INTERNATL MICROBIOL (2000) 3:45–49

RESEARCH ARTICLE

Michael F. Dolan

Graduate Program in Organismic and Evolutionary Biology, University of Massachusetts, Amherst, MA, USA

DNA fluorescent stain accumulates in the Golgi but not in the kinetosomes of amitochondriate protists

Received 27 September 1999
Accepted 27 December 1999

Correspondence to:
Department of Geosciences
University of Massachusetts
Amherst, MA 01003. USA
Tel.: +1-413-5453244
Fax: +1-413-5451200
E-mail: mdolan@geo.umass.edu

Summary Hindgut symbiotic trichomonads (uninucleate *Caduceia versatilis*, and multinucleate *Stephanonympha* sp. and *Snyderella tabogae*) from the dry-wood-eating termite *Cryptotermes cavifrons* (Kalotermitidae) accumulate DAPI (4,6-diamidino-2-phenylindole) in the membranous sacs of the Golgi complex. This form of Golgi complex, typical of protists in the class Parabasalia, is called a parabasal body. Trichomonads contain organellar systems, mastigonts, that consist of four undulipodia (e.g. eukaryotic flagella and cilia), axostylar microtubules, a parabasal body and other structures. These cells bear from one (in the case of *Caduceia*) to hundreds (in the case of *Snyderella*) of mastigonts. These features are characteristic of their protist class (Parabasalia). The nuclei of all three species stained with DNA-specific stains: DAPI, SYTOX, acridine orange, propidium iodide, ethidium bromide and Feulgen, at optimal concentrations, but kinetosomes failed to stain at all. The nuclei, parabasal bodies and symbiotic bacteria (but no microtubular structures) fluoresced in glutaraldehyde-fixed cells stained with 1.45 μM DAPI. Parabasal bodies of *Snyderella* and *Caduceia* treated to remove lipids with Triton X-100, or treated with 5% trichloroacetic acid, lacked DAPI-fluorescence. I conclude that DNA, present as expected in nuclei and bacterial symbionts, is absent from and not associated with calonymphid kinetosomes. The reason for DNA–RNA stain accumulation in the Golgi cisternae is not clear.

Introduction

The hypothetical origin of mitochondria and chloroplasts as bacterial endosymbionts was supported by evidence that these organelles contained their own genomes, similar to those of prokaryotes [7]. That the organelles' proteins are more similar to bacterial proteins than to other proteins in the same cell helped confirm the idea of exogenous origin [20]. The symbiogenetic origin of the eukaryotic flagellum or undulipodium from a spirochete bacterium was further hypothesized by Lynn Margulis, formerly L. Sagan [20, 27]. This hypothesis can be tested by looking for eukaryotic protein homologues, such as tubulin, in spirochetes or by examining extant protists to see if they contain spirochete genes in their nuclei or have DNA of spirochete origin in or associated with their kinetosomes in a manner analogous to the genomes of mitochondria and chloroplasts. Although cytoplasmic tubules have been observed in spirochetes and other groups of bacteria [1], no tubulin homologous protein has been found in spirochetes. A putative prokaryotic tubulin homologue, FtsZ [5], is widespread among eubacteria and archaebacteria.

The search for kinetosome DNA has focused on more modern organisms such as green algae, plants and ciliates. Molecular systematic studies identify early branching eukaryotic lineages (diplomonads, trichomonads [29]) that are amitochondriate and are characterized by a cytoskeleton organized around a karyomastigont, an undulipodial system associated with a nucleus, and other structures, including a parabasal body or Golgi complex in the case of trichomonads. In some of these lineages the mastigonts are disassociated from the nuclei and are called akaryomastigonts.

The multimastigont trichomonad, *Snyderella tabogae*, is an ideal cell to test for kinetosome DNA because it is 100 μm long and its cortex is covered by over a thousand kinetosomes arranged in discrete sets of four, the akaryomastigonts [4]. In contrast to other archaeprotists, its multiple nuclei are separated from its kinetosomes. Therefore, if DNA could be visualized associated with the peripherally situated centriole-kinetosomes (c-k's), the distinctive surface pattern

that resulted could not be confused with other organelles. As representatives of an early branching lineage, trichomonads may retain ancestral characters that are lost from more recently evolved, derived forms. Although *Snyderella* is from the early branching trichomonad lineage, it evolved within the last 100 million years as a symbiont in the hindguts of termites.

Reports of DNA in or associated with the c-k have been controversial for many years. Most tests to find c-k DNA have been negative. Feulgen reaction tests for DNA in the blepharoplasts of plant spermatogenous cells, which generate c-k's de novo, have been negative for the fern *Marsilea* and the cycad *Zamia* [21], and for nine different bryophytes and ferns [33]. The DAPI stain was negative for *Marsilea* blepharoplasts [22]. Unspecified negative results were reported for *Ceratopteris* blepharoplasts [13]. The only positive result, using the Feulgen stain on *Ginkgo* [19], was not supported in a subsequent study [6].

Studies which reported RNA in, or associated with c-k's found no evidence for DNA associated with these same structures. While RNase removed pericentriolar material in PTK_2 cells (rat kangaroo kidney cells), DNase had no effect on the c-k structure [23]. Isolated *Chlamydomonas* and *Tetrahymena* c-k's treated with RNase were prevented from nucleating asters when injected into *Xenopus* eggs, but DNase-treated c-k's functioned normally [11]. RNase-treatment modified *Paramecium* c-k structure, but DNase-treatment did not [3]. Acridine orange-staining material in *Tetrahymena* c-k's was removed by RNase-treatment, but was not removed by DNase [10]. Other studies which found no evidence for RNA in the c-k [12, 16, 17, 25] also found no evidence for DNA there.

Two reports of DNA in ciliate c-k's in *Tetrahymena* [24], and in *Paramecium* [28] were not supported by subsequent studies [10] in *Tetrahymena*, and [30] in *Paramecium*. Because mitochondria are aligned at ciliary bases the false-positive detection of c-k DNA in ciliates are most likely attributable to the presence of regularly-patterned mitochondrial DNA [32]. DNA was reported in the centriole-kinetosomes of *Chlamydomonas reinhardtii* [8]. As these findings could not be confirmed by immunocytochemistry [15], DAPI staining [18] and genetic analysis [14], further experiments documented that the DNA sequence of the earlier study is in the nucleus and is positioned adjacent to the centriole-kinetosomes in interphase [9].

The fluorescent DNA stains acridine orange, DAPI, ethidium bromide, propidium iodide and SYTOX and the brightfield Feulgen stain were used in this study to test for DNA in the kinetosomes of *S. tabogae*, *Stephanonympha* sp. and *Caduceia versatilis*. While these cells lack mitochondria, they all have characteristic sets of bacterial symbionts. *S. tabogae* tends to be covered with small coccoid bacteria. Large rods and spirochetes attach to its posterior. In *Stephanonympha* sp. bacteria are regularly positioned within the axostyle adjacent to each of its nuclei. Intranuclear bacterial symbionts also are present. *C. versatilis* has at least five distinct bacterial symbionts: two epibiotic, two cytoplasmic and one intranuclear [2].

Materials and methods

Cryptotermes cavifrons was obtained from southern Florida. It contains three large trichomonad species: *C. versatilis*, *Stephanonympha* sp., and *S. tabogae*. Termites were kept in glass Petri plates with filter paper as a food source. A drop of distilled water was added to the paper every other day. The filter paper had been saturated in a solution of 10,000 U penicillin and 10 mg streptomycin per ml in 0.9 % NaCl to remove surface bacteria from the cells. Termites were reared on this substrate for at least two weeks before they were sacrificed and their gut contents were broken open in a few drops of Trager's solution [31]. Gut samples from termites that were not fed antibiotics were also used. Cells were pipetted into Eppendorf tubes containing 1 ml of 1% glutaraldehyde in water. Other fixation mixtures contained 0.2% Triton X-100. The hindgut protists of *Incisitermes* nr. *incisus* and *Incisitermes snyderi* were also examined and stained.

The fixed samples were centrifuged at 9 $\times g$ and washed once in 0.1 M phosphate buffered saline (PSA) and then stained in one of the following solutions for 30 min: 1 μM acridine orange, 2 μM DAPI (4,6-diamidino-2-phenylindole, Sigma, St. Louis, MO), 2 μM ethidium bromide, 2 μM of propidium iodide, 1 μM SYTOX (Molecular Probes, Eugene, OR) or 1 μM $DIOC_7$ (3,3-diheptyloxacarbocyanine iodide, Sigma). Cells were washed once with 0.1 M PSA, allowed to settle to the bottom of the tube and then pipetted onto a microscope slide as a wet mount. Coverslips were sealed with fingernail polish. Slides were examined under a Nikon epifluorescence microscope. Photographs were taken with Kodak 160 Tungsten color slide film. Video tape was recorded using a CCD camera and 3/4 inch tapes on a Sony U-matic tape deck.

DNase-treatment Cells were treated with DNase I following an amended procedure of Dipple [3]. Specimens were fixed and washed as above, and then exposed to 1.0–2.0 mg/ml DNase I in 0.001 M PSA containing 0.01M $MgSO_4 \cdot 7H_2O$ at pH 6.6 for 6 hours at 37°C. Cells were then centrifuged and washed in 0.1 M PSA and placed in 5% trichloroacetic acid (TCA) for one hour at 4°C. The specimens were then washed again and prepared as a wet mount. Cells were squashed by gentle pressure on the coverslip with a dissecting needle.

Feulgen stain The termite hindguts were extracted and broken open in a small drop of Trager's solution on a poly-L-lysine-coated coverslip. The gut contents were gently smeared on the coverslip, which was then submerged in a Columbia jar filled

with either 1% glutaraldehyde, Bouin's fixative (picric acid in formalin) or 70% ethanol and fixed for one hour. These coverslips were washed in 0.1 M PSA and then placed in 1 N HCl at 60°C for 5 min. They were then dipped in 1 N HCl at room temperature for one minute and placed in a decolorized basic fuchsin solution for 30 min. The coverslips were removed and placed in three consecutive solutions of potassium metabisulfite (5 ml of 10% aqueous $K_2S_2O_5$ with 5 ml 1 N HCl and 100 ml distilled water). They were then washed in water and dehydrated in a series of ethanol and mounted with Permount (Fisher, Fairlawn, NJ)

Results

Fluorescence microscopy A consistent pattern of staining that coincided with the placement of kinetosomes at the cell cortex was sought. *Snyderella tabogae* contains hundreds of kinetosomes in groups of four over most of its cortex. In all cases, the SYTOX-stained cells lacked any fluorescence in the akaryomastigonts in preparations in which bacteria and nuclei, as expected, were well stained for DNA. Conspicuous fluorescence of the akaryomastigonts was detected in DAPI-, acridine orange-, ethidium bromide- and propidium iodide-stained cells. The clearest and most specific staining of nuclei and akaryomastigonts occurred with DAPI preparations (Fig. 1). DAPI fluorescence was not found at the level of the kinetosomes, but just below it (Fig. 2). The same pattern of fluorescence was observed in cells stained with $DIOC_7$, which binds to lipid-rich structures such as the Golgi complex.

While it was impossible to remove all the nuclear DNA as detected by DAPI, the use of 2.0 mg/ml DNase conspicuously reduced nuclear fluorescence. The enzyme treatment was therefore effective in removing some DNA. The extraction of DNA by DNase requires treatment with cold 5% TCA. After DAPI staining, fluorescence was absent from the DNase-treated akaryomastigonts. However, in DAPI-stained controls lacking DNase but treated for one hour with 5% TCA the akaryomastigonts also did not fluoresce.

The akaryomastigonts of the cells that were fixed with the detergent Triton X-100 in the fixative mixture did not fluorescence with DAPI or any of the other DNA stains (acridine orange, ethidium bromide and propidium iodide). Examination of *Caduceia versatilis*, which has a large parabasal body that coils around the base of its nucleus, showed that the detergent had dissolved some of the parabasal cisternae. Examination of non-detergent-treated lysed cells whose mastigont system remained intact showed that DAPI stained the parabasal bodies of *Snyderella*, *Stephanonympha* and *Caduceia* (Fig. 3).

Varying the DAPI concentration resulted in a pattern of fluorescence of the mastigont component structures (Table 1). Nuclei stained at the lowest concentration, 0.58 μM. The parabasal bodies stained at a higher concentration, 1.45 μM, and the microtubular structures (axonemes and axostyles) stained at the highest concentration, 2.4 μM.

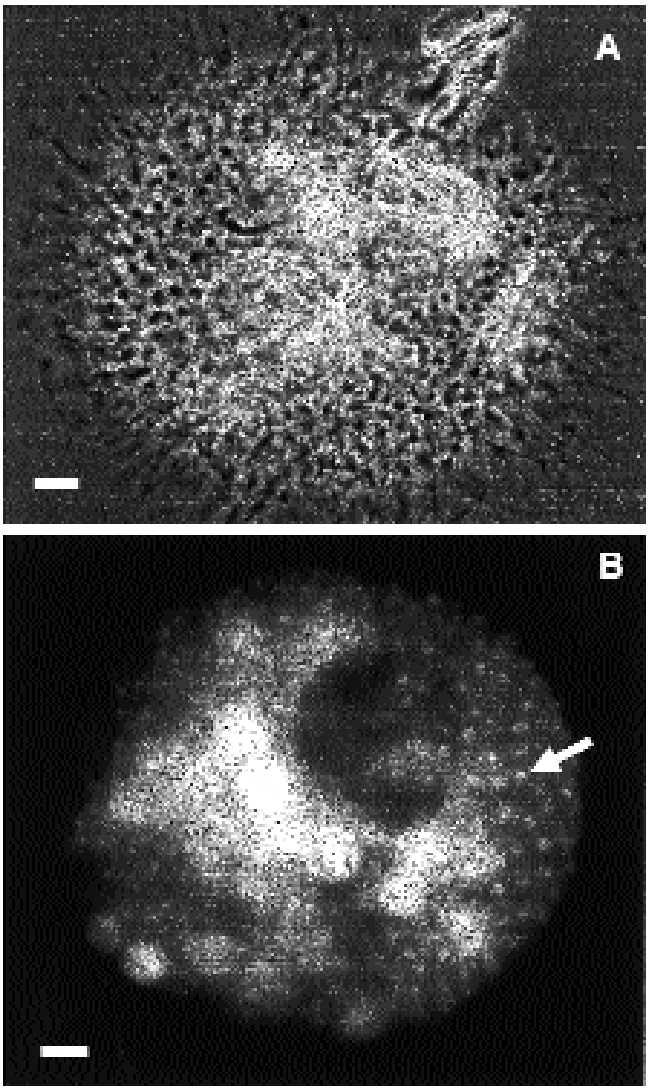

Fig. 1 *Snyderella tabogae.* (A) Phase contrast; (B) DAPI-stained epifluorescence. Akaryomastigonts stained with DAPI (arrow). Bar = 5 μm

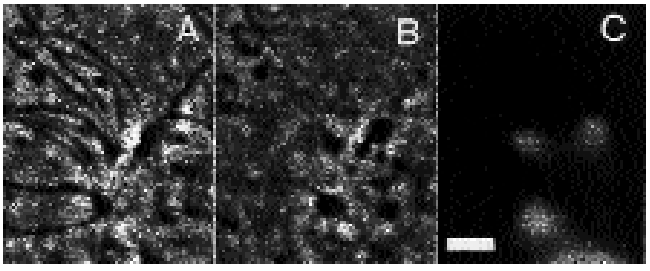

Fig. 2 Three *Snyderella tabogae* akaryomastigonts at the level of the kinetosomes (A), and below them, at the level of the parabasal body (B). DAPI-staining of the three structures in B (C). A and B, phase contrast; C, epifluorescence. Bar = 2 μm

The parabasal body of related calonymphid trichomonads

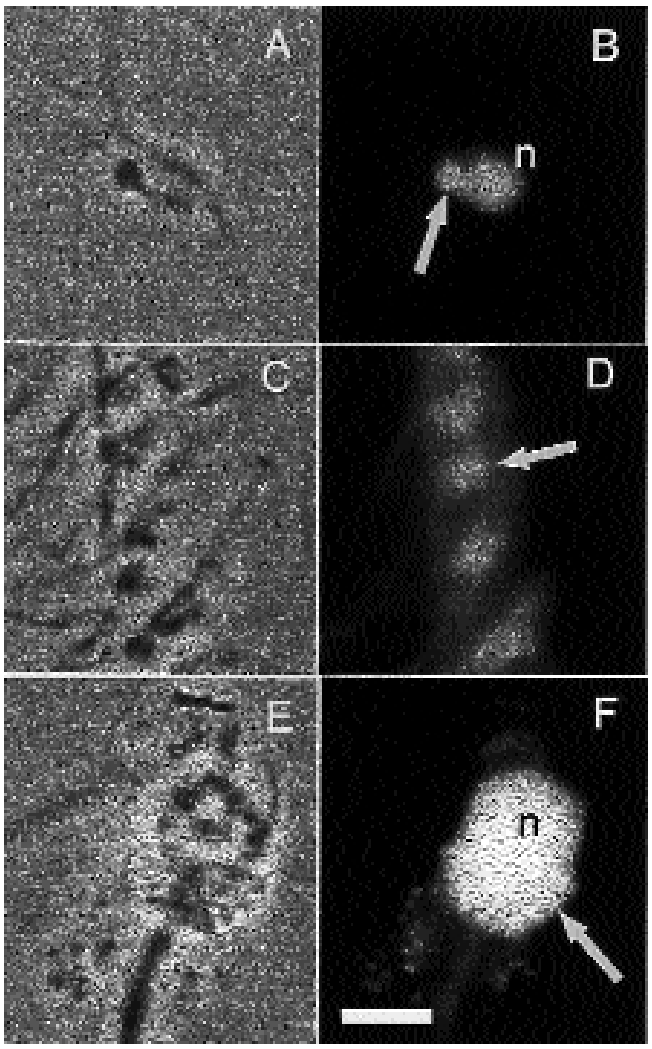

Fig. 3 Isolated mastigonts of *Stephanonympha* sp. (A, B), *Snyderella tabogae* (C, D) and *Caduceia versatilis* (E, F). Note the DAPI-staining of the parabasal bodies (arrows) and the nuclei (N). A, C and E, phase contrast; B, D, and F, DAPI-stained epifluorescence. Bar = 5 μm

(*Coronympha octonaria* and *Metacoronympha senta*) in *Incisitermes* nr. *incisus*, did not stain with DAPI. Neither was such stain seen in other parabasalids, the hypermastigids, *Trichonympha agilis* in *R. flavipes*, and *Trichonympha chattoni* in *I.* nr. *incisus*.

Table 1 *Snyderella tabogae* cell structure fluorescence with DAPI as a function of concentration*

DAPI conc. (μM)	Structures stained
0.29	none
0.58	nuclei and epi- and endosymbiotic bacteria only
1.45	nuclei, bacteria and parabasal bodies
2.40	nuclei, bacteria, parabasal bodies, axostyles

*Cells were not treated with Triton X-100.

Feulgen results Only the nuclei of the three species stained with the Feulgen reagent in cells fixed with picric acid or alcohol. Staining was present in the akaryomastigonts of *S. tabogae* fixed with glutaraldehyde in some cases. Since the Feulgen reagent binds to aldehyde residues, as in acid-treated DNA, this staining was due apparently to aldehyde residue that was bound to or not washed out of Golgi cisternae in the akaryomastigonts.

Discussion

Six different DNA-specific treatments stained the single and multiple nuclei in *C. versatilis*, *Stephanonympha* sp. and *S. tabogae*. Whereas the nucleoids of the regularly associated ecto- and endosymbiotic bacteria stained as well, kinetosomes never stained at all. Neither the DAPI nor the other staining (SYTOX, acridine orange, ethidium bromide, propidium iodide and Feulgen) provide evidence for DNA in or associated with the kinetosomes of *S. tabogae* or any other amitochondriate protist studied here.

By use of detergent-treated and spontaneously isolated mastigont systems of *Snyderella*, *Stephanonympha* and *Caduceia*, it was possible to show that DAPI stained the parabasal bodies, which are densely packed membranous Golgi complexes [2, 26]. The parabasal body apparently also binds or retains other DNA stains except for SYTOX. When the parabasal bodies were treated with detergent they did not stain with DAPI, nor did DAPI staining occur when the cells were pretreated with cold TCA.

The parabasal body is a permanent Golgi complex organized around a fiber that attaches it to the mastigont system in the Parabasalia (trichomonads and hypermastigids). The substances that are usually processed through these organelles have not been described for this class of protists, nor is the chemistry of this membranous-fibrous organelle system known. The same Golgi complex in calonymphids from *Incisitermes snyderi* does not bind DAPI or the other stains, nor do the numerous long, thin parabasal bodies in *Trichonympha*. This suggests that the staining is not due to an accumulation of the stains in the Golgi cisternae, but to binding to material in or on the cisternae. The stain concentration data (see Table 1) suggest a weak binding of DAPI to unidentified material in the parabasal body, analogous to its weak binding to microtubules. While detergent treatment dissolves part of the Golgi cisternae, some membrane is left. The absence of DAPI staining in these partially dissolved cisternae suggests that the basis for the staining is not the membrane itself, but is due to binding to variable quantities of unidentified material within the lumen of the cisternae. The inconsistent results across genera of calonymphids may be due to peculiarities of the microbial ecology of the *Cryptotermes cavifrons* gut versus

that of the two species of *Incisitermes*.

Acknowledgments This work was funded by grants from the Richard Lounsbery Foundation and the NASA Exobiology Program, Office of Space Sciences to Lynn Margulis and by the University of Massachusetts Graduate School.

References

1. Bermudes D, Hinkle G, Margulis L (1994) Do prokaryotes contain microtubules? Microbiol Rev 58:387–400
2. D'Ambrosio U, Dolan MF, Wier AM, Margulis L (1999) Devescovinid with axostyle-based rotary motor ("Rubberneckia"): Taxonomic assignment as *Caduceia versatilis* sp. nov. Eur J Protistol. 35:327–337
3. Dipple RV (1976) Effects of nuclease and protease digestion on the ultrastructure of *Paramecium* basal bodies. J Cell Biol 69:622–637
4. Dolan MF, D'Ambrosio U, Wier AM, Margulis L (2000) Surface kinetosomes and disconnected nuclei of a calonymphid: Ultrastructure and evolutionary significance of *Snyderella tabogae*. Acta Protozool. (In press)
5. Erikson HP, Stoffler D (1996) Protofilaments and rings, two conformations of the tubulin family conserved from bacterial FtsZ to α/β and γ tubulin. J Cell Biol 135:5–8
6. Gifford EM, Lin J (1975) Light microscope and ultrastructural studies of the male gametophyte in *Ginkgo biloba*: The spermatogenous cell. Am J Bot 62:974–981
7. Gilham NW (1994) Organelle genes and genomes. Oxford University Press, New York
8. Hall JL, Ramanis Z, Luck DJL (1989) Basal body/centriolar DNA: Molecular genetics studies in *Chlamydomonas*. Cell 59:121–132
9. Hall JL, Luck DJL (1995) Basal body-associated DNA: *In situ* studies on *Chlamydomomas reinhardtii*. Proc Natl Acad Sci USA 92:5129–5133
10. Hartman H, Puma JP, Gurney T (1974) Evidence for the association of RNA with the ciliary basal bodies of *Tetrahymena*. J Cell Sci 16:241–259
11. Heidemann SR, Sander G, Kirschner MW (1977) Evidence for a functional role of RNA in centrioles. Cell 10:337–350
12. Hirano K-I (1982) Aster formation in sea urchin eggs induced by the injection of enzyme-treated sperm centrioles. Dev Growth Diff 24:273–281
13. Hoffman JC, Vaughn KC (1995) Using the developing spermatogenous cells of *Ceratopteris* to unlock the mysteries of the plant cytoskeleton. Internatl J Plant Sci 156:346–358
14. Johnson DE, Dutcher SK (1991) Molecular studies of linkage group XIX of *Chlamydomonas reinhardtii*: evidence against a basal body location. J Cell Biol 113:339–346
15. Johnson KA, Rosenbaum JL (1990) The basal bodies of *Chlamydomonas reinhardtii* do not contain immunologically detectable DNA. Cell 62:615–619
16. Klotz C, Dabauvalle M-C, Paintrand M, Weber T, Bornens M, Karsenti M (1990) Parthenogenesis in *Xenopus* eggs requires centrosomal integrity. J Cell Biol 110:405–415
17. Kuriyama R (1984) Activity and stability of centrosomes in Chinese hamster ovary cells in nucleation of microtubules *in vitro*. J Cell Sci 66:277–295
18. Kuroiwa T, Yorihuzi T, Yabe N, Ohta T, Uchida H (1990) Absence of DNA in the basal body of *Chlamydomonas reinhardtii* by fluorimetry using a video-intensified microscope photon-counting system. Protoplasma 158:155–164
19. Lee CL (1955) Fertilization in *Ginkgo biloba*. Bot Gaz 117:79–100
20. Margulis L (1993) Symbiosis in cell evolution. 2nd ed. WH Freeman, New York
21. Mizukami I, Gall J (1966) Centriole replication. II. Sperm formation in the fern, *Marsilea* and the cycad, *Zamia*. J Cell Biol 29:97–111
22. Pennell RI, Vondy KP, Bell PR, Hyams JS (1988) Composition and function of the blepharoplast of *Marsilea vestita*. Eur J Cell Biol 46:51–60
23. Pepper DA, Brinkley BR (1980) Tubulin nucleation and assembly in mitotic cells: evidence for nucleic acids in kinetochores and centrosomes. Cell Motil 1:1–15
24. Randall J, Disbrey C (1965) Evidence for the presence of DNA at basal body sites in *Tetrahymena pyriformis*. Proc Roy Soc London B 162:473–491
25. Roobol A, Havercroft JC, Gull, K (1982) Microtubule nucleation by the isolated microtubule-organizing centre of *Physarum polycephalum* myxamoebae. J Cell Sci 55:365–381
26. Rösel J, Radek R, Hausmann K (1996) Ultrastructure of the trichomonad flagellate *Stephanonympha nelumbium*. J Euk Microbiol 43:505–511
27. Sagan L (1967) On the origin of mitosing cells. J Theor Biol 14:225–274
28. Smith-Sonneborn J, Plaut, W (1967) Evidence for the presence of DNA in the pellicle of *Paramecium*. J Cell Sci 2:225–234
29. Sogin ML, Silberman JD, Hinkle G, Morrison HG (1996) Problems with molecular diversity in the eukarya. In: Roberts DM, Sharp P, Alderson G, Collins MA (eds) Society for General Microbiology Symposium. Evolution of microbial life. Cambridge University Press, Cambridge, UK, pp 167–184
30. Sonneborn TM (1970) Determination, development and inheritance of the structure of the cell cortex. In: Padykula HA (ed) Control mechanisms in the expression of cellular phenotype. Academic Press, New York, pp 1–13
31. Trager W (1934) The cultivation of a cellulose-digesting flagellate, *Trichomonas termopsidis*, and of certain other termite protozoa. Biol Bull 66:182–190
32. Younger KB, Banerjee S, Kelleher JK, Winston M, Margulis L (1972) Evidence that the synchronized production of new basal bodies is not associated with DNA synthesis in *Stentor coeruleus*. J Cell Sci 11:621–637
33. Yuasa A (1935) Studies in the cytology of reproductive cells. IV. Feulgen's nucleal staining applied to the blepharoplast. Bot Mag 49:868–875