EFFECTS OF NUCLEASE AND PROTEASE DIGESTION ON THE ULTRASTRUCTURE OF *PARAMECIUM* BASAL BODIES

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ABSTRACT

The action of deoxyribonuclease, ribonuclease, perchloric acid, and pronase on the fine structure of basal bodies of sectioned *Paramecium* was observed as part of a more extensive autoradiographic electron microscope analysis directed toward the problem of basal body DNA. DNase was found to have no detectable effect on basal body fine structure. Pronase first solubilized the linkers and C tubules of the triplets, then attacked the protein portion of the axosome, a localized portion of the ciliary axoneme adjacent to the distal end of the basal body, the rim fiber, and newly described lumen spiral complex. Prolonged pronase treatment disrupted the remaining microtubular elements, basal body plates, and cartwheel. RNase removed material from the axosome and the lumen complex, a conspicuous structure occupying the central portion of the basal body and consisting of a twisted or looped 90-Å diam fiber or, more probably, pair of fibers, in association with large, dense granules. The apparent removal of *both* RNA and protein from this basal body structure by *either* of the two corresponding enzymes suggests an unusual organization of the two components. Observations from this and other laboratories suggest that the basal body RNA is single stranded. Its function is unknown but alternatives are discussed.

The present paper reports the effects of deoxyribonuclease, ribonuclease, perchloric acid, and protease digestion on normal basal body morphology and represents part of a more extensive light microscope-electron microscope (LM-EM) autoradiographic analysis focussing on the intriguing question of basal body DNA. The latter problem traces its roots to the early and important concept of the genetic continuity of centrioles (3) and basal bodies or kinetosomes (16) via division of the parent structure. With the advent of electron microscopy, this hypothesis was invalidated, but the idea that some basal body component might be transmissible and responsible for the property of apparent "self-reproduction" remained viable, catalyzed more recently by the discovery of DNA in other cytoplasmic organelles (27, 28). It was therefore natural that organellar DNA would be sought (and expected) in basal bodies and centrioles. The presence of DNA in basal bodies of *Paramecium* (35) and *Tetrahymena* (25) has indeed been reported, but mounting negative evidence now leaves this an open question (6, 7, 11, 24, 37, 45).[1]

Reports of RNA associated with basal bodies and centrioles have received relatively less atten-

[1] Dippell et al. Unpublished observations.

tion. Ribonuclease-sensitive material apparently has been found in the wall and triplet bases of centrioles (39), but the small amount of RNA detected in chemical assays of isolated basal bodies has not been localized (33, 10, 1, 9). Analysis of the protein components of basal bodies is also limited except for the possible assignment of (*a*) ciliary tubulins to corresponding A and B tubules of the basal body, (*b*) a unique tubulin (43), possibly restricted to the C tubule, and (*c*) ATPase activity (18). Unfortunately, in the majority of these investigations, the characterization and localization of basal body components have suffered due to disruptive techniques resulting in either contamination or destruction of basal body material before analysis.

The present approach seeks to circumvent these difficulties by using sectioned cells rather than pellicle preparations or homogenates and by fixing and enzyme treating cells in such a way as to provide minimal interference with enzymatic activity and maximal preservation of ultrastructure. Evidence is presented for the occurrence of RNA, but not of DNA, in basal bodies and for possible differences in composition among the various structural components and appendages of basal bodies as revealed by their differential response to enzyme action.

MATERIALS AND METHODS

Culture Conditions

Cells of stock 51VIIS *Paramecium tetraurelia* were grown at maximal fission rate (five fissions per day) in bacterized baked lettuce medium buffered with sodium phosphate, pH 6.5–7.1 at 27°C.

Autoradiographic Procedure

Cell synchrony was achieved by individually selecting only dividing animals showing deeply constricted cleavage furrows and placing approximately 30 of these, in groups of 5, into 0.7-ml aliquots of bacterized culture medium containing radioactive isotopes as indicated. No difference in fission rate was detected between the unlabeled vs. exogenously labeled cells and good synchrony was maintained up to the fourth cell cycle. Cells halfway through the fourth cell cycle were harvested, washed three to four times through 1-ml volumes of culture medium, fixed in glutaraldehyde, and subjected to enzymatic digestion before osmium tetroxide postfixation and embedding. Thick (0.3 μm) and thin (900 Å) sections were taken of the same material for LM and EM observation and LM autoradiography.

Ilford K5 nuclear emulsion was used because of its high sensitivity and small grain size. Thick sections were placed on clean slides and dipped into Ilford K5 emulsion dissolved in water in a 1:2 wt/vol ratio, incubated for 36 h at 4°C; developed in D19 Kodak developer for 4 min at 22°C; fixed in standard Kodak fixer; stained in warm 0.5% toluidine blue in 0.25% aqueous sodium borate for 15 s; rinsed and air dried for light microscopy.

Macronuclear and cytoplasmic grain counts were used as an index of nuclease activity. To randomize counting, a macronuclear or cytoplasmic area free from food vacuoles was centered beneath an ocular grid square (16 μm^2) so that the cell structure rather than the grains was sharply focused in Leitz 100× bright-field optics. The grains were then brought into focus and the number within the square recorded. A total of 30 such counts (one square per macronuclear section, three per cytoplasmic section) was made on sections of from 5 to 10 different cells per group, except in the case of ribonuclease-treated cells where only 15 counts were obtained.

Fixation and Embedding

Material was fixed for either 15 min or 30 min in 1.5% phosphate-buffered glutaraldehyde, pH 7.3. No difference in the efficiency of enzymatic action was noted between the procedures; 30-min fixation was selected as standard. Buffered Formalin was eliminated as a fixative because of unacceptable distortion of fine structure. Cells were washed for at least 2 h in several changes of 0.1 M phosphate buffer in order to minimize possible inhibitory effects of fixative residues on enzymatic activity. After enzymatic extration, treated and control cells were washed in buffer, postfixed for 30 min in 1.0% phosphate-buffered osmium tetroxide, pH 7.2, dehydrated, and embedded in Epon 812.

Enzymatic Extraction

Glutaraldehyde-fixed and washed cells were exposed to one of the following enzymes: (*a*) deoxyribonuclease, electrophoretically purified (Worthington Biochemical Corp., Freehold, N. J.), 0.2 mg/ml in 0.001 M phosphate buffer containing 0.01 M $MgSO_4 \cdot 7H_2O$, pH 6.6, 37°C, 6 h: collidine buffer was also used for one experiment with similar results; (*b*) bovine pancreatic ribonuclease A, five times crystallized, protease-free (Nutritional Biochemical Corp., Cleveland, Ohio), 0.1 and 1.0 mg/ml in distilled water, pH adjusted to 6.6 with dibasic sodium phosphate, 37°C, 2.5 h, freed of possible DNase activity by heating for 2 min in boiling water and checked for possible protease contamination by exposing [^{3}H]leucine-labeled cells to the enzyme (no difference in grain counts between control and enzyme-treated cells was observed); (*c*) Pronase, B grade (Calbiochem Corp., San Diego, Calif.), self-digested 0.3 mg/ml in 0.1 M phosphate buffer, pH 7.2, 37°C for 6–18 h. The possibility of RNase as a possible contaminant was checked by

subjecting [^{3}H]uridine-labeled cells to Pronase digestion: grain counts showed no significant reduction in amount of label compared with controls. RNase contamination was also checked by adding substrate RNA to the enzyme solution and measuring in a Brinkman DB-G grating spectrophotometer the amount of nucleotide degradation after incubation and TCA precipitation of undigested nucleotides and enzymes. RNase and substrate RNA alone served as controls. No detectable RNase contamination was found. After enzymatic digestion, animals were washed in 0.1 M phosphate buffer and subjected to 5% TCA, 4°C, for 1 h. All groups were then washed in phosphate buffer and postfixed as described.

Deoxyribonuclease activity was verified in LM by failure of macronuclei to stain with the Feulgen reagent and by removal of thymidine label and, in EM, by the latter plus reduction in the amount of visible DNA-containing material in mitochondria and recently ingested bacteria in the same cell (Figs. 36–39). Ribonuclease activity was judged, in LM, by failure of nucleoli and cytoplasm to stain with Azure B and by removal of tritiated uridine label and in EM by reduction in size and density of cytoplasmic ribosomes and nucleolar ribonucleoprotein particles (Figs. 6–9). Pronase activity was detected by the extent of dye binding of Naphthol Yellow S and removal of [^{3}H]leucine label.

Perchloric Acid Extraction

PCA, 7.0% aqueous solution, 37°C, 2 h, was employed as an alternate RNA extraction procedure according to Pearse (20). Its activity was measured in the same manner as for RNase. Substrate specificity was verified by grain counts of PCA-treated, [^{3}H]leucine-labeled cells and controls.

Scoring of Treated Material and Repeatability of Results

For all experiments, duplicate or triplicate runs were carried out at different times. The results were found to be generally consistent, although, as noted by other investigators, considerable variation in response to the enzymes occurred among different cells of the same group. However, the difference between the control and experimental groups was clearly significant. Prolonged exposure to both enzyme and control solutions produced gross distortion of body form and loss of fine structure. Experiments were therefore terminated immediately before this response, as established by earlier time-course data.

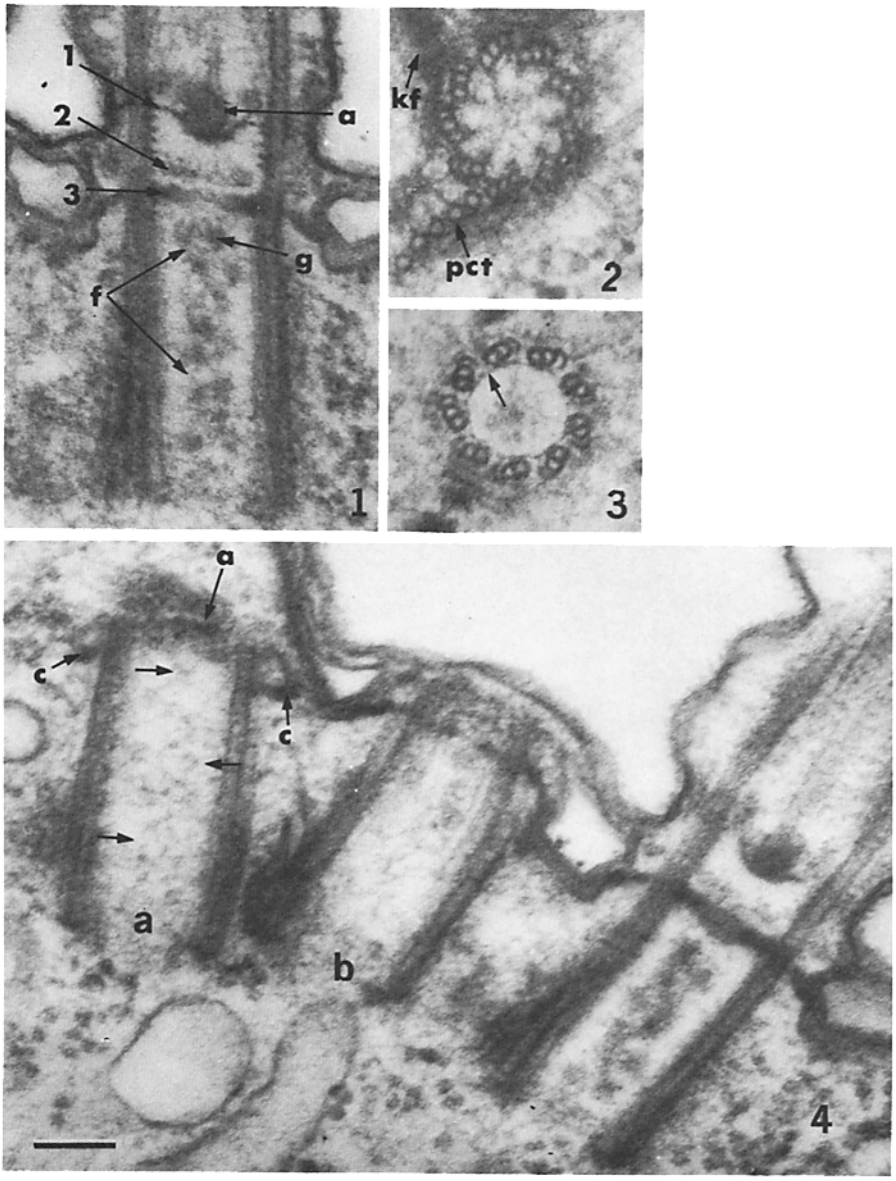
Since all of the nontubular structures being monitored are more or less centrally positioned in the basal body, care was taken to score only those images representing longitudinal sections through the basal body center in which the plane of section passed simultaneously through the axosome, lumen complex, and cartwheel hub.

RESULTS

The Untreated Basal Body

In order to assess the experimental data, it is necessary to become familiar with the major architectural features of the mature basal body, including certain structures not previously described. These key features are summarized here.

The adult *Paramecium* kinetosome, like that of most eucaryotic centrioles and basal bodies, is a cylinder whose wall consists of nine groups of triplet microtubules surrounded by filamentous material and connected proximally by linkers (Figs. 1, 2). A variety of tubular and/or fibrous appendages may be present at its basal external surface, associated with one or more particular, and different, triplet bases (Figs. 2, 14, 15). At its distal end, the *Paramecium* basal body is capped by three transverse partitions (Figs. 1, 10, 32): (*a*) an outermost, axosomal plate separating the basal body from the ciliary axoneme and bearing an axosomal granule from which at least one of the pair of central ciliary microtubules originates; (*b*) a less conspicuous annulate intermediate plate; and (*c*) a dense basal or terminal plate continuous as a collar around the outside of the basal body: for terminology, cf. Pitelka (22). The proximal end of the basal body contains a "cartwheel" structure whose nine spokes connect with the nine triplet sets (Figs. 2, 15, 41). The remaining internal space is occupied by (*d*) a fine fiber coursing the distal two-thirds of the cylinder rim and closely apposed to at least one of the three microtubules of each triplet set (Figs. 3, 40), (*e*) filamentous material distributed through the lumen, forming connections between the cylinder wall and (*f*) a distinctive central, lumen structure not previously reported. The latter is composed of (i) a pair of 90-Å diam fibers or a single, looped fiber arranged in a loose helix, and (ii) large (25–35-nm diam), dense granules more or less regularly spaced along the helix, presenting a staggered image in longitudinal sections through the midregion of the basal body (Figs. 1, 10, 32). (It has thus far been impossible to determine whether one or two fibers are present; since free ends have not been observed the term "pair of fibers" is arbitrarily used here.) This lumen complex extends from the top of the cartwheel to the basal plate and is surrounded by numerous, delicate filaments which connect it with the lumen wall.



FIGURES 1–3 Normal mature basal body morphology. Fig. 1 is a longitudinal section showing the axosome (*a*), axosomal (*1*), intermediate (*2*), and basal plates (*3*), and the lumen spiral complex consisting of a twisted looped fiber or pair of fibers (*f*) and associated large granules (*g*). A nascent basal body whose axis is perpendicular to that of the adult appears at the proximal, left side of the mature basal body. Fig. 2. Cross section through the basal end showing the internal hub and spoke (cartwheel) structure connected to the nine triplet sets of microtubules, the linkers between triplet sets, and two of the accessory structures: the kinetodesmal fiber (*k*) and postciliary tubules (*pct*). Fig. 3. A cross section immediately below the terminal plate. Linkers are absent above the cartwheel, but here the triplet sets of microtubules are connected by a fine fiber (arrow) that courses the inner rim of the cylinder.

FIGURE 4 Late developmental stages of two kinetosomes: (*a*) has not yet surfaced, (*b*) is inserted at the surface but has not produced a cilium. A presumptive axosome can be recognized in (*a*) at the arrow. The material immediately above the axosome is part of the subcortical filamentous network. A collar (*c*) surrounds the distal ends of the tubules. At this stage, the pair of fibers or looped fiber of the spiral complex is evident (arrows in the lumen of *a*) but the large granules have not yet developed. The scale marker in this and subsequent micrographs equals 0.1 μm.

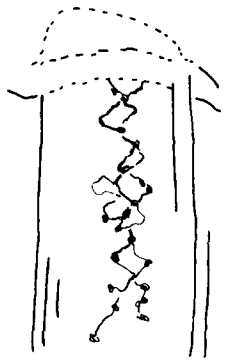


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FIGURE 5 A tracing of the fibers of the spiral complex shown in Fig. 4 (*a*). Figs. 1–5, × 116,000.

Effects of Ribonuclease Digestion

Partial to complete extraction of the lumen complex and reduction in density of the axosome were observed after ribonuclease treatment (Figs. 11–13). Granules of the lumen complex were scored for relative numbers present (many, few, or none) after digestion. The distribution, compared with that of the buffer controls, is summarized in Table I. Ribonuclease action on the pair of fibers was more difficult to score: a single section often did not contain all of the two fibers, and images were fuzzy as a result of treatment. Of 11 basal body sections lacking lumen granules, 6 showed complete removal, and 5, partial removal of the fibers; only fine, randomly disposed filamentous material remained in the lumen. The large granules apparently disappeared first, leaving the fibers intact but the target of subsequent degradation (Figs. 11–13).

The axosome was partially but never completely degraded, even by prolonged RNase treatment (Fig. 11 and Table II), suggesting that this structure is composed of other material in addition to RNA (see section on Pronase digestion). Masking phenomena are of course recognized throughout this study as a possible alternative for failure of a structure to respond to enzyme action.

The basal body collar, three plates, remaining lumen occupants (the wispy, filamentous material, cartwheel, and rim fiber) and the accessory structures all were resistant to RNase (Figs. 11–13, 15, 17). Subsequent data will show that these structures also were DNase resistant but pronase sensitive.

The Effects of Perchloric Acid Extraction

The action of PCA on RNA-containing components of the basal body was comparable to that of RNase, except for greater distortion of fine structure (Figs. 19–22). Activity was checked in the same manner as for RNase. Lumen complex granules usually were diminished first, followed by removal of RNA from the axosome. The fibers of the complex (Figs. 18, 19) also were finally degraded (Fig. 20). The rim fiber and cartwheel structure, as in RNase, were resistant (Figs. 21, 22). Microtubules appeared solid in cross-section, membranes were fuzzy and swollen, and dislocation of cell structures suggested precipitation and displacement of protein. Staining with Naphthol Yellow S (a dye staining dibasic groups of arginine, histidine, and lysine at low pH) showed no detectable shift in protein-dye binding between treated and control groups; this was taken as an indication of no appreciable loss of protein. Grain counts on treated and control cells labeled with tritiated leucine confirmed the staining results.

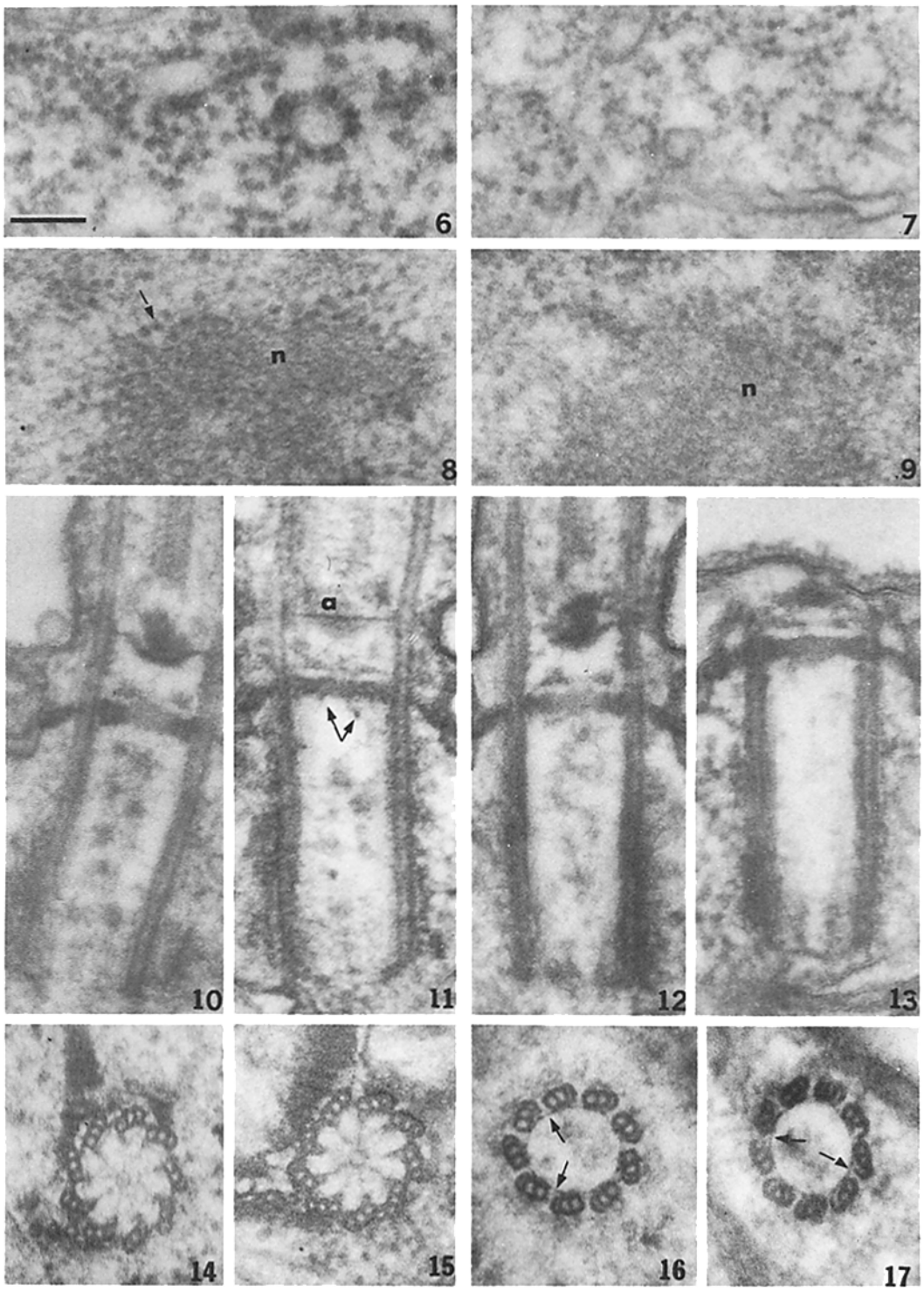
Effects of Pronase Activity

Cells exposed to Pronase showed more variability in degree of response than did cells subjected to either RNase or PCA. As might be expected from the action of a nonspecific protease, cell structures also tended to be more or less distorted, displaced, or solubilized. Nevertheless, repeatable differential sensitivity was observed for different basal body components. Table III summarizes some of the results.

TABLE I

The Effect of RNase on Basal Body Lumen Granules

Experiment no.		No. of basal bodies scored	Percentage of basal bodies containing		
			Many	Few	None
I.	RNase	40	28	50	22
	control	30	80	20	0
II.	RNase	37	19	54	27
	control	21	71	29	0
III.	RNase	122	54	30	16
	control	42	93	7	0



FIGURES 6–17 The action of ribonuclease on basal body fine structure. Figs. 6–9 demonstrate the efficiency of the enzyme via removal of RNA from (*a*) cytoplasmic ribosomes at arrow (Figs. 6, 7), and (*b*) nucleolar ribonucleoprotein particles (Fig. 8). Figs. 6 and 8 are buffer controls. The micrographs show structures occurring in the same cells as the basal bodies in Figs. 10–17. Figs. 10, 14, 16, buffer controls; Figs. 11–13, 15, and 17, representative samples of RNase-treated basal bodies, showing various responses to the enzyme. Fig. 11, partial removal of axosomal material, large bodies, and fibers of the lumen complex: note discontinuity of the latter at arrows. Fig. 12 shows removal of the large bodies but the remains of the fibers of the spiral complex. In Fig. 13, both of these components have been degraded. (From the stage of the cell cycle and location in the cortex, it is presumed that this is an adult basal body that has lost its cilium, but the response to the enzyme is typical also for basal bodies bearing a cilium.) The cartwheel and rim fiber are unaffected by RNase (Figs. 15, 17). × 116,000.

6-H DIGESTION: In more than 50% of the cross-sectional images of basal bodies, linkers between the A and C tubules of adjacent triplets were lacking (Fig. 26). C tubules also were absent (Fig. 24) in contrast to the A and B microtubules which, in most of the sections, remained intact. The presumed structural extensions of these same A and B tubules as ciliary doublets showed solubilization, however, in a localized region immediately distal to the axosomal plate (Fig. 28). Only rarely did the tubules appear disorganized elsewhere along the shaft. The two central ciliary tubules likewise were attacked by Pronase in the same area but appeared in many cases to be more sensitive, as evidenced by solubilization for a farther distance along the shaft. The ciliary membrane in the region opposite the disrupted tubules frequently bulged and appeared to be reduced in density but remained intact.

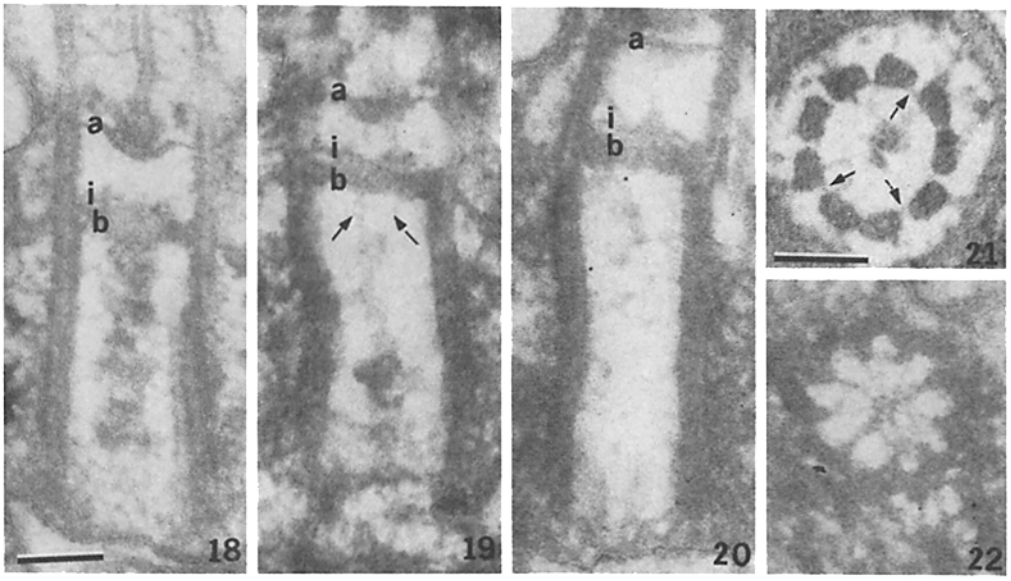
TABLE II

RNase Action on the Basal Body Axosome

Axosome	RNase		Control	
	no.	%	*no.*	%
Unaffected	6	16.6	14	87.5
Density slightly reduced	18	50.0	2	12.5
Density extremely reduced	12	33.3	0	0
Absent	0	0	0	0

The axosome in 90% of the basal bodies was affected by Pronase. The range in reduction in density was from slight to extreme; in a few instances the granule and plate appeared as a dispersed layer of material (see [*a*], Fig. 29). The density of the intermediate plate was markedly reduced, the latter appearing as a very thin, narrow ring of material in some sections (Fig. 28). A differential response to the enzyme was obtained for various parts of the basal plate (Figs. 28, 29): the collar portion, projecting outward from the basal body to the surrounding alveolar membranes, was Pronase sensitive as was the less opaque, central portion within the lumen. The plate material between these two regions (viz. that connected to the inner wall of the cylinder), however, was relatively resistant.

Within the basal body cavity, granules of the lumen complex were present but reduced in size



FIGURES 18–22 The action of perchloric acid on the basal body structures mentioned in the previous legend. The result of the treatment in general parallels that of RNase, but there is more nonspecific distortion of fine structure associated with perchloric acid extraction. Fig. 18, buffer control; Figs. 19–22, treated basal bodies. Fig. 19 shows the removal of the large bodies of the lumen complex but retention of the pair of lumen fibers. In Fig. 20, these, as well as their associated large bodies and the axosome, have been largely degraded. The rim fiber (arrow, Fig. 21) and cartwheel (Fig. 22), as with RNase treatment, are resistant. Figs. 18–20, × 116,000; Figs. 21, 22, × 131,500.

TABLE III

The Effects of 6-h Pronase Treatment on Basal Body Ultrastructure

	Total no. basal bodies observed		Structure unaffected		Density reduced		Structure absent	
	Enzyme	Control	Enzyme	Control	Enzyme	Control	Enzyme	Control
			%	%	%	%	%	%
Axosome	20	20	10 (2)	80 (16)	80 (16)	20 (4)	10 (2)	0 (0)
Terminal plate, central area	25	30	0 (0)	100 (30)	100 (25)	0 (0)	0 (0)	0 (0)
Lumen granules	22	26	0 (0)	92 (24)	86 (19)	8 (2)	13 (3)	0 (0)
Lumen spiral	25	20	20 (5)	65 (13)	16 (4)	30 (6)	64 (16)	5 (1)
Lumen filament component	25	27	0 (0)	100 (27)	100 (25)	0 (0)	0 (0)	0 (0)
Cartwheel	30	30	100 (30)	100 (30)				
A and B tubules	20	20	100 (20)	100 (20)				
C tubule	20	20	0 (0)	100 (20)			70 (14)*	0 (0)

* The remaining six basal bodies showed that some of the nine C tubules were missing.

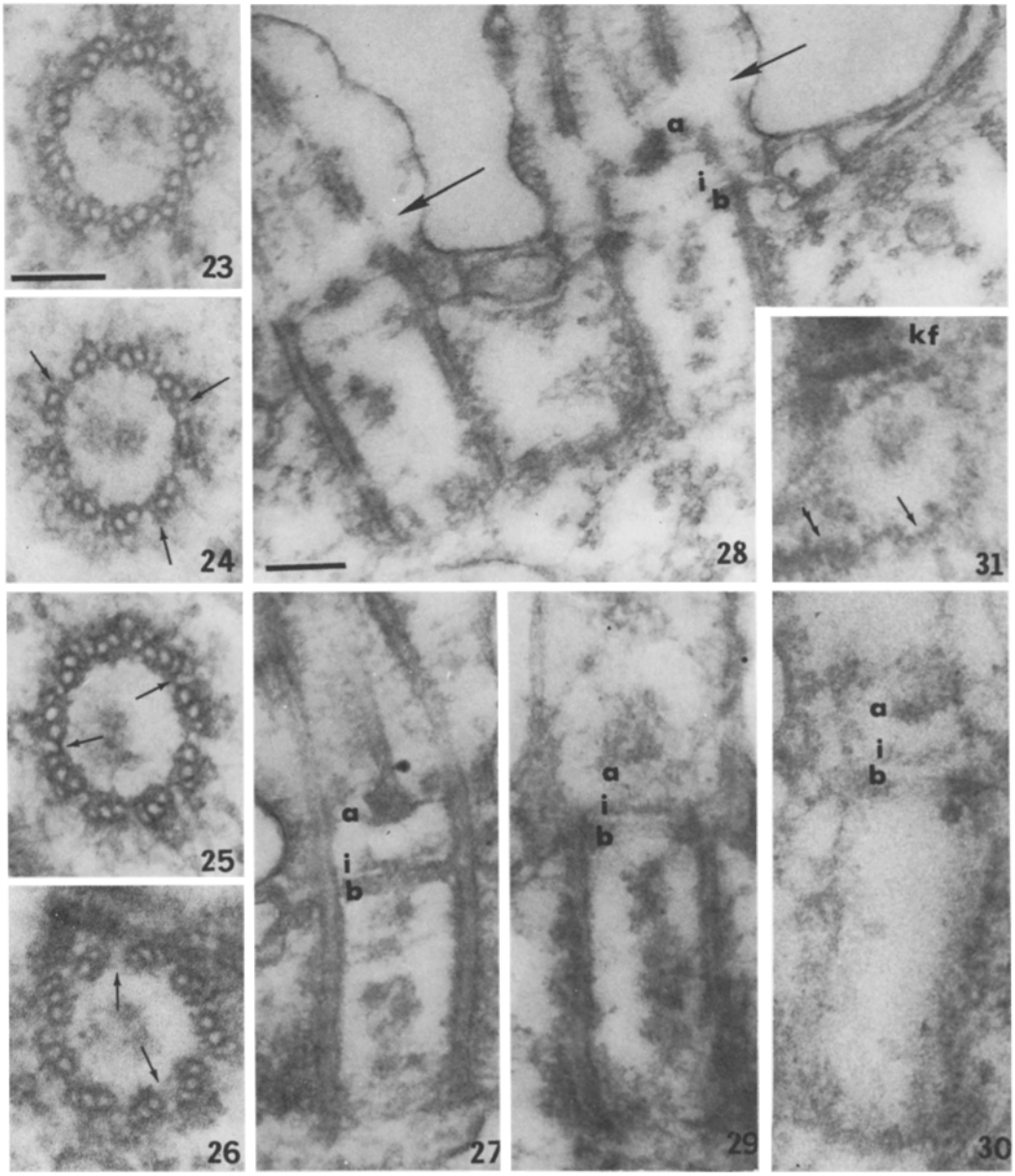
and/or density (Figs. 27, 28). In most instances, the fibers of the complex appeared to be at least partially digested by the enzyme treatment as judged from the absence of one or both of the fibers in the region immediately below the terminal plate (Figs. 27, 28) and by discontinuities observed elsewhere along these strands. The filamentous component extending through the basal body lumen also was absent after Pronase degradation. The rim fiber in both control and treated basal bodies in most instances was tightly appressed against the cylinder wall and difficult to follow, but nine images clearly showed fragmentation of this component. The cartwheel hub was the only lumen occupant clearly resistant to the 6-h treatment.

Among the accessory structures, the kinetodesmal fiber was unaffected, but all of the microtubular arrays (cytoplasmic and cytoskeletal) were in final stages of dissolution. In some instances, complete extraction had taken place, leaving "holes" in the dense, more resistant filamentous matrix surrounding their bases.

18-H DIGESTION: Cells were extremely variable in response to the enzyme and gross distortion of ultrastructure was evident. Nevertheless, some useful information was obtained. The previously resistant A and B tubules of the basal body triplet were now completely extracted, leaving the site of the basal body triplets marked by an enveloping skeleton of filamentous material (Figs. 30, 31). (Limited observations from earlier experiments in which an intermediate, 12-h Pronase digestion was used showed basal bodies with C tubules lacking, B tubules in various states of disintegration, and intact A microtubules.) Ciliary microtubules were largely degraded by the 18-h treatment. The axosome and transverse plates appeared as in the 6-h experiment, but within the lumen a number of basal bodies contained little except remnants of the fine dispersed filamentous component and a distorted cartwheel hub minus its spokes. Disappearance of the lumen granules, which were also RNase sensitive, suggests that the RNA may have been so tightly complexed to the protein as to be solubilized during Pronase extraction. Kinetodesmal fibers, fibrous masses composing microtubular nucleation sites, cell, and alveolar membranes, on the other hand, did not appear to be affected by the enzyme treatment.

Effects of Deoxyribonuclease Digestion

Basal body observations were made on sections of treated, thymidine-labeled cells which showed, in corresponding sections coated with emulsion, approximately 75% removal of macronuclear grains in LM autoradiography and 40% removal of mitochondrial grains in EM autoradiographs. When the ultrastructure was examined, it was found that a detectable but variable amount of aggregated, fibrillar material had been extracted from mitochondria, as well as from recently ingested bateria in food vacuoles of the DNase-treated cells (Figs. 37, 39). These fibers were similar to those identified as DNA (see, e.g., Ris [27], Swift and Wolstenholme [41], Schreil [32]).



FIGURES 23–31 The action of Pronase on basal body structure. Figs. 23 and 25 are buffer controls; Figs. 24 and 26–29, 6-h enzyme treatment; Figs. 30, 31, 18-h treatment. The C tubule and linkers between triplet sets are the first structures to be degraded (Figs. 24 and 26). This is followed by partial removal of axosomal material (*a*) (Figs. 28, 29), elements of the spiral complex (Figs. 27, 28) and axosomal, intermediate, and basal plate material (Fig. 28, *a*, *i*, *b*). Localized solubilization of the axonemal microtubules occurs immediately distal to the axosomal plate (Fig. 28, arrow). Exposure to the enzyme for 18 h (Figs. 30, 31) results in microtubular solubilization leaving the more resistant associated filamentous material as an outlining skeletal structure (arrow). The kinetodesmal fiber (*kf*) remains intact. Figs. 27–30, × 116,000; Figs. 23–26 and 31, × 131,500.

No significant modification of basal body ultrastructure was noted after deoxyribonuclease treatment in approximately 100 basal bodies examined in longitudinal or cross section (Figs. 33, 35, 40, 41). Axosome, transverse plates, and lumen components including the rim fiber, lumen complex, and cartwheel remained intact. Alterations, present in *both* control and treated groups, were limited to (*a*) lumen complex granules which were more disorganized than untreated ones and somewhat reduced in number in approximately 18% of the controls and 2% of the DNase-treated cells, and (*b*) the fine filamentous component of the lumen which, in some basal bodies, appeared at first to be largely extracted but, on closer inspection, was found to be tightly adhering to the cylinder wall and lumen complex. Accessory structures were unaltered.

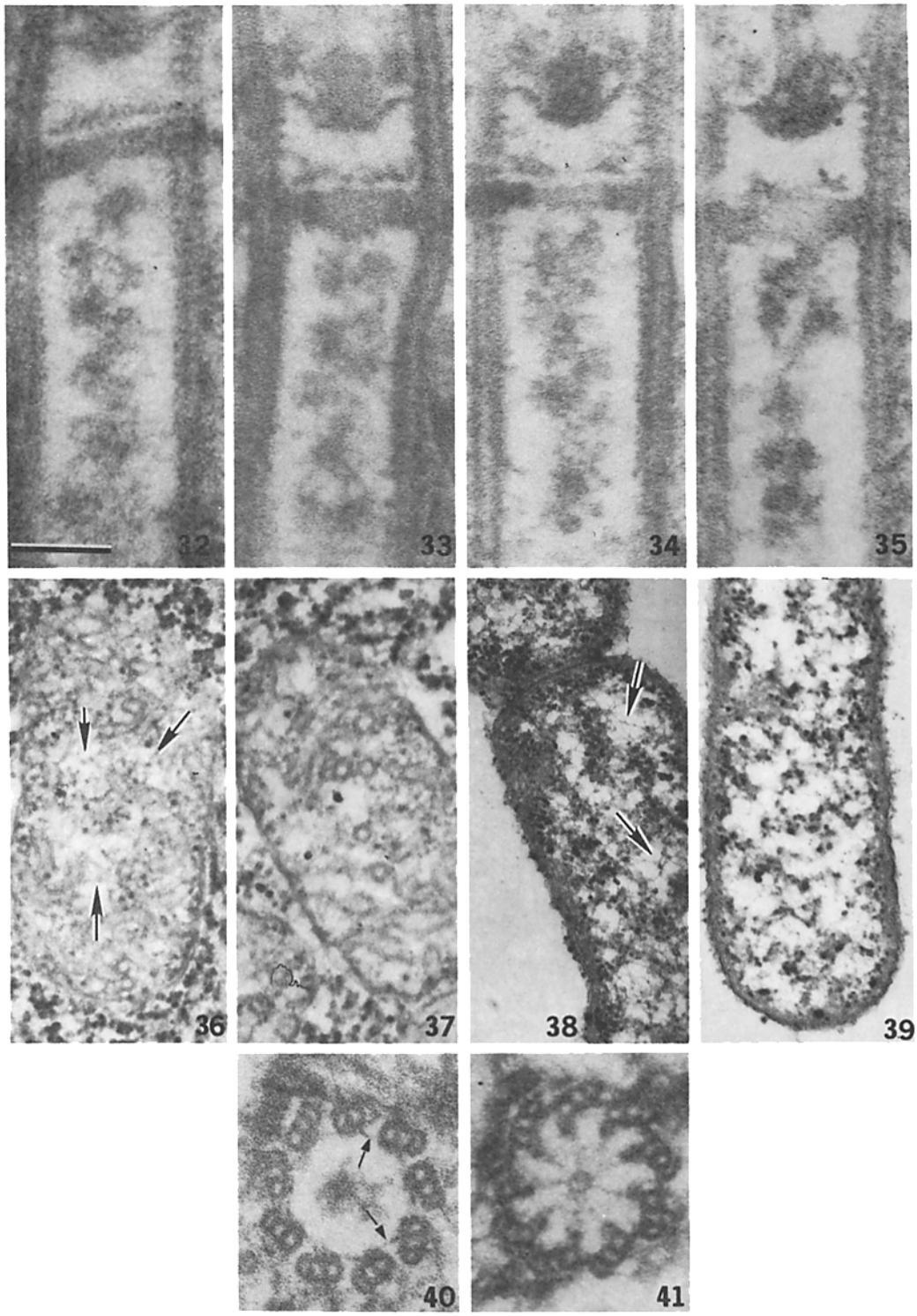
In order to test the possibility of protein masking of the DNA, Pronase hydrolysis was used before DNase treatment. This proved to be disastrous to fine-structure preservation, due at least partly to the required prolonged total incubation period; hence, little information was obtained. However, limited observations from previous, preliminary studies in which trypsin or pepsin extractions preceded DNase treatment, and in which distortion of structure was less evident, indicated no significant difference in ultrastructure between buffer-control and enzyme-digested basal bodies.

DISCUSSION

The data reported here relate to several questions, including the nature and function of basal body RNA, the absence or presence of kinetosomal DNA and its significance, and the heterogeneity of the protein components of the basal body. The results of DNase action are considered first. Admittedly, under conditions of the present experiment, failure to observe removal of DNase-containing material is not unequivocal evidence against its occurrence. One could probably envisage a number of ways in which DNA might remain undetected by enzyme action. The observations are relevant, however, to arguments put forth for the presence of basal body DNA on the basis of fluorescence studies (25, 35; cf. below). In *Tetrahymena*, the amount of DNA per basal body site has been estimated to be approximately equal to that of T2 bacteriophage, viz. 2×10^{-16} g, an inference based on visual comparison of the intensity of basal body fluorescence with that of T2 phage mixed in the same *Tetrahymena* pellicle preparations (25). A considerably lower "minimal" value of 2×10^5 daltons (3.34×10^{-19} g) was reported for the *Paramecium* basal body when calculations were based on autoradiographic grain density and/or specific gravity of thymidine used in exogenous labeling experiments (35). Both groups readily admit that their estimates are subject to considerable error, with the lower value erring on the minimal side. (Calculations estimating a molecular weight less than 10^6 daltons [6] might be a better starting point.) Whether the above quantities of DNA would be sufficient to be detected (if present) would depend largely on the extent of condensation or dispersion of the DNA and on the stage of the cell cycle at which cells were subjected to DNase. Maximal fluorescence and thymidine labeling have been reported for *Paramecium* between the 3rd and 4th h after cell division in a 5-h cycle (34). This clearly is *before* the period of new basal body formation (4).

In *Tetrahymena*, the maximal intensity of fluorescence corresponds to that of T2 phage, which contains tightly packed DNA (25). It was therefore postulated by the authors that the strong basal body fluorescence probably indicated the presence of DNA in a condensed state, while weakly fluorescing or nonfluorescing basal bodies observed in earlier stages of the cell cycle reflected the presence of dispersed (hence, presumably undetectable) DNA.

In the present study, the majority of cells were introduced into DNase within a period ranging from 2 to 3.5 h after cell division, i.e. when no new basal bodies were being produced but at a time when maximal fluorescence was reported (34). The remainder of the cells were exposed to the enzyme at approximately 30 min before cleavage formation. In the latter stage, new basal bodies are present in various stages of maturation. *If* the postulated DNA were present in a condensed state in any of this material *and* in the quantities estimated above, then it would seem reasonable to suppose that its removal might be detected structurally in EM profiles of enzyme-treated basal bodies. This assumption is based on the visualization of DNA fibers in *Paramecium* mitochondria and in recently ingested bacteria within food vacuoles in the same sections in which basal bodies were observed (Figs. 36, 38). Among the various images obtained, neither enzyme-treated mature nor immature basal bodies showed significant



FIGURES 32–41 The action of deoxyribonuclease on basal body fine structure. The activity of the enzyme is detected, in electron micrographs, by removal of DNA-containing material (arrows) from mitochondria (compare Figs. 36, 37) and from recently ingested bacteria (compare Figs. 38, 39). These structures are present in the same cells as the basal bodies in Figs. 32–35 and 40, 41. The response to the enzyme action is representative of that of other cells in this experiment. Figs. 32, 34, 36, and 38 are buffer controls; Figs. 33, 35, 37, 39, 40, and 41 are enzyme treated. No significant differences in alteration of lumen fine structure can be detected between control and DNase-exposed basal bodies. Cross sections show that the rim fiber (arrow, Fig. 40) and cartwheel (Fig. 41) are DNase resistant. × 157,800.

alteration of structure when compared to the buffer controls.

The results of Pronase digestion show the diverse components of the basal body to have different relative stabilities under a given set of degradative conditions. Such differences in reaction to a nonspecific protease provide little information about the molecular composition and/or organization of the various basal body structures, but they might serve as useful, indirect indicators of underlying macromolecular differences. This is suggested, for example, in comparing the present observations and those reporting differential susceptibility of microtubules to heat, cold, and proteolytic enzymes (2) with biochemical data suggesting differences in composition of the A and B tubules (36), or the differential susceptibility to Pronase of the C tubule with biochemical and morphological data indicating uniqueness of the C tubules (30, 42). The puzzling Pronase-sensitivity of the basal portion of the ciliary axoneme, whose tubulin composition and arrangement is assumed to be similar over the length of the cilium, may also reflect some underlying structural or functional specialization, possibly related to the unique plaque structures (Fig. 42) localized in this region (23, 31, 44). These have recently have been shown to be associated with Ca^{2+}-binding sites which may be involved in ciliary function (23).

Of particular interest are the responses to RNase and Pronase of certain basal body constituents which, by speculation or demonstration, involve association with nucleic acids. These are the axosome, elements of the lumen complex, and the rim fiber. (In centrioles, an "internal helix" corresponding to the rim fiber has been suggested as a possible site for DNA localization [39]. The authors found the helix unaffected by RNase; subjection to DNase was not reported.) The present study shows that the rim fiber in the *Paramecium* basal body (not to be confused with the helically disposed fibers of the lumen complex) is resistant to RNase, perchloric acid, and DNase extraction, but is fragmented by Pronase and appears to be composed of protein. The axosome and lumen complex, on the other hand, are DNase resistant but degraded to varying extents by *both* Pronase and RNase treatment. Extraction of the axosome occurs in approximately 10% of the basal bodies subjected to 6-h Pronase digestion; it is reduced in size and electron density by RNase but is never totally removed, even after prolonged exposure to the nuclease (see Tables II and III). The large lumen granules are similarly affected by both enzymes, with the entire complement being removed in 13% of the Pronase-treated cells and in 16–27% of the RNase-treated cells. Discontinuities in the pair of fibers of the complex are observed after *either* Pronase or RNase treatment.

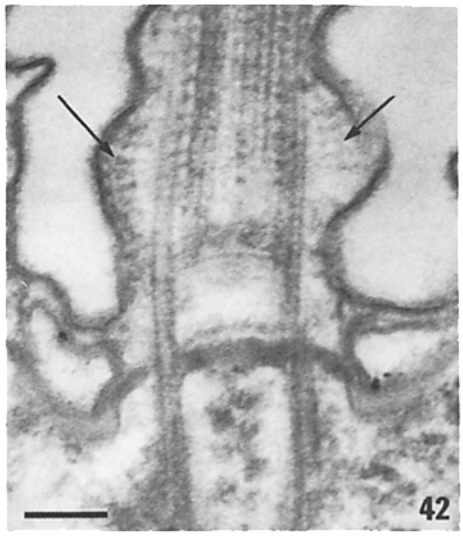


FIGURE 42 A localized structural specialization, ciliary patches, associated with the ciliary membrane in the region of pronase-sensitivity of the axonemal microtubules. See text for description. × 112,000.

The apparent extraction, from the above structures, of RNA-containing material by Pronase and of protein-containing material by RNase is particularly puzzling and intriguing. The results might be explained by possible contamination of the commercial enzyme preparations (17), but it is believed that adequate precautions were taken in the present study to make this unlikely. The action of the enzyme therefore is assumed to be directed at its specific substrate (Pronase attacks the carboxyester linkages of polypeptides but it is unlikely that it would cleave nucleotide phosphodiester linkages). In other systems involving RNA-protein complexes, enzymatic degradation by either Pronase or RNase results in disorganization of the complex but solubilization only of the substrate molecule: e.g., in the assembly of the tobacco mosaic virus (14) or the complexing of protein particles to the poly-A segment of nuclear and cytoplasmic mRNA molecules (15). What is unusual in the present case is the apparent removal of

both RNA and protein by *either* of the two enzymes. The possibility of artifact, of course, cannot be excluded: it could be, for example, that the RNA believed to be associated with the lumen complex fibers might remain intact after RNase or Pronase digestion but in a form undetected by the resolution employed. The latter is less likely to be true for the apparently more abundant and/or more condensed RNase-removable material associated with the large bodies in the lumen. If the observations are valid, they suggest that the integrity of the RNA-containing components is related to an unusual organization of the RNA and protein which could take the form of alternation of protein molecules with small segments of RNA.

A related problem concerns the type, source, and function of the basal body RNA. At least two other investigations have provided reasonably convincing evidence for the association of RNA with basal bodies and centrioles (9, 39). In the case of the Chinese hamster centrioles, the dense, footlike appendage attached to the inner surface of the A subfiber of the triplet was shown to be missing after RNase treatment. In *Tetrahymena*, Hartman et al. (9) report evidence for the presence of RNA in basal bodies on the basis of sucrose gradient sedimentation, DNA-RNA competition-hybridization, and fluorescence (coupled with nuclease) experiments. Localization of the RNA was not attempted. These experiments are particularly relevant to the morphological and cytochemical observations in the present paper. Material for analysis consisted presumably of purified, diethylpyrocarbonate (DEP)-prepared pellicles which contained cellular membranes, basal bodies, but no intact mitochondria. DEP inhibits ribonuclease action and was used to stabilize the internal basal body contents (which most pellicular isolation procedures have failed to do). The results indicated that while 65% of the pellicular RNA could be attributed to ribosomal contamination, the remaining 35% was neither transfer (excluded by the sedimentation data), ribosomal (excluded by competition-hybridization experiments) nor messenger RNA (excluded by long-term labeling methods). The acridine-orange fluorescence studies showed reduction of the yellow-green basal body fluorescence by RNase but not by DNase, and a shift from yellow-green (indicative of double-stranded RNA or DNA) to orange-red (indicative of single-stranded RNA) when staining followed mercaptoethanol or Pronase treatment. Nuclear fluorescence was not shifted, however. The basis for the color of the fluorescence appears to lie primarily in the rigidity and conformation of the structure binding the dye, hence not necessarily in strandedness of the nucleic acid (13). The more rigid, double-stranded nucleic acids allow for greater dye stacking which results in the yellow-green color. From their data, Hartman et al. propose that the basal body contains a single-stranded stable "morphic" RNA held in the proper rigid configuration by protein so as to yield yellow-green fluorescence. (This same reaction has been interpreted as indicative of basal body DNA [25, 34]). Release of the binding constraints by exposure to Pronase or mercaptoethanol was postulated to expose the single-stranded RNA, causing a shift in color of the fluorescence from yellow-green to orange-red. The occurrence of RNA in single-stranded form is supported, in the above described and present experiments, by its sensitivity to ribonuclease A which has absolute specificity for the pyrimidine nucleotides of the single-stranded RNA molecule. Double-stranded RNA would be resistant to degradation by the enzyme. Hartman et al. acknowledged that indirect evidence is never so convincing as direct demonstration, but their results in conjunction with the present observations, do argue for the existence of a single-stranded RNA in the ciliate basal body.

At present, the source and function of the RNA are speculative but amenable to certain types of experimental attack. If, for example, the RNA is considered to be of nuclear origin, it would be expected to be single stranded. If confined to the basal body and self replicative, however, it should appear double stranded at some stage of the cell cycle, depending on the S period of the RNA. It therefore might be possible to distinguish these two alternatives by subjecting pellicles prepared at separate, sequential stages throughout the cell cycle to ribonucleases, such as RNase III, which specifically cleave double-stranded RNA. Monitoring could be accomplished by a variety of standard labeling, isolation, separation, and fluorescence procedures. If RNA replication coincided with or immediately preceded basal body production, exploration might be particularly feasible using either *Paramecium*, in which the timing of initiation of basal body production for the ensuing cell division is known, or *Stentor*, in which during regeneration of oral membranelles thousands of new basal bodies are involved in the production of a new organelle.

The finding of RNA in the basal body is not

surprising to those who see its obvious function as associated with production of microtubular protein. This is an attractive hypothesis and one for which substantial indirect support might be marshalled: for example, (*a*) the demonstration, in *Tetrahymena*, that microtubular proteins required for production of a new oral apparatus are not stored but are newly synthesized in the same cell cycle in which they are assembled into the oral structure (26), (*b*) the dependence of basal body and centriolar formation on DNA-dependent RNA synthesis (40, 45), (*c*) the acquirement, by a centriole (in *Labyrinthula*), of electron-dense opaque material in its lumen when it becomes a basal body (21). In *Paramecium* the two fibers of the lumen complex are recognizable during maturation of the basal body and before it contacts the cell membrane, but the large granules of this complex are not present until the onset of ciliogenesis.[2] Thus, as in the case mentioned above, this material could be implicated in production of a cilium. Alternatively, it could be involved in formation of rootlet structures, although, at least in *Xenopus*, during ciliogenesis, rootlets can develop in the presence of colchicine which inhibits centriolar formation (38). In this instance, the centriole appears unnecessary for rootlet production. But these observations do not clarify the role of the RNA demonstrated by the present study. Neither the mature basal body, with its full complement of RNA-containing structures, nor the newly developing basal bodies still lacking them[2] reveal any identifiable mRNA-ribosomal complex expected if the basal body were the site of tubulin synthesis for new kinetosomes of new or regenerating cilia. Very limited observations have been made also on the fine structure of mature basal bodies in different parts of a predividing cell just initiating basal body production, of basal bodies in 2-, 3-, and 4-h old cells (on a 5-h cell cycle), and of basal bodies of the oral apparatus. The observations are preliminary, but no significant differences in morphology of the RNP structures have been detected which might reflect synthetic or replicative activity. (There are many examples, in other organisms, of large granules and fibers of diverse sorts occupying the lumen of basal bodies and of centrioles which are associated with production and maintenance of cilia and flagella, but these have not been examined cytochemically. On the other hand, there are numerous instances of "empty" basal bodies and centrioles being associated with ciliary or flagellar production.)

It might be instructive to extend the cytochemical experiments in *Paramecium* to include labeling, enzymatic digestion, and EM staining (using RNA-selective techniques) of developmental stages, oral primordium basal bodies preceding and during oral apparatus construction, and basal bodies engaged in ciliary regeneration after deciliation by chemical means. Less ambiguous evidence for the presence of functional mRNA would be expected to be derived, however, from biochemical data such as the demonstration of protein-synthesis capacity of basal body RNA in in vitro systems.

If basal body RNA is not involved in translation, what does it do? It has been postulated that centriolar RNA might play a structural role in determining the length of the centriole (based on analogy to the RNA helix of tobacco mosaic virus [TMV] [39]). Localization of nucleus-derived RNA to a cytoplasmic nucleation site where it might provide a "structural template" for production of a new basal body has been proposed (9). Relevant to this idea is the observation that, in a wide variety of organisms concerned with production of basal bodies, centrioles, and their appended cytoplasmic tubules, the nascent microtubules or microtubular organelles arise in association with a discrete, dense patch of filamentous material. These include the generative disk in *Paramecium* (4), condensation forms in rat trachea epithelium (5), cylindrical cores in chick tracheal epithelium (12), blepharoplasts of the fern *Marsilea* (19), centriolar plaques of yeast (29), and the microtubule-organizing centers associated with mitotic spindles. (This association might not be invariable, but, where nucleation patches are reported to be absent [8], fixation methods might first be checked for removal of cytoplasmic matrix material.) It would be interesting to know, and not too difficult to determine, whether a nucleation site contained RNA at a period before or during microtubular assembly. Certainly, if all of the RNA localized in the *Paramecium* basal body were condensed at some time in the generative disk, it should be readily detectable by nuclease (plus or minus protease) extraction. The sensitive period for detection might be very brief, however, if the RNA extends into the basal body or centriole accompanying microtubular growth.

An intriguing morphological and developmental

[2] Dippell, R. V. Manuscript in preparation.

parallel between certain procentriolar elements and an RNA-containing component of the *Paramecium* kinetosome occurs during centriolar production and development in embryonic chick tracheal epithelial cells (12). During ciliogenesis, the cylindrical cores, around which new procentrioles arise, and the procentrioles themselves, in both early and late developmental stages, contain a central filament (approximately 60 Å in diameter) or pair of filaments resembling the RNP fibers of the spiral complex in the lumen of the basal body. In the cores, which develop from aggregations of material surrounding the original diplosome, the filament is present in the earliest stages of procentriolar formation. In the procentriole (destined to become a ciliary basal body), it appears within the cartwheel hub but extends beyond the basal portion of the procentriole toward (to?) the core. The filament remains in the basal body lumen when the cartwheel subsequently disappears. The authors suggest that the filament might contain "informational macromolecules" for assembly of the cores and centrioles.

The kinds of information mentioned above, while conceptually useful, provide few, if any, clues to the solution of the question raised at the outset of this discussion. If the RNA plays a structural rather than a translational role, then does it provide scaffolding (not indicated by the TMV data) or dictate the order of assembly of the subunits in protofilament formation, or serve as a recognition center for protein aggregations giving rise to nucleation sites? Or does the RNA have some unique morphogenetic function which our present preconceptions about assembly mechanisms have not permitted us to envisage? Certainly, some of these considerations are now open to experimental attack. The evidence for basal body RNA and for its localization to specific centriolar and basal body structures represents a first step toward this analysis.

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