PARTIAL PURIFICATION AND PHOSPHOTUNGSTATE
SOLUBILIZATION OF BASAL BODIES AND KINETODESMAL
FIBERS FROM TETRAHYMENA PYRIFORMIS

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ABSTRACT
Previously devised methods for the isolation of basal bodies from ciliate protozoans were
found to be inadequate for chemical analysis. We have modified and expanded these
procedures and developed a method which gives preparations containing mainly basal
bodies and kinetodesmal fibers. This procedure involved fixation of cells in 30% ETOH
followed by digitonin or Triton X-100 solubilization and homogenization with a Brink-
mann Polytron. This is followed by sucrose gradient centrifugation. Negative staining and
thin sectioning revealed these preparations to be substantially more pure than those of
previous workers. It was also found that neutralized phosphotungstate (PTA) solubilized
many of the components present in fixed Tetrahymena. Neutralized 1.0% PTA solubilized
axonemes, cortical, axonemal, and basal body microtubules as well as kinetodesmal fibers.
These results have been confirmed by both electron microscope observations and gel elec-
trophoresis of 100,000 $g$ supernatants of the PTA extracts. A solution of 0.1% PTA did
not affect the fibers but did solubilize basal bodies. Running 1.0% PTA extracts from our
basal body fractions on sodium dodecyl sulfate (SDS) polyacrylamide gels allowed us to
tentatively identify the peptides of basal bodies and kinetodesmal fibers. The latter struc-
tures appear to consist of a single 21,000 mol wt peptide. These results also suggest that
great caution should be taken in interpreting PTA images, especially of microtubules and
axonemes.

INTRODUCTION
Basal bodies are of interest because they ap-
ppear to have a semiautonomous form of reproduc-
tion and because they appear to be involved in
the generation of cilia and flagella (see review by
Fulton, 1971). Little is known, however, about
the chemistry of this interesting organelle. This
study was initiated to investigate the character-
istics of basal bodies isolated from Tetrahymena
pyriformis using techniques developed by Child
and Mazia (1956), Satir and Rosenbaum (1965),
Argetsinger (1965), Seaman (1960), and Hufnagel
(1969). None of these procedures produced basal
bodies of sufficient purity for chemical analysis
in our hands. We therefore have developed a
technique which yields better purification of
basal bodies than any method of which we are
aware. Using this technique we have analyzed
the protein of a basal body preparation and
have made tentative identification of several basal
body proteins. In the process of assaying the
composition of various isolated organelle fractions using phosphotungstate (PTA) staining we noticed interesting patterns of solubility of various components of basal bodies and cilia which are also described in this report.

MATERIALS AND METHODS

The procedure ultimately developed utilized some of the methodology of Child and Mazia (1956), Seaman (1960), and Argetsinger (1965). The flow diagram for this procedure is given below.

(a) Harvest late log phase cells by centrifugation for 3 min at 3,000 rpm on a GSA Sorvall head (Ivan Sorvall, Inc., Newtown, Ct.).
(b) Wash once with 0.015 M phosphate buffer (as above).
(c) Add 100 vol of cold (-10°C) 30-40% ethanol to washed pellet.
(d) Place in freezer at -20° to -10°C for 2-7 days.
(e) Thaw to a slush and spin down on GSA head at 2,500 g for 3 min.
(f) Wash once in 3 vol of 1% digitonin in 0.4 M KCl at 4,000 g for 5 min.
(g) Resuspend washed pellet in 2-3 vol digitonin-KCl solution and let stand 2-3 h.
(h) Spin down at 7,000 g in GSA head for 7 min and wash three times using 7,000 g spins.
(i) Resuspend washed pellet in 2-3 vol of 0.2 M sucrose and homogenate using a Polytron 10-ST (Brinkmann Instruments, Inc., Westbury, N. Y.) at the highest setting for 2 min. Keep cells in GSA tubes in ice water.
(j) Spin down in GSA head 7,000 g for 8 min. Carefully decant off supernatant and save.
(k) Repeat steps 9 and 10 on pellet and save supernatant. If after the second homogenization and centrifugation the pellet is difficult to separate from the supernatant, discard both.
(l) Pool supernatants and spin down at 50,000 g for 10 min (20,000 rpm SS-34 Sorvall head); this pellet (20 K pellet) can be stored at 1°C for 2-3 days without loss of basal bodies.
(m) Resuspend 20 K pellet in 4-6 vol of 0.2 M sucrose and place over sucrose step gradients (1.4, 1.7, 1.8 M) in 5 or 30 ml cellulose nitrate tubes.
(n) Spin down at 100,000 g for 1.5 h or 150,000 g for 1 h.
(o) Carefully remove the bands which form at the interfaces, dilute with water 1:5, and spin down at 50,000 g for 15 min.
(p) Resuspend pellet from the top of the 1.8 M interface in 0.2 M sucrose and homogenize it with Polytron at no. 5 setting for 30 s.
(q) Place over same step gradient as above, centrifuge, and recover top of 1.8 M step. This is the basal body-rich fraction.

Cell Culture Techniques

_Tetrahymena pyriformis_, syngen 1 cultures were grown in the media of Gorovsky and Woodard (1969) in 4-liter Erlenmyer flasks at 29°C and harvested during late log phase (4-8 × 10^6 cells/ml).

Electrophoresis

The polyacrylamide gel system used was that of Laemmli (1970) and involved the use of 2-mm thick sodium dodecyl sulfate (SDS) slab gels. These gels were run at 15 mA for 6 h, stained in 50% TCA-0.1% Coomassie blue for 30 min, and destained in 10% acetic acid.

PTA Extraction Procedure

A 5 g (wet weight) 20 K pellet was resuspended in 5 ml of 0.2 M sucrose and equal amounts were pipetted into centrifuge tubes. These were then spun down at 50,000 g for 10 min and the resulting pellets were resuspended in equal amounts (20 vol) of 1 and 0.1% PTA (pH 6.8). The PTA was adjusted to the proper pH with KOH before and after resuspension of the 20 K pellets. The PTA extracts for all experiments were centrifuged at 100,000 g for 1 h and the resulting supernatants were then dialyzed against two changes of 100 vol of 0.1% SDS in water. The dialysis step was included because PTA interfered with band resolution on SDS gels. This may be due to the PTA anion competing with SDS for binding sites on the peptides. The extracts were then concentrated with dextran diluted with the buffer of Laemmli (1970), heated to 100°C for 2 min, and run on SDS gels.

Cilia and Axoneme Isolation

Cilia were isolated by the procedure of Gibbons (1965). Membranes were removed by the technique of Stephens (1970).

Electron Microscopy

Negative staining was carried out essentially according to the technique of Brenner and Horne (1959). A drop of the particle suspension in distilled water was mixed on a carbon-coated Formvar film with an equal drop of 2% neutral PTA or 2% ammonium molybdate. The excess fluid was drawn off with filter paper and the suspension air dried. To preserve structures soluble in the stain, the suspension was fixed briefly with a neutral, 0.5% solution of glutaraldehyde, and then washed repeatedly by centrifugation and resuspension in 0.2 M sucrose until the suspension would spread evenly on a carbon Formvar-coated grid. Usually the sucrose was diluted with distilled water before
RESULTS

Attempts to isolate and purify basal bodies from *Tetrahymena*, using previously published techniques (Child and Mazia, 1956; Seaman, 1960; Satir and Rosenbaum, 1965; Hufnagel, 1969; Argetsinger, 1965), resulted in preparations which appeared to be composed of homogeneous, small particles in the phase-contrast microscope. Examination of these preparations in the electron microscope, however, by either negative staining or thin-section techniques (see Fig. 1a), showed them to be heavily contaminated with mitochondria, cilia, microtubules (both cortical and axonemes), kinetodesmal fibers, pellicular fragments, fragments of the endoplasmic reticulum (ER), and DNA. Basal bodies proved to be a minor component in these fractions. One of the causes for this extreme contamination appeared to be the mild homogenization provided by the Potter Elvejhem type Teflon-glass homogenizer, which failed to release the organelles from the sticky web of pellicle and DNA formed when the cells were disrupted. Another source of variation and contamination in these procedures was fixation of the cells in 40% ethanol at -20°C. If the solution warms up slightly during the freezing or recovery of the cells, fixation is so complete that subsequent treatment with digitonin fails to solubilize the pellicle and membranous organelles, and the basal body fraction is heavily contaminated as described above. Such preparations contained large amounts of amorphous debris which appeared to be denatured proteins, perhaps from partially solubilized membranes.

The technique we have developed, which is described in Materials and Methods, has several important modifications. Cells are fixed in 30% ethanol at -20°C. Fixation is necessary. Basal body structure is maintained if the cells are disrupted without ethanol fixation, but separation of the basal bodies from DNA, membranes, and cell debris is very difficult. The length of fixation (from 1 to 7 days) does not seem to be critical. The suspension will freeze at -20°C but this is not deleterious. Solubility of the membranous organelles in digitonin after this mild fixation is rapid and complete.

Another important step in our isolation procedure is homogenization of the pellicular ghosts in a Polytron homogenizer at top speed (about 20,000 rpm) for 2 min. This treatment produces both high shear and ultrasonic vibrations which appear to be very effective in releasing basal bodies from contaminating particles. Before this treatment the basal bodies are tightly bound to both the kinetodesmal fibers and the collars, or epiplastic rings, by which they are attached to the pellicle. This complex sediments at relatively low centrifugal forces (7,000 g for 10 min) before homogenization. After homogenization, however, centrifugation at 50,000 g for 10 min is required to sediment the basal bodies which appear to be mostly free of attachment to other organelles. Basal bodies withstand this vigorous treatment; we have seen no indication of disruption by shear or ultrasonic forces. The cilary axoneme is sheared from the basal body slightly above the basal plate and the axonemal microtubules are disrupted by this treatment.

An example of a 50,000 g precipitate (20 K pellet) is shown in Fig. 1b. This fraction can be seen to contain basal bodies, granular membranous vesicles, unidentifiable debris, and fragments of pellicle. The thickened basal rings are embedded, together with the parasomal sac rings, in a thin, smooth fragment of epipalium which has a much lighter density than the granular or spongy fragments which we interpret as being derived from some other part of the pellicle (see Fig. 3d). Some basal bodies remain attached to these collars. There is also considerable material which we interpret as fragments of denatured DNA because of its similarity to the appearance of purified DNA in this type of negative stain as well as its sensitivity to DNase. An important source of error in this use of negative staining as an assay of basal body morphology and purification is the solubility of many proteinaceous structures in neutral PTA. If the basal body preparation is fixed in glutaraldehyde before negative staining, the cylindrical organization of the basal body microtubules is preserved. Fixation also preserves kinetodesmal fibers (see Fig. 2a). If the suspension is mixed with the PTA on the grid and then dried within a matter of a few seconds, as was the case in Fig. 1b, the triplet microtubules of the basal body are still present, although splayed.

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Purification and Phosphotungstate Solubilization
FIGURE 1a Negatively stained preparation of a basal body fraction (4,000 g ppt.) isolated according to the technique of Argetsinger (1965). Note mitochondria (M), microtubules (T), membrane vesicles (V). No fixation. X 25,000.

FIGURE 1b Negatively stained preparation of 20 K pellet described in Materials and Methods. Note basal bodies (BB), epiplasmic collars (C), membranous vesicles (MV), and DNA. Stained with neutral potassium PTA and dried quickly so that basal body microtubules are partially preserved. X 25,000.
Fig. 2b shows another example of a basal body at higher magnification after brief exposure to neutral PTA. The basal plate is clearly exposed and nine sets of microtubules are present, although some degradation has already occurred. As previously described by Wolfe (1970), the third or "C" tubule in each group can be seen to be only about one-third as long as the other microtubules, is located at the proximal end of the basal body, and is striated with a spacing of about 80 Å. If the basal bodies are exposed to the PTA for 30-60 s, as was the case in Fig. 2c, all of the microtubules are completely dissolved except for the short striated tubules, and the fine structure of the basal plate is revealed. Evidence that the microtubules are solubilized rather than simply washed off the grid will be discussed in a later section.

Microtubules and kinetodesmal fibers are less soluble in ammonium molybdate than PTA, but both structures show deterioration in this stain. An example of a partially dissolved kinetodesmal fiber is shown in Fig. 2d. Fine filaments or fibrils can be seen. Glycogen is observed on the grid with this method, but pellicular fragments are not.

Fixed and embedded material, of course, eliminates the problems of stain-soluble components, but the basal bodies are so rarely oriented perpendicular to the plane of sectioning that it is difficult to assess their numbers and state of preservation conveniently. For some unknown reason, the basal bodies in these preparations do not pack together tightly upon centrifugation, so that sections through pellets show widely spaced basal bodies with apparently nothing around them. The time required for fixation, embedding, and sectioning also militates against use of these procedures for routine assays of isolation procedures, and almost all fractions prepared in this study were examined by negative staining. However, sectioned material did reveal that the basal bodies after isolation contain apparently normal microtubules attached to the basal plate.

Although we believe that the 20 K pellet described above represents a more highly purified basal body preparation than those previously described, the level of contamination clearly makes it unsuitable for direct chemical analysis. An attempt at further purification using sucrose gradients was therefore initiated. Linear sucrose gradients between 1.4 and 1.8 M sucrose produced a diffuse mixture of the components of the 20 K pellet throughout the gradient. Discontinuous sucrose steps, however, of 1.4, 1.7, and 1.8 M yielded some separation of basal bodies and contaminants. The granular, membranous vesicles accumulate at the top of the 1.4 M layer (see Fig. 3a). Epiplastic collars, mostly without basal bodies, accumulate at the 1.4-1.7 M interface (see Fig. 3b). At high magnification a regular pattern of striations with approximately 100 Å center-to-center spacing can be seen on these collars. Most of the basal bodies accumulate at the 1.7-1.8 M interface (see Fig. 3c). This band also contains a few collars, some pellicle material, kinetodesmal fibers, and axonemal microtubules.

In the unfixed basal body preparations each basal body layer represents a solubilized basal body which would have been observed intact if the preparation had been pretreated with glutaraldehyde. Ovoid or spongy masses of material, which we believe to be derived from the pellicle, sediment through the 1.8 M sucrose to the bottom of the tube (see Fig. 3d).

A slight improvement in purification can be obtained by recovering the band at the 1.7-1.8 M interface and running it again on a second gradient. The recovery of basal bodies is low, however, because they tend to be trapped by the pellicle material and carried into the 1.8 M sucrose layer. We found it necessary to start with at least 100 ml of fresh packed cells in order to obtain enough material (approximately 5 mg protein) for biochemical analysis.

Fig. 5a1 and 2 show a SDS-acrylamide gel of proteins from the membrane layer and the basal body layer, respectively, of a discontinuous sucrose gradient. A larger number of proteins are present in these fractions. Since microtubules kinetodesmal fibers, and fragments of pellicle also occur in the basal body fraction, it is not clear which are basal body proteins. However, it is apparent that we are getting considerable separation (compare Fig. 5a1 with 5a2). Some proteins which we feel may be basal body specific are revealed by the differential solubility of these components in PTA. Basal body doublet microtubules are soluble in 0.1% neutral PTA, whereas kinetodesmal fibers and the third triplet microtubules are not. With the exception of the third triplet microtubule, these structures are soluble in 1% neutral PTA at least as judged by the criteria that they will not sediment at 100,000 g for 1 h. An example of these PTA extracts is shown in Fig. 5b. There are four major proteins between 27,000-32,000 mol wt present in both
extracts which we believe may be basal body proteins. There is also a major band at 21,000 (arrow, Fig. 5 b 1) and a minor component at 40,000 mol wt, in the 1% PTA extract only, which we believe may be kinetodesmal fiber protein. Both extracts also have bands at about 60,000 mol wt which we believe correspond to tubulin. No proteins are extracted by PTA from the top two gradient layers although the gel patterns from PTA extracts are identical for the 20 K pellet and band 3 (basal body fraction). This further strengthens our belief that the four major protein bands come from the basal bodies.

A very large number of proteins can also be extracted by PTA from axonemes. Fig. 5 c shows an acrylamide gel of PTA extract from demembranated Tetrahymena cilia, and Figs. 4 a and 4 b show thin-section micrographs of this fraction before and after PTA extraction. The protein solvent properties of PTA depend strongly on pH. The axonemal proteins shown in Fig. 5 c 1 were extracted at pH 7.0. Fig. 5 c 2 shows proteins extracted from an equal amount of axonemes at pH 5.0 in PTA. Fig. 5 c 3 shows proteins extracted from an equal amount of axonemes by H2O.

Probably among the basal body proteins solubilized by SDS but not by PTA are the components of the basal plate. An example of a plate obtained by PTA extraction of basal body microtubules is shown at high magnification in Fig. 6. Much of the details of this structure were shown by Wolfe (1970) in his study of oral apparatus basal bodies, but some additional structures are revealed here. The second concentric ring from the outside, which is about 0.2 µm in diameter and is the site of attachment of the microtubule doublets to the plate, can be seen to be composed of double filaments which cross or are helically wound between the radial spokes to the others. The third ring has a scalloped circumference and the fourth ring is attached by radial spokes that are offset with respect to the spokes of the other three rings.

DISCUSSION

Ever since basal body isolation methods were developed, the study of this organelle has been greatly hampered by questions concerning the purity of the preparations used. In a recent review (Fulton, 1971), this problem is discussed at length and it is concluded that we still do not know whether DNA is present in basal bodies or centrioles. Much of this confusion is due to the fact that it is very difficult to attain chemically pure basal body preparations. These preparations are often so contaminated as to make chemical analysis fruitless.

The three essential features of the procedure described here are: (a) fixation at 30% ETOH to enhance later digitonin solubilization; (b) the use of the Polytron to free the basal bodies from pellicle, basal rings (or collars), and kinetodesmal fibers; and (c) the use of step gradients to remove membranes, basal rings, and pellicle. The last two features of this method deserve further consideration. It is surprising that the Polytron, run at its highest speed, does not break up the basal bodies. In fact we found the basal bodies to be very stable to homogenization, room temperature (24°C), and prolonged digitonin or Triton solubilization. This resistance allows various preparations to be stored over long periods of time. This was true even when no ethanol fixation was used. Therefore the basal body microtubules can be classed along with flagellar and axoneme tubules as being much more stable than typical cytoplasmic microtubules which break down readily under conditions of cold, pressure, neutral pH, and colchicine.

Lastly, the failure of linear gradients to clearly separate basal bodies from other contaminants can be attributed, we believe, to a great hetero-

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**Figure 2 a** Basal bodies and kinetodesmal fiber from 20 K pellet negatively stained after fixation in glutaraldehyde. Note basal plate (BP) near distal end of basal body. X 30,000.

**Figure 2 b** Basal body negatively stained with neutral PTA without previous fixation. Dried quickly to partially preserve microtubule structure. Note striated third tubule at proximal end of basal body. X 100,000.

**Figure 2 c** Basal plates and striated tubules remaining after long (1–2 min) exposure to neutral PTA. X 75,000.

**Figure 2 d** Partially disrupted kinetodesmal fiber stained without fixation in 3% ammonium molybdate. X 100,000.
FIGURE 3 Negatively stained samples from a sucrose density gradient centrifugation of a 20 K pellet. (a) membranes at 0.5–1.4 M interface; (b) collars collected at 1.4–1.7 M interface; (c) basal plates and striated tubules from basal bodies and collars collected at 1.7–1.8 M interface not extensive disruption or basal bodies due to use of PTA; (d) pellicular fragments sedimenting through 1.8 M sucrose. No fixation. × 25,000.
The use of PTA to elucidate biological structure is now accepted as a standard technique in electron microscopy. The results presented here clearly show that this method can be accompanied by serious artifacts. Henley (1970) and Chasey (1969) first demonstrated that PTA had a disruptive effect on ciliary and flagellar microtubules. Hufnagel (1969) reported that numerous *Paramecium* structures (including basal bodies and kinetodesmal fibers) are soluble in 4% neutral PTA. She suggested that this property be utilized to characterize the proteins of these components. This we have attempted to do. Our results indicate that neutralized PTA is actually much more disruptive than previously suggested. Complete solubilization of axoneme microtubules, basal body tubules, kinetodesmal fibers, and, in fact, practically all axoneme proteins occurs at the relatively low concentration of 1% PTA. Furthermore, this solubilization occurs within seconds. The fact that only partial disruption occurs...
when the PTA is applied and dried very rapidly only emphasizes that great caution must be used in interpreting PTA negatively stained images. For example, the classic studies of Grimstone and Klug (1966), Pease (1963), and André and Thiéry (1963) may have to be reinterpreted in light of the disruptive effects of PTA.

Protein Analysis of Basal Bodies and Kinetodesmal Fibers

On the basis of acrylamide gels of SDS and PTA extracts of 20 K pellets and basal body fractions, we suggest that the major protein component of kinetodesmal fibers is a 21,000 wt peptide. The peptides composing basal bodies are less well defined but certainly are few in number. The differences between gels from whole basal body fractions and 1% PTA extracts probably reflect the presence of basal plates and pellicular contaminants in the basal body fraction.

Concerning the presence of DNA in basal bodies, it is our belief that previous efforts to answer this question have been ineffective due to the high degree of contamination present. We feel that our preparations are also inadequate for DNA or RNA analysis due to large amounts of nucleic acid present throughout the isolation procedure. This material which can be recog-
nized as stain repellent, branched, tapering fibers in PTA-stained grids, is presumably of nuclear origin. Until a basal body isolation procedure is developed in which the nuclei are removed intact, the question of nucleic acid in basal bodies will remain unanswered.

Observations of basal bodies which have been partially disrupted by negative staining reveal many structural details for which it is not yet possible to assign functions. Our results confirm the observations of Wolfe (1970) that the third tubule in each of the nine triplets in the basal body is about 0.2 µm long and is striated perpendicular to the axis of the organelle. A great deal of fine structure is seen in the isolated basal plates. The finest of the fibers which make up this interesting structure are only 20-30 Å in diameter. The location of this plate at the junction of the basal body and the cilium make it a logical candidate for a role in ciliary formation and function. The axonemal microtubules always shear about 0.1 µm above the basal plate. Perhaps the plate confers rigidity to the basal body structure.

The resistance of the basal ring, the opening of the parasomal sac, and the associated collar, or epiplasm, to digitonin solubilization makes it
seem unlikely that these structures are membranous as was suggested by Hufnagel (1969). Although we have called the granular sheets which collected on top of our gradients membranes, we have no evidence that they are not merely digitonin micelles artificially created by the isolation procedure. They do have unit membrane structure when fixed and sectioned, however.

In agreement with Henley (1970) and Hufnagel (1969) we observe that the kinetodesmal fibers are composed of aggregates of thin (50 Å) subfibrils. The longitudinal spacing of the striations in these fibers, after fixation and drying, appears to be about 410–430 Å in our preparations. If they are composed of collagen, as has been suggested (Afzelius, 1969), the molecular weight of the protein subunits appears to be low. It seems likely that the low molecular weight of the electrophoretic band which we have equated with the kinetodesmal fibers is not produced by the PTA extraction since other proteins, such as tubulin, which have known molecular weights, are not affected by this extraction (Fig. 5).

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