ELECTRON MICROSCOPE STUDIES OF

SPERMATOZOA OF *RHYNCHOSCIARA* SP.

I. Disruption of Microtubules by Various Treatments

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ABSTRACT

The flagellar complex of the unusual motile spermatozoon of the fungus gnat, *Rhynchosciara* sp , does not conform to the usual "9 + 2" filament pattern but rather consists of over 350 pairs of filaments (doublet microtubules) distributed in a spiral array. Experiments were designed to disrupt and extract flagellar microtubular components from spermatozoa of the fungus gnat. Pepsin, chymotrypsin, potassium iodide, urea, and heat were used to extract specific portions of microtubule walls Such experiments provide information on the composition of the wall and the existence of wall sites selectively sensitive to various treatments Results obtained include: (*a*) doublet microtubules are comprised at least in part of protein, and all subunits are probably not identical; (*b*) a portion of the B subfiber is apparently more sensitive to disruption than other portions of the doublet microtubule; and (*c*) the ac cessory singlet microtubules may be chemically different from the doublet microtubules

INTRODUCTION

Most motile cilia and sperm flagella have a common "9+2" pattern of microtubules, where nine peripheral doublet microtubules encircle a central or axial pair of singlet microtubules and comprise the "axoneme " Microtubules in spermatozoa of *Rhynchosciara* sp., a fungus gnat, are unique in their arrangement in that the flagellum contains over 350 doublet microtubules (15, 30) distributed in a spiral array similar to that of the Sciaridae studied by Phillips (18, 19, 20, 21) and Makielski (12). As far as could be determined no other spermatozoon flagellum has been investigated which contains such a large number of doublet microtubules Since most motile cilia and flagella possess the 9 + 2 pattern, it is likely that this configuration is a favorable one for motility Therefore, cilia and

flagella showing deviations from the 9+2 pattern are interesting not only as cytological curiosities, but, more important, for evaluating deviations from the 9+2 pattern which provide for motility.

It has been shown by Behnke and Forer (2), Burton (5, 6), and Shay (30) that microtubules differ in sensitivity to various treatments. Behnke and Forer (2) have shown differences in the sensitivities of portions of microtubular elements of crane fly and rat spermatozoa in terms of their responses to temperature, colchicine, and pepsin They concluded that there are at least four classes of microtubules: (*a*) the A subfiber of the doublet microtubule, (*b*) the B subfiber of the doublet microtubule, (*c*) the central and accessory singlet microtubules of cilia and flagella, and (*d*) cyto-

plasmic and mitotic microtubules There are numerous studies which suggest that these four classes of microtubules may appropriately be further subdivided For example, Jacobs et al. (9) have reported that the two central singlet flagellar microtubules of *Chlamydomonas* are not identical In a recent review article, Behnke (3) has stated that there are insufficient morphological, biochemical, and functional data available to allow a definitive classification of microtubules, except into two general groups· (*a*) a stable variety, and (*b*) a labile variety.

The present work provides further information on microtubules found in the unique axial filament complex of *Rhynchosciara* sp Experiments reported here demonstrate differences in microtubular response to various agents such as pepsin, chymotrypsin, potassium iodide, urea, and to heat Such studies support the thesis that all types of microtubules and portions of individual microtubules are not chemically identical Methods of chemical extraction or "dissection" may provide information on the composition and packing of the subunits of microtubular elements and may also provide information on the existence of wall sites selectively sensitive to various treatments

MATERIALS AND METHODS

Rhynchosciara sp. belong to the oldest suborder, Nematocera, of the dipteran order of insects, and Mattingly and Parker have described the general characteristics of their life cycle (13, 14). Dr. C. Pavan, of the University of Texas at Austin, has had success in developing culture methods for fungus gnats, and those used in the present study were kindly provided by him. The eggs laid by a single female hatch together and the larvae develop in very close synchrony which made it possible to study the differentiation of the spermatid into the mature spermatozoon.

Testes were isolated in cold Shen's saline (16) and fixed for electronmicroscopy in 3–6% glutaraldehyde (29) in 0.05 M cacodylate buffer at a final pH of 7.2–7.4. The glutaraldehyde used was purified over charcoal and $BaCO_3$ and was then assayed for its aldehyde content by a modification of the method of Siggia and Maxcy (32). Material was fixed in glutaraldehyde for 1 hr and then washed for 1 hr in four 15-min changes of cold cacodylate buffer. Material was then placed in 1% osmium tetroxide buffered with cacodylate for 1 hr at room temperature, and then dehydrated in a graded alcohol series to propylene oxide Final embedding was done in Epon 813 (11). Silver or gray sections (500–1000 A thick) were cut on a Porter Blum MT-1 microtome with a diamond knife and

were picked up on 150-mesh grids coated with parlodion and a film of amorphous carbon Sections were then stained with lead citrate for 5 min (25), washed, and stained with a saturated (aqueous) uranyl acetate solution for 1 hr. An RCA EMU-3H electron microscope, equipped with a 35–40 μ aperture and operated at 50 kv, was used to examine the material. Negatives were made on Kodak projector slide plates at magnifications up to about 24,000 and were subsequently enlarged using Schneider Companon lenses (Schneider Instrument Co , Cincinnati, Ohio). For purposes of critical measurements, the microscope was calibrated with a carbon grating replica.

All experiments were repeated at least twice and usually three times, with controls run simultaneously with the experimental groups. It is important to mention that the methods described for *Rhynchosciara* are repeatable with very little variation, and all micrographs used to illustrate the results of various treatments are representative.

Pepsin Treatment

Sections of mature spermatozoa were exposed to pepsin-HCl in the manner described by Behnke and Forer (2), who modified the method originally described by Bernhard et al. (4). Grids covered with sections were floated facedown for 10 min on a drop of 10% H_2O_2, rinsed in distilled water, and dried. They were then floated on drops of 0 5% pepsin (Difco Laboratories (Detroit, Mich.), 1:10,000 or Sigma Chemical Co (St Louis, Mo), 1:10,000) in 0 1 *N* HCl on dental wax inside a moist chamber at 37°C for 5–60 min. Control sections were treated with H_2O_2 simultaneously with experimental sections cut from the same block, and then floated on 0.1 *N* HCl without pepsin alongside their experimental counterparts.

Chymotrypsin Treatment

Mature spermatozoa were placed for 30–60 min in 0.1% alpha-chymotrypsin (Sigma Chemical Co., Type II) in Tris-HCl buffer (pH 8.05) at room temperature. As controls, other spermatozoa were placed in Tris-HCl buffer without chymotrypsin.

Potassium Iodide Treatment

Mature spermatozoa were placed for 1–24 hr in 0 6 M KI in 0.03 M Tris-HCl buffer (pH 7 3) at room temperature. As controls, spermatozoa were placed in Tris-HCl without KI.

Urea Treatment

Mature spermatozoa were placed in 3 M or 8 M urea (Mann Research Labs. Inc. (New York) ultrapure) in Tris-HCl buffer (pH 7.0) for 15–180 min at room
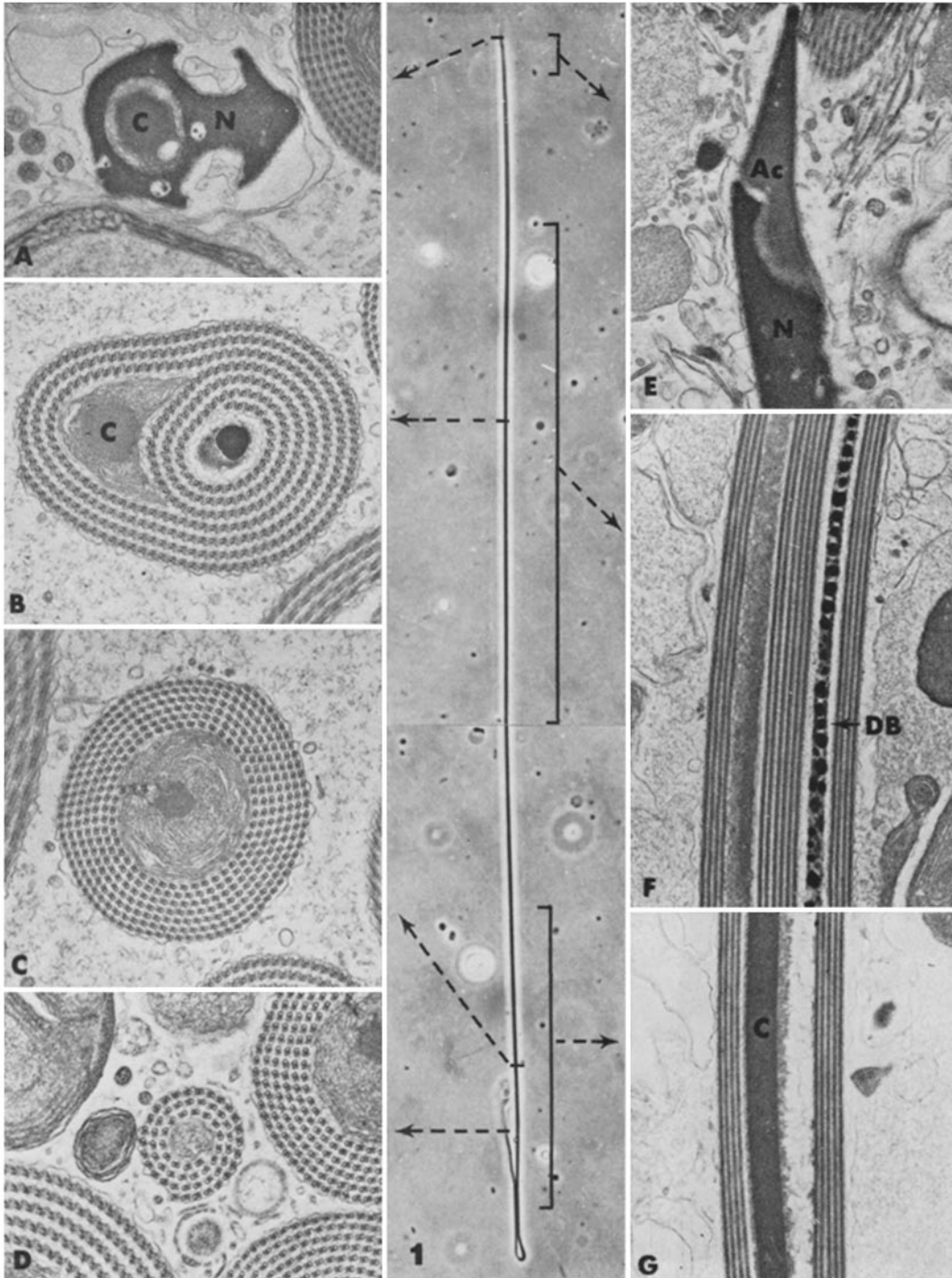
Figure 1 Phase contrast photomicrograph of a mature spermatozoon *Rhynchosciara* sp. A, Cross-section through the nucleus (N). Note the mitochondrial crystalloidal element (C). × 31,000. B, Cross-section revealing the mitochondrial crystalloidal element (C), the dense bodies, and the spiral array of doublet and singlet microtubules. × 31,000 C, Cross-section revealing the mitochondrial crystalloidal element and the doublet microtubules. × 31,000. D, Cross-section near the tip of the flagellum revealing only a few doublet microtubules. × 31,000. E, Longitudinal-section through the acrosome (Ac) and part of the nucleus (N). × 28,000. F, Longitudinal-section revealing the mitochondrial elements, the dense bodies (DB), and microtubules. × 13,420. G, Longitudinal-section revealing the mitochondrial crystalloidal element (C) and the microtubules. × 15,750.

temperature. Controls were placed in buffer without urea.

Heat Treatment

Mature spermatozoa were placed in Shen's saline in a water bath (50°C) for 15–180 min

In all experiments, except for pepsin treatment, the testes were dissected and placed in a specific medium which was intended to disrupt microtubular components. In most experiments the supernatant fluid was discarded and the remaining pellet was prepared for electron microscopy. By this technique, I was able to determine what portions of the microtubules were disrupted.

OBSERVATIONS

The mature spermatozoon of *Rhynchosciara* sp (Fig 1) consists of an acrosome (Fig 1E), a nucleus (Figs 1A and 1E), and a highly complex tail (Figs 1B, 1C, 1D, 1F, and 1G). The tail or flagellar complex in *Rhynchosciara* consists of over 350 doublet microtubules arranged in a spiral array (Figs 1B, 1C, and 1D). In addition, a portion of the flagellum contains a singlet microtubule peripheral to each doublet microtubule (Fig. 1B) The singlet microtubules, also called accessory singlet microtubules, occur only from the base of the nucleus to about half way down the flagellum The dimensions of the doublet microtubules (330–375 A × 250 A) and singlet microtubules (250–300 A) do not significantly differ from those observed in spermatozoa of other insects In a portion of the flagellum there is also a structure composed of dense bodies (Figs. 1B and 1F) The function of these dense bodies is unknown, but also extending almost the entire length of the flagellum is a mitochondrial derivative (Figs 1B, 1C, 1F, and 1G) A portion of the mitochondrial derivative contains a crystalloidal element (Figs 1A, 1B, 1F, and 1G) which extends from almost the end of the tail into the nucleus region (Fig. 1A) but remains separated from the nucleoplasm by mitochondrial and nuclear membranes

Pepsin Treatment

When sections of mature spermatozoa are exposed to 0 5% pepsin-HCl, the doublet microtubules, the mitochondrial crystalloid, and the dense bodies are digested (Fig 2A) Sections from the same block treated identically except for the elimination of pepsin show no evidence of diges
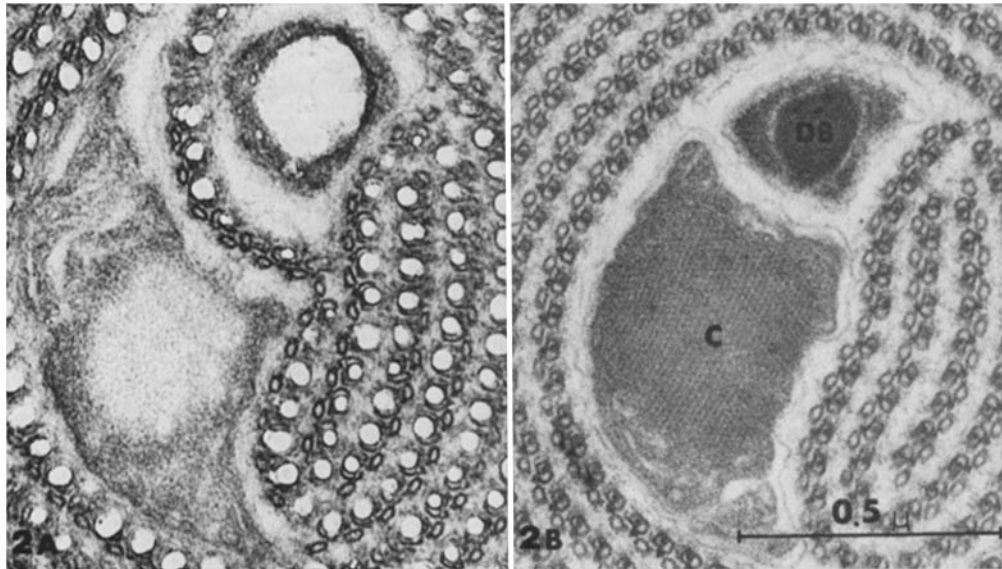

FIGURE 2A Cross-section through the flagellum of a mature spermatozoon after 1 hr of exposure to 0.5% pepsin-HCl. Note that the mitochondrial crystalloid (C), the dense bodies (DB), and the doublet microtubules are missing, although the singlet microtubules are still present × 71,000

FIGURE 2B Control section from the same block treated identically to Fig 2A except for the deletion of pepsin. × 71,000.

tion (Fig. 2B). The accessory singlet microtubules in the pepsin-treated sections are resistant to such treatment and remain intact even after 1 hr of incubation (Fig. 2A). The doublet microtubules were not digested in an all-or-none fashion, but in a specific sequence relative to the length of exposure to pepsin. Fig. 3A shows an untreated doublet microtubule The tubule bearing the arms is designated the A subfiber and the other the B subfiber In *Rhynchosciara* the A subfiber can also be identified because it contains a dense structure on the side adjacent to the B subfiber. When sections were incubated in pepsin-HCl for 5 min (Figs. 3B, 3C, and 3D), the dense part of the A subfiber becomes electron-transparent (Fig 3B), and this is usually followed by the appearance of a "hole" or transparent region in this area (Fig. 3C) Sometimes there appears to be an association between one of the arms on the A subfiber and this dense area (Fig. 3D). After 15 min of digestion

a larger portion of the A subfiber is missing (Figs 3E and 3F), and after 30 min the entire A subfiber and sometimes part of the B subfiber disappear (Figs. 3G and 3H). Finally, after 1 hr of digestion, entire doublet microtubules are missing (Fig. 3I).

Chymotrypsin Treatment

When mature spermatozoa are incubated in 0 1% chymotrypsin, the first noticeable effect is a disorientation of the spiral array of microtubules (Fig 4). As incubation time increases (30 min), the arms on the A subfiber disappear (Fig. 7A) and a portion of the B subfiber is disrupted (Figs. 5, 7B, and 7C). Even though the arms are no longer present on the A subfiber, one can be sure that it is the B subfiber that is disrupted because the A subfiber retains a 50–70 A electron-opaque particle in its core on the side adjacent to the B
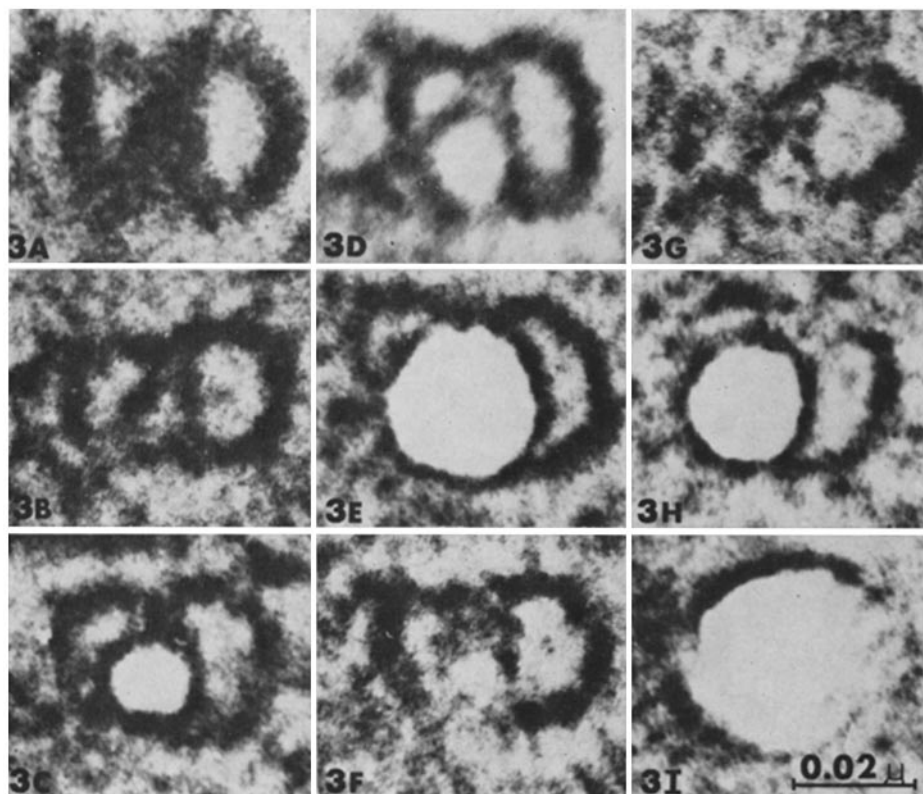

Figure 3 Composite revealing that the doublet microtubules are not digested in an all-or-none fashion. × 777,000. Fig. 3A, control, 30-min exposure to HCl without pepsin; Figs. 3B, 3C, and 3D, 5-min exposure to pepsin-HCl; Figs. 3E and 3F, 15-min exposure to pepsin-HCl; Figs. 3G and 3H, 30-min exposure to pepsin-HCl; Fig. 3I, 60-min exposure to pepsin-HCl.

subfiber (Figs. 7A, 7B, 7C, and 7D), and the A subfiber is comprised of a complete tubule whereas the B subfiber is comprised of a C-shaped profile adherent to the A subfiber (Fig. 7B) After 1 hr of incubation in chymotrypsin, only the A subfiber usually remains intact (Figs 6 and 7D)

Potassium Iodide Treatment

When mature spermatozoa are incubated in 0.6 M KI for up to 9 hr, a specific portion of the B subfiber is disrupted (Figs 8A and 8B) The spiral orientation is lost but some orientation of the whole complex is retained (Fig 8A). In the proteinase experiments previously described the arms on the A subfiber were usually among the first structures to be disrupted. In the KI experiments, however, the arms remain intact for up to 9 hr of incubation (Fig 8B, arrow) When spermatozoa are incubated in 0 6 M KI for 24 hr, the doublet microtubules lose their arms and are disrupted into various configurations as in Fig 9A Fig. 9B shows spermatozoa incubated for 24 hr in Tris-HCl buffer but without KI.

Urea Treatment

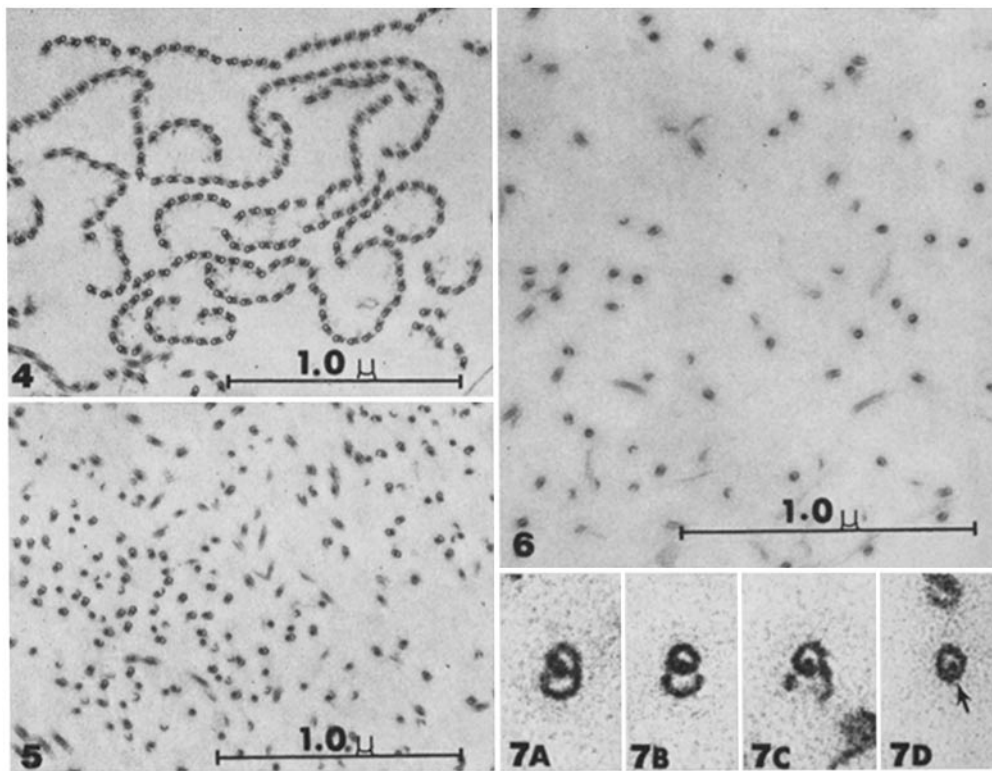When spermatozoa are incubated in 8 M urea for 15 min, all portions of the doublet and acces-

FIGURE 4   Cross-section of doublet microtubules after brief incubation in 0 1% chymotrypsin. Note that the spiral orientation is disrupted. × 32,000.

FIGURE 5   30-min exposure to chymotrypsin. Note that intact doublets and disrupted ones are present. The arms on the A subfiber are missing. × 32,000.

FIGURE 6   60-min exposure to chymotrypsin. Only the A subfiber is not disrupted. The extra dense portion of the A subfiber is still present (see arrow, Fig. 7D). × 40,000.

FIGURES 7A–7D   A possible sequence showing how the doublet microtubules are disrupted by chymotrypsin × 150,000. 7A, 15 min; 7B, 30 min; 7C, 30 min, 7D, 60 min.
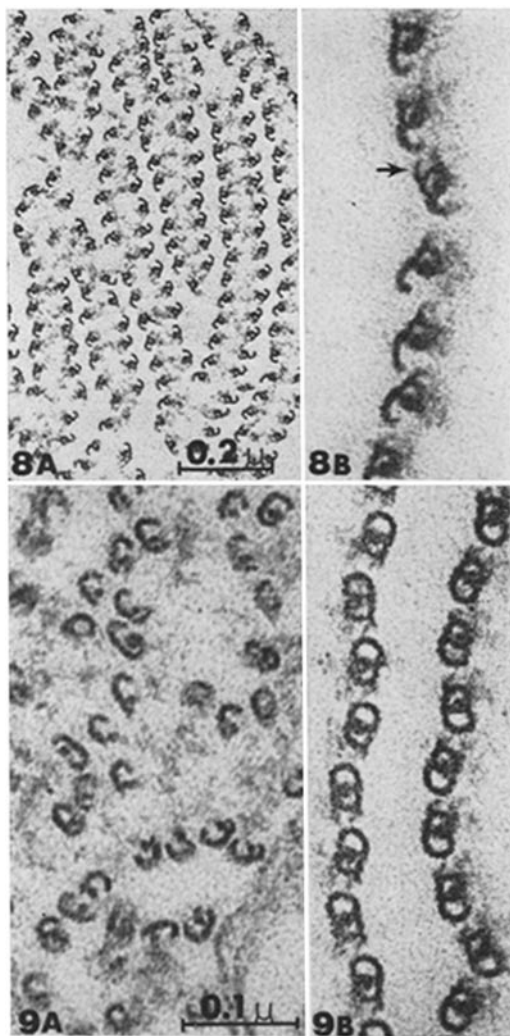
FIGURE 8A Transverse-section through sperm incubated in 0.6 M KI for 1 hr. Note that the spiral orientation of the flagellar microtubules is not entirely disrupted. × 57,000.

FIGURE 8B 0.6 M KI for 1 hr. Note the disruption of a portion of the B subfiber, although the arms on the A subfiber are still present (arrow). × 150,000

FIGURE 9A 24-hr incubation in 0 6 M KI. Note that the doublet microtubules lose their arms and are disrupted into various configurations. × 150,000

FIGURE 9B Spermatozoa treated like those in Fig. 9A, except for the deletion of KI. × 150,000.

sory singlet microtubules are disrupted (Fig 10), but the mitochondrial cystalloid (C) and the dense bodies (DB) are still present. Upon reducing the concentration of urea to 3 M, the effect after 90

min is a disruption of a portion of the B subfiber of the doublet microtubule (Fig. 11). Areas can also be observed where the accessory singlet microtubules and a portion of the A subfiber are disrupted (Fig 11, arrows) After 180 min in 3 M urea, complete doublet microtubules are not usually observed, and normally only a small portion of the A and B subfibers remain (Fig 12).

Heat Treatment

Mature spermatozoa incubated at 50°C for 15 min reveal no apparent morphological changes (Fig. 13) Examination of spermatozoa incubated for 30 or 60 min at 50°C reveals a loss of arms and sometimes a disruption in a portion of the B subfiber (Fig. 14) After 120 min of incubation, doublet microtubules are completely disrupted, but most of the accessory singlet microtubules are intact (Fig. 15) After 180 min of incubation, doublet microtubules and most of the singlet microtubules are disrupted (Fig. 16).

DISCUSSION

The digestion by chymotrypsin or pepsin of the elements studied indicates that they are wholly or in part protein When Epon-embedded sections of mature spermatozoa of *Rhynchosciara* are incubated in 0 5% pepsin-HCl, a dense portion of the A subfiber is the first to be digested, and according to Burton (6), the early digestion of this portion suggests that this structure is somehow different from the remainder of the doublet microtubule. The dense portion of the A subfiber has been described before by Gibbons (8) in the cilia of the gills of a mollusc, by André (1) and Cameron (7) in different spermatozoa of insects, and by Reese (24) in the olfactory cilia of the frog. According to Cameron (7) this dense structure arises in the lumen of the A subfiber independently of either arm, but in close association with the partition between the subfibers It is only later, when the spermatozoa near maturity and the dense portions of the A subfibers have increased in size, that there appears to be any association with one of the arms of the subfiber In *Rhynchosciara* the dense thickening is apparently associated with the partition between the two subfibers, sometimes appearing as a small triangle and usually seen on the inner (centripetal) side of the doublet microtubule (Fig 3A). After brief exposure to pepsin digestion this dense triangular structure becomes somewhat electron-transparent, and apparent
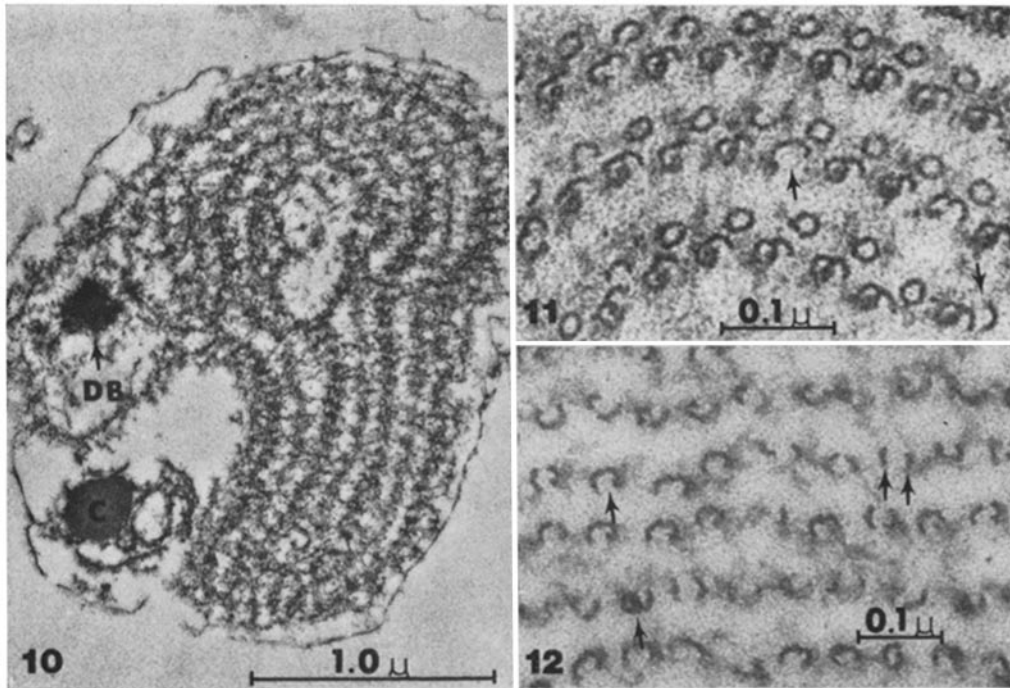
FIGURE 10   Transverse-section through the sperm incubated in 8 M urea for 15 min. Note disruption of the flagellar microtubules although the mitochondrial crystalloidal element (C) and the dense bodies (DB) appear relatively unchanged. × 32,000.

FIGURE 11   Flagellar microtubules after 90 min in 3 M urea. Note that a portion of the B subfiber is disrupted Areas can be observed where the A subfiber and singlet microtubules are also disrupted (arrows). × 142,000.

FIGURE 12   180 min in 3 M urea. Note that usually only a small portion of the A and B subfibers remain. (arrows). × 112,000.

connections between this material and one of the arms are sometimes observed (Fig 3D). However, the significance of this material and its apparent association with one of the arms is unknown

Incubation of spermatozoa in pepsin for 30–60 min shows that the B subfiber is more resistant to digestion than the A subfiber, and according to Behnke and Forer (2) and Burton (6) this may mean that the proteins of the two subfibers are not identical Also, the accessory singlet microtubules are not affected by pepsin, even after periods of incubation that digest entire doublet microtubules The simplest conclusion to be drawn from this is that the chemical composition of the doublet microtubules is probably different from that of the accessory singlet microtubules and that the doublets are more sensitive to the

action of pepsin Pepsin-HCl also digested the crystalloidal portion of the mitochondrial derivative and a portion of the dense bodies, which suggests that these structures are comprised of protein

Early effects of digestion with chymotrypsin were disorientation of the spiral array, loss of the arm material of the doublet microtubules, and disruption of a specific portion of the B subfiber This is the same portion of the B subfiber that Behnke and Forer (2) indicated was heat labile in the 9+2 axonemes of the crane fly. After longer periods of incubation in chymotrypsin, only the A subfiber remains intact which indicates that the A and B subfibers are probably not identical Almost the same results were observed when trypsin was used to disrupt microtubules, except that occasionally the A subfiber was also disrupted
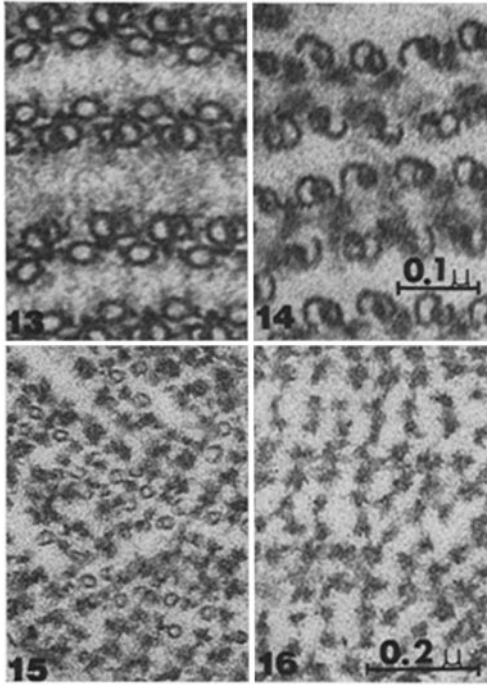
Potassium iodide has been used to extract one

FIGURE 13 Mature spermatozoa incubated at 50° C for 15 min. There do not appear to be any morphological changes. × 110,000.

FIGURE 14 50° C for 30 min. Note the loss of arms and occasional disruption of a portion of the B subfiber. × 110,000.

FIGURE 15 50° C for 120 min. Note disruption of the doublet microtubules although the accessory singlet microtubules appear relatively unchanged. × 65,000.

FIGURE 16 50° C for 180 min. Note that all the flagellar singlet and doublet microtubules are disrupted. × 65,000.

of the contractile proteins, actin, from muscle preparations (23, 38) When spermatozoa of *Rhynchosciara* are incubated in 0.6 M KI, a portion of the B subfiber is disrupted, although the arms and the remainder of the doublet microtubule remain intact for up to 9 hr of incubation Again, this is the same portion of the B subfiber that Behnke and Forer (2) showed to be heat labile. With the compelling evidence that microtubular protein is different from muscle actin (33, 34, 35, 36), one can only conclude from the KI experiments that the same reagents that apparently remove actin from muscle also remove a specific portion of the B subfiber from doublet microtubules.

The mechanism whereby urea denatures protein

is not well understood, but it is currently thought that urea interferes with normal hydrogen bonding Rash et al (22) have shown that urea can selectively extract Z band material from the heart muscle of the chick and have suggested that there may be localization of the proteins actin and tropomyosin in this region Szent-Gyorgyi and Joseph (37) indicated that urea affects the shape and size of protein molecules and that high urea-concentrations can depolymerize actin. Because urea and KI have been shown to extract the same protein, it is not surprising that the early effects of these chemicals on *Rhynchosciara* flagella are manifest on a specific portion of the B subfiber of the doublet element. Fig. 10 reveals this labile portion of the B subfiber relative to the accessory singlet microtubules. Again, one cannot conclude from the KI and urea experiments that flagellar microtubules contain actin, as the specificity for actin of these chemicals is questionable

Roth (27) found that urea inhibited reformation of microtubules during rewarming after cold-degradation, but did not affect microtubules formed before urea treatment. However, Shigenaka et al. (31) found that dilute concentrations of urea disrupt the microtubules in heliozoan axopodia and that recovery is possible after removal of the urea. Burton (6) incubated spermatozoa of the parasitic flatworm, *Haematoloechus medioplexus*, in urea and observed dissociation of doublet and singlet microtubules as well as an early alteration of the prominent helical wall pattern in the singlet elements. In *Rhynchosciara*, some orientation of the whole flagellar complex was retained even after incubation in 8 M urea. However, when incubated in 3 M urea the doublet microtubules were digested in a specific sequence relative to the length of exposure to urea

Incubation of microtubules at 50°C results in disruption of cytoplasmic and mitotic microtubules as well as doublet and accessory singlet microtubules of flagella and cilia (2) In *Rhynchosciara*, the accessory singlet microtubules are much more resistant to heat disruption than the doublet microtubules Stephens (36) incubated spermatozoa of the sea urchin at 37–40°C and noted that after 2 min the entire B subfiber was dissolved but that the A subfiber remained intact. Utilizing this technique, he was able to isolate subfibers and show that the proteins of the A subfiber and B subfiber have different electrophoretic mobilities and amino acid compositions, and he suggested

that the A subfiber contains the protein A tubulin and the B subfiber, B tubulin. Witman (39) and Olmsted et al. (17) have recently demonstrated by disc electrophoresis that there are probably more than one type of protein in a subfiber, which suggests that the chemistry of these microtubules may be even more complicated than thought.

It is significant to mention that the microtubules of typical 9+2 flagellar axonemes and the microtubules located in the unique flagellar complex of *Rhynchosciara* have similar solubility properties Previous reports have indicated that the relative stabilities of different microtubules and their responses to particular treatments may be determined in part by the attachment of varying numbers of microtubular links to the microtubules, or to the relative position of different microtubules in the axoneme (10, 28) Another viewpoint is that the relative stabilities of different microtubules to particular treatments may be largely determined by the protein composition of the microtubular subunits (2, 26). The singlet and doublet microtubules in the flagellar complex or *Rhynchosciara* are in different relative positions and have different links than the microtubules in the 9+2 axonemes studied by other investigators However, the solubility properties of the microtubules in the flagellar complex of *Rhynchosciara* are, for the most part, similar to those of the microtubules in typical 9+2 axonemes. Therefore, extrinsic factors, such as links, are perhaps not so important in determining stability as is the protein composition of the subunits of the microtubules. For example, many treatments in the present study resulted in the disruption of one specific part of the B subfiber before other portions of the B subfiber. This may indicate that the subunits that comprise the B subfiber are not all identical as previously suggested by Witman (39) and Olmsted et al. (17) in the isolated A subfiber from the flagellum of *Chlamydomonas* However, one cannot exclude the possibility that the portion of the B subfiber that is more stable to experimental treatments is due in part to other fators such as the proximity and the possible links to the accessory singlet microtubules. However, if this is the case, one might wonder why the accessory singlet microtubules, which appear to have few links, are so stable in the pepsin and heat treatment experiments as compared to the doublet microtubules, whereas in the urea experiments the singlet microtubules were among the first structures to be disrupted.

Experiments in which microtubular elements have been disrupted or extracted have provided information on the labile nature of certain portions of microtubules and have added further evidence for the existence of morphological and chemical differences between various types of microtubules, as well as differences between the A and B subfibers of doublet microtubules. Eventually, it is hoped, such information may help provide an understanding of the mechanism(s) of flagellar motility and of microtubular function

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