CHARACTERIZATION OF TEMPERATURE-SENSITIVE, FERTILIZATION-DEFECTIVE MUTANTS OF THE NEMATODE *CAENORHABDITIS ELEGANS*

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ABSTRACT

The isolation and characterization of three *Caenorhabditis elegans* temperature-sensitive mutants that are defective at fertilization are described. All three are alleles of the gene *fer-1*. At the restrictive temperature of 25°, mutant hermaphrodites make sperm and oocytes in normal numbers. No oocytes are fertilized, although they pass through the spermatheca and uterus normally. The oocytes can be fertilized by sperm transferred by wild-type males, indicating that the mutant defect is in the sperm. The temperature-sensitive period for the mutants coincides with spermatogenesis. Sperm made by mutants at 25° cannot be distinguished from wild-type sperm by light microscopy. The sperm do contact oocytes in mutant hermaphrodites, but do not fertilize. Mutant sperm appear to be nonmotile. Mutant males are also sterile when grown at 25°. They transfer normal numbers of sperm to hermaphrodites at mating, but these sperm fail to migrate to the spermatheca and are infertile. The phenotype of these mutants is consistent with a primary defect in sperm motility, but the cause of this defect is not known.

ONE can investigate how the action of individual gene products enables a sperm to recognize and fertilize an egg by isolating and characterizing mutants that are sterile because their sperm fail to fertilize. Among such mutants should be some defective in gene products that specify the structure and distribution of components on the surface of the sperm and some that are defective in gene products necessary for sperm motility. The availability of mutants altered in these genes products would allow morphological and biochemical comparison of mutant and wild-type sperm that could identify the gene products and establish their normal function.

We have sought to obtain such sperm fertilization-defective mutants in the nematode *Caenorhabditis elegans* because of its many advantages for mutant isolation and genetic manipulation (BRENNER 1974). Its self-fertilizing hermaphroditic mode of reproduction is particularly convenient for identification of

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fertilization defective mutants because hermaphrodite sterility cannot be due to male behavioral or copulatory defects. Therefore, a substantial fraction of hermaphrodite sterility mutants should have defective gametes. HIRSH and VANDERSLICE (1976) have shown that this is the case for Caenorhabditis temperature-sensitive sterile mutants by isolating mutants that were defective in both spermatogenesis and oogenesis. Many of these mutants prevented the accumulation of mature gametes, but some of them accumulated nonfunctional sperm. In this paper we describe the isolation and characterization of three additional mutants with defective sperm and show that the mutants are sterile both as males and as hermaphrodites. This means that the males, which make many more sperm than hermaphrodites, can be used as a source of sperm for eventual isolation and biochemical characterization.

MATERIALS AND METHODS

Strains

The nematode used for these studies was *Caenorhabditis elegans* var. Bristol from the stock collection of Sydney Brenner (strain N2). The following marker strains were also obtained from Brenner's collection: E61, *dpy-5(e61)I* and E51, *unc-13(e51)I* (BRENNER 1974). The strains E1034 and E1035 were isolated by LEWIS and HODGKIN (1977) and generously provided to us by LEWIS, as was the strain X-M1. Their genotype is described in RESULTS. Strains mutant in gene *che-1* were isolated in our laboratory (Ward 1976) or kindly provided by D. DUSENBERY. Strain HC17, *isx-1(hc17*ts*)IV*, has a temperature-sensitive mutation that abolishes spermatogenesis in hermaphrodites and causes males to develop an intersexual phenotype. Its isolation and phenotypic characterization will be described in detail elsewhere (G. NELSON, K. LEW, J. MIWA and S. WARD, in preparation). HC-D7 is *isx-1(hc17*ts*); dpy-11(e224)*. The isolation and genotypic characterization of fertilization defective strains HC1, HC24 and HC13 is described in RESULTS. HC-D11 is *hc1e61*.

Chemotactic assays

The chemotactic behavior of individual worms was assessed in a radial attractant gradient established by diffusion in a thin film of 1.5% agarose. In this medium the worm leaves tracks that can be recorded photographically (WARD 1973). Such tracks were examined to see if the worm oriented up the gradient and if the worm reached the center.

Populations of worms were assayed quantitatively for their chemotactic response to various attractants as described by WARD (1973).

Light and electron microscopy

Live specimens were held between a cover slip and a thin slab of agar on a slide and observed using a Zeiss Universal microscope with Nomarski (differential interference contrast) optics (SULSTON 1976). For Feulgen staining to observe nuclei, worms were transferred to a drop of 10% ovalbumin on a slide coated with Zein (extracted from corn meal with ethanol) and fixed in place with Carnoy's fixative. They were then hydrated, hydrolyzed in 5N HCl at room temperature for 30 min, stained in Schiff reagent for two hr, washed, dehydrated and mounted in DPX or Permount. Sperm distributions were counted by bright-field observation with a green filter and recorded on camera lucida drawings.

Sperm morphology was determined by squashing males or hermaphrodites under a cover slip in buffered saline (33 mM KH_2PO_4; 63 mM Na_2HPO_4; 136 mM NaCl; 1 mM $MgCl_2$) and observing the expelled sperm with dark-field or Nomarski optics. Methods for electron microscopy were essentially as described in WARD *et al.* (1975).

Genetics

Complementation between chemotaxis-defective phenotypes was tested by determining the phenotype of the heterozygotes produced by the cross of two homozygous parents, using males to identify outcross progeny. Complementation between sterile phenotypes was tested by crossing homozygous mutant males reared at permissive temperature with homozygous hermaphrodites reared at restrictive temperatures; only heterozygous outcross progeny are produced from such courses. The heterozygous hermaphrodites were scored for sterility.

Map positions were determined relative to standard morphological and behavioral markers by two- and three-factor crosses as described in the text.

Temperature shift experiments

For temperature shift experiments, populations were synchronized by gently rinsing worms from a plate, leaving the eggs behind. Larvae hatching during the next one-hr interval were then collected and aliquots transferred to plates at $16 \pm .5$ or $25 \pm .1°$ and transferred to the other temperature at appropriate times. The growth rate of wild type and the strains E1034 and E1035 and HC1 at different temperatures was found to be in the ratio 1:1.3:2 for 16, 20 and 25°, as observed for wild type by HIRSH, OPPENHEIM and KLASS (1976). For plotting and tabulation, all data at different temperatures were normalized to "equivalent hours at 25°." We call the critical period of temperature sensitivity the temperature-sensitive period (TSP), following the nomenclature of SUZUKI (1970).

RESULTS

1. Mutant isolation

The two strains, E1034 and E1035, had been independently selected for nonchemotaxis toward NaCl, and they fail to orient or accumulate in gradients of NaCl or KCl (LEWIS and HODGKIN 1977). The two strains have a second phenotype: anatomical alterations of the dendritic terminals of specific sensory neurons (LEWIS and HODGKIN 1977). We discovered a third phenotype in both strains E1034 and E1035 when testing for temperature sensitivity of their chemotactic behavior. The mutant strains grow normally at 16° or 20°, but are sterile when grown at 25°. Instead of viable zygotes, the mutant hermaphrodites produce only unfertilized oocytes.

We began experiments to quantify and analyze this sterile phenotype on both of the original strains. Initial attempts to segregate the phenotypes were unsuccessful, indicating that all three phenotypes were linked on chromosome *I*. As characterization of the phenotypes proceeded, several observations suggested that the temperature-sensitive (ts) phenotype was due to a different mutation from the behavioral phenotype: (1) the behavioral phenotype was not temperature sensitive; neither 16° nor 25°-grown strains accumulated in response to gradients of KCl; (2) the anatomical defect was not temperature sensitive; 3 of 7 sensory neurons reconstructed were defective in E1034 grown at 25°, whereas 4 of 8 were defective when grown at 16°; (3) strains carrying other alleles of *che-1* were not sterile at 25°.

The sterile and chemotaxis-defective phenotypes were finally separated by recombination in the course of construction of a linked double mutation between the temperature-sensitive mutation in E1034 and a morphological mutant, *dpy-5(e61)I*. The double mutant strain (HC-D11) was dumpy and sterile, but

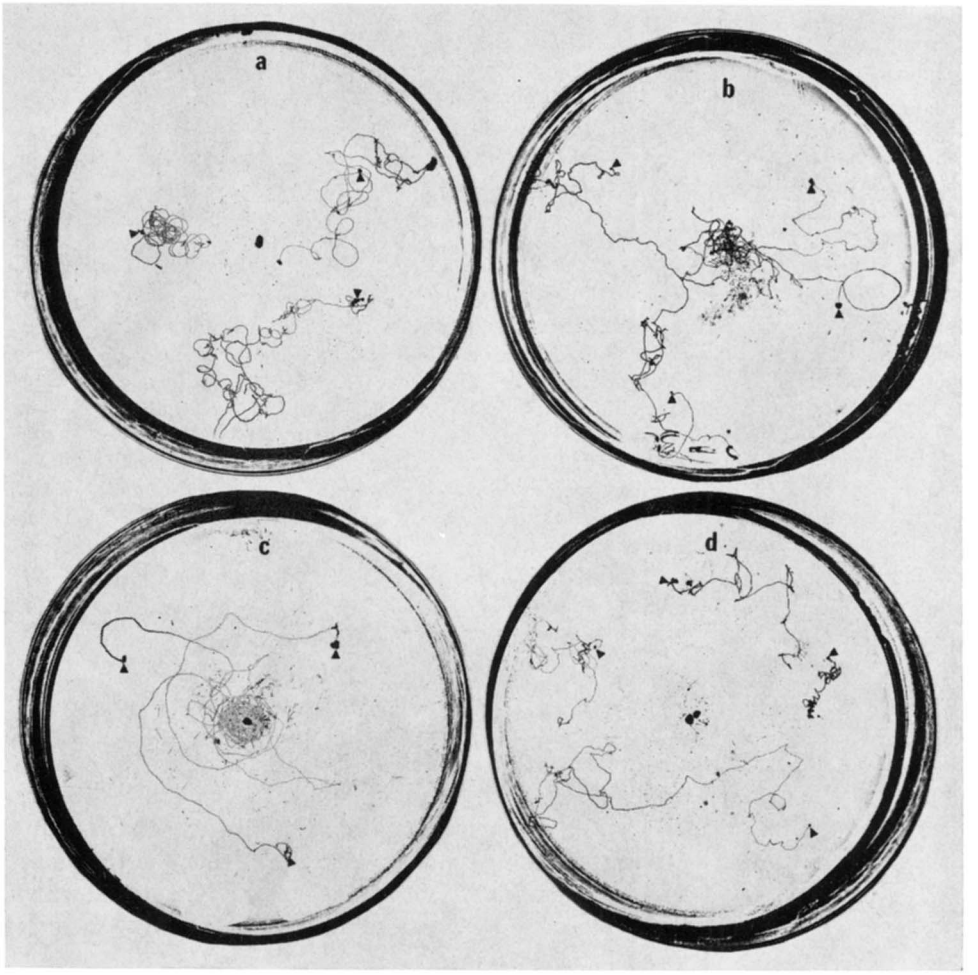

FIGURE 1.—Chemotaxis defective phenotypes. Tracks of worms responding to a preformed radial gradient of KCl from 100 mM at the center to about 0.1 mM on the side are shown. Three or four young adult hermaphrodites were placed at the points marked by arrows and allowed to leave tracks for 35 minutes. (a) E1034; (b) HC-D11; (c) HC1; (d) X-M1. The spiraling of some of the HC1 tracks is not typical of strain HC1 and occurs occasionally with wild type as well, presumably due to slight variations in gradient shape or worm handling. Tracks of more than thirty worms of each genotype were analyzed to confirm the phenotype. In addition, quantitative accumulation assays agreed with the tracking results (data not shown).

it had normal chemotaxis, as shown in Figure 1b. Figure 1a shows the chemotaxis-defective phenotype of E1034. The mutation causing sterility was then separated from the dumpy mutation by recombination. The strain bearing this sterile mutation is designed HC1, and the mutant allele, *hc1*ts. As will be shown below, the sterility is due to a temperature-sensitive defect in the sperm that prevents fertilization; the gene defined by this allele is called *fer-1*.

Figure 1c shows that strain *HC1* has normal chemotaxis. Strain *HC1* fails to complement its parent strain, E1034, for fertility at 25°. We presume that the parent strain contained the temperature-sensitive mutation *hc1*ts, as well as the mutation causing defective chemotaxis, *e1034*. As discussed below, strain E1035 appears to have similar or identical double mutations.

Two additional temperature-sensitive alleles of *fer-1* have been obtained. HC24, *fer-1*(*hc24*ts), was isolated by screening mutagenized populations for temperature-sensitive mutants that produce unfertilized oocytes. This mutant isolation has yielded mutants in at least five additional fertilization-defective genes and will be described in detail elsewhere (Y. ARGON, J. MIWA and S. WARD, in preparation). Strain HC13, *fer-1*(*hc13*ts), was obtained by doubly backcrossing the sterile mutant strain A13 provided to us by R. S. EDGAR. A13 was obtained in a general mutant isolation for temperature-sensitive lethals and steriles (R. S. EDGAR, personal communication).

Detailed data for the phenotypic characterization of only one of the *fer-1* alleles, *hc1*, are presented below.

2. Genetics

LEWIS and HODGKIN (1977) showed that the sterile mutation in strains E1034 and E1035 mapped 5.2 units to the right of *dpy-5* on chromosome *I*. We have confirmed this map position in the following way. First, segregation from the three-factor cross between the *fer-1* mutant and the double mutation *dpy-5 unc-13* showed that the *fer-1* gene was to the right of *dpy-5*. Second, the map distance from *dpy-5* was determined by segregation from the *cis* heterozygote, *dpy-5 fer-1*/+ +, at 16°. Of 520 dumpy progeny cloned, 37 were fertile. Therefore the *fer-1* gene maps 3.6 ± 0.5 units to the right of *dpy-5*, at 16°. Recombination frequency increases about two-fold with increase in temperature from 16° to 25°in this chromosomal region (D. BAILLIE and M. PRIDHAM, personal communication). LEWIS and HODGKIN maintained their heterozygotes at 25°, so that our map position agrees with theirs within experimental error.

We attempted to obtain wild-type recombinants between the fertilization-defective mutations in E1034/E1035 heterozygotes. No wild-type recombinants were obtained among 10^6 progeny screened. Therefore, these two strains contain either very closely linked or identical temperature-sensitive mutations. We have been unable to revert the sterile phenotype in either strain.

The chemotaxis-defective phenotype of the strains E1034 and E1035 has not been obtained separate from the sterile phenotype. It is likely that the *che-1* gene maps between *dpy-5* and *fer-1* because a dumpy-sterile double mutant constructed by J. LEWIS (X-M1) retains the chemotaxis defective phenotype (Figure 1d), whereas our construction of a similar double mutation separated the sterile from the chemotaxis phenotype (Figure 1b). If both these dumpy-sterile strains arose from single recombination events, the *che-1* must lie between *dpy-5* and *fer-1*. The two multiple-mutant strains have nearly identical sterile phenotypes, so that the presence of the chemotaxis-defective mutation appears to have little effect on the sterile phenotype.

Both HC24 and HC13 fail to complement HC1 for fertility at 25°. In addition, HC24 has been found to map to the same locus, using crosses similar to those described above for HC1.

3. Quantitation of the sterile phenotype

The sterile phenotype in these mutant strains can be quantitated by comparing unfertilized oocyte and progeny production. Unfertilized oocytes are readily recognized by their squashy, disc-like shape, their brownish pigmentation and their uptake of the dye trypan blue (S. WARD and J. CARREL, unpublished). Table 1 summarizes the phenotypes of strains E1034, HC1 and wild type. At 16°, E1034 produces nearly as many progeny as the wild type, and HC1 is indistinguishable from wild type. At 25°, however, neither mutant strain produces progeny; instead they lay unfertilized oocytes. Since the sterile phenotype of the strains was hardly altered by segregation from the chemotaxis defect, data from phenotypic characterization of either strain and strain E1035 is reported below.

Caenorhabditis elegans is normally a self-fertilizing hermaphrodite. As the adults mature, they first accumulate sperm in a spermatheca and then produce oocytes. Oocytes are fertilized as they pass through the spermatheca (NIGON, 1949; HIRSH, OPPENHEIM and KLASS 1976). The absence of zygotes in the mutant strains could be due to a defect either in the sperm or in the oocyte. To distinguish these possibilities, mutant hermaphrodites were grown at 25°, so that they would produce only oocytes, and then they were mated with an excess of wild-type males to determine if mutant oocytes could be fertilized by wild-type sperm. The efficiency of mating varies, but in many such matings fertile

TABLE 1

Quantitation of sterile phenotype

Strain	Temp. (°C)	Progeny Worm	Oocytes Worm
Wild	16	284 ± 37	45
E1034	16	225 ± 34	43
HC1	16	302 ± 21	17
Wild	25	253 ± 14	14
E1034	25	0.1	242 ± 36
HC1	25	<0.06	205 ± 49
E1034 + wild ♂	25	240	

Mutant or wild-type worms were grown from conception at the temperature indicated, two worms per plate. After maturity the worms were transferred to fresh plates at 24 hr intervals. Each plate was scored for unfertilized oocytes or F_1 progeny 24 hr after removing the parents. This ensured that only the F_1 generation was scored. Each number shown is the mean and standard deviation of four such plates. The oocyte counts of wild type or mutants at 16° varied from 0–120. They were very sensitive to growth conditions and handling so a standard deviation was not calculated. The number of progeny shown for E1034 hermaphrodites mated by wild-type males is the maximum that we have observed in several individual matings. Mating efficiency varies widely but at least 50 progeny were obtained from each mated hermaphrodites in many matings. Mating HC1 hermaphrodites grown at 25° with wild-type males gives similar progeny yields, as does mating with strain E1034.

progeny were always produced, together with some oocytes. As many as 240 progeny were obtained from a single mated hermaphrodite, indicating that essentially all the oocytes produced at 25° are capable of being fertilized (Table 1). Two observations showed that all these progeny resulted from fertilization by the wild-type sperm: (1) about half of them were males, indicating that they must have been fertilized by sperm from a male, since the *XO* male produces half nullo-*X* gametes that yield male progeny; and (2) all the hermaphordite progeny tested were fertile at 25°, but they segregated one-quarter sterile offspring, indicating that they must have been heterozygous for the mutant and wild-type allele.

These results show that the oocytes laid by the mutant hermaphrodites at 25° are capable of being fertilized if the mutant is provided with wild-type sperm. Therefore, the sterile defect in the mutants must be due to a defect in the sperm, and not due to a defect in the oocyte. The fertility of heterozygous hermaphrodites grown at 25° and the sterility of one quarter of their progeny indicates that the sterile defect is recessive and shows no maternal effect.

The fertility of males of strains E1034, E1035 and HC1 was tested by raising them at 25° and then mating them to hermaphrodites marked by either dumpy or sterile mutations. No more than five outcross progeny per hermaphrodite was ever obtained and the average was less than one. Control experiments with wild-type males grown at 25° or mutant males grown at 16 or 20° invariably yielded more than 50 outcross progeny per hermaphrodite. These results show that the temperature-sensitive mutation in these strains sterilizes males as well as hermaphrodites. This is consistent with a sperm defect.

4. Mutant spermatogenesis and sperm

Light microscopic comparison of the process of spermatogenesis in the mutants at 25° and 16° failed to detect any morphological difference in the chromosomal events of spermatogenesis at the two temperatures (Figure 2a, b, c, d). Spermatids or sperm can be recognized and counted in Feulgen-stained preparations because of their condensed chromatin. The total number of sperm or spermatids accumulated in the mutants grown at 25° (211 ± 35, and Table 3) is not different from that in the wild type grown at 25° (196 ± 32, $n = 7$). Therefore, the sterility of mutants at 25° is not due to an arrest of spermatogenesis.

Caenorhabditis sperm resemble those found in other nematodes. They are not flagellated, but have pseudopods and appear to move by amoeboid motion (BEAMS and SEKHON 1972; WARD 1977). Unlike other nematode sperm, Caenorhabditis sperm have a striking morpohological specialization after they have been extruded into buffer. They project many filamentous processes that sometimes grow to as long as 40 microns within an hour after extrusion from a male (WARD 1977). Figure 3 shows light micrographs of mutant and wild-type sperm focused on the filamentous processes. These processes appear in both mutant and wild-type sperm and the sperm look similar. Examination of more than a thousand sperm at various times after extrusion from 25°-grown mutant males and hermaphrodites indicates that, with our methods, the mutant sperm cannot be

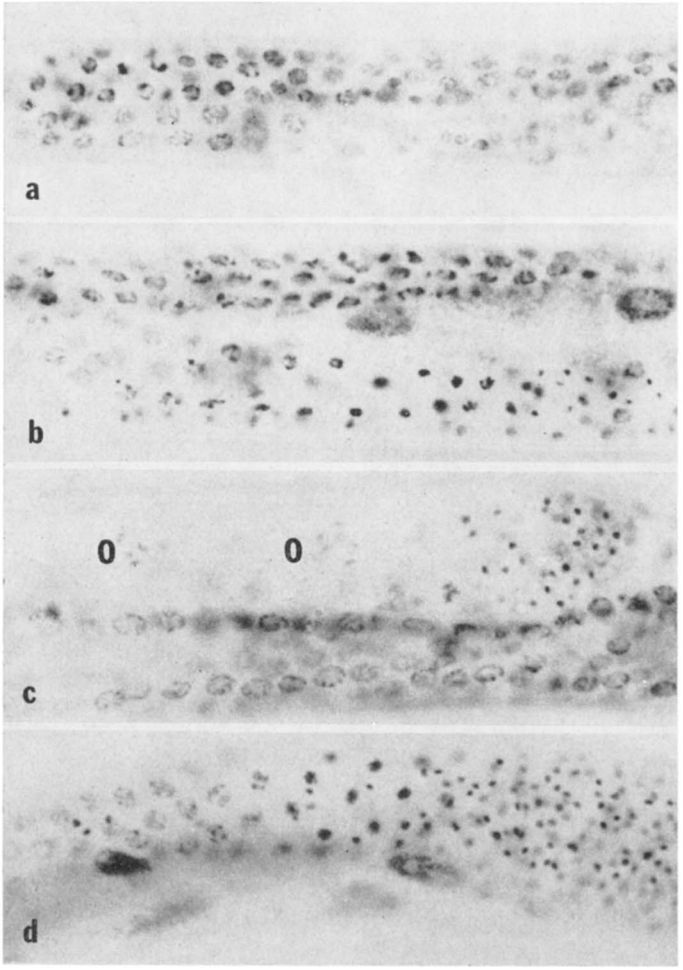

FIGURE 2.—Mutant spermatogenesis. Light micrographs of one of the gonads of Feulgen-stained whole mounts of E1034 worms grown from eggs at 25° are shown. The gonad is "U" shaped with gametes maturing along the U from the distal arm toward the vulva. (a) hermaphrodite, 37 ± 1 hr after hatching, distal arm above; (b) hermaphrodite, 44 ± 1 hr, (c) hermaphrodite 48 ± 1 hr, distal arm below; (d) virgin male about 72 hr. The large nuclei with partially condensed chromatin are primary spermatocytes, mostly in pachytene I. The somewhat smaller nuclei with more condensed chromatin are more mature primary and a few secondary spermatocytes. The small dense spots of chromatin are spermatid and mature sperm nuclei. (See HIRSH, OPPENHEIMER and KLASS 1976 and KLASS, WOLF and HIRSH 1976 for wild-type comparison). The large diffuse nuclei in (b) and (d) are gut cell nuclei and the nuclei labelled O are oocytes arrested at diakinesis I prior to fertilization. Magnification = 1200×.

distinguished by light microscopy from the sperm of wild-type grown at 25° or mutants grown at 16°.

5. Temperature-sensitive period

In order to determine the time period during development that is temperature sensitive for fertility (the TSP), the mutants were grown at 16° (the permissive

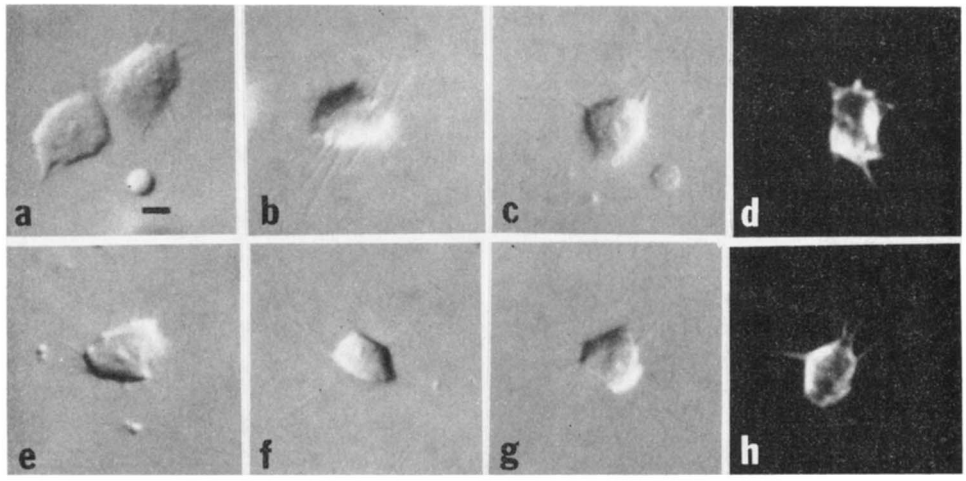

FIGURE 3.—Mutant and wild-type sperm. Sperm were extruded from HC1 and wild-type virgin males grown from eggs at 25° for about 60 hr after hatching. (a-d) wild-type, (e-h) HC1. Nomarski differential interference contrast optics except for (d) and (h), which are dark field. Focus is on filaments. All magnifications 1600×. Bar = 2 microns.

temperature) or 25° (the restrictive temperature) and then shifted to the other temperature at different ages. The shifted worms were allowed to develop past their reproductive period and then the total number of progeny and unfertilized oocytes produced was counted. In parallel with each shift, 8–10 worms were fixed and Feulgen stained for light microscopic examination to determine the exact stage of maturation of the gonad at the time of the shift.

The results of the shift experiment are summarized in Figure 4. Worms that are shifted up from 16° to 25° before 38 hr after hatching (normalized to growth at 25°) produce no progeny. Worms shifted down from 25° to 16° before 38 hr yield normal progeny. Worms shifted either way from 36 to 44 hours produce intermediate numbers of progeny. Worms shifted after 44 hr are unaffected by the temperature shift: 25°-grown worms produce only oocytes and 16°-grown worms produce viable progeny. Therefore the TSP for the sterile phenotype is between 36 and 44 hr of post-embryonic development. This was further confirmed by observing that worms shifted up to 25° at 35 hr and down at 48 hr are sterile, whereas worms shifted conversely are fertile.

Figure 4 also indicates the duration of spermatogenesis and the time course of spermatid accumulation during the temperature-shift experiments, based on the Feulgen stained specimens. The initiation of the TSP coincides with the initiation of meiosis and precedes spermatid formation. Figure 2a, b, and c were light micrographs of the Feulgen-stained gonads at the time of the TSP, showing that spermatocytes are undergoing meiotic maturation during the TSP.

Quantitative assessment of sperm fertility in males is not as simple as in hermaphrodites because the determination of fertility depends on the efficiency of mating and sperm transfer. Mating efficiency varies substantially from experiment to experiment for individual males. Nonetheless, an attempt was made to

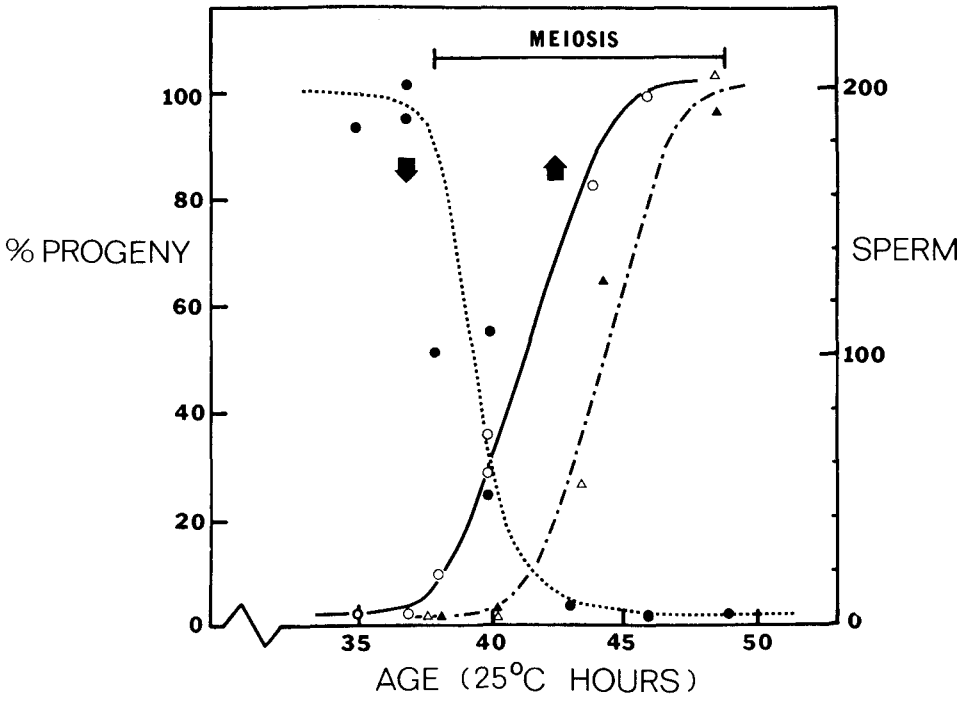

FIGURE 4.—Temperature shift of strain E1034. The progeny produced by worms shifted from a synchronized culture at the age indicated is shown as % of control value of 240 per hermaphrodite. All ages are normalized to 25° hours. Each point is the average progeny of five worms. - - - ● - - - shift down; —○— shift up. For down shifts the shifted worms were transferred to two successive plates to ensure that only F_1 progeny were scored. The shifted plates were also scored for unfertilized oocyte production which followed the inverse of progeny production (data not shown).

At each time point, 8–10 worms from each shift were fixed and Feulgen stained to determine their stage of spermatogenesis at the time of the shift. The bar at the top indicated the duration of spermatogenesis and spermiogenesis from the initiation of the first meiotic prophase to the accumulation of mature sperm. The number of mature sperm or spermatids at each time is indicated by the dash-dotted line and the right hand scale; ▽ = shift up, ▼ = shift down. Identical results have been obtained with strain E1035. Strain HC1 has been found to have the same TSP although exact quantitation was not repeated.

determine the TSP for male fertility. Males shifted from 16 to 25° at 38 hr (normalized to 25°) produced about 20% as many outcross progeny as the unshifted control value of 250/male. Males shifted up at 70 hr produced about 80% as many progeny. Males shifted down from 25° at 38 hr produced 90% of the control outcross progeny; and males shifted down at 55 hr produced only 30%. The variation in progeny produced from plate to plate was greater than a factor of two. These results, nevertheless, indicate that TSP for male fertility extends throughout the male's adult life as does male spermatogenesis. Sperm made at 16° in the mutants appear to be fertile whether they are made early or late during spermatogenesis.

6. Determination of the transmission ratio

Although the temperature-shift experiments indicated that the TSP in the hermaphrodite was during meiosis, it is still possible that the gene product might have some post-meiotic expression. If this were so, it might be revealed in a heterozygote of mutant and wild type grown at 25° by the reduced fertility of the sperm carrying the mutant allele. Such a reduction would be shown by a transmission ratio of the mutant allele of less than 0.5. This was tested by examining the phenotype of the progeny produced both in selfing of *hc1*ts/+ hermaphrodites and in outcrossing with *hc1*ts/+ males. The data are summarized in Table 2. This table shows that as many ova were fertilized by sperm bearing the mutant allele as by sperm bearing the wild-type allele. This was true in both selfcross and outcross experiments. This is consistent with the TSP during meiosis preceding formation of the haploid sperm.

7. Mutant fertilization

The process of fertilization in *C. elegans* has been described briefly by NIGON (1949) and HIRSH, OPPENHEIM and KLASS (1976) and in more detail by our laboratory (S. WARD and J. CARREL, unpublished).

A summary of our observations is given here. After spermatogenesis is nearly complete, sperm accumulate in the spermatheca. Oocytes mature through meiosis I as they pass down the oviduct and arrest at diakinesis I as they reach the spermatheca. The sperm then begin to contact the oocyte, and fifteen or twenty sperm accumulate on the leading surface of the mature oocyte. The oocyte with its surrounding cap of sperm is squeezed into the spermatheca by contractions of the oviduct sheath. The oocyte contacts many sperm in the spermatheca before passing through a constriction that separates the spermatheca from the uterus. The passages from the oviduct to uterus takes a minute or so. The oocyte enters

TABLE 2

Transmission ratio

Male genotype	Hermaphrodite genotype	Progeny genotype *fer*/*fer*	*+*/*fer*	*+*/*+*	?	Transmission ratio
fer/+	*fer*/*fer*	51	51	0	2	0.5 ± 0.1
——	*fer*/+	22	44	23	3	0.5 ± 0.1

The heterozygous males and hermaphrodites were obtained by mating wild-type males to E1034 hermaphrodites raised at 25°. For the determination of the male sperm-transmission ratio, several mating plates of about ten heterozygous males and two 25°-raised mutant hermaphrodites were prepared. The progeny of this mating were transferred individually to plates at 25° and scored for sterility. Individuals that were sterile were assumed to be homozygous and thus fertilized by sperm bearing the mutant allele, whereas individuals that produced progeny were assumed to have been fertilized by sperm bearing the wild-type allele. A few individuals produced nothing, presumably due to damage in handling. The transmission ratio is the fraction of zygotes fertilized by the mutant-bearing sperm. To measure the transmission ratio of hermaphrodite sperm, heterozygous hermaphrodites were cloned at 25° and their progeny examined for sterility. Those that were fertile were followed for a second generation to determine if they were heterozygotes or wild type.

the uterus with a cap of sperm around its front end. These sperm abruptly migrate from the front to the back of the oocyte, returning through the constriction to the region of the spermatheca. Only one sperm actually fertilizes the oocyte, sometimes during passage into the uterus, and every sperm fertilizes an oocyte. After the supernumary sperm have migrated off the zygote, the oocyte nucleus begins its reductive divisions and the egg shell is formed. Two polar bodies are extruded and then the oocyte and sperm pronuclei fuse and cleavage commences.

A wild-type hermaphrodite produces more oocytes than sperm. When the sperm have been exhausted, the oocytes pass through the oviduct and spermatheca into the uterus without fertilization. No shell is formed, but the oocyte nucleus still undergoes its reductive divisions. Shortly thereafter the nucleus of the unfertilized oocyte begins to undergo chromosome replication, becoming manyfold polyploid and enlarging.

At 16° spermatogenesis and fertilization in hermaphrodites of strains E1034, E1035 and HC1 are identical to wild-type. At 25°, spermatogenesis is normal by light microscopy but fertilization is not. When the oocyte passes from the oviduct through the spermatheca in a mutant grown at 25°, sperm contact the oocyte apparently normally (Figure 5). These sperm are then carried through the constriction of the spermatheca into the uterus as in wild type. Fertilization, however, does not occur.

In addition, the mutant sperm do not move back to the spermatheca from the front of the oocytes. Instead the sperm are carried with the oocyte into the uterus and eventually expelled along with the unfertilized oocytes as they are laid. This movement of sperm into the uterus has been observed in live animals, but it can also be recognized by comparing sperm locations in Feulgen stained preparations

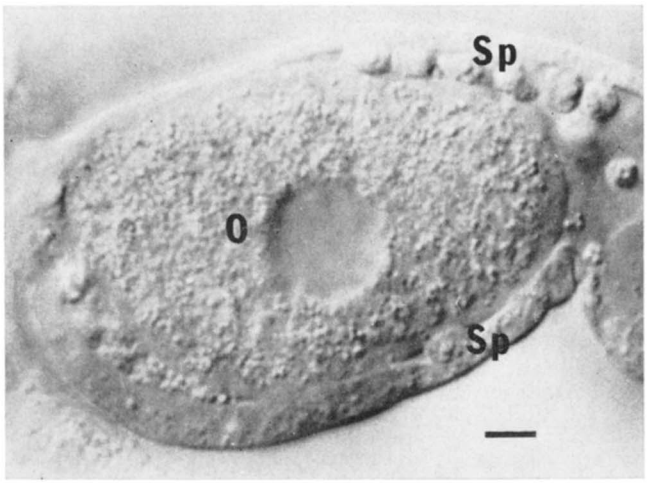

FIGURE 5.—Mutant sperm contacting an oocyte. Light micrograph with differential interference contrast optics of sperm contacting an oocyte. Dissected from an E1035 hermaphrodite grown at 25°. Sp = sperm; O = oocyte. Magnification 1400× . Bar = 5 microns.

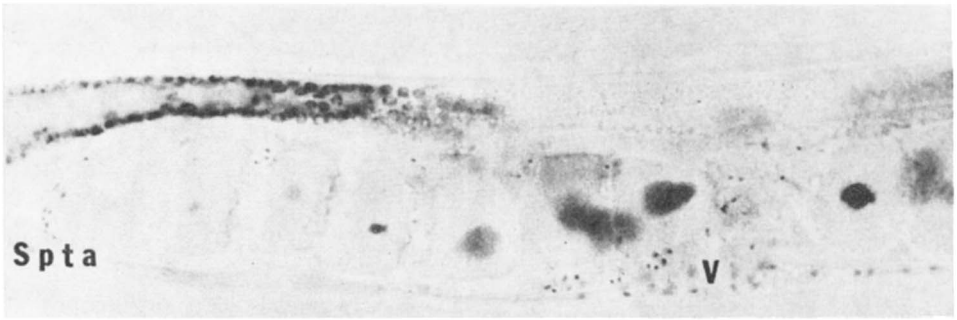

FIGURE 6.—Fate of mutant sperm and oocytes. Light micrograph of a Feulgen stained E1034 hermaphrodite raised at 25° for 52 hr after hatching. Note large Feulgen positive nuclei of unfertilized oocytes (compare with oocyte nuclei in Figure 2c). The nuclei of sperm can be seen scattered among the oocytes in the uterus. Spta = spermatheca, V = region of the vulva. Magnification = 800×.

after oocytes have been laid (Figure 6). The sperm distribution can be quantified in these specimens as shown in Table 3. This table shows that by the time five oocytes have passed through each spermatheca, more than half of the total sperm have been carried into the uterus to the vicinity of the vulva. By the time 50 or so oocytes have been laid, more than half of these sperm have been expelled. In a wild type or mutant grown at 16°, no more than three sperm have ever been observed in the region of the vulva in over a hundred animals examined; most had none.

Two observations are consistent with the conclusion that the mutant oocytes at 25° are not fertilized: (1) no shell is formed around the oocytes after passage through the spermatheca; (2) no sperm pronucleus can be seen inside the oocytes after Feulgen staining. In whole mounts of wild type or in the mutants grown

TABLE 3

Sperm distribution in E1034 grown at 25°

25° age (hours)	No. worms	Oocytes	Sperm in spermatheca	Sperm in uterus	Total sperm
42	10	0	211 ± 35	0	211 ± 35
45	5	10 ± 4	85 ± 35	95 ± 44	180 ± 40
52	7	20 ± 6	85 ± 30	53 ± 18	138 ± 25
64	4	75	51 ± 13	9 ± 8	60 ± 11
88	4	168	13 ± 17	14 ± 21	27 ± 19

Synchronized populations of worms grown at 25° were transferred to separate plates, 4–10 worms per plate. At the post-hatching age shown, the worms were fixed, Feulgen stained and examined with the light microscope. "Oocytes" is the average of the total oocytes produced (laid plus *in utero*) per worm. Sperm in spermatheca is the average number of sperm per worm in or near the spermatheca as in wild type. For the time point at 42 hr sperm is the sum of sperm plus 4× the spermatocytes, since not all sperm have matured and nearly all the immature sperm are primary spermatocytes that will yield four spermatozoa. Sperm in uterus is the average number of sperm found in the uterus per worm. Many of these sperm are collected among the oocytes around the vulva at the early time points. Uncertainties indicated are standard deviations of the mean.

at 16°, the sperm pronucleus can be identified in about ⅓ of oocytes adjacent to the spermatheca. (In the other ⅔, nuclei have already fused or they are not clearly visible).

The unfertilized oocytes in the mutants at 25° behave identically to unfertilized oocytes in old wild-type adults that have exhausted their sperm: they undergo their reductive divisions and then began chromosome replication, becoming many-fold polyploid by the time they are laid. The oocytes in the gonads in Figure 6 show this dramatically by their large, intensely Feulgen-positive nuclei.

When wild-type males mate with hermaphrodites, their sperm are deposited in the region of the vulva. The sperm then migrate up the uterus to the spermatheca (WARD 1977). Observation of 25°-grown E1034, E1035 and HC1 mutant males showed that they had apparently normal mating behavior. They contacted hermaphrodites and copulated normally. In order to determine if HC1 25°-grown males transfer sperm and to determine whether the sperm reach the spermatheca, mutant males were mated to hermaphrodites that had no sperm of their own. The mated hermaphrodites were then Feulgen stained to see if sperm had been transferred.

A representative camera lucida drawing of a hermaphrodite mated by 25°-grown mutant males is shown in Figure 7, together with a control. The sperm transfer is quantified in Table 4. It can be seen that the 25°-grown mutant males do transfer sperm to the region of the hermaphrodite vulva. The number of sperm transferred by 25°-grown mutant males is similar to 16°-grown mutant

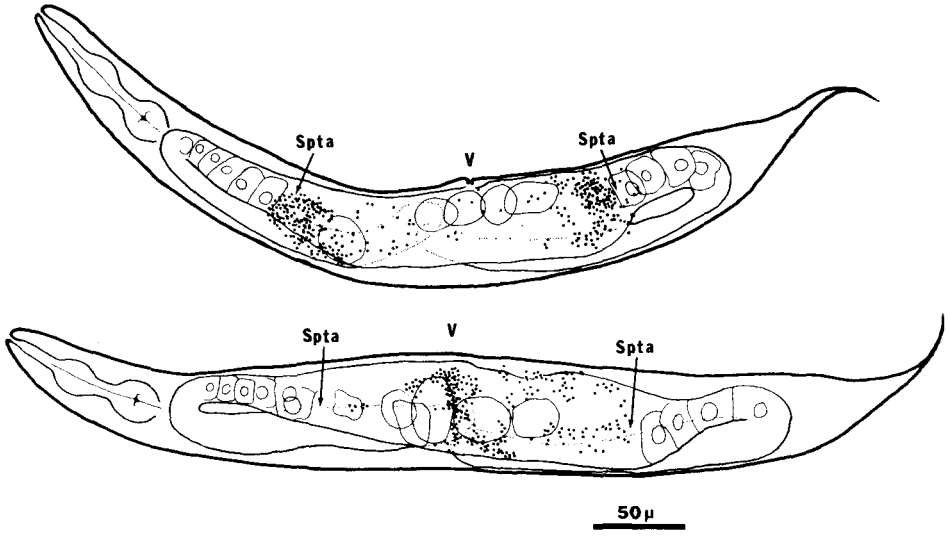

FIGURE 7.—Male sperm transfer. Camera lucida drawings of HC-D7 hermaphrodites grown at 25° and mated with males. HC-D7 contains the temperature-sensitive mutation *hc17*ts that completely blocks spermatogenesis at 25° and also contains the dumpy mutation *e1*. The transferred sperm are indicated by block dots. (a) control, mated by wild-type males; (b) experimental, mated by HC1 males raised at 25°. Labelling as in Figure 6.

TABLE 4

Male sperm transfer

Male strain	Hermaphrodites with sperm	Sperm/ hermaphrodite	Sperm in spermatheca (%)
Wild (25°)	3/4	212 ± 84	39 ± 30
HC1 (16 or 20°)	10/15	328 ± 277	63 ± 34
HC1 (25°)	5/7	237 ± 151	0.6 ± 1

Several mating plates were established with 6 HC-D7 hermaphrodites reared at 25° plus 15–20 males reared at the temperature indicated. Males were kept virgin for two or three days before mating. Mating plates with males reared at 25° were incubated at 25°, whereas those with males reared at 16 or 20° were incubated at 20°. After six to eight hours of mating, all the hermaphrodites were fixed, Feulgen stained and examined to determine how many sperm had been transferred and where they were located. Unmated controls had no sperm because the *hc17*ts mutation blocks spermatogenesis. (G. NELSON, personal communication) Hermaphrodites with sperm is the fraction of hermaphrodites scored that had received more than 30 sperm. This fraction varies substantially from plate to plate and has little significance in itself. Total sperm transferred is the average sperm in the hermaphrodites with more than 30 sperm. The sperm in spermatheca is the % of transferred sperm in or near the spermatheca (see Figure 7).

or wild-type controls. Few sperm of the 25°-grown mutant male reach the spermatheca, however. The sperm remain in the region of the vulva and are expelled when eggs or oocytes are laid (Figure 7; Table 4).

Further evidence that HC1 males raised at 25° transfer their sperm is obtained by comparing the sperm remaining in males maintained at 25° in the presence of many hermaphrodites with that found in virgin males of the same age. Data from two separate matings show that males allowed to mate with hermaphrodites have on the average 1100 ± 365 sperm ($N = 8$), whereas virgin controls all had more than 2100 sperm ($N = 9$). Large variation and reduced sperm number is found with wild-type males in the presence of hermaphrodites as well (data not shown), indicating that varying numbers of sperm have been transferred to hermaphrodites during mating.

8. Phenotype of additional fer-1 *alleles*

Both strains HC13 and HC24 have phenotypes similar to that of HC1. At 25° the hermaphrodites lay large numbers of unfertilized oocytes that can be fertilized by wild-type sperm. HC24 is leaky, producing about 15 progeny at 25°. Hermaphrodites of both alleles have normal numbers of sperm, and these sperm are swept out of the spermatheca by the passage of oocytes. The males are also sterile at 25°, although they make sperm in normal numbers and these sperm are transferred during mating. The TSP for hermaphrodite sterility overlaps that of HC1, but is not exactly the same. These results show that the fertilization-defective phenotype of HC1 is a property of other mutations in the *fer-1* gene and not just specific to the *hc1*ts allele.

DISCUSSION

1. Origin of HC1

The temperature-sensitive sterile mutation *hc1*ts was an apparently adventitious mutation in a stock originally selected for nonchemotaxis. The mutation

causing the chemotaxis defect has not been precisely mapped, but it probably lies between *dpy-5* and the sterile mutation. The presence of the chemotaxis-defective mutation has little effect on the phenotype of the sterile mutation, except to reduce the progeny slightly. This reduction may not even be due to the chemotaxis defective mutation directly, but to other mutations carried in the original strain that were eliminated in the course of isolation of HC1.

The isolation of linked double mutations is a problem inherent in the isolation of mutants from heavily mutagenized stocks. The average forward-mutation frequency using chemical mutagenesis by EMS, as in the isolation of these mutants, is estimated to be 5×10^{-4} mutations per gene per gamete (BRENNER 1974; LEWIS and HODGKIN 1977). It is expected, then, that mutant strains isolated using chemical mutagenesis should contain multiple mutations. Although the mutants were backcrossed to wild type to eliminate unlinked mutations, E1034 and E1035 still contained multiple mutations because they were tightly linked. It is surprising that two strains isolated independently would contain identical double mutations, as these apparently do. The absence of recombinants between the sterile mutations in an E1034/E1035 heterozygote argues that the sterile mutations in these strains are close together in the same gene and could be identical. It might be that E1034 and E1035 are not in fact independent, but represent progeny of the same mutant clone that were somehow isolated twice.

Since two other alleles have been obtained that have a sterile phenotype similar to *hc1*ts, this phenotype must be a property of the defective *fer-1* gene.

2. *Phenotype of* fer-1 *mutants*

We designate *hc1*ts, *hc24*ts, and *hc13*ts as fertilization defective mutations (*fer*) because they make both sperm and oocytes in normal numbers, but the oocytes are not fertilized by the sperm. Some mutants of this phenotype, including these, would be a subset of mutants that have been designated as spermatogenesis defective, *sp* by HIRSH and VANDERSLICE 1976. Sperm fertilization-defective mutants have been obtained in other organisms including Drosophila, mice and men (KIEFER 1973; AYLES *et al.* 1973; ROMRELL 1975; HESS 1975; KIEFER 1969; HARTL 1973; BENNETT 1975; AFZELIUS *et al.* 1975). Since all these organisms have flagellated sperm, there may be no relation of these mutants to the nematode mutants.

The temperature-shift experiments for HC1 show that it behaves as a "classical" temperature-sensitive mutant with overlapping up and down shifts (SUZUKI 1970; HIRSH *et al.* 1976). The TSP coincides with the time of meiotic maturation of the sperm. The midpoint of the TSP is during the last moult. This is convenient for certain experiments because animals in the middle of their TSP can be identified in an unsynchronized population by looking for moulting animals. The end of the TSP precedes the accumulation of many mature sperm, so that the TSP probably ends before spermiogenesis. This is difficult to prove because spermatogenesis and spermiogenesis go on simultaneously.

We assume that the temperature sensitivity is due to a gene product that is made temperature sensitive by a mutation, but we have no direct evidence for

this. We do not know if the TSP corresponds to the time of synthesis, the time of utilization of the gene product, or both. Many new proteins are synthesized in spermatocytes of other organisms during spermatogenesis, and this is presumably true in Caenorhabditis as well.

Our experiments do not reveal whether the defective gene product is produced autonomously by the spermatocytes or made by some other cell. The precise correspondence of the TSP to meiosis makes it likely that the gene product is made in the spermatocytes themselves, but does not prove this.

Many temperature-sensitive mutations that affect somatic development, oogenesis, or sexual determination are pleiotropic. They cause various phenotypes depending on the time and duration of exposure to high temperature (*e.g.*, VANDERSLICE and HIRSH 1976; KLASS, WOLF and HIRSH 1976; POODRY, HALL and SUZUKI 1973). Unlike these mutants, HC1 is remarkably specific. Mutant growth, maturation, and behavior and oocyte production are all indistinguishable from wild type at 25°. The production of oocytes is a particularly sensitive indicator of metabolic function because so much metabolic effort is invested in the production of oocytes (HIRSH, OPPENHEIM and KLASS 1976). Anything that interferes with normal metabolism, even slightly, will reduce oocyte production. Many *C. elegans* mutant strains of all phenotypes produce fewer oocytes than wild type (unpublished observations). Since *hc1*ts produces a normal yield of oocytes when grown at 25°, its one phenotype appears to be the fertilization defect.

In addition to the specificity of the phenotype at 25°, the mutant is sharply temperature sensitive. At 16°, the mutant is indistinguishable from wild type, at 20° it is nearly normal, but a further increase of only 5° to 25° completely abolishes progeny production.

We do not know at exactly which step in fertilization the mutant is defective. The mutant sperm contact the oocyte, but it is not possible to determine from *in vivo* observations whether their affinity for the oocyte is normal or not. The lack of a detectable sperm nucleus inside the oocyte and the presence of sperm in the uterus argue that penetration of the egg does not take place.

The observations that mutant male sperm fail to reach the spermatheca and that mutant hermaphrodite sperm fail to return to the spermatheca after being swept into the uterus suggest that the primary sperm defect might be a defect in motility. The inability to fertilize oocytes could then be due to the inability of the sperm to move over the surface of the oocyte to allow penetration. Alternatively, the sperm could stick to the surface of the oocyte or the uterus thus preventing their movement, or perhaps they fail to respond to signals initiating movement.

We are currently trying to distinguish between these possibilities by examining the normal and mutant sperm with the electron microscope, by comparing them immunologically and biochemically, and by trying to develop a reliable method of *in vitro* fertilization.

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