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The Initiation of Spermiogenesis in the Nematode *Caenorhabditis elegans*

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Spermiogenesis in nematodes involves the activation of sessile spherical spermatids to motile bipolar amoeboid spermatozoa. In *Caenorhabditis elegans* males spermiogenesis is normally induced by copulation. Spermatids transferred to hermaphrodites as well as some of those left behind in the male become spermatozoa a few minutes after mating. Spermiogenesis can also be induced *in vitro* by the ionophore monensin (G. A. Nelson and S. Ward, 1980, *Cell* **19**, 457-464) and by weak bases such as triethanolamine. Both triethanolamine and monensin cause a rapid increase in intracellular pH from 7.1 to 7.5 or 8.0. This pH increase precedes the subsequent morphological events of spermiogenesis. Triethanolamine or monensin must be present throughout spermiogenesis for all cells to form pseudopods, but once pseudopods are formed the inducers are unnecessary for subsequent motility. The pH induced spermiogenesis is inhibited by drugs that block mitochondria or glycolysis. Protease treatment can also induce spermiogenesis without increasing intracellular pH, apparently bypassing the pH-dependent steps in activation and the requirement for glycolysis. These results show that the initiation of spermiogenesis in *C. elegans*, like some steps in egg activation and the initiation of sea urchin sperm motility, can be induced by an increase in intracellular pH, but this pH change can be bypassed by proteolysis.

INTRODUCTION

During nematode spermiogenesis, sessile spherical spermatids are activated to motile bipolar spermatozoa. In the parasitic nematode *Ascaris lumbricoides* this activation can be induced *in vitro* by a substance extracted from the glandular vas deferens (Foor and McMahan, 1973; Burghardt and Foor, 1978) or by proteases (Abbas and Cain, 1979). In the bacteria-feeding soil nematode *Caenorhabditis elegans* we have shown that spermatids can be activated *in vitro* by exposure to the Na^+ and K^+ transporting ionophore, monensin (Nelson and Ward 1980). During activation sperm organelles became confined to one side of the cell, membranous organelles (MO) fuse with the plasma membrane, a pseudopod extends from the side opposite these fusions and pseudopod motility begins. Since monensin acts by exchanging Na^+ or K^+ for protons so that it can alter intracellular pH (Pressman, 1976), and intracellular pH changes participate in several developmental processes including sea urchin egg activation (reveiwed in Epel, 1980) and initiation of sperm motility (Nishioka and Cross, 1978; Lee *et al.*, 1980), we investigated whether or not internal pH changes could cause the activation of *C. elegans* spermatids.

In this paper we first describe the natural *in vivo* activation of *C. elegans* spermatids by mating and then show that activation *in vitro* can be induced by an increase in intracellular pH caused either by monensin or by weak bases. Proteases, however, can activate the cells without altering intracellular pH.

MATERIALS AND METHODS

A. Worm Strains and Culture

Caenorhabditis elegans (Bristol) strain CB1490: *him-5(e1490)* was used as a source of sperm for all experiments because it produces males at high frequency (Hodgkin *et al.*, 1979). The males have normal fertility and their sperm have normal motility and morphology (Hodgkin *et al.*, 1979; Nelson *et al.*, 1982). Stocks were maintained on petri plates seeded with *Escherichia coli* as described by Brenner (1974). For mating experiments males were picked when immature and maintained in the absence of hermaphrodites. Matings were carried out as described by Ward and Carrel (1979), using 5-10 males and 10-20 *isx-1(hc17ts)* hermaphrodites which have no sperm of their own (Nelson *et al.*, 1978).

B. Sperm Isolation

Sperm isolation was essentially as described in Nelson *et al.* (1982) except that the medium used for squashing was altered by eliminating bisulfite and lowering

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the pH to 6.5 to minimize the activation of spermatids during squashing. Briefly, CB1490 males were grown in liquid culture on *E. coli*. Males were isolated by fractionation through filters. Sperm were released by squashing the males and the sperm were isolated by filtration and centrifugation. They were resuspended in sperm medium (SM) (Nelson and Ward, 1980). Final sperm preparations invariably contained more than 90% spermatids at the start of an experiment and were used within 3 hr of isolation.

C. In Vitro Spermiogenesis

Spermatids were centrifuged and resuspended at 10^7 cells/ml in the desired buffer and then 45 μl was distributed to wells of small microtiter plates (Colab, 96 well). Five microliters of test activator at the appropriate concentration was added to each well. After 20 min cells were fixed by addition of 25 μl of 3% glutaraldehyde and 3% formaldehyde in the test buffer. The fraction of cells with pseudopods was determined by transferring a drop of cells to a microscope slide and scoring at least 200 cells using a Plan 40 objective with Nomarski optics. Although there is slight subjectivity in deciding whether a pseudopod is present on some cells, the reproducibility of repeated scorings by the same investigator is within 5% and between investigators is within 8%. Spermatid activation experiments that required continuous observation of the cells were done by perfusing solutions through microscope slide chambers as previously described (Roberts and Ward, 1982). Control experiments showed that the fraction of spermatids activated on slides was similar to that found in microwells.

The background fraction of spermatozoa in unactivated controls always stayed less than 10%. As described in Results (Table 4) the fraction of remaining cells that could be converted to spermatozoa with pseudopods varied from batch to batch of cells and was less for TEA and monensin than for Pronase activation. In order to facilitate comparison between experiments, all activation data presented are normalized by converting the fraction of spermatozoa observed to percentage of the maximal fraction of spermatozoa obtained for that batch of cells. Error bars on graphs are the standard error of the mean for 3–10 independent measurements on at least two batches of cells unless stated otherwise.

D. Proteases

Pronase (Boehringer-Mannheim) is a mixture of proteases, but at the pH range used here its predominant activity is an endopeptidase that cleaves adjacent to hydrophobic amino acids (Narahashi, 1970). Trypsin, chymotrypsin, and soybean trypsin inhibitor were obtained from Worthington Biochemicals. Stock solutions of proteases were prepared in 1 m*M* HCl and stored frozen. They were diluted on ice just before use. Proteolytic activity was assayed by release of 5% cold trichloroacetic acid-soluble dye from azoalbumin (Sigma Chemical Co.) or by release of acid-soluble counts from ^{35}S-labeled *in vitro* synthesized proteins which had first been acid precipitated and resuspended.

E. Intracellular pH Determination

Intracellular pH was measured by determining the partitioning of a weak acid or base into the cells essentially as described by Gillies and Deamer (1979a). [^{14}C]Methylamine (New England Nuclear) was used for all experiments and in addition, [^{14}C]5,5-dimethyloxazolidine-2,4dione (DMO) (New England Nuclear) was used for the spermatid pH measurements shown in Table 2. Cells, 3–5 $\times$ 10^7/ml, were combined with the ^{14}C-labeled marker and ^3H_2O and equilibrated for 5 min. Two hundred-microliter samples were then centrifuged in a Beckman microfuge before or after addition of activator. The supernatant was carefully removed with a capillary pipet and an aliquot was sampled. Both supernatant and pellet were dissolved in Aquasol (New England Nuclear) and their radioactivity was determined in a Beckman LS5000 liquid scintillation spectrophotometer using automatic quench compensation. The extracellular volume in the pellet was determined from parallel controls containing [^{14}C]inulin (New England Nuclear) and ^3H_2O. The external volume varied from 43 to 60% of the total pellet volume (^3H_2O accessible) depending on the number of cells used in the assay. The internal volume did not vary more than 8% in control cells during activation to spermatozoa. Since this variation is within the experimental error this indicates that the internal volume of the spermatids does not change appreciably during activation to spermatozoa, and only the total pellet volume of spermatids was routinely measured. The intracellular pH was calculated from the concentration of weak base or acid inside and outside the cells as described by Gillies and Deamer (1979a).

RESULTS

A. Copulation Induces Spermiogenesis in Vivo

Spermatogenesis arrests in young virgin *C. elegans* males with the accumulation of spermatids. When males copulate spermiogenesis is initiated in the spermatids transferred to a hermaphrodite and they become spermatozoa (Ward and Carrel, 1979). Some spermatids remaining behind in the male testis also become spermatozoa. Three hours of mating to hermaphrodites stimulates more than 60% of the spermatids left in a

male to become spermatozoa (Fig. 1). Even in the absence of hermaphrodites, as males age for several days on a petri plate some spermatids mature to spermatozoa (Fig. 1). During this time, males attempt to copulate with each other and themselves and evert their copulatory spicules. These attempted copulations apparently cause the maturation of spermatids because the spermatids do not mature in mutant males that are too paralyzed to attempt copulation (Fig. 1) (Nelson *et al.*, 1982). In addition, spermatozoa are only rarely found in males grown in liquid, presumably because they cannot attempt copulation without a surface to move against.

TEM examination of both the sperm transferred to a hermaphrodite during copulation and the sperm remaining behind in the male confirms that both populations have been induced to mature into normal spermatozoa with pseudopods extended and membranous organelles fused with the plasma membrane (Table 1). From both light and electron microscopic examination of males following copulation it is seen that the sperm closest to the vas deferens become spermatozoa while those farther away remain spermatids.

The mechanism of activation by copulation is unknown and we have been unable to isolate a natural inducer of spermiogenesis from males. However, several alternative methods of inducing spermiogenesis *in vitro* are described in the following sections.

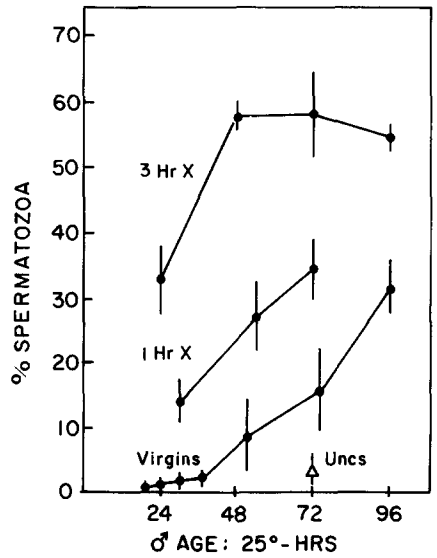

FIG. 1. Activation of spermatids by mating. Larval *him-5* males were grown in the absence of hermaphrodites at 25°C. Males were mated with hermaphrodites for the times indicated on the abscissa and then removed and dissected into SM to score the fraction of sperm with pseudopods. The mean of several experiments totaling more than 6000 sperm is plotted with the standard deviation as error bar. The lower curve shows virgin males; the middle curve shows the effect of 1-hr mating with excess hermaphrodites; the upper curve shows the effect of 3 hr of mating. The open triangle shows sperm from uncoordinated males that are too paralyzed to attempt copulation.

TABLE 1
IN VIVO SPERM ACTIVATION

Male sperm source	Total sperm	Sperm with pseudopods (%)	Total MO	Fused MO (%)
Virgin	134	0 (0)	503	2 (0.5)
Transferred	79	52 (66)	435	191 (44)
Mated	50	39 (78)	241	133 (55)

Note. Sectioned males and hermaphrodites were examined by TEM. Virgin wild-type males, 48 hr old, were mated to *isx-1* hermaphrodites which have no sperm of their own. Virgin is sperm in unmated males; transferred is sperm transferred to the hermaphrodites and found in the uterus or spermatheca (see Ward and Carrel (1979) for micrographs); mated is sperm left behind in the male after copulation. MO is the membranous organelle that fuses with the cell membrane during spermiogenesis (Ward *et al.*, 1981).

B. Weak Bases Induce Spermiogenesis in Vitro

Spermatids can be activated to spermatozoa *in vitro* by treatment with monensin (Nelson and Ward, 1980). This activation could be caused by alteration of internal ion concentrations, alterations in membrane potential, or alterations in internal pH. If internal pH changes were responsible then it should be possible to activate spermatids by exposing them to weak acids or weak bases which will diffuse across membranes in their uncharged form and alter the internal pH (reviewed in Gillies and Deamer, 1979a; Roos and Keifer, 1982).

Exposure of spermatids to 0.1 *M* sodium acetate had no effect but exposure to ammonium chloride (40 m*M* at pH 7.5) did activate up to 80% of the spermatids. The activation observed was erratic, however, and was only seen in a narrow concentration range. At NH_3 concentrations above 1.5 m*M* spermatids became irregular in shape and often formed long protrusions without forming pseudopods. This suggests that NH_3 or NH_4^+ is toxic to the cells so other weak bases were tried.

Triethanolamine (TEA) gave the best results. TEA has a pK_a of only 7.8 (Hodgman, 1958; and our titration in SM) so a substantial fraction of the total TEA will be unprotonated and able to penetrate cells at pH values below 8.0. As shown by the solid line in Fig. 2, TEA was a potent activator of spermiogenesis with an optimal unprotonated base concentration of 25 m*M*.

To determine if the TEA concentration that induced spermiogenesis did indeed change the internal pH, the intracellular pH was measured by determining the partition of an isotopically labeled weak base between the intracellular and extracellular volumes. Prior to activation the internal pH of spermatids is about 7.1 (Table 2). Measurement using the weak acid DMO or the weak

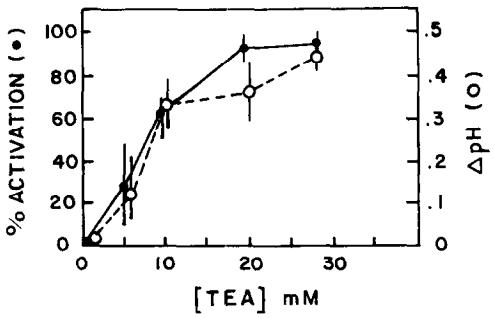

FIG. 2. Activation by triethanolamine (TEA). Sperm medium was adjusted to pH 7.8 and varying concentrations of TEA were added. The abscissa shows the concentration of unprotonated TEA calculated from the Henderson-Hasselbach equation. The solid line is the percentage of cells activated to form pseudopods. The dashed line shows increase in intracellular pH (difference between cells treated with TEA and untreated controls) of the same population of cells measured 4-8 min after TEA addition. Each point is the average of 6-20 measurements on 3-10 different batches of cells.

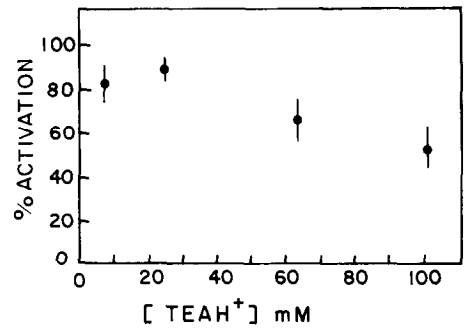

FIG. 3. Activation as a function of $TEAH^+$. Activation as in Fig. 2, but the pH for each point from left to right was 8.1, 7.7, 7.4, 7.2. The amount of added TEA was adjusted so that the TEA concentration would be 27 m*M* at each point. The abscissa plots the calculated concentration of $TEAH^+$ at each pH.

base MA give comparable results and the internal pH was largely independent of external pH between 7.0 and 7.8 (Table 2). However, when spermatids are treated with TEA, the intracellular pH increases in parallel with the fraction of spermatids activated by TEA (Fig. 2).

The activation shown in Fig. 2 is plotted as a function of unprotonated TEA concentration. However, both the protonated ($TEAH^+$) and unprotonated TEA concentrations varied in this experiment. To see which amine form was responsible for activation, the ratio of TEA to $TEAH^+$ was varied by adjusting the external pH. The concentration of total TEA added was then varied in such a way that the unprotonated amine concentration remained constant while the protonated amine varied. Under these conditions the activation is largely independent of the $TEAH^+$ concentration (Fig. 3). If anything it is reduced at higher concentrations. Therefore it is the unprotonated TEA which is the activator, consistent with a direct effect of TEA on the intracellular pH.

TABLE 2
INTRACELLULAR pH OF SPERMATIDS

Reagent	Extracellular pH	Intracellular pH ± SD (*N*)
DMO	7.0	7.1 ± 0.04 (5)
MA	7.0	6.9 ± 0.2 (5)
MA	7.8	7.2 ± 0.1 (22)

Note. Intracellular pH was determined as described under Materials and Methods. Measurements were made from 5 to 15 min after adding the ^{14}C-labeled reagent. There was no significant effect of time so measurements were combined in one average. *N* is the number of independent determinations.

The activation of spermatids by monensin is also accompanied by an increase in intracellular pH (Fig. 4). The sensitivity of cells to monensin activation and the magnitude of the pH change observed are dependent on the extracellular pH. As shown in Fig. 4 at an extracellular pH of 7.8 a 1-unit pH increase is observed and maximal activation can occur at 40 n*M* monensin. At pH 7.0, the pH increases only 0.4 ± 0.1 ($N = 8$) and maximal activation requires 200 n*M* monensin.

The kinetics of the pH change induced by TEA is indicated in Fig. 5. As rapidly as a measurement can be taken following addition of TEA (2 min) the pH increase is complete. No detectable signs of spermatid activation are seen this soon after TEA addition when cells are monitored continuously with time-lapse video recording, so the pH change precedes the visible events of spermiogenesis.

In order to determine whether just a brief exposure to TEA would trigger spermiogenesis, the duration of TEA exposure was varied. Figure 6 shows that the fraction of spermatids activated to spermatozoa is depen-

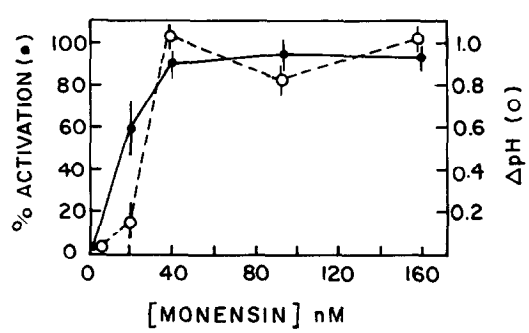

FIG. 4. Activation by monensin. Solid line shows the percentage of cells activated to form pseudopods in sperm medium at pH 7.8. The dashed line shows the increase in intracellular pH measured 4-8 min after monensin addition. Each point is the average of 4-16 measurements, on 2-8 batches of cells.

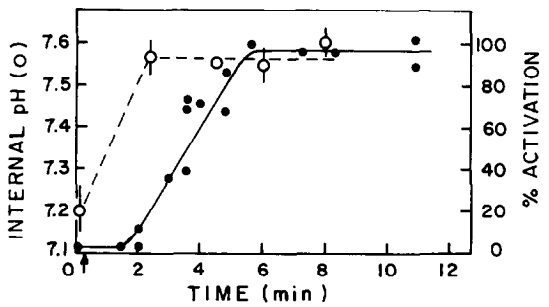

FIG. 5. Time course of intracellular pH changes (dotted line) and activation (solid line) in response to TEA. All experiments in SM pH 7.75, TEA was added to make unpronated TEA to 30 m*M* just after taking $T = 0$ point.

dent on the duration of TEA exposure and that the optimal time of exposure is about the same time that it takes to complete spermiogenesis, 6–8 min. Once spermatozoa have formed, TEA can be removed without altering pseudopod morphology. This was confirmed by examination of continuous time-lapse video recordings of spermatozoa while TEA was washed out. There was no detectable alteration in pseudopod motility or shape during or after the removal of TEA. As indicated by the triangles in Fig. 6 and by direct observation, a similar result is obtained when monensin activation is terminated by washing out all Na^+ and K^+ ions by replacement with choline. Therefore TEA or monensin must be present throughout spermiogenesis for all cells to form pseudopods, but once pseudopods are formed, the activators are unnecessary for pseudopod maintenance and motility.

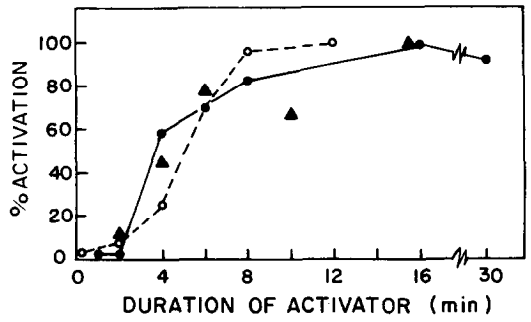

FIG. 6. Duration of TEA exposure necessary to activate spermatids. Solid line shows the percentage activation for spermatids exposed to 27 m*M* unprotonated TEA at pH 7.8 for the times indicated on the abscissa. All cells were in microscope slide chambers and the TEA was removed by perfusion with SM pH 7.0. Cells were fixed 20 min after the initial addition of TEA. Points are the mean of two independent determinations. Dotted line shows the kinetics of activation in the continuous presence of TEA of the same batch of cells recorded with time-lapse video tape, 60 cells total. Triangles show the activation with 0.5 μM monensin which was terminated by perfusion with sperm medium containing choline in place of Na^+ and K^+.

C. Proteases can Activate Spermiogenesis in Vitro

Abbas and Cain (1979) observed that *Ascaris* spermatids could be activated to spermatozoa by protease treatment. This is true for *C. elegans* spermatids as well. The concentration dependence for activation by Pronase, trypsin, and chymotrypsin is shown in Fig. 7. The mixture of enzymes present in Pronase is about 25× as effective as either trypsin or chymotrypsin. As shown by the control points on Fig. 7, Pronase activation is abolished by boiling the enzyme and trypsin activity is blocked by addition of trypsin inhibitor. This shows that the activation is due to the proteolytic activity of these enzyme preparations.

When the intracellular pH was measured during Pronase activation no significant pH change was observed preceding or following activation to spermatozoa (Fig. 8). Activation by Pronase is independent of external pH; the concentration of Pronase necessary to activate 50% of a population of spermatids was 26, 20, and 20 μg/ml at pH 7.0, 7.4, and 7.8, respectively. Reasoning that normal *in vivo* spermiogenesis or TEA triggered spermiogenesis might involve the activation of endogenous sperm proteases which could be mimicked by Pronase, we tried to detect protease activity in spermatids and spermatozoa using either added azoalbumin or ^{35}S-labeled polypeptides as substrates. No proteolytic activity could be found in intact or Triton-lysed spermatids or spermatozoa at a concentration of 7×10^7 cells/ml, even when spermatids were activated by TEA in the presence of the exogenous substrates. Reconstruction experiments with pronase added to the lysed cells showed that we would have detected less than 1 μg/ml. Since 7×10^7 cells is 1.1 mg protein/ml (Nelson *et al.*, 1982) protease activity, if present, must be less than 0.1%. An additional attempt to determine if sperm proteolytic activity was involved in sperm activation was to try to inhibit the activation by TEA with the protease inhib-

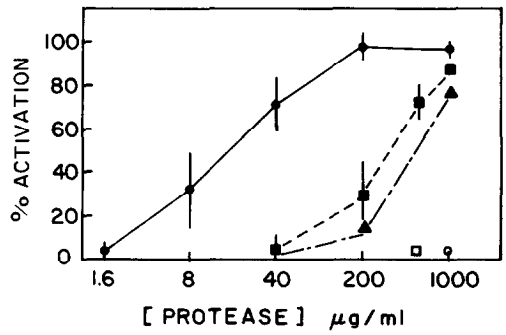

FIG. 7. Protease activation. The percentage activation is plotted against protease concentration on a logarithmic scale. Solid circles, pronase; squares, trypsin; triangles, chymotrypsin; open square, 500 μg/ml trypsin plus 500 μg/ml soybean trypsin inhibitor; open circle 1000 μg/ml Pronase, boiled 5 min before adding.

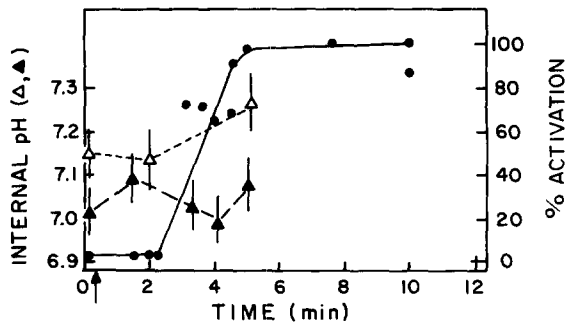

FIG. 8. Time course of intracellular pH and activation with Pronase. Pronase at 200 μg/ml was added to cells just after time zero point was taken. Triangles show the intracellular pH of two batches of cells. Circles show the activation of the same batches of sperm.

itor phenylmethylsulfonyl fluoride (PMSF). One millimolar PMSF only reduced the activation by 7%, an insignificant change.

D. Metabolic Requirements for Spermiogenesis

We have examined the metabolic requirements for both TEA and Pronase activation by using drugs that interfere with either aerobic or anaerobic metabolism. Three mitochondrial poisons which block aerobic metabolism at different sites, oligomycin, dinitrophenol, and sodium azide, were all found to inhibit both TEA- and Pronase-induced spermiogenesis with a similar concentration dependence (Fig. 9). In contrast, an inhibitor of anaerobic metabolism, 2-deoxyglucose, prevents activation by TEA but not by pronase (Fig. 9). This inhibition appears to be specific because it is overcome by addition of glucose but not fructose.

E. Ionic Requirements for Activation

We have examined some of the ionic requirements of both TEA and Pronase activation. Neither TEA nor pronase activation is affected by elimination of calcium from the external medium (Table 3). This is consistent with the observation that calcium ionophores do not initiate spermiogenesis (Nelson and Ward, 1980). TEA activation is somewhat reduced if Na^+ and K^+ are replaced by choline in the medium (Table 3). Similar replacement totally abolishes monensin activation (Nelson and Ward, 1980). Pronase activation is sensitive to the concentration of external K^+ ions, however (Fig. 10).

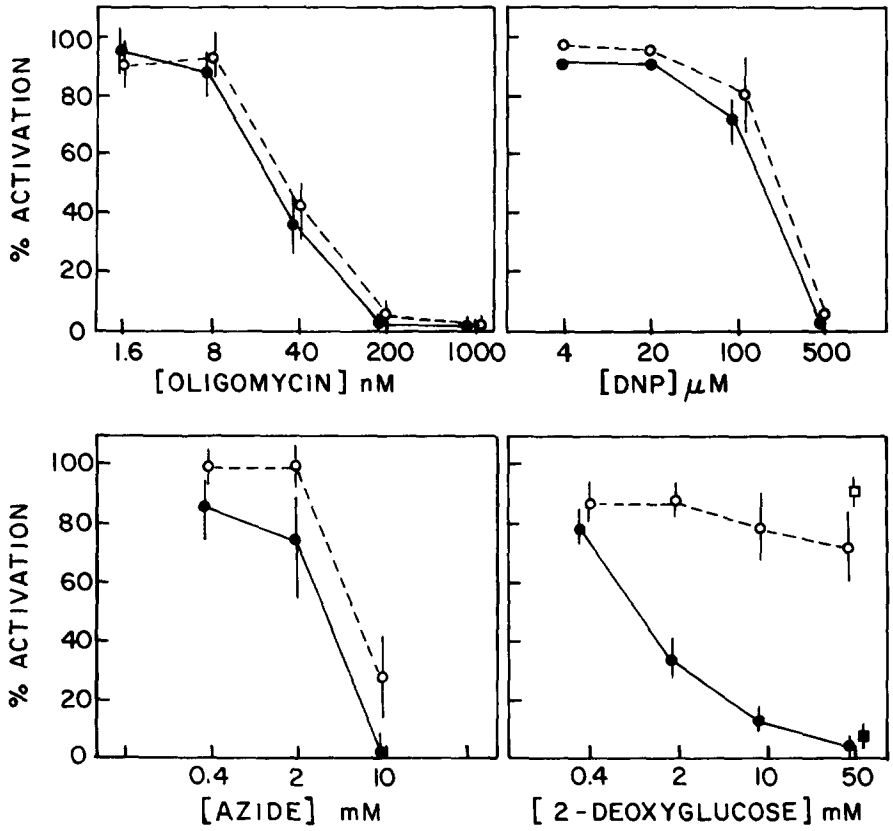

FIG. 9. Effect of metabolic inhibitors. Drugs were added at the concentration indicated 5 min prior to adding 30 m*M* TEA (solid circles) or 200 μg/ml Pronase (open circles). For 2-deoxyglucose inhibition the effect of 10 m*M* glucose (solid square) and 10 m*M* fructose (open square) on the inhibition of TEA activation is indicated.

TABLE 3
IONIC DEPENDENCE OF ACTIVATION

Medium	Activator	Activation
Na^+, K^+, Ca^{2+}	TEA	100 ± 4
Na^+, K^+, EGTA	TEA	90 ± 5
Choline$^+$, Ca^{2+}	TEA	67 ± 3
Na^+, K^+, Ca^{2+}	Pronase	100 ± 10
Na^+, K^+, EGTA	Pronase	104 ± 6

Note. Normal sperm medium (Na^+, K^+, Ca^{2+}) contains 50 m*M* Na^+, 25 m*M* K^+, 5 m*M* Ca^{2+}, 1 m*M* Mg^{2+}, and 5 m*M* Hepes buffer. Na^+, K^+, EGTA is sperm medium with no added calcium and 1 m*M* EGTA. Choline$^+$, Ca^{2+} is sperm medium with both Na^+ and K^+ replaced by 75 m*M* choline$^+$. Activation shows the mean normalized fraction of sperm with pseudopods ± the standard error from four measurements.

A 25-fold reduction in activation is observed when K^+ ions are replaced by choline in the medium. No such effect is seen when Na^+ ions are replaced by choline (Fig. 10). The effect of ions is not due to direct effects on Pronase activity because the proteolytic activity measured by hydrolysis of azoalbumin decreased only 5% when K^+ was removed from the medium. These results show that in addition to not altering intracellular pH pronase activation differs from TEA activation by its sensitivity to K^+ in the external medium.

F. Comparison of Sperm Activated by TEA and Pronase

In all the experiments presented so far the process of activation was followed by scoring for pseudopod extension. Normal spermiogenesis also includes the fusion of an intracellular organelle, the membranous organelle (MO) with the plasma membrane (Wolf *et al.*, 1978; Ward *et al.*, 1981), and the rearrangement of surface components (Roberts and Ward, 1982) and internal organelles. We have examined spermatozoa for MO fusions by fixing cells at various times following addition of activator and then staining them with rhodamine-conjugated wheatgerm agglutinin (RWGA). This stains the newly fused MO's in a characteristic dotted pattern (Argon, 1979). Figures 11 and 12 show that MO fusion does occur during both TEA and Pronase activation. During TEA activation, however, MO fusion usually precedes pseudopod formation (Figs. 11c, d; Fig. 12a) whereas during Pronase activation these two events are coincident (Fig. 12b). MO fusion also precedes pseudopod formation following monensin activation (Nelson and Ward, 1980; Argon, 1979).

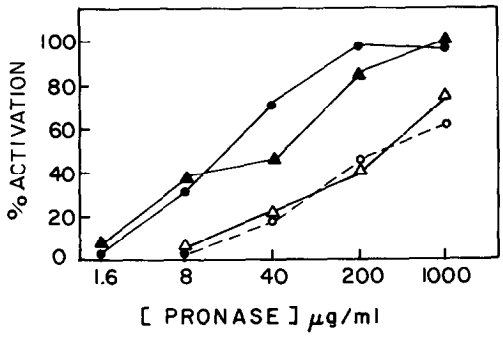

FIG. 10. Ionic dependence of pronase activation. Solid circles, sperm medium pH 7.0; open circles, sperm medium with Na^+ and K^+ replaced by choline; open triangles, sperm medium with K^+ replaced with choline; solid triangles, sperm medium with Na^+ replaced with choline.

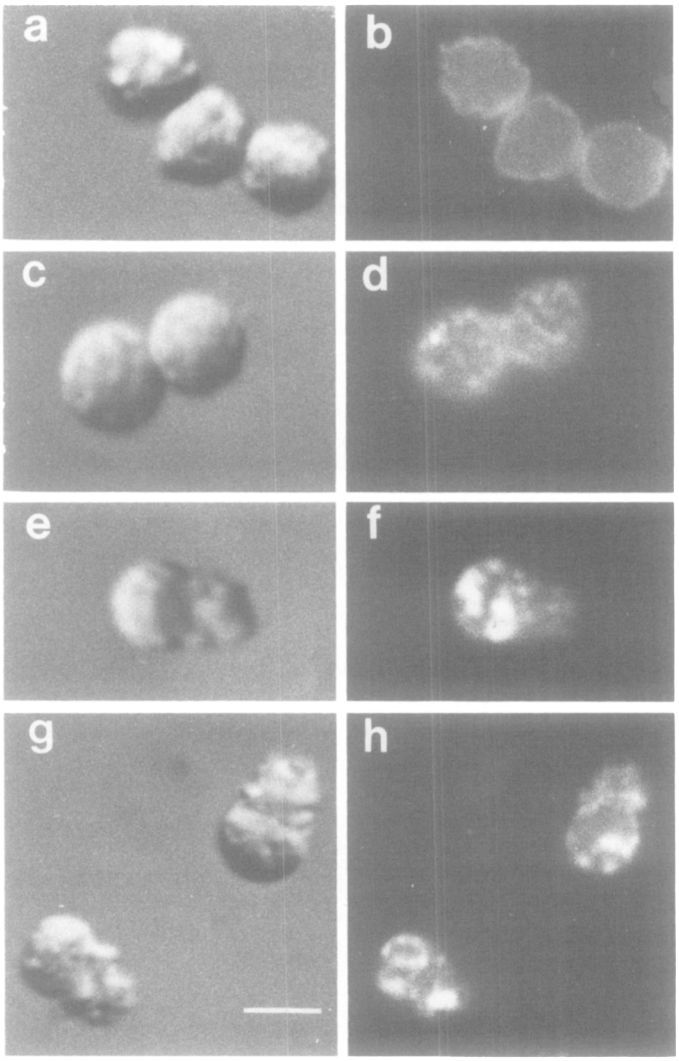

FIG. 11. MO fusions during spermiogenesis. *In vitro* activated sperm were fixed and stained with RWGA. a, c, e, g are Nomarski optics; b, d, f, h are fluorescence. Focus is slightly above the center of the cells to emphasize the surface fluorescence. a, b are spermatids prior to activation (note the even ring of stain). c, d are 3 min after TEA activation showing the dotted staining characteristic of fused MO's, but the cells have no pseudopods. e, f are spermatozoa with both pseudopod and fused MO's 3 min after TEA. g, h are spermatozoa with fused MO's 6 min after Pronase treatment.

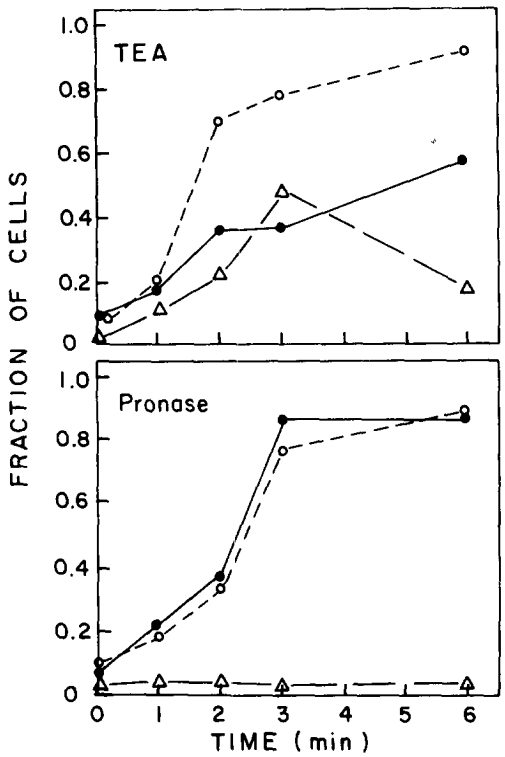

FIG. 12. Kinetics of MO fusion and pseudopod formation. Spermatids activated by 30 m*M* TEA (top) or 20 μg/ml Pronase (bottom) were stained as for Fig. 11. Solid circles are cells with pseudopods; open circles are all cells with more than four dots of fluorescence; triangles are only cells with more than four dots of fluorescence but without pseudopods as in Figs. 11c, d.

It is not possible to determine if the spermatozoa resulting from *in vitro* activation are fertile because reliable *in vitro* fertilization has not been achieved. However, the light microscopic appearance of pseudopodial movements of *in vitro* activated spermatozoa is indistinguishable from that activated *in vivo*. The rearrangement of lectin receptors found during monensin-induced spermiogenesis (Roberts and Ward, 1982) is also seen following TEA or Pronase activation (data not shown). The appearance of TEA and Pronase-activated spermatozoa in the electron microscope (Fig. 13) is also identical to cells activated *in vivo* (Ward *et al.*, 1981, Fig. 4) or by monensin (Nelson and Ward, 1980a, Fig. 8) except that Pronase removes the MO contents and neither TEA nor Pronase causes the electron-dense appearance of the laminar membranes that is caused by monensin.

Both Pronase and TEA cause pseudopod formation on a higher fraction of spermatids than does monensin (Table 4). Since some spermatids do activate and fuse their MO's with the plasma membrane without pseudopod extension (Figs. 12, 13), scoring activation just by pseudopod extension underestimates the fraction of spermatids activated by TEA and monensin.

DISCUSSION

The mating experiments reported here show that copulation induces spermiogenesis in *C. elegans* males caus-

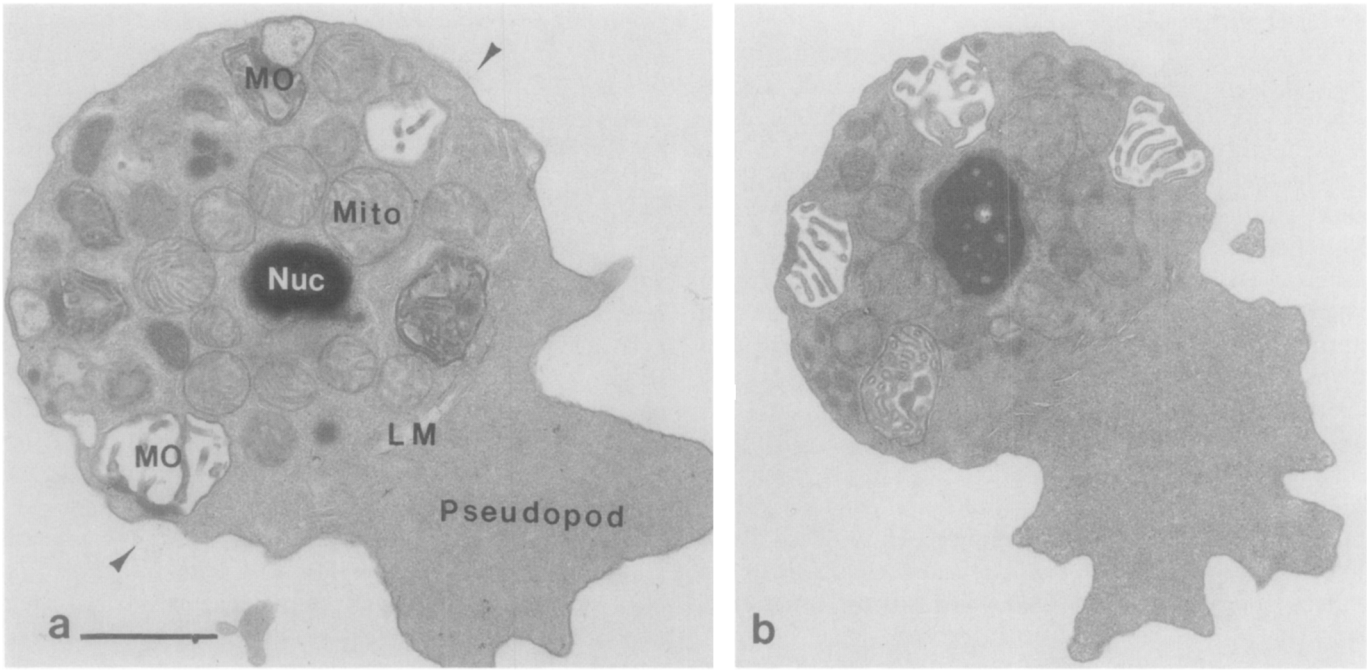

FIG. 13. *In vitro* activated spermatozoa. Transmission electron micrographs of spermatozoa. (a) TEA activated; (b) Pronase activated. Nuc, nucleus; MO, membranous organelle; Mito, mitochondria; LM, laminar membranes. Note the unfused MO on upper right in (a) and a fused MO in lower left. Arrows show fibrous contents of fused MO's extending from the cell surface.

TABLE 4
COMPARISON OF THE MAXIMAL FRACTION OF SPERMATIDS ACTIVATED TO SPERMATOZOA WITH DIFFERENT ACTIVATORS

Activator	Sperm preparations	Fraction activated
Monensin	13	0.52 ± 0.21
TEA	14	0.68 ± 0.13
Pronase	16	0.81 ± 0.08

Note. The fraction activated is the mean and standard deviation for the number of different sperm preparations shown.

ing accumulated spermatids to activate to spermatozoa. Since attempted copulations with each other and themselves stimulate spermatid activation in males, the presence of hermaphrodites is unnecessary. This means that something in the males alone is sufficient to cause spermiogenesis, although hermaphrodites could still contribute as well. An unidentified substance from the vas deferens stimulates spermiogenesis in *Ascaris* males (Burghardt and Foor, 1978), but we have been unable to obtain an extract from *C. elegans* males that would activate spermatids *in vitro* (Nelson and Ward, 1980). It may be that such an activity in *C. elegans* is unstable or masked in the whole worm extract. A sample of *Ascaris* vas deferens extract kindly provided by Sol Sepsenwol was also inactive on *C. elegans* although it still activated *Ascaris* spermatids. It is possible that normal activation in *C. elegans* might be caused by contact with the vas deferans or some other physical signal not associated with a soluble substance.

We have found a number of ways to activate spermiogenesis artificially *in vitro*. Weak bases and monensin both act by an apparently identical mechanism, increasing intracellular pH. Four results support this conclusion. First, an increase in intracellular pH is observed following both monensin and TEA treatment. This increase precedes the cellular rearrangements associated with spermiogenesis. Second, TEA activation is proportional to the concentration of unprotonated amine in solution and not dependent on the protonated form. This is consistent with TEA diffusing across the spermatid membrane in its uncharged form and increasing the intracellular pH as it associates with protons inside the cell. Third, the increase in pH following TEA activation parallels the fraction of cells activated. Fourth, the increase in intracellular pH observed is not just a consequence of spermiogenesis because Pronase causes spermiogenesis without increasing intracellular pH.

Pronase activation differs from TEA and monensin activation in several ways. Pronase activation does not cause an increase in intracellular pH; it is sensitive to K^+; it is not blocked by 2-deoxyglucose; and it induces MO fusion and pseudopod formation simultaneously. In spite of these differences both activators produce motile spermatozoa at a similar rate and the spermatozoa are morphologically normal with normal motility.

We cannot obtain enough spermatozoa from mated males to determine if the intracellular pH is increased during normal spermiogenesis, so the normal mechanism of activation could be proteolysis, or increased intracellular pH, or another mechanism. In any case, the spermatozoa resulting from *in vitro* activation are indistinguishable from normal spermatozoa in motility and morphology by light, scanning, and transmission electron microscopy.

One hypothesis to relate the different activators is that the increase in intracellular pH somehow causes the release of a sperm protease at the surface of the cell. Proteolysis of some sperm surface protein could then cause the membrane to begin movements and perhaps cause a change in ionic permeability that induces intracellular rearrangements and initiates aerobic metabolism. This hypothesis could explain the inhibition of TEA activation by 2-deoxyglucose if the release of the protease were dependent on ATP generated by glycolysis. It would also explain how Pronase works from outside the cell. So far all attempts to identify the hypothesized sperm protease have been negative. Preliminary experiments to demonstrate proteolysis of iodinated spermatid surface components during TEA activation have been negative (S. Ward, unpublished). If TEA activation does result in proteolysis the effect must be on minor components. One puzzling observation that does not fit the hypothesis is the dependence of Pronase, but not TEA activation, on K^+ ions. Perhaps the Pronase-induced activation is dependent on membrane potential changes induced by changes in K^+ permeability.

Both Pronase- and TEA-induced spermiogenesis are blocked by mitochondrial inhibitors showing that aerobic metabolism is necessary for spermiogenesis, presumably to generate ATP to power the cellular movements. The complete inhibition of either pronase or TEA activation by azide, however, requires a 10-fold higher concentration (10 m*M*) than that necessary to inhibit pseudopod motility once the pseudopod has extended (Roberts and Ward, 1982). When pseudopod formation in the presence of 1 m*M* azide is observed on time-lapse video tapes it is seen that the pseudopod extends normally, but only a few projections move for a few seconds and then the pseudopod is immotile. This indicates that pseudopod formation is not dependent on normal pseudopod motility, a conclusion supported by the observation that *fer-4* mutant sperm and most *fer-2* mutant sperm form immotile pseudopods (Ward *et al.*, 1981).

Increases in intracellular pH have been observed during egg activation following fertilization (Johnson *et al.*,

1976, reviewed in Epel, 1980) during the initiation of motility in sea urchin spermatozoa (Nishioka and Cross, 1978; Lee *et al.*, 1980) and have been found correlated with the initiation and termination of DNA synthesis in Tetrahymena (Gillies and Deamer, 1979b; see also Nuccitelli and Deamer (1982) for reviews of pH changes in cells). In the case of egg activation alteration of intracellular pH correlates with alteration in protein synthesis suggesting that pH changes directly control protein synthesis (Winkler *et al.*, 1980). The change in internal pH observed during *C. elegans* sperm activation cannot act through an effect on macromolecular synthesis as in sea urchin eggs because there is no RNA or DNA synthesis in sperm and the only protein synthesis, mitochondrial, is unnecessary for activation (S. Ward and E. Hogan, in preparation).

We do not know the sequence of steps leading from the pH change to the cellular movements of spermiogenesis. Nor do we know the relationship between TEA or Pronase activation to the natural mechanism of activation. Further studies of the biochemical changes occurring during spermiogenesis are in progress. Since mutants have been isolated in which spermatids fail to activate *in vivo* and *in vitro*, (Ward *et al.*, 1981; T. Roberts and S. Ward, unpublished) it should now be possible to dissect the mechanism of activation in finer detail.

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