are between 2 and $5\ \mu\text{m}$ in diameter, about half the volume of each being filled by a highly prominent nucleolus. A photomicrograph of a Feulgen stained gonad from *C. elegans* var. Bristol is shown in Fig. 7. The morphology is much the same as that in the

French variant studied by Nigon and Brun (1955). It is apparent that most of the nuclei in the terminal 25% of the distal arm adjacent to the cul de sac are interphase nuclei. Among these interphase nuclei are mitotic figures. Prophase, metaphase, and anaphase nuclei are observed. We can count 12 chromosomes in some of these nuclei, which corresponds to the diploid number of linkage groups established by Brenner (1974). In the adjacent region of the distal arm there is a narrow zone of nuclei where the chromosomes appear more condensed. The nuclei in the remainder of the distal arm all the way to the loop contain meiotic figures in the pachytene stage of meiosis I.

Loop. In the region of the loop, oocytes form and move into the proximal arm at the same rate as they pass through the spermatheca and enter the uterus. The loop represents a zone of transition from the circumferential arrangement of nuclei characteristic of the distal arm to the single file of oocytes seen in the proximal arm. This transition involves the following redistributions of nuclei, core cytoplasm, and plasma membrane: (1) Nuclei are not present on the inner side of the bend; (2) the number of peripheral nuclei adjacent to the outer side of the bend is reduced to only two or three; and (3) there is a marked increase in volume of core cytoplasm surrounding each nucleus, accompanied by an increase in the integrity of the surrounding membranes. Thus, oocytes appear to be the result of the packaging of a single peripheral nucleus with a volume of core cytoplasm. The first portion of the loop contains 15 to 20 nuclei that display a clear progression from the meiotic stages of pachytene to diakinesis (Figs. 7 and 9). Plasma membrane grows across the diameter of the core from the periphery of the

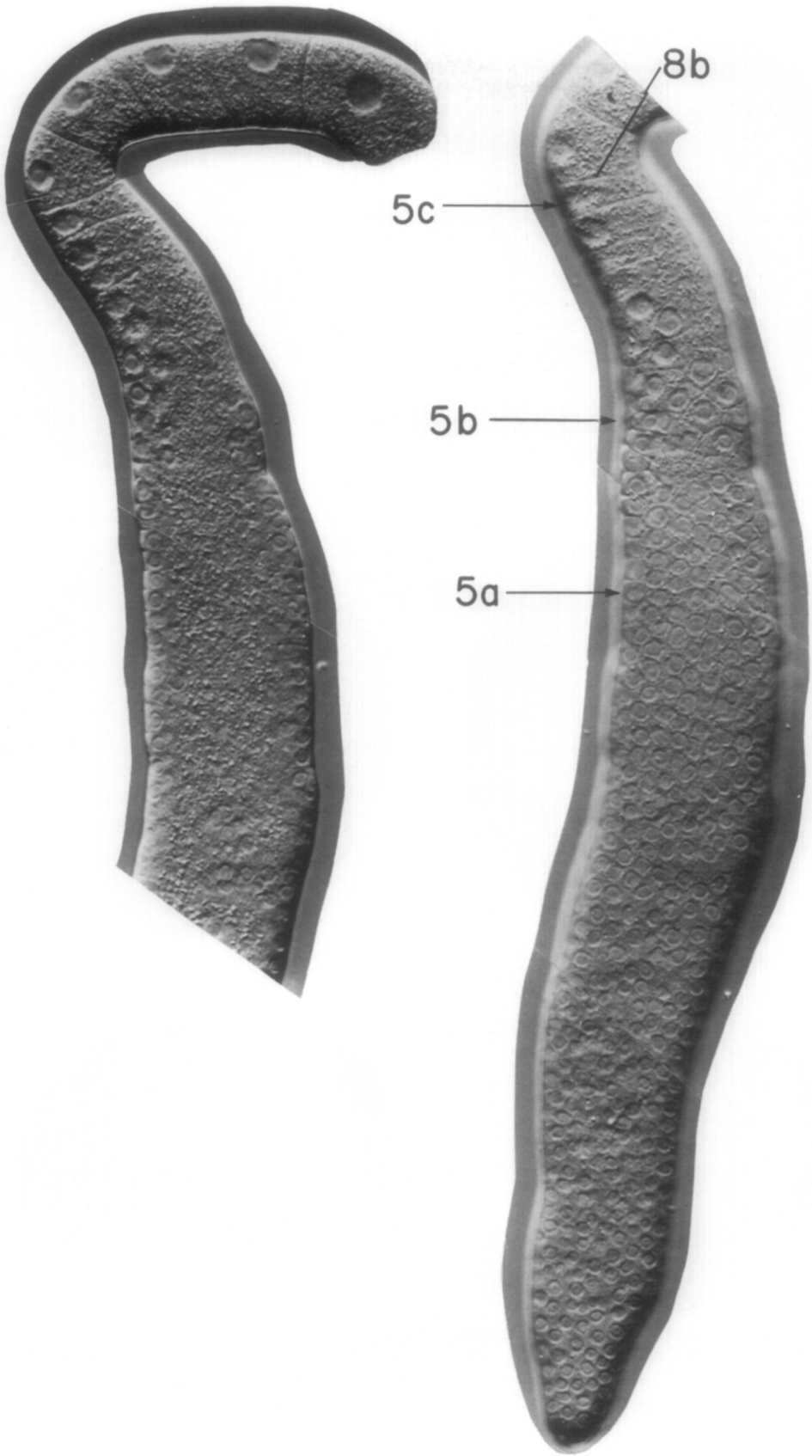
loop as if to slice off a section of core (Fig. 8).

Proximal arm. The proximal arm of the ovary consists of a single-file of enlarging oocytes. Each of these cells contains a single nucleus which shows typical chromosome patterns of late meiotic prophase (Fig. 9). Six chromosomal figures can be distinguished in each oocyte nucleus corresponding to the haploid number of linkage groups. We presume each of these figures to be a bivalent chromosome. There are approximately 10 to 14 oocytes increasing in size from the loop to the spermatheca in each ovary. All oocytes are halted, at diakinesis of meiosis I. Meiosis resumes only upon fertilization. The spermatheca has been examined only by light microscopy in whole worms. It appears as a hollow cylindrical structure with apertures on either end (Fig. 15b). These apertures are considerably smaller than mature oocytes, so that as an oocyte enters and exits the spermatheca, the shape of the oocyte is deformed. Sperm can be seen inside the spermatheca (Figure 9).

The diameter of a distal arm cell, measured from the surface focal plane, is 6 to 8 μm . Treated as a sphere, this is a volume of 163 μm^3 . An average oocyte has 250 times this volume. The volume of a distal arm and its loop is equivalent to the volume of 11 eggs. Therefore, the volume of both sides of an adult reproductive system is equivalent to 22 eggs. At 20°C, a worm will lay about 300 fertilized eggs over a 3-day period. The worm therefore turns over the entire volume of its gonad as eggs every 6.5 hr.

Epithelial sheath. The distal arm, the loop, and the proximal arm are each surrounded by very thin cells (Fig. 5 and 10). In general, these epithelial cells contain more rough endoplasmic reticulum than

FIG. 4. Photomicrographs of a dissected gonad from a *C. elegans* hermaphrodite. The loop region is at the top and the distal region of the gonad is at the bottom of the photograph. Nomarski interference contrast optics were used to obtain a surface focal plane (right) and a midfocal plane of the gonad (left). $\times 500$. The locations of the thin sections used in electron microscopy and illustrated in Figs. 5 and 8 are indicated.



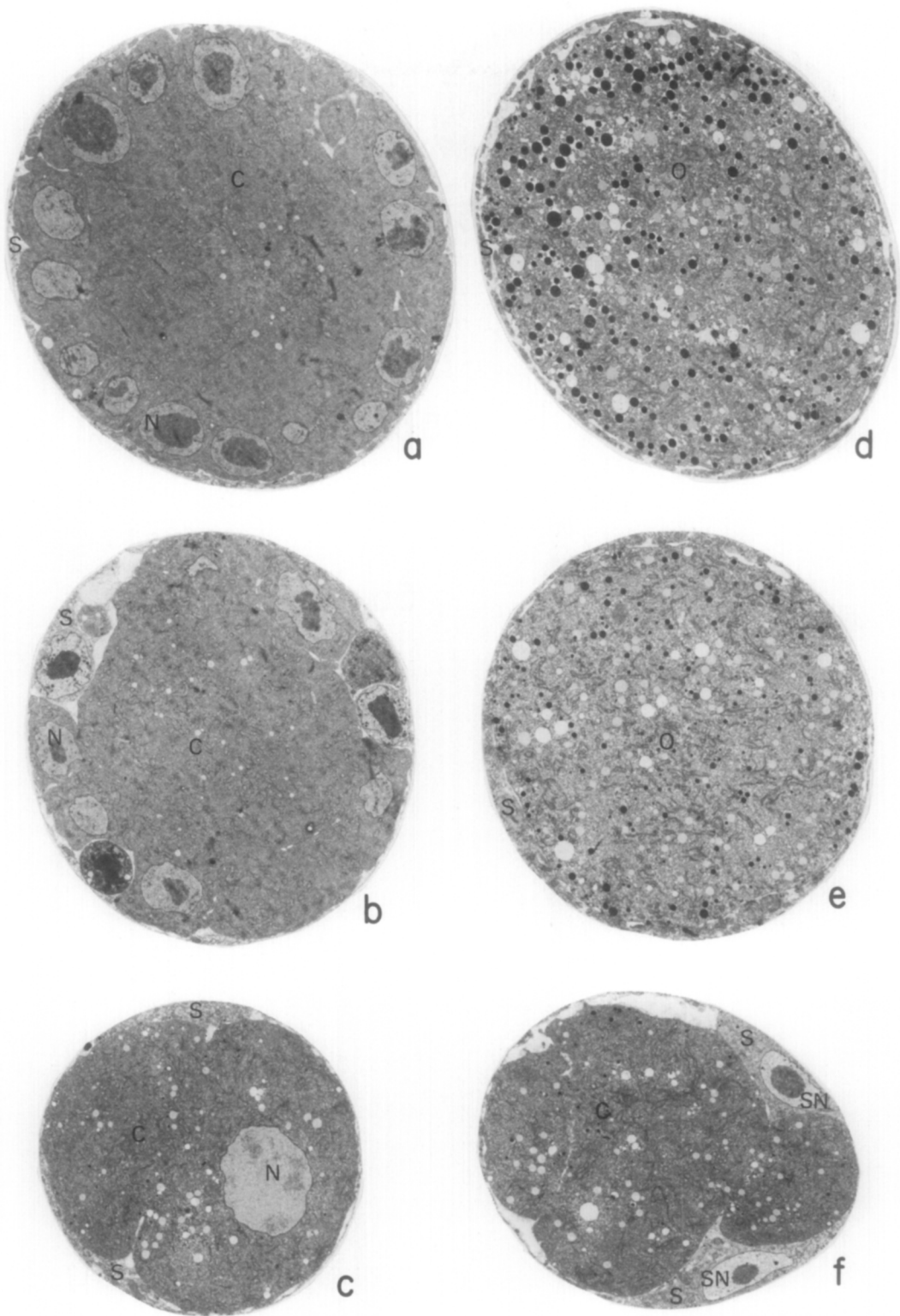


FIG. 5. Electron micrographs of cross sections through the gonad: (a) Section through the distal arm 145 μm from the bend (250 μm from tip); (b) 90 μm from the bend (310 μm from tip); (c) 30 μm from the bend (470 μm from tip); (d,e,f) sections through the proximal arm at corresponding distances from the bend. S, epithelial sheath cell; N, nucleus; SN, sheath cell nucleus; C, core cytoplasm; O, oocyte. $\times 2350$.

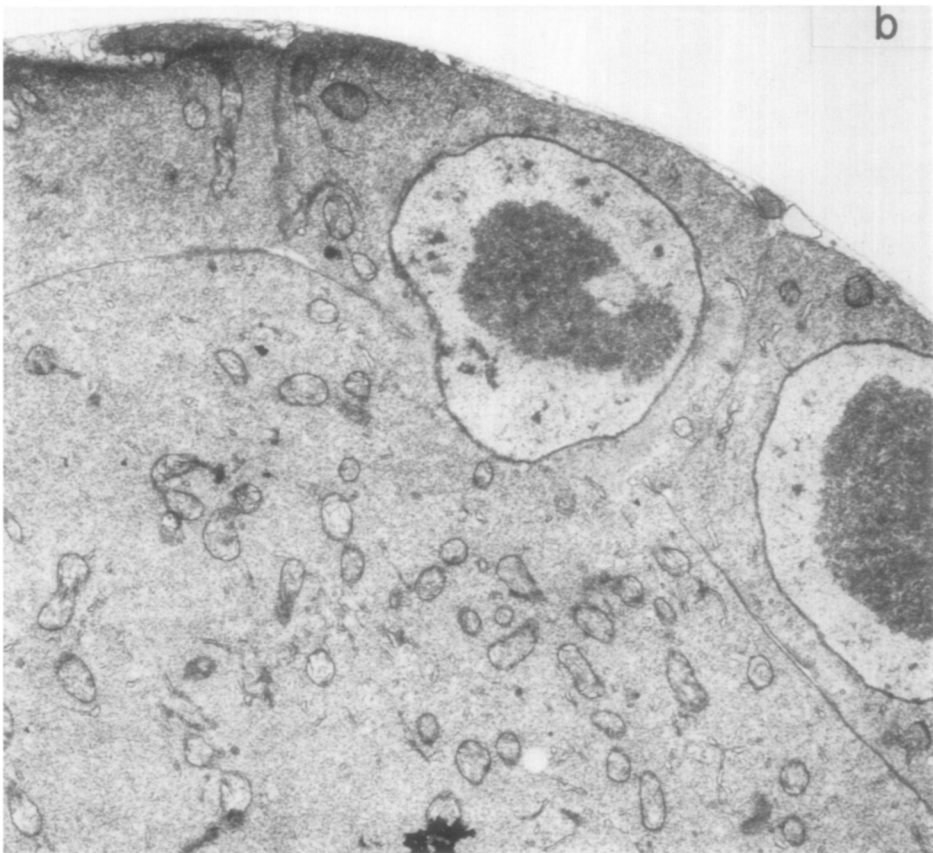
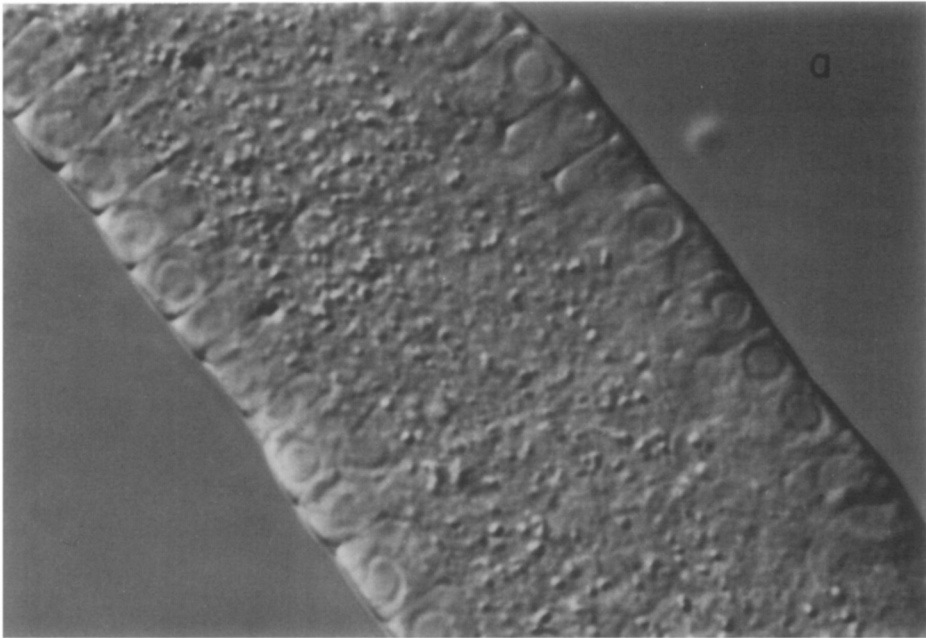


FIG. 6. Morphology of the distal arm of gonad (a) Midfocal plane photomicrograph of the distal arm of the gonad showing the central cytoplasmic core, the peripheral nuclei, and the incomplete cell membranes at the periphery. Nomarski-Zeiss interference contrast optics. (b) Electron micrograph of periphery of distal arm showing incomplete plasma membranes. The section is $80\ \mu\text{m}$ from the bend of the gonad ($320\ \mu\text{m}$ from distal tip). (a), $\times 1500$; (b), $\times 8200$.

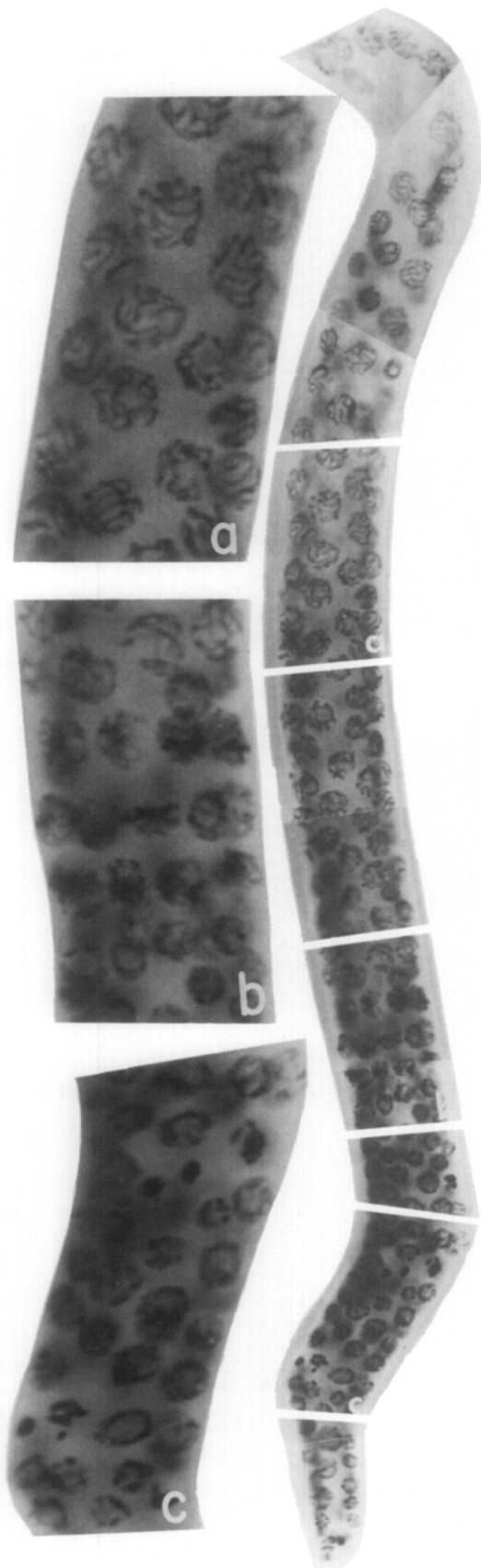


FIG. 7. Feulgen-stained gonad of *C. elegans* hermaphrodite ($\times 780$). Enlargements of individual zones are shown on the left ($\times 1700$). Bright-field optics with a 546 nm interference filter. (a) Region of pachytene nuclei; (b) interface of mitotic and meiotic regions; (c) region of mitosis.

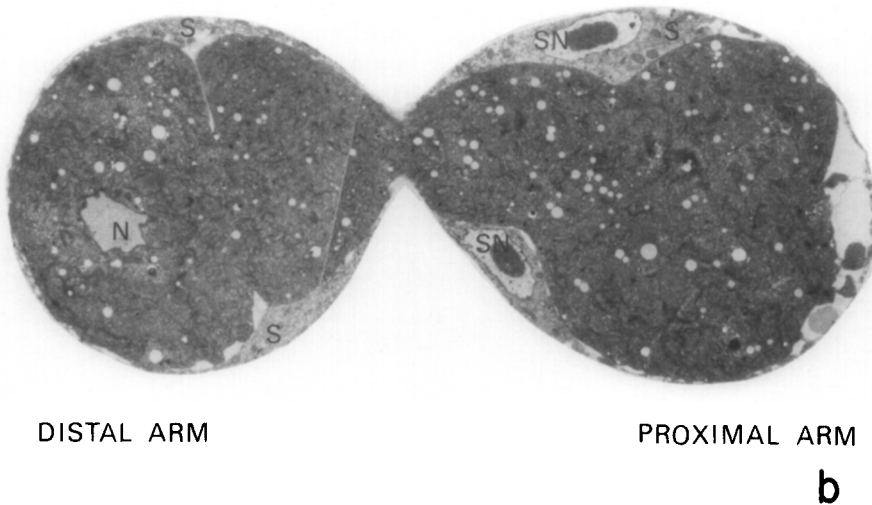
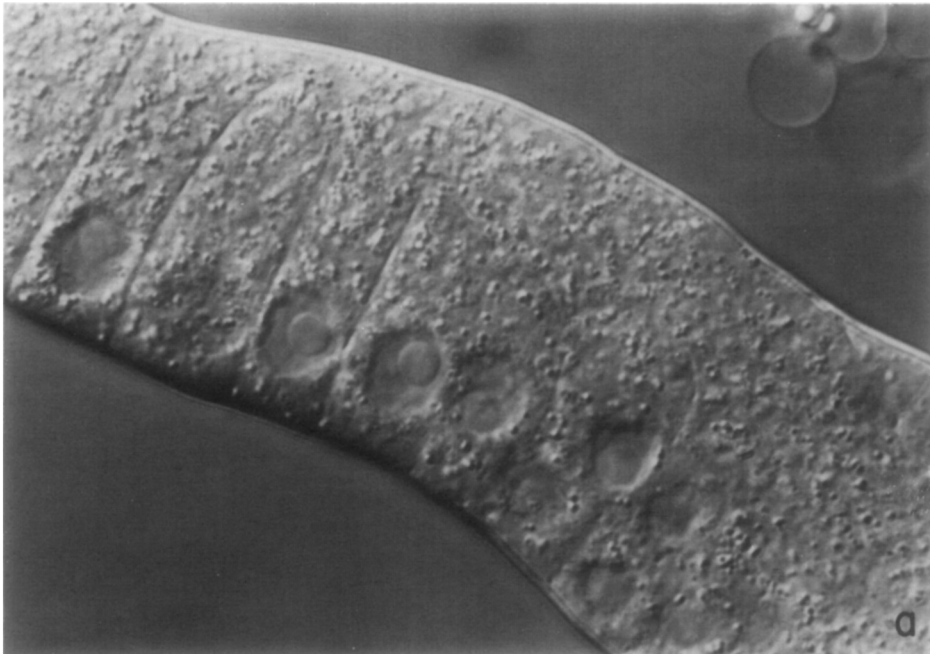


FIG. 8. The loop of the hermaphrodite gonad. (a) Photomicrograph of the loop showing the membrane partitions where oocytes form. Zeiss-Nomarski interference contrast optics. (b) Electron micrograph of a cross section within the loop region showing membrane growth across the cytoplasmic core of distal arm to form oocyte. S, epithelial sheath cell; N, nucleus. (a), $\times 1500$; (b), $\times 2550$.

the ovary or the oocytes. Frequently, Golgi bodies are visible and vesicular bodies are seen. The role of the epithelial sheath cells *vis á vis* the underlying cells is unknown at this time. Clearly, they are the primary

interface with the coelom of the nematode into which nutrients are transported by the gut cells. Therefore, they must be active in transport of nutrients to the ovary, but what functions they perform in the

synthesis of specific components for the ovary or for the oocytes remain unknown. The epithelial sheath cells surrounding the distal arm have basically the same morphology as those surrounding the oocytes in the proximal arm with one important difference. The cells of the proximal arm are myoepithelial cells containing arrays of thick and thin filaments. These are

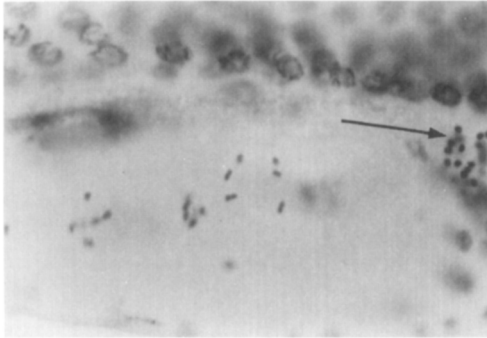


FIG. 9. Chromosomes of the oocyte nuclei within the proximal arm of the gonad. The oocytes are aligned from left to right. The oocyte chromosomes are in diakinesis. The distal arm nuclei are visible above the oocytes. The sperm nuclei are visible (arrow). Feulgen-stained preparation. Bright-field optics with a 546 nm interference filter. $\times 970$.

seen in cross section, and therefore, the filaments run longitudinally along the long axis of the oviduct. Their presence suggests a contractile nature of the proximal arm epithelial sheath cells to force the oocytes through the spermatheca.

The studies on the structure of the adult gonad of wild-type *C. elegans* have provided (1) the evidence that the reproductive system in *C. elegans* has a relatively simple morphology that lends itself to models and specific experiments to understand fundamental processes of oogenesis, and (2) a basic picture of its normal morphology, which can be used for comparison of mutant phenotypes.

Embryogenesis. The major features of early embryogenesis can be characterized since the surface of a fertilized egg is transparent. The fertilized egg is a prolate spheroid, $38 \times 56 \mu\text{m}$. The female pronucleus is eccentrically placed along the long axis. The male pronucleus first appears at the opposite edge of the egg (Fig. 11). Immediately upon fertilization, the female pronucleus resumes its meiosis (Fig. 12). Two polar bodies are extruded from the

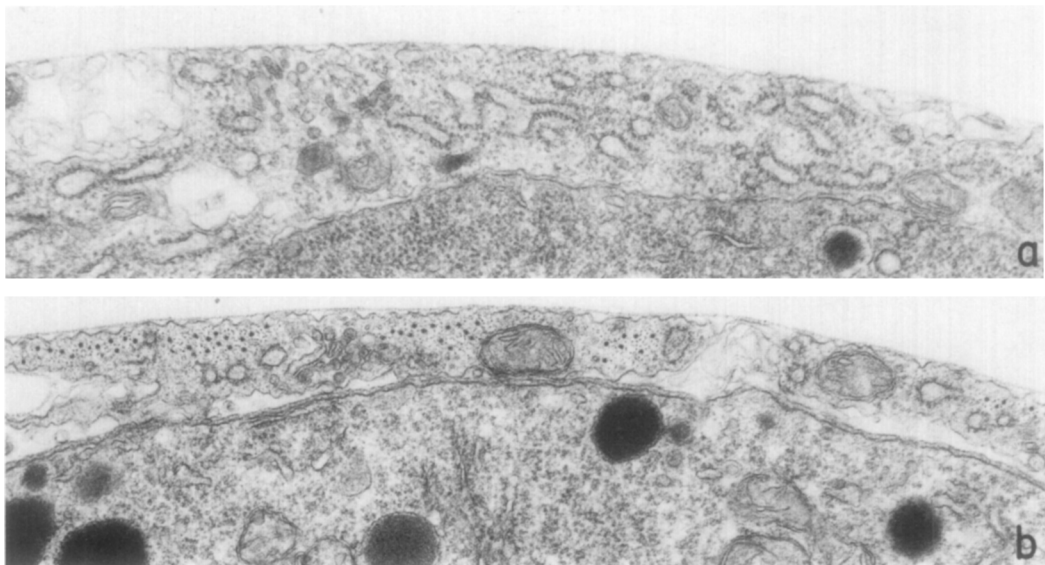


FIG. 10. Electron micrographs of the sheath cells. (a) Sheath cell around the distal arm (ovary). The section is $30 \mu\text{m}$ from the bend ($370 \mu\text{m}$ from distal tip). (b) Sheath cell around the proximal arm (oviduct). The section is $90 \mu\text{m}$ from the bend. $\times 27,500$.

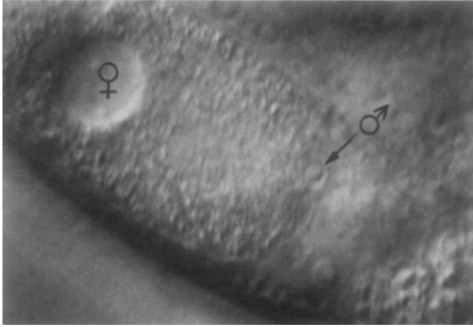


FIG. 11. Fertilization. The female pronucleus (♀) prior to polar body formation. The sperm has entered the egg on the right and the male pronucleus (♂) resides near the point of entry. Zeiss-Nomarski interference contrast optics. $\times 900$.

egg cytoplasm. In the later stages, the second polar body is easier to see than the first. Three polar bodies are never observed. Both polar bodies emerge from the end of the egg near the female pronucleus. A highly refractile shell then develops on the surface, and by analogy with studies on the *Ascaris* egg shell, it is likely to be chitin synthesized by the egg (Foor, 1967). This shell is useful for distinguishing fertilized eggs from oocytes even outside the animal. After fertilization, considerable surface membrane and cytoplasmic activity ensues in the egg. These activities have been described by Nigon *et al.* (1960), who believed that this movement causes the female pronucleus to move toward the male pronucleus to form the fertilization nucleus.

Stages in early embryogenesis are shown in Fig. 13. At the time that the two pronuclei approach each other, a pseudo-cleavage appears on the side of the egg, then recedes (Figs. 13c,d). The newly formed fertilization nucleus now resides closer to the end of the egg where the male pronucleus originally resided. Thus, although both pronuclei move toward each other, the female pronucleus makes the greater excursion. Asters form along the long axis of the egg and the first cleavage begins (Figs. 13e,f). The first cleavage furrow is transverse to the long axis of the

egg, and since the fertilization nucleus is eccentrically placed, this first cleavage divides the egg asymmetrically. The larger blastomere corresponds to the end that originally contained the female pronucleus. The nuclei then reform in the two-cell zygote and after approximately 10 to 15 min, the second cleavage begins. First, the large blastomere divides with a cleavage furrow oblique to the long axis of the egg (Figs. 13g,h). As the large blastomere cleaves, it rotates and then the small blastomere begins to divide with its cleavage furrow approximately perpendicular to the long axis of the egg (Fig. 13i). The four-cell zygote that results has a rhomboid shape (Fig. 13j). In a similar manner,

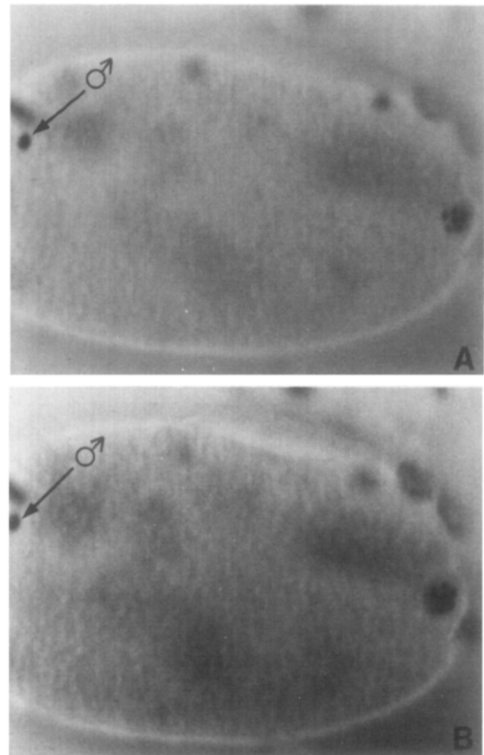


FIG. 12. Polar body formation. The sperm (♂) has entered the egg on the left. The oocyte nucleus is undergoing the first meiotic division. The chromosomes of the first polar body are visible in one focal plane (A) and the chromosomes of the oocyte nucleus are evident in the other focal plane (B). Feulgen staining. Bright-field optics with 546 nm interference filter. $\times 1800$.

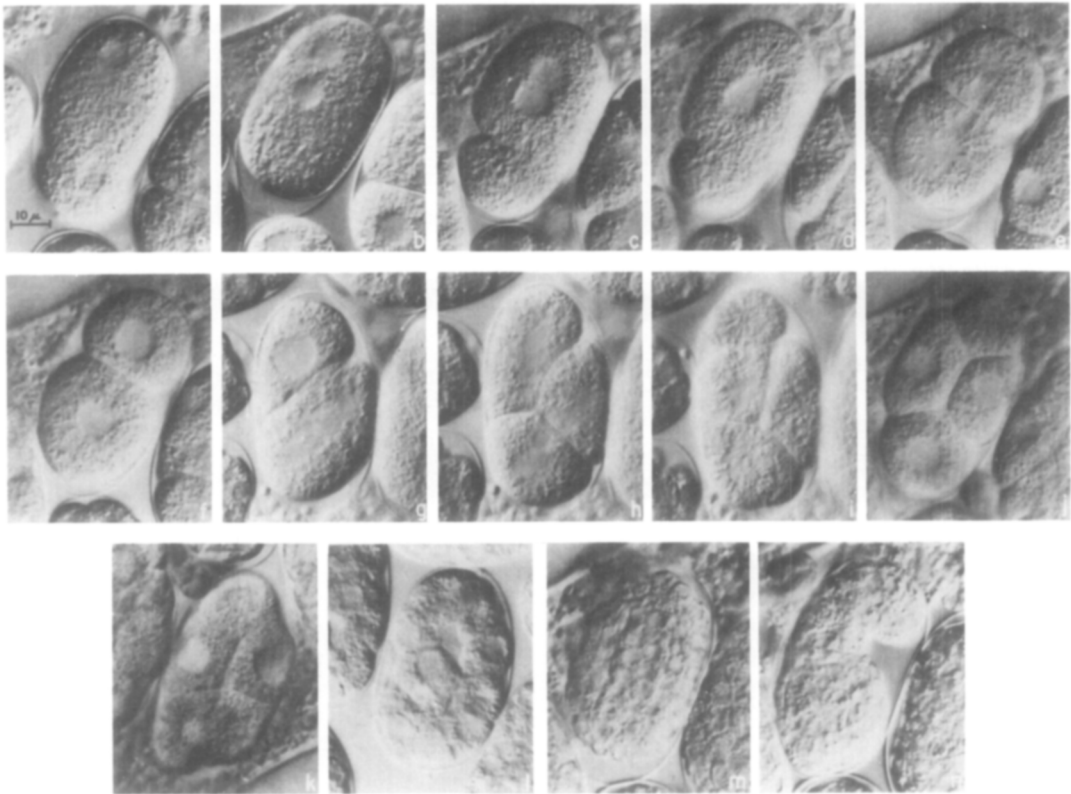


FIG. 13. Embryogenesis in *C. elegans* (see text). Photomicrographs were taken with Zeiss-Nomarski interference contrast optics at room temperature (20–22°C). Elapsed times, in minutes, are: (a) 0; (b) 39; (c) 80; (d) 91; (e) 111; (f) 128; (j) 148; (k) 165; (l) 210; (m) 360; (n) 900.

cleavages up to the 22 blastomere stage can be followed easily. Thereafter, it is more difficult but certainly not impossible to follow cleavages. The zygotes exit through the vulva at approximately the 30-cell stage (Fig. 13l). Shortly thereafter, gastrulation begins. The later stages of embryogenesis, represented by the transition from Fig. 13m to Fig. 13n, convert a spheroid embryo to an asymmetrical structure. The anterior–posterior polarity is easily distinguished. The larger end of the embryonic worm (Fig. 13n) will become the anterior end of the larva. The cleavage stage at which zygotes are laid is very dependent on the growth conditions and age of the worms. Starving or older worms tend to retain their eggs, and in the complete absence of food, larvae will hatch inside the gravid parent.

In summary, the normal adult reproduc-

tive system in *C. elegans* displays a complete linear axis of development from formation and maturation of oocytes, through fertilization, and early embryogenesis.

GONADOGENESIS

Several key features are recognizable during gonadogenesis that help define the exact stage of development of larval worms. Such features prove to be useful in characterization of some mutant phenotypes at higher resolution. The major events visible during gonadogenesis are listed in Table 1 and particular stages are shown in Fig. 14. When the first stage larva (L1) hatches from the egg, it contains four distinct round cells on its ventral side which proliferate to form the entire reproductive system within 45 hr at 25°C. By 6 hr after hatching, the gonad consists of six

cells arranged in a single-layered rhomboid pattern with three nuclei lying in the upper focal plane and the other three lying in a lower focal plane (Figs. 14a, b). Note that since the worm normally lies and crawls on its side, these two focal planes correspond to the sides of the animal and not the dorsal and ventral surfaces. The bilateral nature of the gonad persists as the two halves of the gonad grow outward from the central patch of tissue following the symmetry established at the six-nucleus stage. At 12 hr, 10 nuclei are seen and at 15 hr, 30 nuclei are seen. The developing gonadial tissue at this time becomes less planar and more cylindrical. By 26 hr after hatching, the tips of the growing gonads double back and start to grow 180° from their previous direction. By 30 hr after hatching, the tips of the gonads are meeting above the position of the original cells. Also at this time, the tissue changes from a homogeneous distribution of nuclei into the morphologically distinct regions characteristic of the adult gonad. The core of cytoplasm forms down the middle of the distal arm of the ovary and spermatogenesis occurs in the proximal arm.

During the early stages of gonadogenesis, the proximal and distal arms have similar morphology, namely, a homogeneous distribution of nuclei, as is seen with the light microscope in the distal arm of the adult (Fig. 4). However, at 30 hr of development, the nuclei in the proximal arm condense, the cells enlarge slightly, first assume a columnar appearance, then become more spherical, and then start to dissociate from each other (Fig. 15). These cells become the spherical sperm, thus leaving the proximal arm between the loop and the spermatheca hollow. The surface of this hollow cylinder is made up of the thin sheath cells. This hollow cylinder is occupied later, at 36 hr, by the maturing oocytes. Adults appear to contain all their mature sperm cells within the spermatheca prior to the appearance of mature oocytes in the proximal arm. At the pres-

TABLE 1
DEVELOPMENT OF THE REPRODUCTIVE SYSTEM OF *C. elegans*^a

		Hours at 25°C	
		0	Hatch
		0-6	4 primordial gonadial cells (p cells)
L1		6-11	6 p cells
L2		11.5	First larval molt
		12-13	10 p cells
L2		14-18	30 p cells
L3		18.5	Second larval molt
		20-22	~60 p cells. hypodermal cells enlarge
		24-26	~120 p cells
L3		26	Gonad begins 180° turn
L4		26	Third larval molt
		28-32	Hypodermal cells form vulva and vagina
			Distal arm core formation
			Proximal arm nuclei condense
		32-35	Sperm formation in proximal arm; proximal arm hollow
			Vulva and vagina near completion but opening covered by cuticle
			Core near completion in distal arm
L4			Spermathecae formation
Adult		35.5	Fourth larval molt
		35-37	Vulva open to exterior
			Uterine canal completion
			Spermathecae formed
			Oocytes appear
		37	Fertilized eggs present
		45-46	Egg laying begins

^a A synchronous population was prepared from worms that hatched within a two hour period at 25°C (see Methods). The worms were grown on petri plates with bacterial lawns. Groups of worms were removed at various times, anesthetized, and examined with the compound microscope. The major features at each time are denoted in the table.

ent time, we know very little of the sequence of morphological events associated with spermatogenesis. However, the presence or absence of mature sperm within the spermathecae can be useful for characterizing phenotypes of certain mutants. Sperm of *C. elegans* are round cells ap-

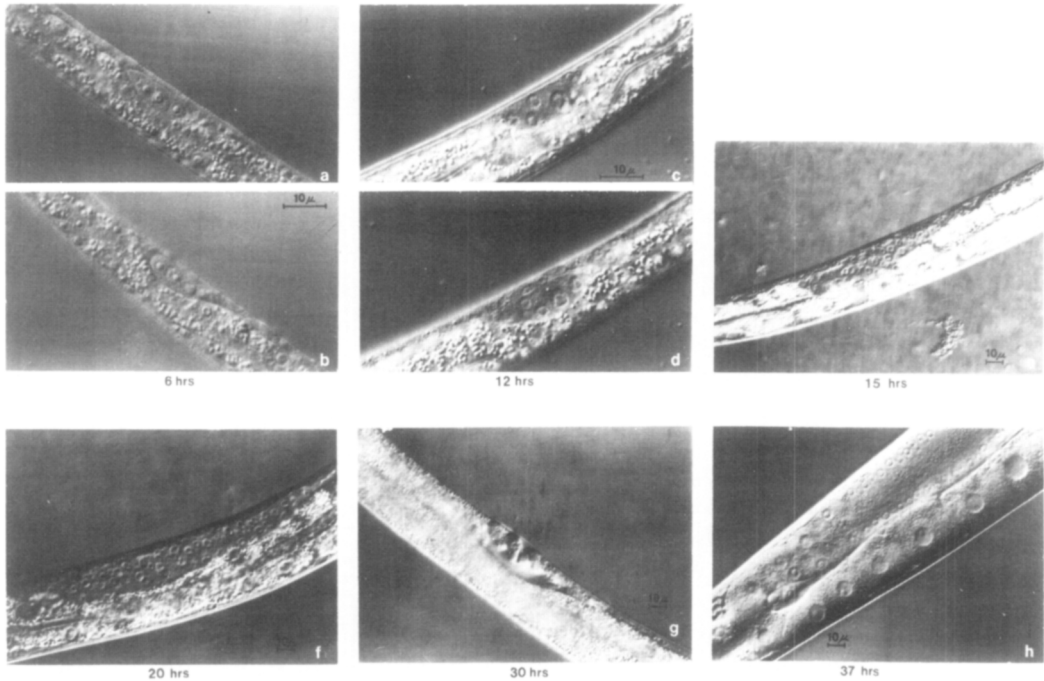


FIG. 14. Gonadogenesis in wild-type (N2) *C. elegans* hermaphrodites. A synchronous population of worms was distributed to plates at 25°C 2 hr after hatching. Photographs were taken at the indicated times using Zeiss-Nomarski interference contrast optics. (a) and (b) are two focal planes of the same nematode showing the three primordial gonadal cells present at each focal level. (c) and (d) are also two different focal planes of the one nematode. The focal plane of (g) is adjusted to show the rearrangement of the hypodermal cells and the formation of the vulva and vagina. Oocytes are aligned in the proximal arm and the core has formed in the distal arm of the gonad shown in (h).

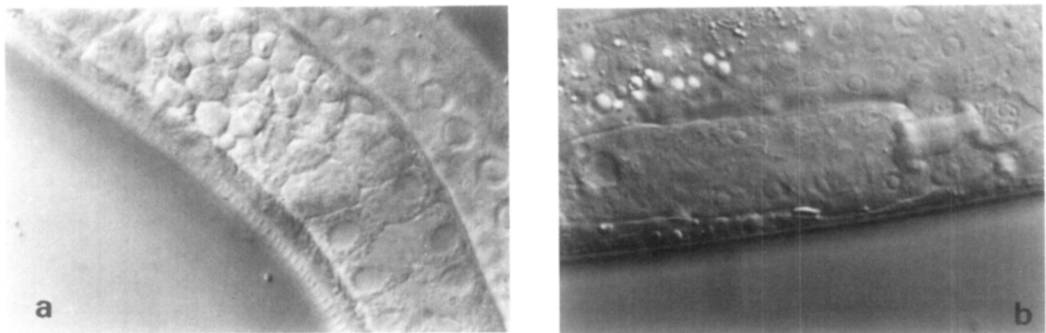


FIG. 15. Spermatogenesis and spermatheca in the proximal arm of the gonad of a *C. elegans* hermaphrodite. (a) A view of the proximal arm between the loop and the spermatheca showing the differentiation of the sperm. (b) The spermatheca has formed. The cells to the left of the spermatheca, between the loop and the spermatheca of the proximal arm, are in the first stages of differentiation to form sperm. The area to the right of the spermatheca will become the uterus. The distal arm is located above the proximal arm. Zeiss-Nomarski interference contrast optics. (a), $\times 950$; (b), $\times 800$.

proximately 5 to 6 μm in diameter with a distinct nucleus that is 1 to 2 μm in diameter.

Parallel with the development of the go-

nads is the development of the vagina, vulva, and uterus. The worm hatches with no easily detectable secondary sex characteristics. At about 20 hr after hatching at

25°C, the hypodermal cells lying directly under the position of the original primordial gonadial cells thicken and become very prominent. By 24 hr, a slight invagination of the hypodermal layer is seen. This invagination increases, forming the lumen of the uterus by 33 hr. The opening of the vulva to the exterior occurs only in the last molt. Thus, these structures form entirely during the fourth larval stage. It is unclear from which cells the uterine wall is derived, but it appears to form from the gonadial tissue overlying the developing vagina. Finally, the first fertilized eggs appear at 37 hr and egg laying begins at 45 hr.

DISCUSSION

The function of the adult gonad in *C. elegans* is to produce 280 fertilized eggs at the rate of approximately four per hour. The structure of this gonad is organized to this end. The overall picture that emerges of the adult reproductive system suggests that the cytoplasmic core of the distal arm is synthesized at a very high rate, moves down the distal arm toward the loop and in the process becomes oocyte cytoplasm. At the loop, this core cytoplasm is packaged into oocytes.

The gaps in the membranes and the uniformly high density of ribosomes throughout the distal arm suggest that a large amount of synthesis in the periphery is contributing to the core. There are three components in the periphery of the distal arm that could contribute: the cytoplasm which surrounds each nucleus, the nuclei themselves, and the epithelial sheath cells. A central question that arises is whether the synthesis in the distal arm is under the direction of the nuclei that surround the core. In the adult, most of the nuclei that surround the core are in pachytene stage of the first meiosis, a stage in which transcription occurs in several organisms (Davidson, 1968). In addition, the nuclei contain large nucleoli that could be actively producing ribosomes in their sur-

rounding cytoplasm. These ribosomes could then be transported through the gaps in the membranes to the central core. It could also be that the peripheral cytoplasm contains stable messages, and that products are being synthesized under the direction of these messages, and that such products are donated to the core.

It is intriguing to know whether there is regional specialization of the synthetic activities along the length of the distal arm. Certainly the texture of the cytoplasm of the core differs in the region of the loop. The cytoplasm has the appearance of that characteristically seen in oocytes. Either there is synthesis of large amounts of material such as yolk in this region or there is a coalescence of material synthesized all the way along the distal arm to form the droplets and particles seen in the electron micrographs. How regional specialization might be laid down is unknown at this time.

What produces the force for the movement of the core cytoplasm down the distal arm is as yet unknown, although there are several possibilities. It could be mitotic pressure generated by those cell divisions occurring in the terminal 25% of the distal arm. On the other hand, it could also be due to the large amounts of synthesis of material in a confined cylindrical tissue that creates a pressure which moves the oocytes down the cylinder. The sheath cells of the distal arm contain no contractile elements when viewed in the electron microscope.

It is clear that the core cytoplasm is packaged into an oocyte in the region of the loop. In addition, a nucleus is included, destined to become the oocyte nucleus. Whereas the studies thus far have outlined the basic morphology of the events in the loop, several questions remain that are currently under study. For example, the exact origin of the membrane that slices across the core at the loop region is not known. Whether this is a preexisting plasma membrane of one of the surround-

ing areas, or whether it is *de novo* synthesis of a new membrane from a specialized region of the loop is yet to be elucidated. Certainly further studies with electron microscopy should facilitate answering these questions. It is most likely, although not proven yet, that each oocyte nucleus is derived from one of the peripheral nuclei of the distal arm in the region of the loop. This seems to be the case because as one observes the nuclei before the loop, in the loop, then past the loop in the young oocytes, there is a progression from pachytene to diakinesis of meiosis. Time-lapse microscopy should help elucidate the morphology of the sequence of events that place a future oocyte nucleus into the core in the region of the loop.

Although many of the structural questions can be answered with microscopic observation alone, biochemical studies are indispensable to understand the important aspects of the function of the gonad and to observe the dynamic processes occurring in time. To this end, an *in vitro* system is being developed. At the present time, it is possible to dissect the gonad out of a gravid adult into a medium containing radioactive precursors, allow incorporation to occur, then perform autoradiography on the dissected gonad. Ultimately, it would be valuable to develop a medium that supports long-term survival and function of the gonad removed from the worm.

One of the important problems faced by any oogenetic system is how to manufacture a large egg in a short time, i.e., how to produce enough cytoplasm and specific cytoplasmic components to build a cell of the size required of an egg in a time considerably shorter than would normally be possible under the direction of a single genome. Different systems have solved this problem in different ways but they all involve the effective increase of the number of genomes or the size of the genome contributing to production of egg cytoplasm. Gene amplification, syncytia, nurse cells, etc., all have this function. *C. elegans* has about 1300 nuclei in each

ovary producing cytoplasm for each egg, and thus, can produce a cell which represents a 200-fold volumetric increase over its precursor cell every forty minutes. It is, in fact, a highly effective solution to the problem.

The morphological changes during the development of the reproductive system of *C. elegans* establish a set of landmarks for identifying developmental stages of wild type worms. These also will be useful in characterization of mutants with aberrant development. These studies have also raised several further questions concerning wild-type development which we are currently pursuing. It is apparent from the present studies that each primordial gonadal cell is visible with interference light microscopy during gonadogenesis. This now raises the possibility of deciphering the cell lineages during the formation of the entire reproductive system.

Another question raised by the present studies is when during gonadogenesis is the onset of meiosis. It is clear that Feulgen staining of the adult presents a clear distinction of meiotic and mitotic nuclei in the adult. By staining worms in a synchronous population at different stages of development, it should be possible to determine when the distal arm cells enter meiosis.

Probably the most difficult question is that of the relationship of gonadogenesis and oogenesis to embryogenesis, i.e., the problem of the production of a highly patterned and structured egg whose cytoplasm contains the necessary information to produce an adult organism. It is apparent that the initial cleavage is asymmetric, producing a large blastomere destined to become the anterior end of the worm. If one believes that the egg is assembled with a pattern of molecular inhomogeneities, the questions remain: Where does this pattern come from? What makes it? How is it maintained? If at the time the egg is made, its cytoplasm is already substantially patterned, then we might expect the core cytoplasm itself to be patterned,

and thus, the peripheral regions to be directing the synthesis of differential cytoplasmic components dependent on their positions in space. This, of course, begs the question of how such a pattern of differentially synthesizing nuclei might itself be established. One possibility is that the egg membrane formed at the loop establishes the asymmetry within the egg. This then raises the question of the existence of an ordered pattern of membrane components established in the loop. Another possibility is that the oocyte is packaged as an unpatterned, random volume of core cytoplasm which subsequently, during the 10 or so hours after it is made and before it is fertilized, produces, through rearrangement and self-structurings of its cytoplasm, its own asymmetries and inhomogeneities.

These morphological studies suggest contributions of ooplasmic components by the distal arm of the ovary. One might now expect that much of early embryogenesis relies on these maternal contributions to the egg. Further, functions necessary for the formation of the gonad might also play a role in embryogenesis. Studies on particular maternal effect mutants (Vanderslice and Hirsh, 1976) imply that this is the case.

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