

The Localization of Protein Carboxyl-Methylase in Sperm Tails

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ABSTRACT Protein carboxyl-methylase (PCM), an enzyme known to be involved in exocytotic secretion and chemotaxis, has been studied in rat and rabbit spermatozoa. PCM activity and its substrate methyl acceptor protein(s) (MAP) were demonstrated in the supernate after solubilization of the sperm cell membrane by detergent (Triton X-100). A protein methylesterase that hydrolyzes methyl ester bonds created by PCM was demonstrated in rabbit but not in rat spermatozoa. This enzyme was not solubilized by nonionic detergent.

The specific activities of PCM in rat spermatozoa from caput and cauda epididymis were similar and lower than that found in testis. By contrast, MAP substrates were low in testis and increased in parallel with sperm maturation in the epididymis. Multiple MAP were demonstrated in spermatozoa by polyacrylamide gel electrophoresis. The pattern of these proteins was similar in spermatozoa from different portions of the reproductive tract. Fractionation of heads and tails of rat spermatozoa on sucrose gradients indicated that PCM was found exclusively in the tail fraction, whereas MAP was detected both in head and tail fractions.

The presence of all the components of the protein carboxyl-methylation system in spermatozoa and the localization of PCM and some of its substrates in the sperm tail are consistent with their involvement in sperm cell motility.

Protein carboxyl-methylase (PCM)¹ is one of several enzymes that produces a reversible posttranslational modification of protein structure that, in turn, leads to altered function. This enzyme methylates the free carboxyl groups of glutamate or aspartate residues in protein substrates (methyl acceptor proteins, MAP) using *S*-adenosyl methionine (SAM) as a methyl donor (14–16, 18, 23). The methyl ester bonds are hydrolyzed, either enzymatically by a protein methylesterase (PME) (9) or spontaneously in neutral and alkaline pH, to yield methanol. A consequence of these reactions is the reversible neutralization of the negative charge on substrate proteins.

The methanol-forming enzyme was first discovered in 1965 (1) and was later identified as PCM (14, 27). This enzyme has been associated with several cellular functions, including exocytotic secretion (4–8, 34) and chemotaxis (19–21, 29–33). Experiments on bacterial chemotaxis showed that methylation of several membrane proteins was stimulated by chemotactic

agents in wild-type organisms (19, 20, 31–33), but not in nonchemotactic mutants (20). Furthermore, the relationship between protein carboxyl-methylation and chemotaxis has also been demonstrated in leukocytes, monocytes (29, 30), and macrophages (21).

Although PCM is ubiquitous in mammalian cells, the highest activities are found in endocrine glands and in nervous tissues. Because this enzyme activity is especially high in testis (16), it was of interest to determine whether it was primarily localized in the secretory or germinal elements. A study of testicular cells fractionated on a bovine serum albumin gradient demonstrated that the highest activities of both PCM and MAP were in spermatids with relatively little activity in Sertoli's cells (10). Additional studies showed that PCM activity in the young rat increased in parallel with germinal development and was low in animals with x-ray-induced or genetically determined germ cell deficiency (10).

In view of the observation that protein carboxyl-methylation was localized in germ cells, we postulated that the neutralization of negative charge brought about by this reaction could be

¹ PCM: *S*-adenosyl-L-methionine:protein carboxyl-*O*-methyltransferase, EC 2.1.1.24.

involved in the motility of spermatozoa. If this postulate is correct, then PCM activity should be found in the sperm tail of motile spermatozoa. The present study was conducted to investigate this possibility.

MATERIALS AND METHODS

Materials

S-adenosyl-L-[methyl-³H]methionine ([methyl-³H]SAM), 10–15 Ci/mmol, was obtained from New England Nuclear (Boston, Mass.). Pig-skin gelatin, bovine serum albumin, Triton X-100, and cetyl-pyridinium-chloride (CPC) were purchased from Sigma Chemical Co. (St. Louis, Mo.); sucrose (ultrapure grade) from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.); S-adenosyl-L-homocysteine (SAH) from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); and Coomassie Brilliant Blue R-250 from Bio-Rad Laboratories (Richmond, Calif.).

Testis

Testes were obtained from sexually mature rats (100- to 140-d-old Sprague-Dawley rats from Charles River, Wilmington, Mass.) and mature rabbits (New Zealand white from Marland Farms, Hewitt, N. J.). Before assay, a 10% homogenate was prepared in 0.3 M sucrose with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.).

Isolation of Spermatozoa

Rat spermatozoa were obtained from caput and cauda epididymis as follows: Caput epididymis was minced in a petri dish containing warm Krebs-Ringer bicarbonate (30°C). The petri dish was then placed for 15 min in a humidified air/CO₂ (95%/5%) incubator (Heinicke Instruments, Inc., Hollywood, Fla.) at 37°C. After this incubation, all the visible contaminant particles were taken out by filtration through a 50- μ m mesh nylon filter. To isolate caudal spermatozoa, the epididymis was dissected free from the testis and transected distal to the midportion. The vas was cannulated and perfused with buffer to extrude the spermatozoa out of the cut end of the epididymis.

Rabbit spermatozoa were obtained by ejaculation and centrifuged at low speed (600 g) to sediment the cellular debris.

In all cases, spermatozoa were collected and washed in warm Krebs-Ringer bicarbonate buffer (30°C). They were examined by phase-contrast microscopy for motility and then centrifuged at 800 g for 10 min. The pellet was resuspended in buffer and spun at 800 g for 10 min. This washing procedure was repeated.

Separation of Sperm Heads and Tails

Sperm heads and tails were separated by equilibrium density gradient centrifugation according to Calvin (2). Motile cauda spermatozoa were isolated from rats and washed in 0.02 M Na-phosphate buffer, pH 6.0 (buffer P), then passed through a 50- μ m mesh nylon filter. This and all other operations were then performed at 0°–4°C. The samples were sonicated for 2 min at 55 W with a 3-mm-diameter probe (sonifier cell disruptor, Branson Sonic Power Co., Plainview, N. Y.). The sperm suspension was counted, diluted, and a sample of $\sim 3 \times 10^6$ cells/ml was mixed into 1.80 M sucrose and layered over a step gradient composed of 13 ml of 2.20 M and 13 ml of 2.05 M sucrose solutions in buffer P. The gradients were centrifuged at 91,000 g for 60 min in nitrocellulose tubes. The tails sedimented to the interface between the 1.80 M and 2.05 M sucrose solutions, intact spermatozoa to the interface between the 2.05 and the 2.20 M sucrose, and the heads to the bottom of the tube. The tails were collected with a syringe, resuspended in buffer P, and sedimented at 90,000 g for 30 min. Finally, both tail and head pellets were resuspended in 0.3 M sucrose.

The effect of sonication and the purity of the fractions were monitored by dark-field, light, and transmission electron microscopy. All samples were either assayed the same day or frozen at –20°C (after determining that PCM activity and MAP capacity were not affected by freezing).

Electron Microscopy

Head or tail fractions were pelleted at room temperature at 1,000 g for 15 min and fixed for 14–20 h in 3% glutaraldehyde buffered at pH 7.2 with 0.2 M collidine. Specimens were rinsed with several changes of buffer and postfixed for 1–2 h in collidine buffered with 1% OsO₄. After alcohol dehydration, samples were embedded in Epon. Sections were stained with 3% aqueous uranyl acetate for 2 h and examined with a Philips 300 electron microscope.

Purification of PCM

PCM was purified from human erythrocytes as previously described (9, 15, 17) by use of successive ammonium sulfate precipitations, Sephadex G-100 chromatography, and hydroxylapatite chromatography. This fractionation scheme produced a 2,500-fold increase in specific activity. Because this enzyme preparation was devoid of MAP and because it was equally effective at methylating testicular and spermatozoal proteins, it was used as a reagent in the MAP assay.

Measurement of PCM Activity and MAP Capacity

PCM activity was assayed as previously described (5, 8, 10). The enzymatic activity was measured at 37°C in 50 mM sodium acetate buffer, pH 6.0, using 5 μ M [methyl-³H]SAM as a methyl donor, gelatin (10 mg/ml) as an exogenous MAP, and Triton X-100 (0.1% vol/vol). The capacity of sperm cell proteins to be carboxyl-methylated was measured using similar conditions, except that 1 μ g of purified PCM replaced the gelatin (1 mg PCM = 10,000 pmol [³H]methyl groups transferred per 10 min of incubation = 1,000 U of enzyme activity [3]). In both assays the results were expressed as picomoles of [³H]methyl groups transferred per milligram of protein per 10 min of incubation.

PME Activity

The PME activity was measured as previously described (9). Protein [³H]methyl ester substrates were enzymatically synthesized in the following mixture: 10 μ l of 0.25 M Na-acetate buffer, pH 5.2, 10 μ l of gelatin (50 mg/ml), 20 μ l of purified PCM and 10 μ l of [methyl-³H]SAM, 3.5 μ M (2.5 μ Ci/10 μ l), and Triton X-100 (0.1%). After a 10-min incubation at 37°C, the reaction was stopped by the addition of 10 μ l of 10 mM SAH. The reaction mixture then contained 12 pmol of gelatin [³H]methyl esters. Samples for PME assay (0–20 μ l) were then added, and incubation proceeded for 20 min more at 37°C. Boiled samples were run in a parallel set of tubes to determine the spontaneous rate of methyl ester hydrolysis. The results were expressed as percent of hydrolysis or in picomoles per milligram of protein per 10 min of incubation.

Protein Determination

Protein concentrations were measured by the method of Lowry et al. (24), using bovine serum albumin as a standard.

Electrophoretic Analysis of the MAP

Sperm cell proteins were methylated with [methyl-³H]SAM by purified PCM and electrophorized under acidic conditions in the presence of the detergent CPC in 10% acrylamide gels as previously described (7). The gels were stained with 0.25% Coomassie Brilliant Blue, destained, and sliced in 1-mm fractions. Each slice was incubated overnight with 10 ml of Liquifluor-toluene mixture containing 3% of Protosol (New England Nuclear, Boston, Mass.). The radioactivity was measured in a liquid scintillation spectrometer.

RESULTS

Effect of the Solubilization of Sperm Cell Membrane by Detergent

Because PCM has been associated with cell motility and because a large fraction of the PCM activity and MAP capacity in testis is in spermatids, we first determined whether this enzyme was present in spermatozoa. A nonionic detergent was used in the assays because it can dissolve sperm membranes without altering the machinery of movement (13). After treatment of intact rat and rabbit sperm with detergent (Triton X-100 at a final concentration of 0.1%), PCM activity and MAP capacity were predominant in the soluble fraction (Table I). The results obtained with detergent-solubilized sperm were the same as those after sonication.

PME Activity

Because PME is a newly identified component of the protein carboxyl-methylation system in mammals, this enzyme activity

TABLE I
Effect of Treatment by Triton X-100 on PCM Activity, MAP Capacity, and PME Activity in Rat and Rabbit Sperm

	Soluble fraction	Insoluble fraction
	pmol/ mg protein	pmol/ mg protein
PCM activity		
Rat caudal sperm	28.5 ± 8.3* (5)‡	8.8 ± 3.0 (5)
Rabbit ejaculated sperm	4.4 ± 0.9 (5)	2.1 ± 0.4 (5)
MAP capacity		
Rat caudal sperm	50.3 ± 16.9 (3)	19.5 ± 4.4 (3)
Rabbit ejaculated sperm	67.1 ± 10.6 (5)	33.3 ± 4.5 (5)
PME activity		
Rat caudal sperm	N.D.§	N.D.
Rabbit ejaculated sperm	1.0 (2)	10.0 (2)

Rat and rabbit sperm were treated by detergent (Triton X-100, 0.1%) and centrifuged at 10,000 g for 30 min to separate the soluble fraction from the insoluble. The pellet was resuspended in 0.5 ml of 0.3 M sucrose. PCM activity, MAP capacity, and PME activity were determined as described in Materials and Methods.

* Mean ± SE.

‡ Number of experiments.

§ N.D., not detectable.

was examined in spermatozoa (Fig. 1). The PME activity in rabbit spermatozoa was high (33 pmol/10 min per milligram of protein) relative to that reported for other tissues (9), but was undetectable in the rat sperm. Although this enzyme was present in both rat and rabbit testes, the activities were low (1 and 2 pmol per milligram of protein, respectively). The presence of an inhibitor of PME activity in rabbit testis was excluded by an experiment in which samples of testis and sperm were mixed. After treatment by detergent, PME activity was located predominantly in the nonsoluble fraction (Table I).

PCM Activity and MAP Capacity in Rat Testis and Epididymis

The PCM activity and MAP capacity in testis are compared in Table II with those of spermatozoa from different portions of the epididymis. Because PCM and MAP are predominantly in the detergent-soluble fraction, assays were performed both on solubilized spermatozoa (Triton X-100, 0.1%) and on high-speed-produced supernate obtained from these detergent-treated spermatozoa. The specific activity of PCM was much lower in rat spermatozoa than in the testis. Enzyme-specific activities in spermatozoa from different portions of the epididymis were in the same range with slightly higher values in those from caput. By contrast, the MAP capacity in the high-speed-produced supernate increased between testis and cauda epididymis in parallel with sperm maturation (Table II).

The PCM activity and MAP capacity (131.9 and 11.7 pmol per milligram of protein, respectively) in the high-speed-produced supernate of rabbit testis were similar to those of rat. In rabbit sperm as in rat, the MAP capacity was also higher (38.3 pmol per milligram of protein) and the PCM activity lower (6 pmol per milligram of protein) than in testis cytosol.

Analysis of the MAP by Polyacrylamide Gel Electrophoresis (PAGE)

Rat spermatozoa from caput and cauda epididymis were first treated by detergent (Triton X-100, 0.1%) and then reacted

with [methyl-³H]SAM in the presence of excess PCM; the methylated proteins were fractionated on CPC-PAGE (Fig. 2). The patterns of methylated proteins were similar, regardless of the origin of the rat sperm. In addition, the pattern of methylated proteins in rabbit spermatozoa was similar to that of rat.

Localization of the Enzymatic System

Having demonstrated PCM and MAP in spermatozoa, we next determined the distribution of these activities in fractionated heads and tails. The head and tail fractions were highly purified and the cellular components were well preserved, as judged by light and transmission electron microscopy (Figs. 3 and 4). In particular, the acrosomes were present and generally intact, even though the plasma membrane was absent. Structures of the tail, including dense fibers, mitochondria, flagellar tubules, and fibrous sheath, appeared intact.

>95% of the recovered PCM activity was in the tail fractions (12.6 ± 1.7 pmol per milligram of protein; *n* = 5). Little or no activity was recovered in the head fraction. For each experiment a sample of nonfractionated sperm from the same animal was assayed, and the PCM specific activity was found to be similar to that of the tail fragments (16.8 ± 5 pmol per milligram of protein; *n* = 4). By contrast, the MAP capacity was higher in the head (61.5 ± 9.8 pmol per milligram of protein; *n* = 3) than in the tail fraction (21.1 ± 4.3, *n* = 3).

DISCUSSION

Rapid and transient modification of the protein charge brought about by the addition of methyl groups to the free carboxyl

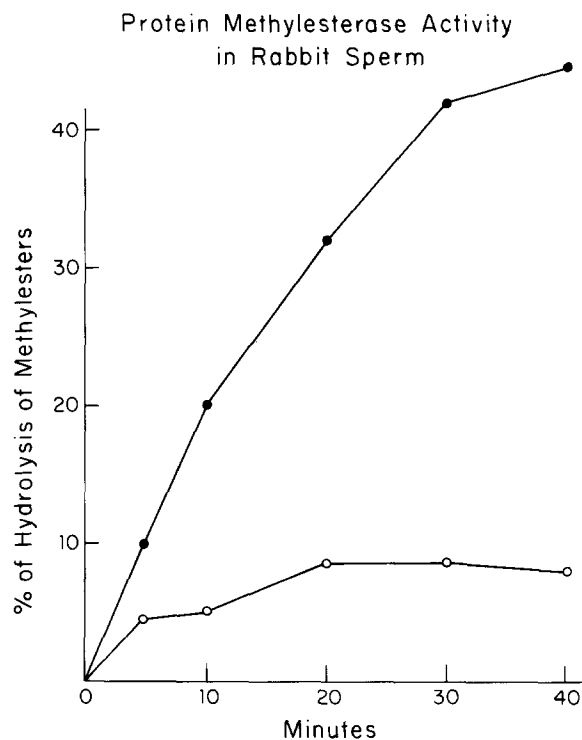


FIGURE 1 PME activity in rabbit sperm. Gelatin was methylated with purified PCM and [methyl-³H]SAM. The reaction was stopped by adding SAH (10 mM). Fresh (●) or boiled (○) rabbit sperm were then added and the mixture was further incubated at 37°C. The reaction was stopped at various times and the amount of [³H]-methyl esters determined as described in Materials and Methods. The results are expressed as percent of hydrolysis with time.

TABLE II
PCM Activity and MAP Capacity in Rat Testis and Spermatozoa

	PCM Activity		MAP Capacity	
	Homogenate	High-speed-produced supernate	Homogenate	High-speed-produced supernate
	<i>pmol/mg protein</i>		<i>pmol/mg protein</i>	
Testis	87.0 ± 9.8* (8)‡	137.3 ± 13.4 (8)	7.8 ± 0.6 (8)	5.7 ± 0.6 (6)
Caput epididymal spermatozoa	26.8 ± 3.0 (8)	86.5 ± 14.6 (9)	15.6 ± 3.1 (8)	35.4 ± 7.8 (9)
Cauda epididymal spermatozoa	19.4 ± 2.8 (8)	58.6 ± 8.4 (8)	16.7 ± 2.6 (8)	45.4 ± 10.2 (8)

Rat spermatozoa were assayed for PCM activity and MAP capacity, either as a homogenate containing both detergent-soluble and -insoluble fractions or after an ultracentrifugation (105,000 *g*) of the detergent-treated spermatozoa (*high-speed-produced supernate*).

* Mean ± SE.

‡ Number of experiments.

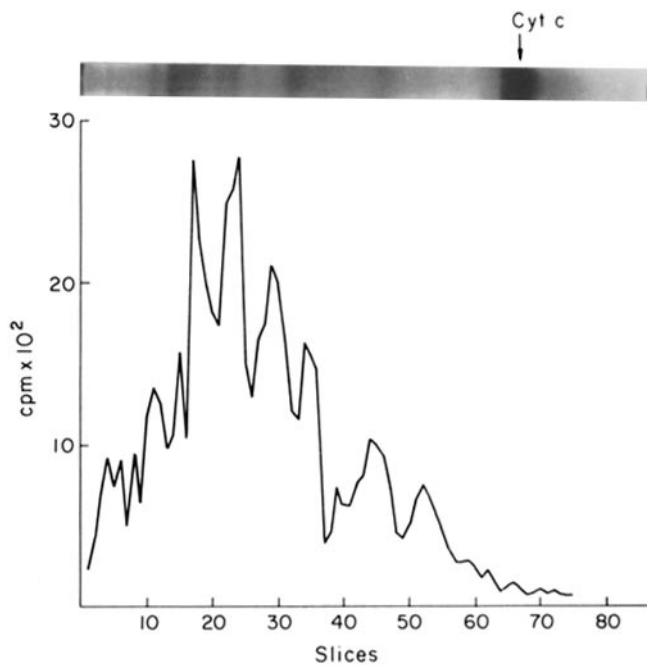


FIGURE 2 PAGE analysis of rat sperm MAP. Rat and rabbit sperm proteins were methylated with [methyl-³H]SAM by PCM and electrophorized at pH 2.2 in the presence of the detergent CPC in 10% acrylamide gels. The gels were stained with 0.25% Coomassie Brilliant Blue and then sliced for measurement of radioactivity by liquid scintillation counting. Cytochrome *c* (*Cyt c*) was added to the samples as a marker (7).

groups by PCM has been associated with the function of exocrine and endocrine glands (4–8, 34). Interestingly, in anterior pituitary where the enzyme was first discovered (1), the function of this enzyme is still uncertain. Although PCM could play a role in the storage of secretory granules, as has been proposed in platelets (28) and adrenal medulla (5, 7), it could also modulate hormone secretion or biological activity directly, as all pituitary polypeptidic hormones are excellent substrates *in vitro* (3). In this respect, the degree of methylation of prolactin is inversely correlated with its biological activity (22). It was initially assumed that the high PCM activity in testis would be associated with its endocrine or exocrine secretion. Recent studies demonstrating that both enzyme and substrates are preferentially localized in germ cells raised the possibility that protein carboxyl-methylation was important for the function of spermatozoa. It was suggested that PCM was involved in sperm cell motion or in the acrosomal fusion to the plasma membrane (10).

In the present study, these latter experiments were extended by demonstrating the components of the carboxyl-methylation system in mammalian spermatozoa. In the rat, maturation of spermatozoa in the epididymis seems correlated with an increase in MAP capacity. The ratio of MAP/PCM increases 10-fold from testis to cauda epididymal spermatozoa. The presence of PCM and its substrates in the soluble extract after treatment by detergent is in agreement with studies in other tissues (5, 11). By contrast, in rabbit the MAP/PCM ratio increases 60-fold between testis and ejaculated spermatozoa. Rabbit spermatozoa were also distinguished from those of rat by the presence of PME activity. The significance of these species differences must await further study.

The localization of the PCM activity in sperm tail suggests its involvement in motility. This hypothesis was prompted by the fact that PCM is involved in chemotaxis of bacteria and leukocytes (19, 20, 29, 30–33). This postulate was subsequently strengthened by the observations that patients with immotile spermatozoa have very low PCM activity compared with normal controls (12). Although the exact function of PCM in the sperm tail remains to be elucidated, the rapidity with which the reaction occurs and its reversibility suggest that it could act as a “switch” for reversal of the beat. Alternatively, protein carboxyl-methylation could be involved in sperm chemotaxis. Although chemotaxis has not been demonstrated in mammalian spermatozoa, it has been clearly demonstrated in spermatozoa of some marine invertebrates (25, 26).

Certainly protein carboxyl-methylation plays a part in the control of behavior at the cellular level in many systems (11, 33). One possible approach to understanding these mechanisms is the characterization of the specific substrates. It has been shown here that several proteins are substrates in spermatozoa. The analysis and exact location of these proteins should help to clarify the role of protein carboxyl-methylation in signal transduction. It is of note that several substrates for PCM were observed in sperm heads. Although no enzyme activity was recovered in this fraction, its presence in sperm heads cannot be excluded, because the plasma membrane was lost during purification of heads and tails.

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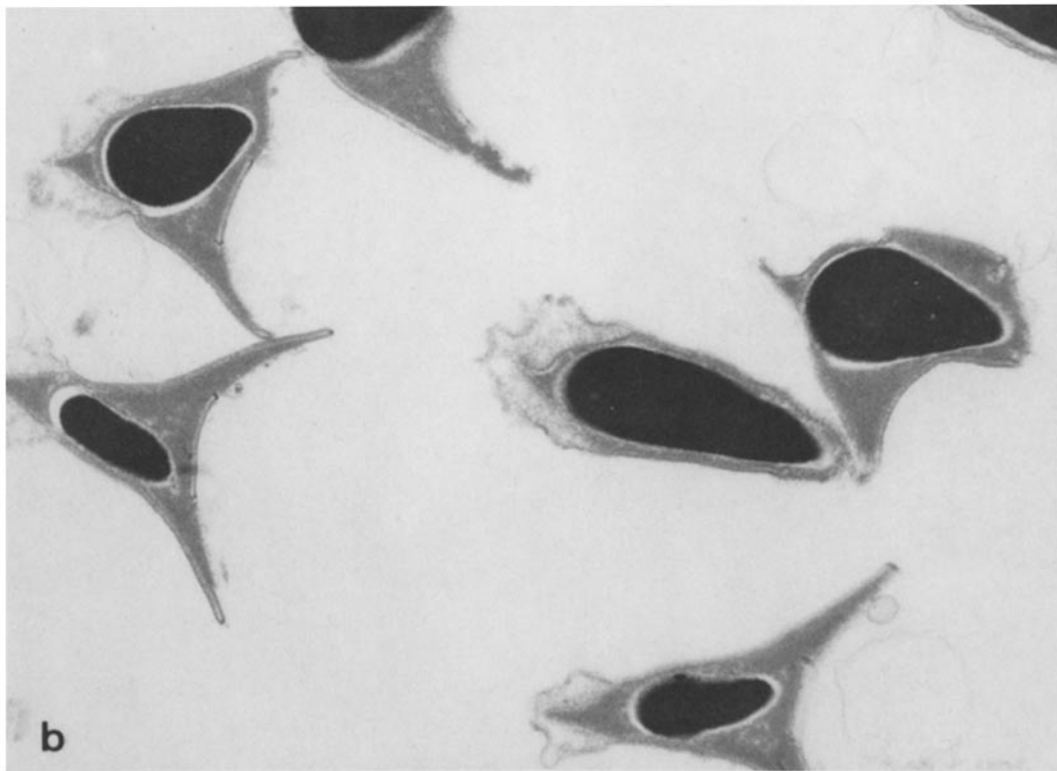
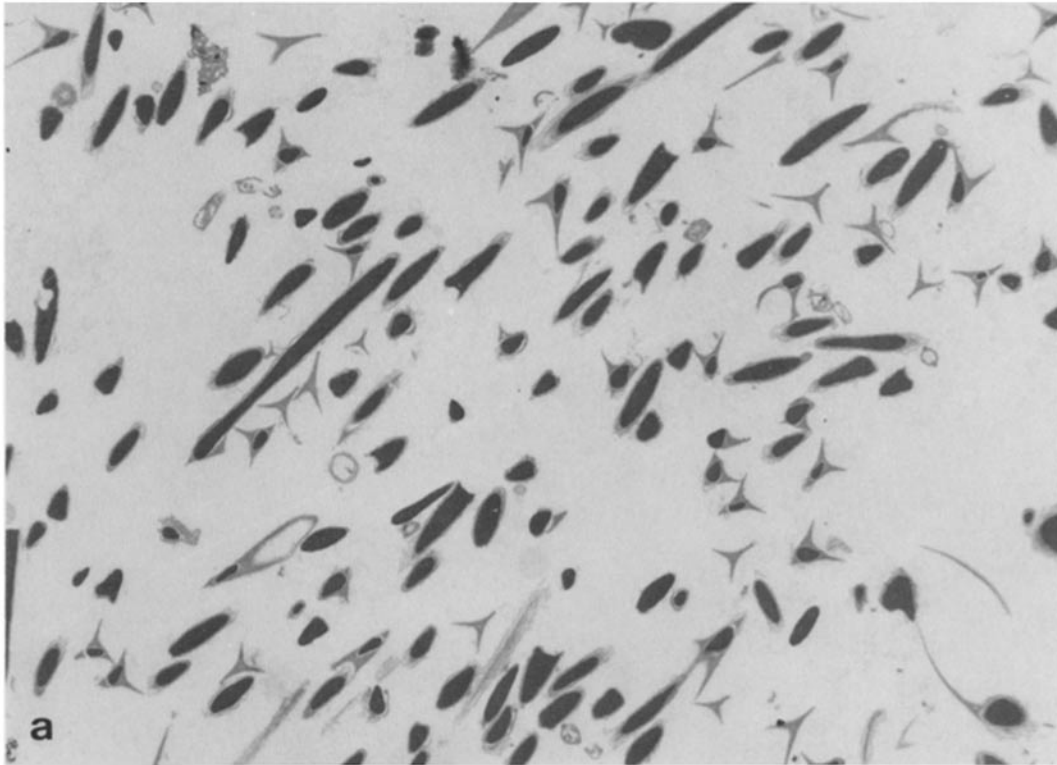


FIGURE 3 (a) The head fraction contains only very slight contamination with tail elements and no observable contamination with cell debris. $\times 4,000$. (b) Acrosomes appear intact by morphological criteria. The plasma membrane is missing. $\times 30,000$.

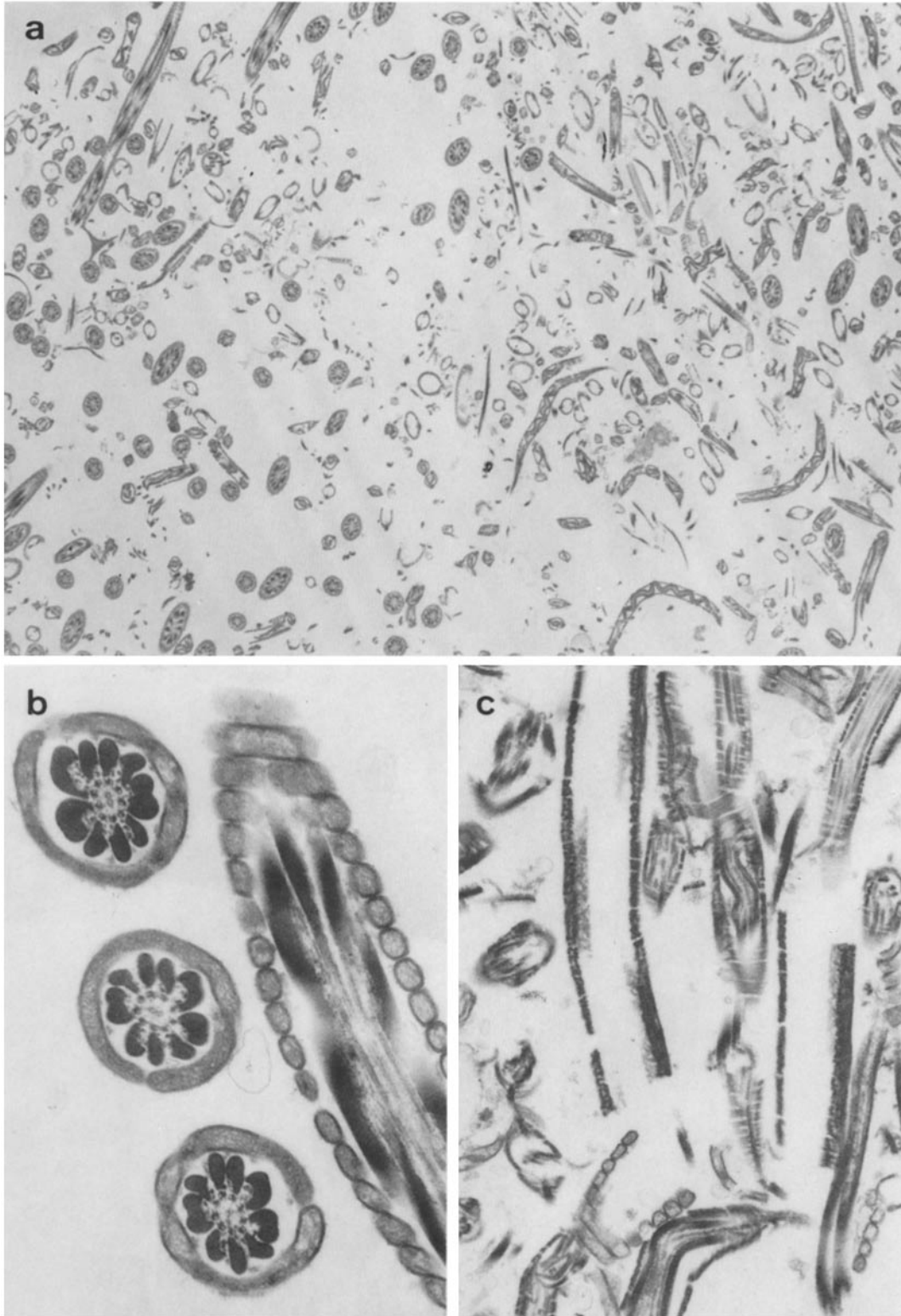


FIGURE 4 (a) The tail fraction is highly pure. $\times 4,000$. (b) The midpiece appears intact except for the missing plasma membrane. $\times 25,000$. (c) The fibrous sheath maintains the segmented appearance although the principal piece is fragmented by the sonication. $\times 18,000$.

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