

SURFACE ORGANIZATION AND COMPOSITION OF *EUGLENA*

II. Flagellar Mastigonemes

G. B. BOUCK, A. ROGALSKI, and A. VALAITIS

From the Department of Biological Sciences, University of Illinois, Chicago, Illinois 60680

ABSTRACT

The surface of the *Euglena* flagellum is coated with about 30,000 fine filaments of two distinct types. The longer of these nontubular mastigonemes (about 3 μm) appear to be attached to the paraflagellar rod whereas the shorter nontubular mastigonemes (about 1.5 μm) are the centrifugally arranged portions of a larger complex, which consists of an attached unit parallel to and outside of the flagellar membrane. Units are arranged laterally in near registration and longitudinally overlap by one-half of a unit length. Rows of mastigoneme units are firmly attached to the axoneme microtubules or to the paraflagellar rod as evidenced by their persistence after removal of the flagellar membrane with neutral detergents. SDS-acrylamide gels of whole flagella revealed about 30 polypeptides, of which two gave strong positive staining with the periodic acid-Schiff (PAS) procedure. At least one of these two bands (glycoproteins) has been equated with the surface mastigonemes by parallel analysis of isolated and purified mastigonemes, particularly after phenol extraction. The faster moving glycoprotein has been selectively removed from whole flagella and from the mastigoneme fraction with low concentrations of neutral detergents at neutral or high pH. The larger glycoprotein was found to be polydisperse when electrophoresed through 1% agarose/SDS gels. Thin-layer chromatography of hydrolysates of whole flagella or of isolated mastigonemes has indicated that the major carbohydrate moiety is the pentose sugar, xylose, with possibly a small amount of glucose and an unknown minor component.

KEY WORDS mastigonemes · flagella
surface · peptido-xylan · *Euglena*

The relatively stable surface of *Euglena* has facilitated the mapping of the insertion of new surface antigens by means of antibody-labeled surface markers (14). These studies indicate that surface materials are interpolated in a regular pattern between preexisting spiral strips, and suggest the presence of surface domains in which antigens do not freely diffuse laterally, unlike the mobile antigens in many other membrane systems (31).

The membrane of the *Euglena* flagellum while continuous with the plasma membrane of the cell surface is also a region of membrane specialization which consists in part of well-defined, microscopically visible components which do not disperse throughout the nonflagellar regions of the cell surface. It is the purpose of this report to detail the organization, distribution, and composition of these flagellar surface components, with the ultimate aim of understanding the underlying mechanisms which segregate and maintain surfaces on the flagellum and elsewhere on the cell.

In general, two distinct types of flagellar surface appendages (mastigonemes) have been identified from a variety of organisms bearing at least one anteriorly directed flagellum. Tubular mastigonemes, characterized the most extensively, are found (a) to be relatively uniform structurally among different organisms but differ in detail from phylum to phylum, (b) to consist of one or more proteins or glycoproteins (3, 6, 7), (c) to originate within the perinuclear continuum-endoplasmic reticulum-Golgi apparatus compartments (cf reference 4), (d) to be firmly attached to the axoneme microtubules (19) and, (e) to reverse the normal thrust of the flagellum so that the organism is capable of movement in the same direction wave propagation (16). Nontubular mastigonemes may be present on the same flagellum as tubular mastigonemes or present alone on the flagellar surface of many flagellates. These mastigonemes, although not well characterized, are also glycoprotein (39), but they do not reverse the thrust of the flagellum. The widespread distribution of nontubular mastigonemes, their extraflagellar location, and, in some cases, their extensive disposition along the flagellar surface all suggest that these mastigonemes assume a major role in flagellar movement and must be incorporated into realistic models of flagellar development and function in lower organisms.

The nontubular mastigonemes of many Euglenoids form both a short, thick felt and an array of longer elements on the locomotory or emergent flagellum (17). The thickness of the felt has obscured details of mastigoneme attachment in whole flagella, but in section (20) or in frayed preparation (17) the mastigonemes are reported to be arranged along a spiral band which extends the length of the flagellum. In the present study, which employs a combination of sections, negative staining, and selective solubilization of the surface membrane and isolated mastigonemes, it has been found that the external felt consists of numerous, elaborate but uniform "units" of mastigonemes arranged parallel to the flagellar surface. Some of these units are attached to the underlying axonemal microtubules. SDS-acrylamide and SDS-agarose gels together with thin-layer chromatography of acid-hydrolyzed mastigonemes has revealed that mastigonemes consist of at least one major glycoprotein in which the predominant sugar is xylose. This large glycoprotein is polydisperse and may consist of two or more additional glycoproteins. The absence of presumptive intracellular

mastigonemes during flagellar regeneration or during cell division suggests that the assembly of these mastigonemes may be different than that of nontubular mastigonemes.

MATERIALS AND METHODS

Culture and flagella isolation

Euglena gracilis was grown under constant illumination in acetate-containing medium (8) either in 1-liter Ehrlenmeyer flasks or in larger quantities of 16 liters in 20-liter carboys. 5 Days to 1 wk after inoculation, cells were harvested in 500-ml batches at low centrifugal force to avoid the premature deflagellation induced by the trauma of excessive packing. Pellets from several batches were combined and deflagellated in 4-ml portions by agitation in a fluted glass tube on a Vortex homogenizer (Scientific Industries, Inc., Bohemia, N.Y.) (29) or by resuspension in ice-cold fresh medium for 1-2 h. The latter method gave consistently cleaner preparations and was adopted for most of the biochemical work. Whole cells were removed from the suspension by centrifugation for 2-4 min at top speed (1,500 g) in an International clinical centrifuge (International Scientific Instruments, Inc., Mountain View, Calif.). The supernate containing the flagella was recentrifuged at 17,500 rpm in the SS-34 rotor of a Sorvall RC2B centrifuge (Du Pont Instruments-Sorvall DuPont Co., Wilmington, Del.), thereby yielding a whitish pellet.

Removal of Flagellar Membrane and

Isolation of Mastigonemes

The flagellar membrane was readily removed by a variety of methods and different neutral and anionic detergents. The neutral detergents Nonidet P-40 (Shell Chemical Co., New York, N.Y.) at 0.25% wt/vol and Triton X-100 (Spectrographic grade, Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.) at 0.25% wt/vol were used together or separately in 0.1 M piperazine *N,N'*-bis[2-ethane sulfonic acid] (PIPES) buffer at a pH of 7.0. The anionic detergent Sarkosyl (sodium lauryl sarcosinate, Ciba-Geigy Corp., Piscataway, N.J.) at 1.5% wt/vol in PIPES buffer or water alone solubilized all electron microscopically visible axonemal components except mastigonemes, if left overnight. Mastigonemes could then be collected by resuspension in distilled water and centrifugation at 40,000 rpm in the SW50.1 rotor of a Spinco 65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Usually the pellet was resuspended again in distilled water or buffer to remove residual Sarkosyl.

Preparation of Flagella and

Mastigonemes for Electrophoresis

Whole flagella were reduced in 8 M urea and 1 mM mercaptoethanol and alkylated in iodoacetate essentially

as described by Renaud et al. (28). Samples were then dialyzed overnight against reservoir buffer [0.1 M sodium phosphate, pH 7.2, 1% sodium dodecyl sulfate (SDS)]. Purified mastigonemes did not readily dissolve in SDS or 8 M urea even when placed in a boiling water bath for 5 min. However, aqueous solutions of phenol were used with success as follows (cf references 18, 41). Purified mastigonemes were stirred in a cold suspension of 50% phenol in water for 15 min. The suspension was centrifuged at 7,000 rpm in the Sorvall HB-4 rotor for 15 min, producing a sharp separation of the phenol and water. The aqueous phase and the precipitate at the phenol/water interface were dialyzed against distilled water overnight at 4°C. The dialysate usually dissolved completely, but any remaining residue was pelleted by centrifugation and solubilized in a drop or two of mercaptoethanol. The fractions were combined and dialyzed overnight against two changes of one-tenth strength reservoir buffer at 4°C. The solution was then made 1% in SDS and 5 mM in mercaptoethanol.

Acrylamide and Agarose Gels

SDS-acrylamide gels and buffers were prepared according to Weber et al. (37) in 5-mm diameter glass tubes or in 1-mm thick slab gels cast in a Hoefer (Hoefer Scientific Instruments, San Francisco, Calif.) slab gel apparatus. Agarose (low EEO, Sigma, or Seakem LE agarose, Marine Colloids, Inc., Rockland, Me.) was dissolved in reservoir buffer after the precautions outlined in Peacock and Dingman (25). Gels were cast in a prewarmed (to 45°C) Hoefer apparatus and allowed to solidify at room temperature. Tube gels were run without stacking gels at 2 mA/gel whereas slab gels were operated at 75 mA for about 4 h. Pre-cooled water was circulated in the Hoefer apparatus to avoid overheating. Sample viscosity was increased by addition of a few sucrose crystals, and the running front was tracked with bromophenol blue. After electrophoresis, acrylamide gels were fixed in 50% wt/vol trichloroacetic acid (TCA) for at least 2 h, followed by staining for proteins with 0.1% Coomassie blue in 50% TCA or for carbohydrates with the periodic acid-Schiff (PAS) procedure. Agarose gels will not fix in TCA and were therefore soaked in 7.5% acetic acid overnight. Coomassie blue and PAS staining were carried out as with acrylamide gels, except that 7.5% acetic acid was substituted for TCA as a dye solvent. After destaining in 7.5% acetic acid, agarose gels were photographed and then dried on flat glass plates. By careful scraping, the dried gel could be removed and mounted on index cards for permanent record. These gels shrank somewhat during drying but retained the relative positions of stained components. Tubular gels were scanned at 665 nm for Coomassie blue and at 550 nm for PAS in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a linear transport and modified by insertion of a 16 × Zeiss apochromat objective between light source and the gel.

Thin-Layer Chromatography

Samples of Sarkosyl-purified mastigonemes were resuspended in 1 N hydrochloric acid and sealed under vacuum in 5 ml ampules. Hydrolysis was carried out by placing the sealed ampule in a boiling water bath for periods of 15 min to 24 h. After hydrolysis, the solution was neutralized by mixing with Dowex-1 resin (bicarbonate form) (Dow Chemical Co.) for 5 min, followed by centrifugation to remove the resin beads. The supernate was then deproteinized (32) and applied to thin-layer plates prepared as follows: a slurry consisting of 35 g of microcrystalline cellulose DS-O (Sargent-Welch Scientific Co. Skokie, Ill.) in 180 ml of distilled water was spread in a uniform layer 250 nm thick on glass plates 20 cm square, using a mechanical spreader (VWR Scientific Inc., Subsidiary of UNIVAR, San Francisco, Calif.). The plates were dried overnight at 25°C. Ascending chromatography was performed using several solvent systems: butanol-pyridine-water (6:4:3, reference 12 or 5:3:2, reference 13) or ethyl acetate-pyridine-acetic acid-water (5:5:1:3, reference 5). Sugar detection was accomplished with alkaline silver nitrate (35) or aniline phthalate (24). Since the developed plates frequently faded rapidly, a contact print was made immediately after sugar detection, and spots were carefully outlined on the print. Subsequently, drawings were made from tracings of the contact print or, when possible, from the original plate. Sugar standards were purchased (Sigma Chemical Co., St. Louis, Mo.), and *Euglena* pellicle was prepared by the method described previously (14). *Euglena* starch (paramylon) was obtained from the pellet produced during the final isopycnic centrifugation of pellicle purification. The pellet, after resuspension in distilled water and recentrifugation, contained nearly pure starch. Both starch and pellicle were hydrolyzed and neutralized under conditions identical to those used to treat mastigonemes.

Electron Microscopy

Whole cells were fixed, stained, and sectioned as described previously (14). Isolated flagella were centrifuged into a pellet and subsequently handled as small compact fragments. During embedding, an effort was made to orient the fragments so that sectioning could be performed perpendicular to the direction of flagellar orientation. Negative staining was carried out in 1% aqueous uranyl acetate, and electron microscopy was performed in a Hitachi 11E electron microscope.

RESULTS

The emergent flagellum of *Euglena* is about 25 μm long and consists internally of the usual axonemal microtubules together with a characteristic paraflagellar rod (20). The latter structure, which has an ordered paracrystalline structure, assumes no consistent orientation with respect to the central

pair of axonemal microtubules. Externally a massive coating of fine nontubular mastigonemes extends from the flagellar surface, but this coat is too thick to characterize clearly details of the flagellar surface in whole flagella negatively stained with uranyl acetate. However, after rapid treatment with a mixture of 0.1% Triton X-100 and Nonidet P-40, the flagellum flattens sufficiently so that mastigoneme attachment and structure can be readily determined. Such preparations (Fig. 1) reveal that the external felt described by earlier workers consists of numerous, fine, nontubular mastigonemes which individually range up to about $1.5 \mu\text{m}$ in length and about 50 \AA in diameter. No specific orientation of mastigonemes can be seen in such preparations. Longer (up to $3.5 \mu\text{m}$) and thicker (ca. 100 \AA) nontubular mastigonemes became easily detached in such preparations, but when mild detergent treatment is carried out directly on flagella self-stuck to carbon-coated EM grids occasional evidence is obtained which suggests that these longer mastigonemes arise singly or in small clusters of three to four attached to the paraflagellar rod (Figs. 2 and 3). These findings are consistent with earlier interpretations of the unilateral distribution of longer mastigonemes observed in shadow-cast material (17). The longer nontubular mastigonemes are flexible in appearance, often extend into a still finer distal filament, and taper proximally at the point of attachment to the paraflagellar rod.

The relationship of the short nontubular mastigonemes to the axoneme can be better resolved in intact or detergent-treated flagella embedded and sectioned. In median longitudinal or glancing section (Figs. 4 and 5), it is immediately apparent that mastigonemes are arranged in tiers, about $1,200\text{--}1,400 \text{ \AA}$ apart, which presumably correspond to portions of the spiral band noted earlier by others (17, 20). The wavy appearance of the membrane reflects the greater tenacity of portions of the flagellar membrane to the underlying axonemal microtubules. In longitudinal view, a fine intraflagellar filament connecting each attachment can be found occasionally (Fig. 4). Flagella prepared with the membrane still intact show, in glancing section, a network of regularly spaced components lying parallel to the long axis of the flagellum (Fig. 5). A study of this and other tangential views of the flagellar surface suggests that the network lies outside the flagellar membrane, and that the intraflagellar filament is distinct from this network.

After treatment of isolated flagella with low concentrations (0.1–0.5%) of neutral detergents, the surface membrane is generally removed but, in many cases, the mastigonemes remain attached to the flagellum even after fixation and embedment (Figs. 6*a–b* and 7). Tangential views of these extracted flagella show the same pattern of ordered surface components (Fig. 6*a*), while in median section these components can be seen to form a layer at about the level of the flagellar membrane before its removal. Extending centrifugally from these surface components are the short nontubular mastigonemes which can be identified at regular intervals of about $1,200\text{--}1,400 \text{ \AA}$. Each tuft or tier appears to be anchored, by means of a detergent-resistant fraction, to the underlying axonemal microtubules or to the flagellar paracrystal in that region where the paracrystal overlies the microtubule doublet (Fig. 6*b*). The nature of that anchorage is difficult to resolve, but in transverse section fine connections to the axonemal doublets are occasionally preserved after detergent treatment (Fig. 7).

Complete dissociation of the axonemal microtubules and membrane with 1.5% Sarkosyl releases the surface components either in large segments (Fig. 8) or in smaller fractions (Figs. 9 and 10). The regular periodicity seen in sections can now be recognized in these negatively stained fragments at about $1,400\text{-\AA}$ intervals. Nearly complete dissociation of the fragments produces images which resolve the ultimate mastigoneme "unit." Each unit which is about $2,800 \text{ \AA}$ long has a well-defined and remarkably uniform organization (Fig. 10), consisting at one end (herein referred to as the proximal end) of a cluster of fuzz attached to a small loop of about 100-\AA outside diameter, which in turn is attached to a shaft with several distinct regions. Near the lower portion of the shaft a second larger loop overlies the shaft. This loop often becomes dislodged from the shaft axis (Fig. 9) and may lie at some angle from the shaft held by attachment only at one point, indicating that shaft continuity is not dependent on the large loop. The distal one-third of the shaft possesses fine lateral elements which apparently serve to maintain the near-registration of adjacent units (Fig. 10). At the distal pole, the shaft is modified into a dendritic collection of elements some of which extend as the filaments of the short nontubular mastigonemes. Units are aligned in slightly oblique rows laterally and overlap in the longitudinal direction by about one-half

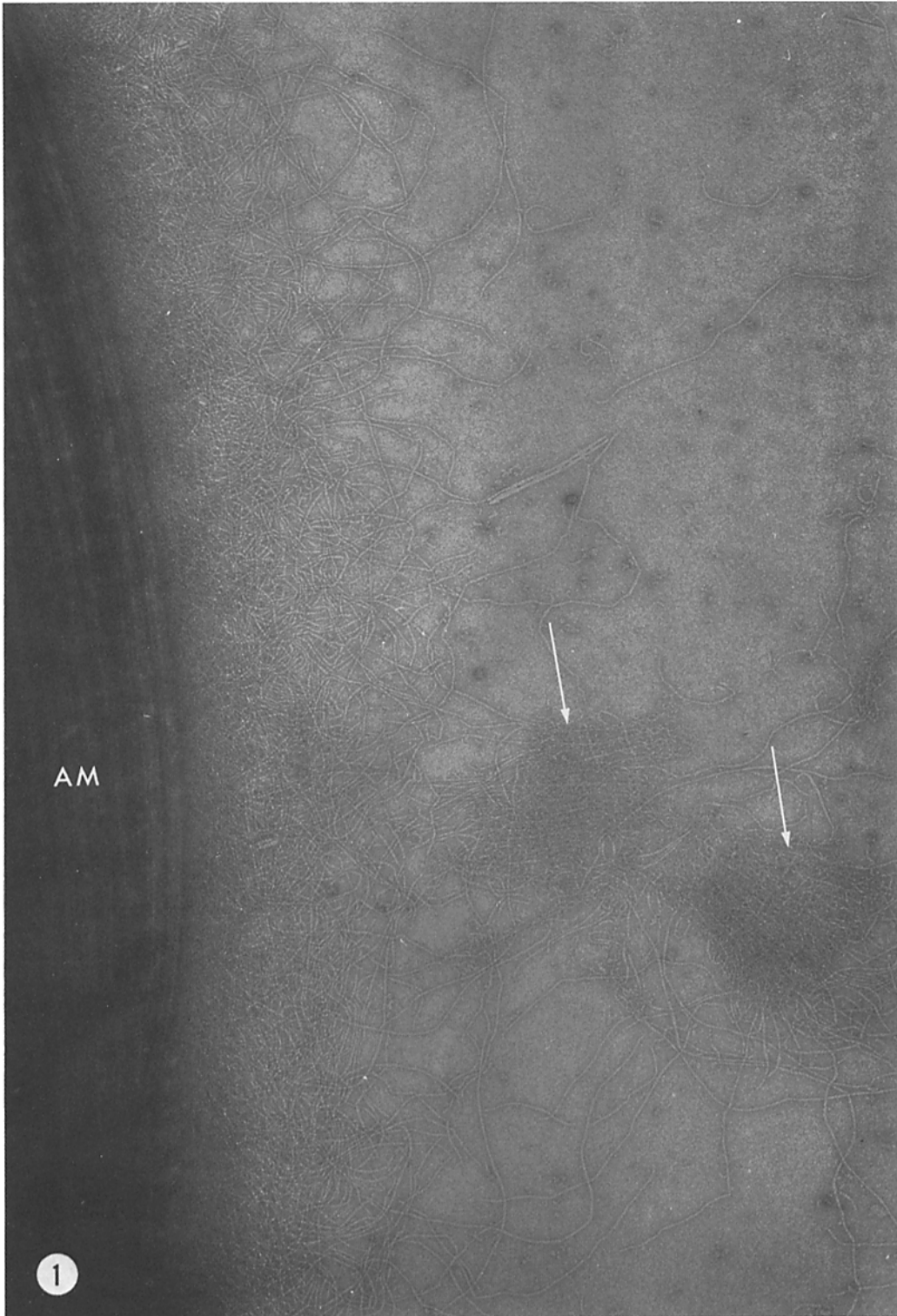
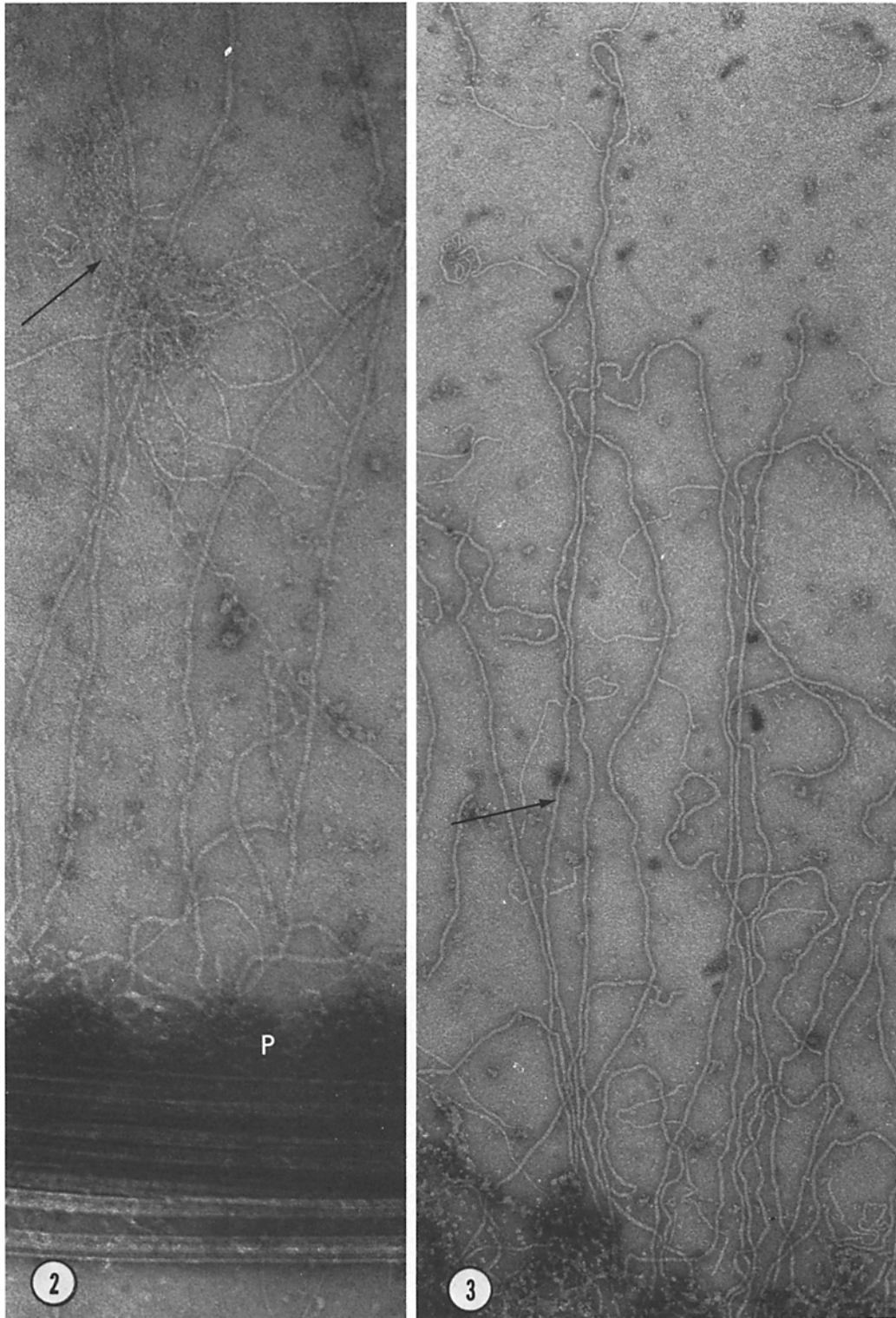


FIGURE 1 Negatively stained, detergent-treated, flattened flagellum of *Euglena gracilis* illustrating the profusion of short nontubular mastigoneme filaments. Several clusters of mastigoneme units have separated from the flagellar surface (arrows). *AM*: axoneme microtubules. $\times 54,000$.



FIGURES 2-3 Long nontubular mastigonemes of *Euglena* prepared by treatment of flagella with detergent while attached to the carbon-coated grid. In Fig. 2, mastigonemes appear to be attached directly to remnants of the paraflagellar rod (*P*). Note terminal (distal) narrowing of mastigonemes and general flexible appearance of whole structure. Relative size of short nontubular mastigoneme (arrow) is evident. In Fig. 3, the entire length of one mastigoneme (arrow) can be measured at about 3.2 μm . Fig. 2, $\times 72,000$. Fig. 3, $\times 46,000$.

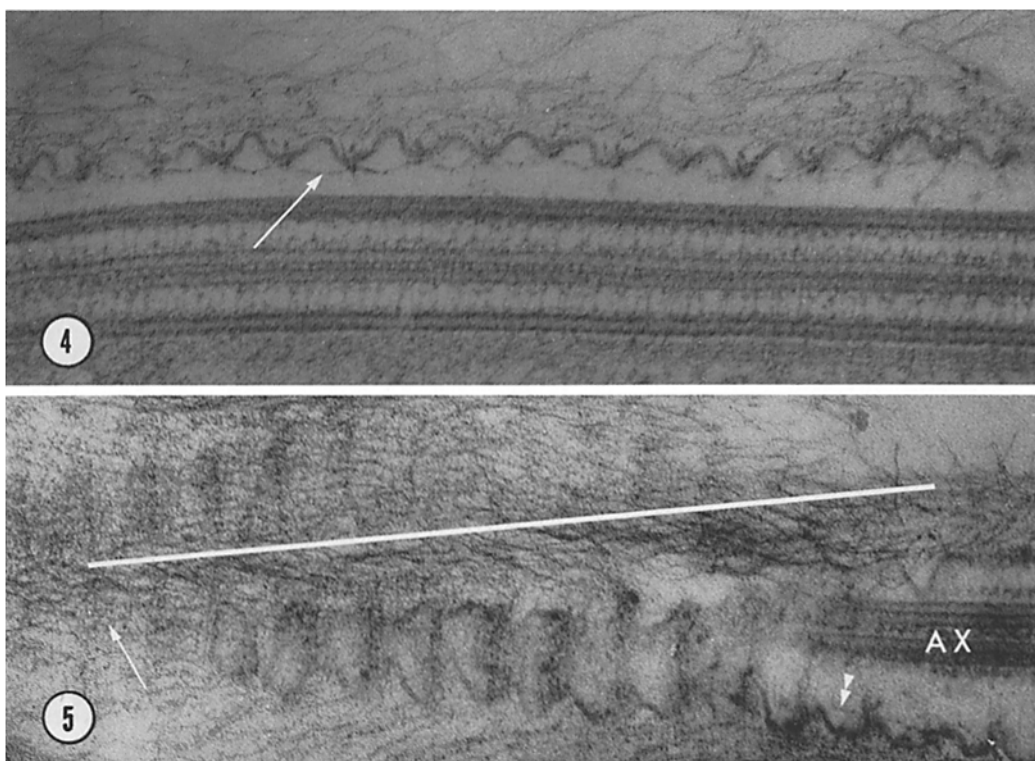


FIGURE 4 Portion of a sectioned flagellum with membrane still intact but separated from the axoneme during preparation. Wavy appearance of membrane is probably due to greater adherence of those parts of the surface from which mastigoneme filaments arise. Fine internal connecting filament (arrow) is not present in all preparations, and may represent a longitudinal element binding mastigoneme units to the axoneme. $\times 74,000$.

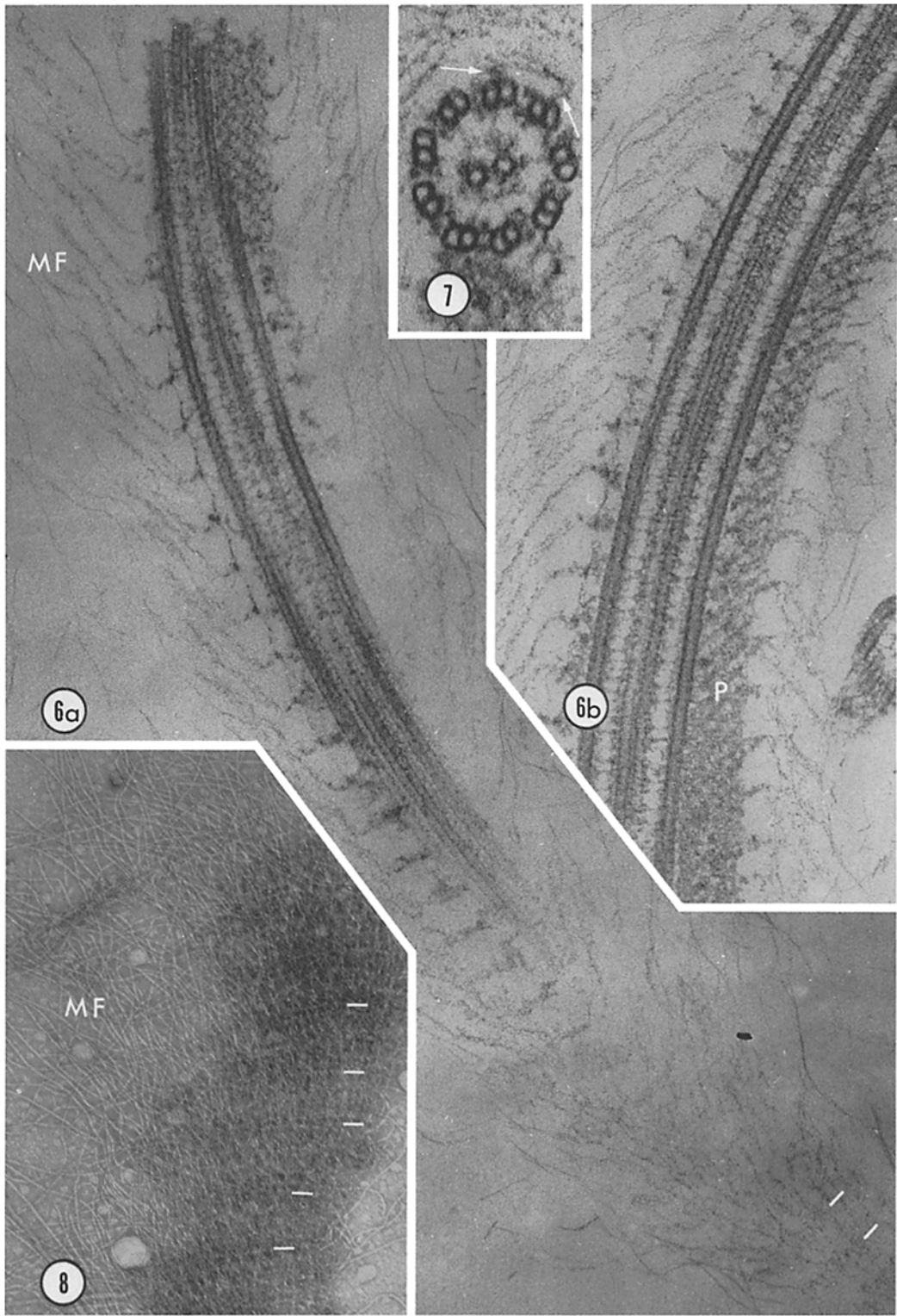
FIGURE 5 In this tangential view of two flagella (separated by white line), the extraflagellar location of mastigoneme units can be seen. In lower flagellum, from right to left, progressively more centrifugal portions of the flagellum are revealed, and more of the network is exposed. A small portion of the intraflagellar filament is visible at double arrowhead. $\times 78,000$.

of a unit length (about $1,400 \text{ \AA}$). The point of attachment to the flagellar axoneme appears to be at the point at which the mastigoneme filaments arise from the units (cf. Figs. 6a and b). However, since mastigoneme units overlap by half a length, which is about the position of the large loop on the shaft from the next tier of units on the flagellum, it is also possible that the loop itself provides the attachment.

Origin of Mastigonemes

Attempts to follow the origin and development of nontubular mastigonemes were made by sectioning cells after mechanical deflagellation or during the course of flagella duplication during cell division. The Golgi complex (cf reference 23) and endoplasmic reticulum (ER) were carefully

scrutinized to find evidence for presumptive mastigonemes similar to those characteristic of early mastigoneme ontogeny in regenerating cells with tubular mastigonemes. In no case was there evidence for recognizable mastigonemes in any cell compartment in *Euglena*. However, the reservoir chamber from which the flagella arise is lined with fine appendages similar to the nontubular mastigonemes found on the flagellum. These appendages are present regardless of the stage of flagellar regeneration and are often confined to the extreme peripheral region of the reservoir due to the presence of membrane-limited sacs (Fig. 11). The origin of these sacs is not clear, but they may be products of the contractile vacuole system which is reported to empty its contents into the reservoir (17).



Acrylamide Gels of Flagella and Purified Mastigonemes

Electrophoresis in 7.5% acrylamide SDS gels of isolated whole flagella produced ~30 Coomassie blue-staining bands. Flagellar tubulin (from axonemal microtubules) was identified by coelectrophoresis with pig brain tubulin prepared by two cycles of polymerization/depolymerization (2), and by comparison of relative mobilities with mobilities of protein standards with known molecular weights. As expected, flagellar tubulin comprises one of the most prominent bands, migrating well into the gel (Fig. 12a). An equally prominent staining band barely enters the gel surface even after reduction and alkylation of the sample. Similar preparations stained with PAS (Fig. 12b) yield two prominent bands: one band is identical to the slow migrating band in protein-stained gels, the other PAS-positive band is only faintly visible in the corresponding region of the Coomassie blue-stained gel. After phenol extraction of whole flagella, the aqueous phase almost selectively contains high molecular weight components, one of which stains strongly with Coomassie blue (Fig. 12c), whereas PAS staining of similar gels produces two prominent bands (Fig. 12d). A few minor additional PAS-positive bands not seen in the SDS/urea-treated flagella are evident in phenol-insoluble extracts.

To further separate the two major PAS-positive components, whole flagella were treated at 4°C in a solution containing 0.3 M Tris, pH 8.0 and 0.1% Nonidet P-40. After 30 min, the solution was centrifuged at 17,500 rpm in the SS-34 Sorvall rotor, the pellet (*N*-pellet) was reduced and alkylated as usual, while the supernate from the detergent treatment was centrifuged over 40% wt/vol sucrose at 25,000 rpm for 30 min in the SW50.1 Spinco rotor in order to remove any

contaminating whole axonemes. The supernate (*N*-supernate) was also reduced and alkylated, and both *N*-pellet and *N*-supernate were electrophoresed on SDS 7.5% acrylamide gels. The *N*-pellet produced a pattern of bands, after protein staining, similar to that of unextracted whole flagella, except for the loss of several low molecular weight peptides (Fig. 12g). However, PAS staining of similar gels of *N*-pellets revealed the loss of one of the major PAS-positive bands (Fig. 12e), whereas the *N*-supernate when run on gels and stained with PAS produced the complementary, single, faster moving PAS-positive band (Fig. 12f).

Acrylamide and Agarose SDS Gels of Isolated Mastigonemes

In order to identify specific bands on whole flagella gels, a mastigoneme fraction was isolated by Sarkosyl treatment (cf. Materials and Methods), solubilized, and electrophoresed in a manner similar to that for whole flagella. The pattern obtained after phenol extraction of these fractions alone was virtually identical to that obtained from PAS-stained whole flagella (Fig. 13a); i.e., two prominent bands, one migrating a short distance into the gel, the other well resolved. Efforts to remove the faster migrating PAS-positive band from these isolates by the use of low concentrations of Nonidet P-40 in Tris buffer (as above) produced essentially the same result obtained with whole flagella. Much, but usually not all, of the faster migrating band was removed (Fig. 13b), and the remaining insoluble fraction (*N*-pellet) consisted primarily of a large glycoprotein (Fig. 13b-c) unresolved in this gel system.

The uppermost poorly resolved band was further characterized by applying phenol-purified fractions to a series of increasingly porous acryl-

FIGURE 6 (*a* and *b*) Fixed, embedded, and sectioned flagellum after treatment with 0.25% Nonidet and 0.25% Triton X-100 in PIPES buffer at neutral pH. Membrane is removed but fibrous material remains at membrane level. Mastigoneme filaments (*MF*) arise centrifugally from the flagellar surface. In tangential view (lower right), the fine network of fibers can be seen in tiers (hash marks) corresponding to the complex of material which gives rise to mastigoneme filaments. Note attachment of mastigoneme tiers directly to both axoneme microtubules and the paraflagellar rod (*P*). (*a*) $\times 64,000$. (*b*) $\times 76,000$.

FIGURE 7 Transverse section of flagellum prepared as in Fig. 6a and b. Note fine connections (arrows) to axoneme doublets. $\times 116,000$.

FIGURE 8 Negatively stained portion of mastigoneme network obtained by treatment of flagella with 1.5% Sarkosyl. Regular periodicity (hash marks) indicates portion of complex from which mastigoneme filaments (*MF*) arise. $\times 64,000$.

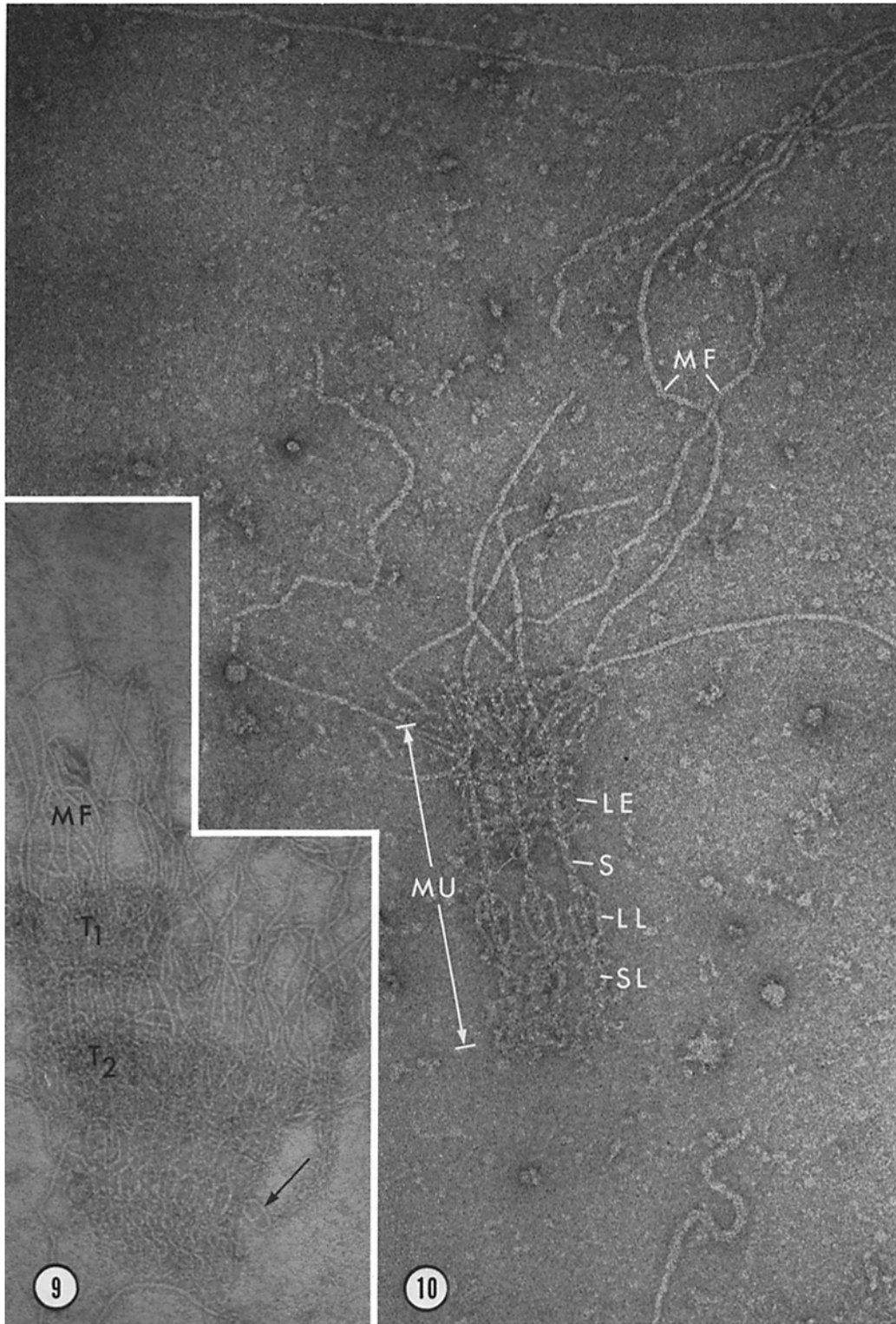


FIGURE 9 Portion of two tiers of mastigoneme units. Uppermost tier (T_1) is only half as wide as lower tier (T_2), thereby illustrating how units overlap and how tiers give rise to mastigoneme filaments (MF). Upper tier has separated slightly from lower tier in this preparation so that distance between tiers is greater than on the flagellar surface. A large loop of the mastigoneme unit has become partially dislodged from the unit shaft in this preparation (arrow). $\times 85,000$.

FIGURE 10 Cluster of three mastigoneme units (MU) with distal extension into mastigoneme filaments (MF). Small loop of unit (SL), large loop of unit (LL), lateral elements (LE), and unit shaft (S) can all be identified in this preparation. $\times 141,000$.



FIGURE 11 Transverse section through a dividing cell in which two sets of flagella (F_1 and F_2) can be seen extending into the reservoir (R). Filamentous material can be seen lining the reservoir membrane. This material is similar in appearance to the nontubular mastigoneme filaments on the flagellum, RS, reservoir sacs. $\times 51,000$.

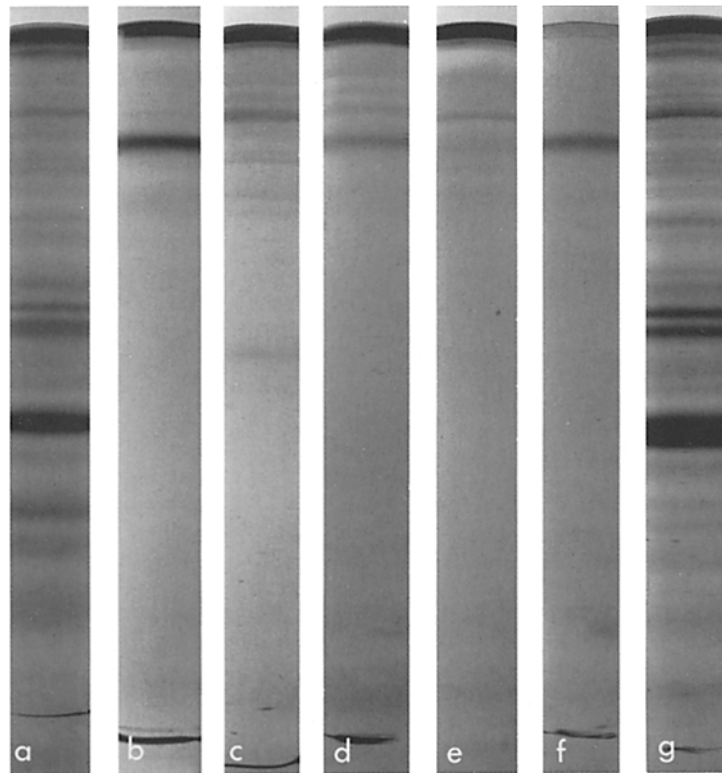


FIGURE 12 SDS-acrylamide gels: (a) Whole flagella—Coomassie blue; (b) whole flagella—PAS; (c) whole flagella, aqueous phase after phenol extraction—Coomassie blue; (d) same as gel c but PAS; (e) whole flagella after extraction with Tris/Nonidet—PAS; (f) extract from gel e—PAS; and (g) whole flagella after extraction with Tris/Nonidet P-40—Coomassie blue. Gel in a was loaded with same amount of protein as gel in b. Gels c and d were also loaded with identical amounts of protein.

amide/agarose and finally pure SDS 1% agarose gels. All the PAS-positive material migrated into the gel only when 1% agarose SDS gels were used, and the inclusion of mercaptoethanol in the sample was essential for complete entry. The bands that were obtained were diffuse but could be resolved into three or four zones after electrophoresis and staining with PAS (Fig. 14). Coomassie blue staining of similar gels was weak and variable with different runs. Absorbance scans of whole and detergent extracted flagella are presented in Figs. 15–17.

Thin-Layer Chromatography (TLC)

Hydrolysis of purified mastigonemes for 3 h in 1 N HCl followed by neutralization and development on thin-layer cellulose plates, using a butanol-pyridine-water solvent, produced a single prominent and several minor spots after spraying with alkaline silver nitrate. Co-chromatography

with known standards indicates that the mastigoneme hydrolysate contained a predominant sugar with a migration pattern nearly identical with that of the pentose sugar xylose (Figs. 18 and 19). Changing the hydrolysis time from 3 h to 15 min or reducing the concentration of HCl to 0.1 N produced the same pattern of major and minor spots. To assess the degree of possible cellular contamination, two other *Euglena* components with known carbohydrate content were run simultaneously (Fig. 18). One of the minor mastigoneme spots may be coincident with starch or a pellicle-derived glucose, but the major xylose area was not present in either starch or pellicle hydrolysates. To distinguish xylose from fucose which migrates similarly in this solvent system, a second solvent consisting of pyridine/acetic acid/water and different developing reagent (aniline phthalate) was utilized. This combination, although less sensitive in revealing minor spots, produced chromatograms in which xylose and

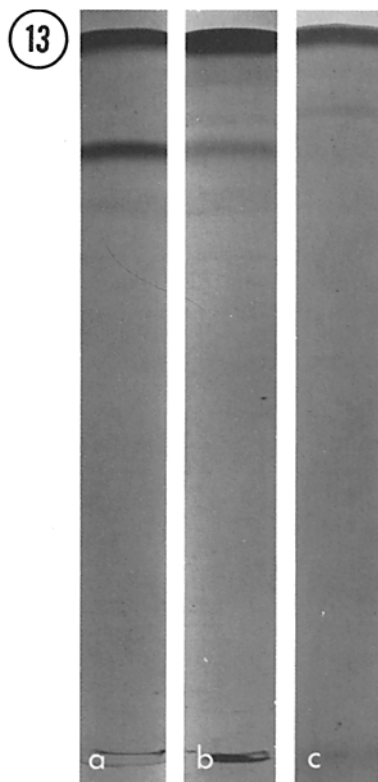


FIGURE 13 (a) Whole flagella-PAS; (b) Fraction mastigoneme extracted with Tris/Nonidet P-40-PAS; and (c) same as gel b-Coomassie blue.

fucose could be distinguished both in migration pattern and in their color reactions. These chromatograms illustrate (Fig. 20) that the mastigoneme hydrolysate still comigrates with xylose and produces the characteristic red color of an aldopentose. In neither solvent system could positive identity of the fast moving minor spot be identified from available standards.

DISCUSSION

Despite the profusion of mastigonemes on the Euglenoid flagellum, earlier studies have clearly indicated that these components are not randomly distributed. Leedale (17) has suggested for images of shadow-cast material that the longer mastigonemes are arranged only along one side of the flagellum, and Mignot (20) has shown in sectioned flagella that mastigonemes are attached in a spiral band. Furthermore, a surface network oriented parallel to the flagellar surface is clearly illustrated in the studies of Piccinni et al. (27) after partial dissociation of the flagellar membrane with the



FIGURE 14 SDS 1% agarose slab gel of phenol-purified mastigonemes. Three broadly diffuse bands (arrowheads) have entered the gel.

neutral detergent digitonin. These separate studies together with the results of this report can be reconciled in a comprehensive model of the Euglenoid flagellar surface (Figs. 21-24). Thus, it is proposed that the flagellar membrane is overlaid by a network of numerous, laterally aligned, longitudinally overlapping mastigoneme units which may produce a two-start helix running from base to tip. However, it should be cautioned that neither the polarity of the units nor the presence of helical orientation has yet been directly demonstrated. Each mastigoneme unit has a similar and remarkable substructure consisting of additional loops and fine elements. It seems probable that the precise near-alignment is maintained by the lateral arms of each unit, and that the larger loop may provide adherence between overlapping tiers of units and possibly also in the binding of the mastigonemes to the axonemal microtubules. The distal portion of each unit is elaborated into relatively long mastigoneme filaments which arise more or less perpendicular to the surface and

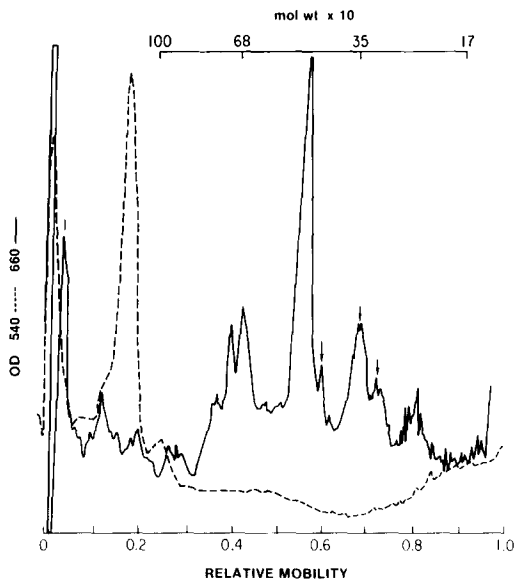


FIGURE 15 Absorption scans of acrylamide gels of whole flagella. Continuous line is gel stained with Coomassie blue; broken line is gel stained with PAS. Molecular weight standards are myoglobin (17,000 mol wt), pepsin (35,000 mol wt), bovine serum albumin (68,000 mol wt), and phosphorylase *a* (100,000 mol wt). Arrows indicate bands reduced or absent after flagella are extracted with Tris/Nonidet P-40.

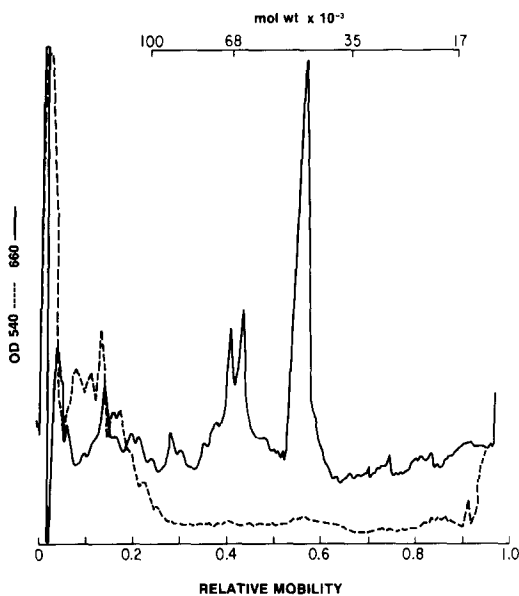


FIGURE 16 Scan of whole flagella extracted with Tris/Nonidet P-40. Continuous line is Coomassie blue stained; broken line is PAS.

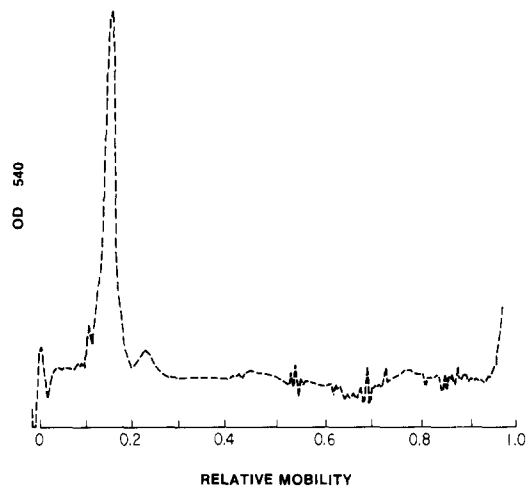


FIGURE 17 Scan of PAS-stained gel of extract obtained by treating whole flagella with Tris/Nonidet P-40. Recovery in nearly pure form of PAS band removed from whole flagella (Fig. 16) is evident.

account in part for the thick coating of extraflagellar mastigonemes.¹

The relationship of the mastigonemes in spiral tiers suggested above has been tested by superimposing tracings (Fig. 22*a*) from negatively stained preparations. Such a reconstruction appears in Fig. 22*b*, in which separate tracings from a cluster of three adjoining units have been half overlapped. Comparison of this reconstruction with isolated portions of the mastigoneme complex (Fig. 9) suggests that the model produces an image consistent with the appearance of the flagellar surface. It is not yet entirely clear how the mastigoneme units are attached to the axoneme, but images such as Fig. 7 suggest that attachment is confined to the axonemal doublet microtubules. The intraflagellar filament occasionally observed

¹ A note on terminology: the term mastigoneme (synonymous with "Flimmer" or "flagellar hair" of some workers) has been adopted throughout this report, as proposed in reference 4, to designate the essentially filamentous surface component distinct from the flagellar membrane. Differentiated portions of the mastigoneme whether tubular or nontubular are treated as modifying; hence, here the complete short nontubular mastigoneme consists of the mastigoneme unit and mastigoneme filament. The mastigoneme unit is further distinguished by mastigoneme unit loops, shaft, etc. The complete long nontubular mastigoneme consists of only the mastigoneme filament.

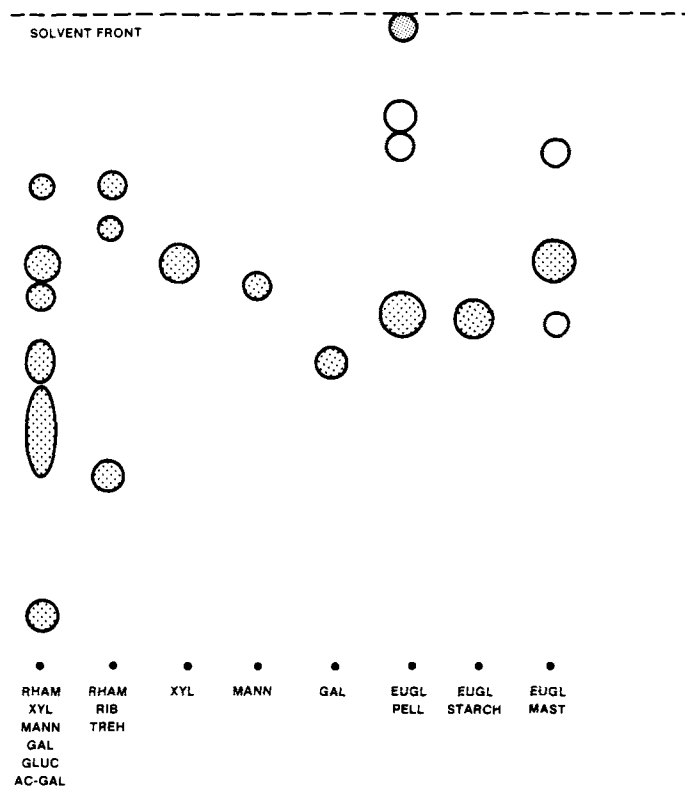


FIGURE 18 Tracing from TLC cellulose plate developed in butanol-pyridine-water (6:4:3), detected with alkaline silver nitrate. Coarse stippling indicates prominent spots, open circles are faint but clearly detectable spots, and fine stippling indicates barely visible area. Note that prominent spot obtained from *Euglena mastigonemes* coincides with authentic xylose, and one minor spot coincides with hydrolyzed *Euglena* starch (glucose). GLUC equals glucuronic acid.

(Fig. 4) may in fact represent a longitudinal view of this region. Mastigoneme attachment appears to extend through the membrane (cf. also reference 20 and 27) as evidenced by the stability of attachment after removal of the membrane (Figs. 6a and b), and by the appearance of the intact membrane in section (Fig. 4).

A calculation of the number of mastigoneme units per flagellum underscores the magnitude of the organizational problem and biochemical requirements of the regenerating flagellum. Assuming that mastigoneme units are uniformly distributed throughout the flagellum, then in excess of 7,000 U (Table I) would be required for each newly synthesized flagellum. Since each unit provides the anchor for three to five mastigoneme filaments, the total number of short nontubular mastigonemes can be estimated at about 30,000. If one adds to this the longer nontubular mastigonemes which appear to attach directly to the

paraflagellar rod, the requirements for surface growth are impressive. Thus, it is particularly difficult to explain the absence of visible assembled mastigoneme components intracellularly, especially during the periods of flagellar regeneration. It seems unlikely that such components would be overlooked, in view of their size (up to 3 μm), number, and complexity. Thus, while the developmental pathway of tubular mastigonemes seems to follow the perinuclear continuum/Golgi complex route (4), at least the final assembly of nontubular mastigonemes must occur within the reservoir from which the flagellum arises, or directly on the flagellar surface. That the reservoir is lined with fine filaments suggests that polymerization or assembly of mastigonemes may take place as precursors are released into this area.

Surface polymerization of mastigonemes in *Euglena* in the reservoir or membrane adjacent to the flagellum would be consistent with the unsus-

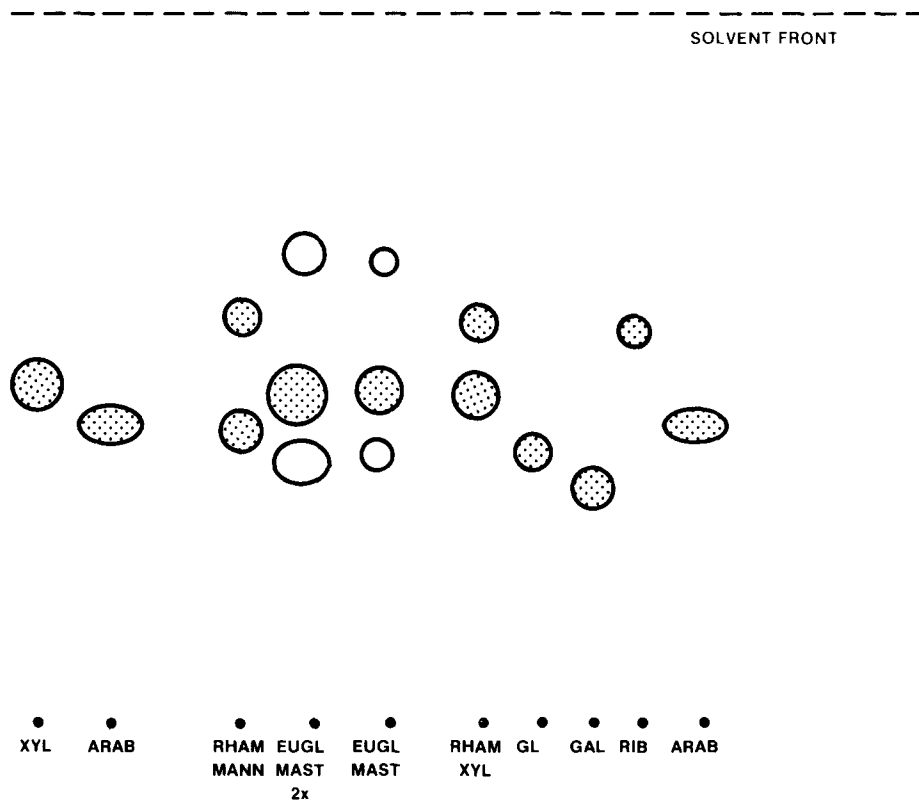


FIGURE 19 TLC tracing using a solvent of butanol-pyridine-water (5:3:2) and detection with alkaline silver nitrate. Symbols as in Fig. 20. Increasing concentration of mastigonemes did not reveal additional spots. Lower mastigoneme minor spot comigrates with glucose (GL) whereas major spot migrates with xylose (XYL).

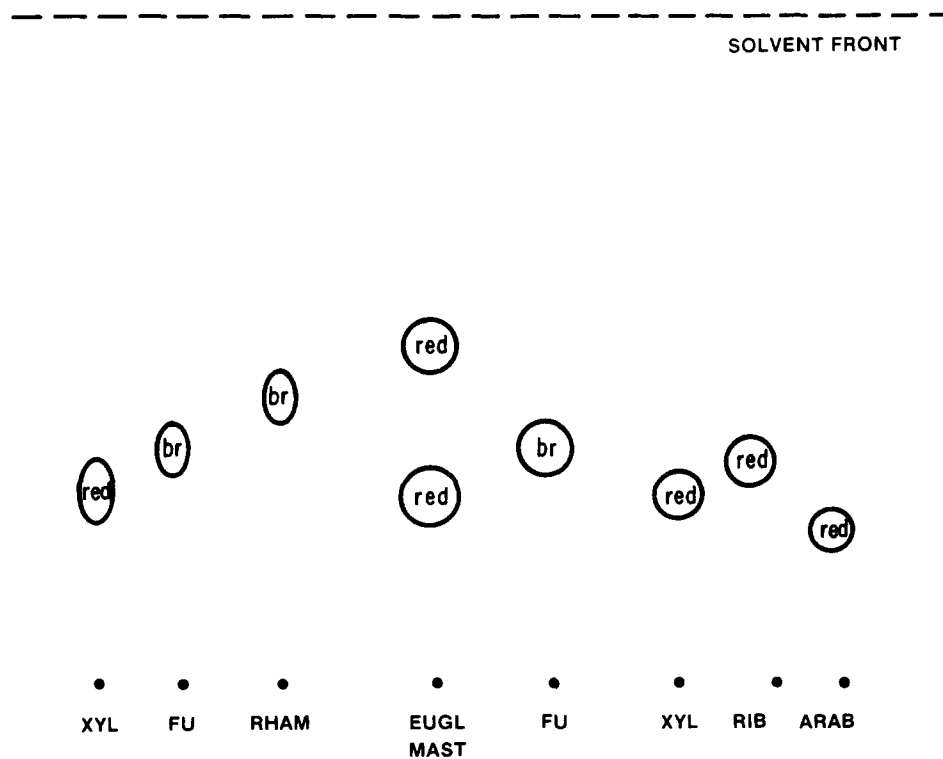


FIGURE 20 Tracing of TLC plate developed in a mixture of ethyl acetate-pyridine-acetic acid-water (5:5:1:3), and detected with aniline phthalate. Aldopentoses (xylose) and methylated aldopentoses stain red; aldohexoses (fucose, rhamnose) stain brown. A clear separation of xylose and fucose can also be achieved with this solvent system.

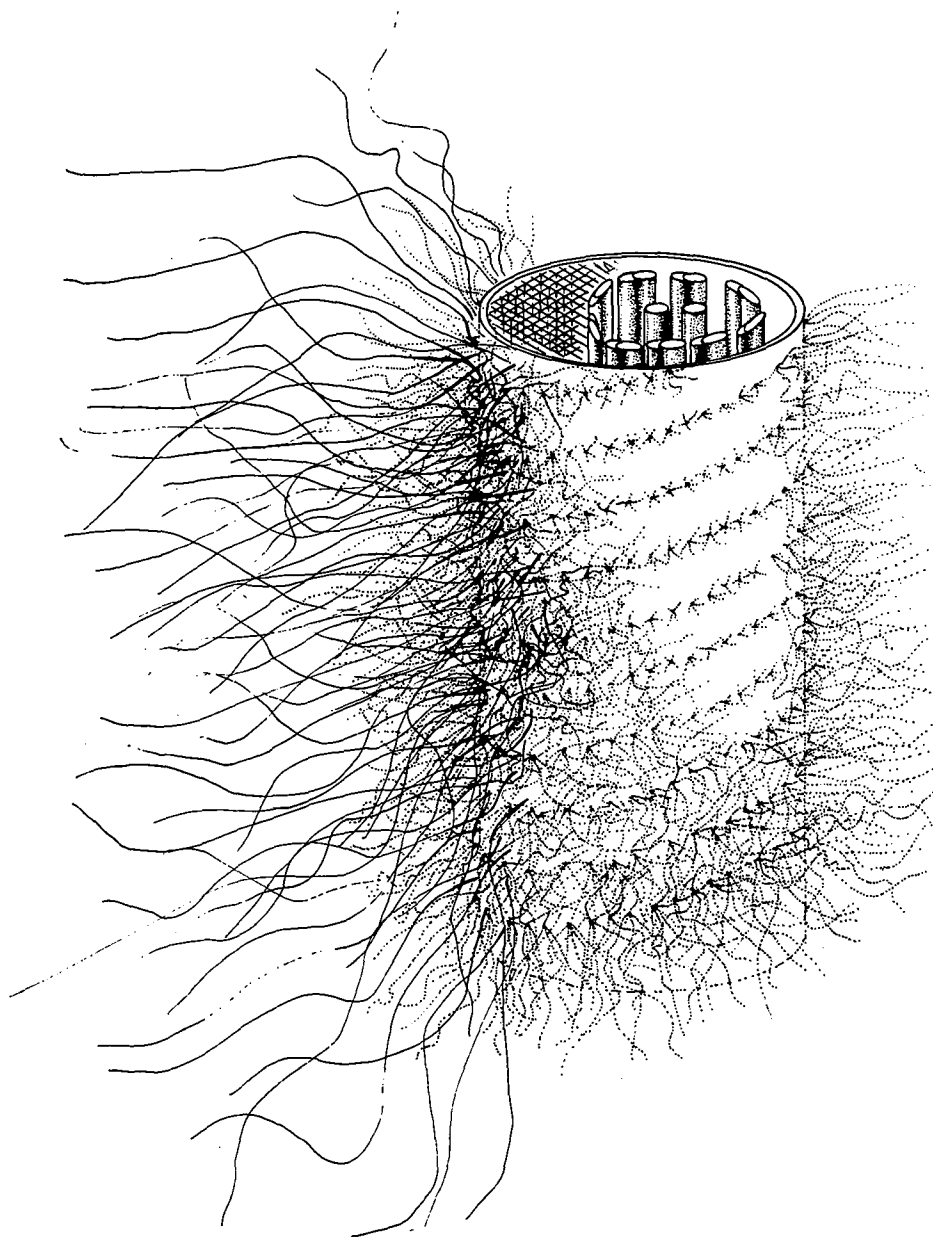
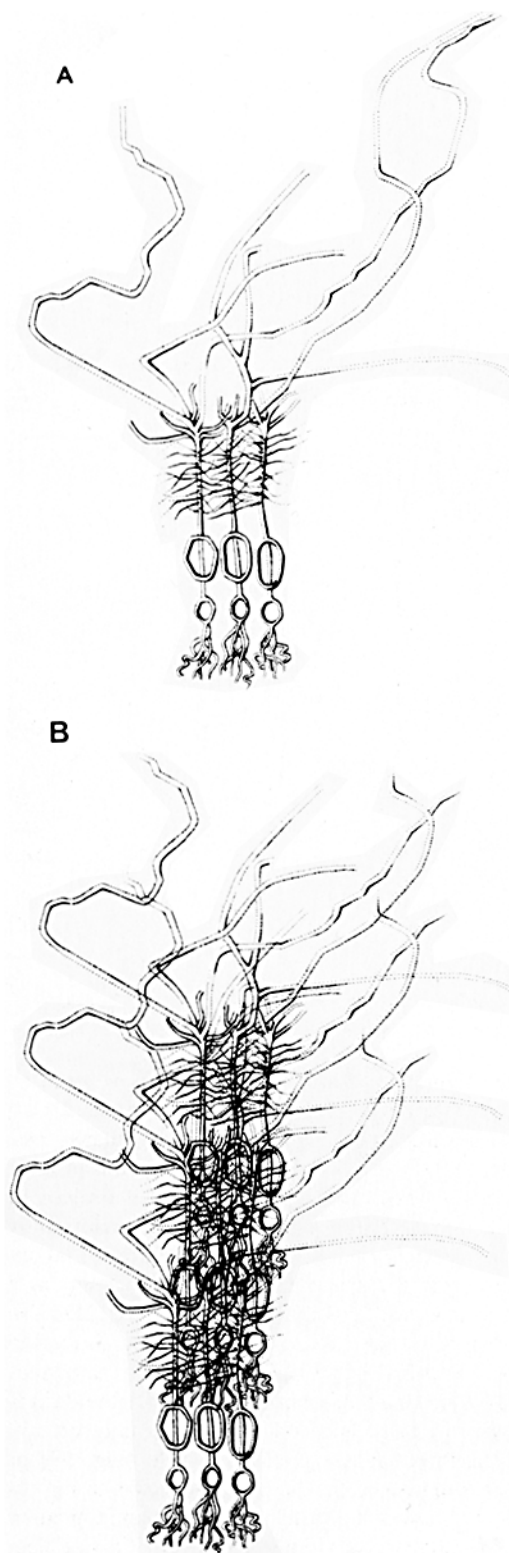


FIGURE 21 Diagram illustrating possible arrangement of short and long nontubular mastigonemes along the flagellar surface. Mastigoneme filaments have been omitted in the central region to illustrate a possible two-start helix comprised of spiral tiers of mastigoneme units.

cessful search in other organisms, e.g., *Chlamydomonas*, for cytoplasmic mastigoneme precursors. The problem awaits further resolution, however, especially in view of the as yet unproven identity of the reservoir filaments, and also because in Dinoflagellates with nontubular mastigo-

nemes intracellular presumptive mastigonemes are clearly present (cf. reference 11). Furthermore, the significance of filaments identified with nonspecific glycoprotein stain within the Golgi complex of a Euglenoid (21) remains to be determined.



Mastigoneme Composition

Of the 30 or more proteins which can be identified on SDS-acrylamide gels of reduced and alkylated whole flagella (Figs. 12*a* and 15), only two appear to be strongly PAS-positive. This characteristic together with their retention in the aqueous phase after phenol extraction suggests that there are two major glycoproteins in *Euglena* flagella. Phenol, which is generally recognized as a good protein solvent, excludes nucleic acids, polysaccharides, and glycoproteins (38). Thus, in a biphasic phenol/water mixture, glycoproteins should accumulate in the aqueous phase and proteins in the phenol phase. Furthermore, proteins not soluble in urea nor SDS can often be made urea/SDS soluble after pretreatment with phenol (30). Phenol/water has proven to be an excellent mixture for extracting glycoproteins of mastigonemes and flagella in the present study, especially since mastigonemes are only partially soluble in SDS/urea/mercaptoethanol. Comparison of the phenol exclusion products obtained from whole flagella and from mastigoneme fractions indicates that essentially the same major glycoproteins are present in the aqueous phase from both preparations. Thus, it appears that extraction of mastigoneme glycoproteins can be obtained without purification simply by treatment of whole flagella with phenol/water. Furthermore, the faster moving of the major glycoproteins from the mastigoneme fraction is soluble in low concentration of neutral detergents, a fact which should facilitate its purification and ultimate characterization. Such selective solubilization suggests that nonpolar hydrophobic interactions may be the principal forces holding together aggregates of these complexes (cf. reference 40). It is of further interest that the anionic detergent Sarkosyl has no apparent effect on mastigoneme glycoproteins or mastigoneme integrity, and thus a combination of Sarkosyl and neutral detergents can be used to alternately isolate and solubilize mastigoneme fractions. The high molecular weight glycoprotein characteristic of a variety of other flagellar membranes (cf. reference 33) may be coincident in *Euglena* with the fast moving PAS-positive band

FIGURE 22 (A and B) Upper image is a tracing of Fig. 10 which was photographically superimposed at half-overlapping intervals three times to produce the lower composite. This reconstruction is similar to the appearance of negatively stained, isolated fragments of the flagellar surface.

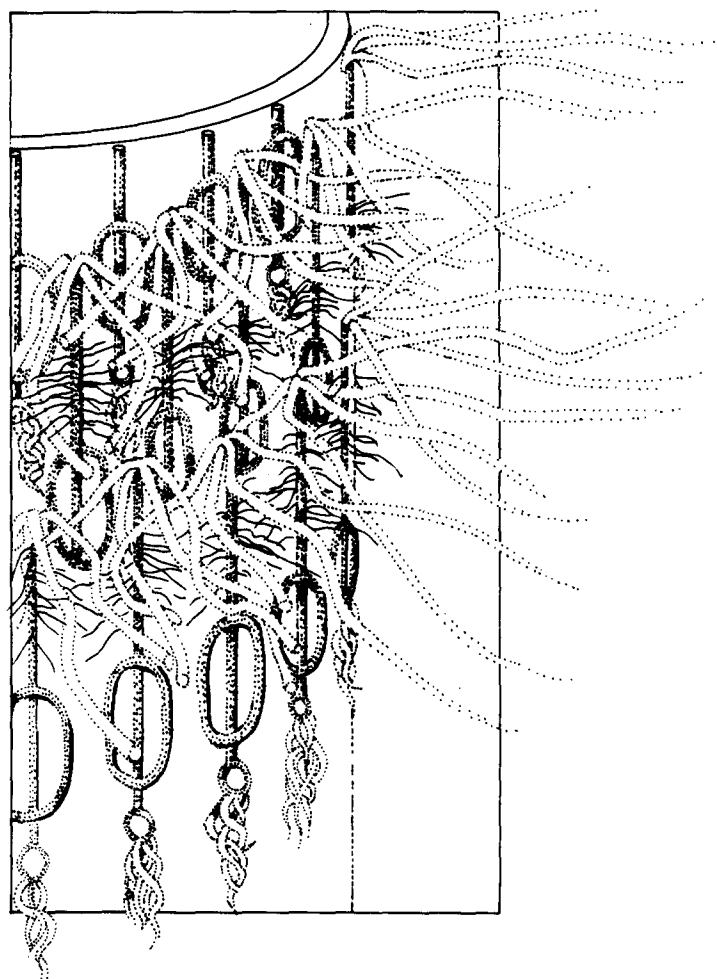


FIGURE 23 Details of lateral alignment and half overlap of mastigonemes outside the flagellar membrane. Staggered appearance is suggested from tangential sections of flagellar surfaces.

obtained from whole flagella after phenol extraction (Fig. 12 *d*).

Attempts to resolve the large glycoprotein generally found near the starting zone of 7.5% acrylamide gels by using SDS 1% agarose gels revealed that this component probably consists of several subspecies of glycoproteins. The broad bands so obtained suggest that the glycoproteins are polydisperse, and the requirement for mercaptoethanol suggests that at least part of the tendency to aggregate is due to the presence of disulfide bonds. These properties are remarkably similar to those of complex mucous glycoproteins obtained from various animal sources and fractionated on agarose gels (15).

The carbohydrate moiety of mastigoneme glycoproteins has not been previously identified, but

the thin-layer chromatographic analysis reported here indicates that the major sugar obtained from both whole flagella hydrolysates and isolated mastigoneme hydrolysates is the aldopentose sugar xylose. Since altering the length of hydrolysis or the concentration of HCl did not significantly change the chromatography pattern, it would appear that the sugars are present principally as a polyxylan with possibly some glucose residues also present. A fast moving minor spot remains yet to be identified, but its migration pattern and color reactions are consistent with a methylated derivative of xylose. Since xylose is not a major constituent of either the *Euglena* pellicle (Fig. 18) or *Euglena* starch, there seems little reason to suspect that xylose is the result of cellular contamination of flagellar preparations. However, it is not clear

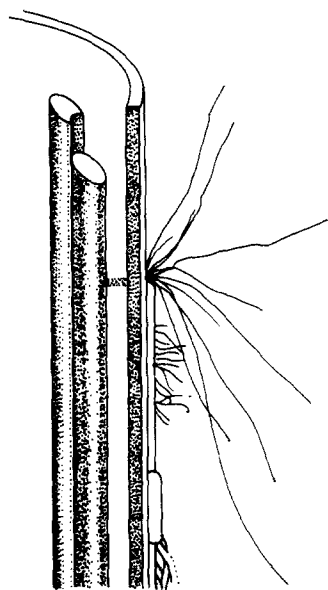


FIGURE 24 Relationship of mastigoneme units to flagellar membrane and axonemal microtubules is schematized in this drawing. However, the nature of the attachment of units to axonemal microtubules has not yet been entirely resolved.

whether glucose detected in small quantities represents such contamination or is a real component of the flagellum. A xylose-containing fraction has previously been obtained from *Euglena* cells and *Euglena* medium that binds vitamin B₁₂ (9). This binding fraction is strikingly similar superficially to mastigonemes, i.e., large size, extracellular location, but it seems unlikely that the two fractions are related.

Although xylose is not generally recognized as a common constituent of membrane glycoproteins, xylans are found broadly distributed in extracellular polysaccharides and/or glycoproteins in the cell walls of higher plants. The hemicelluloses of secondary walls are largely polyxylans (34). A key component in linking crystalline cellulose to pectins in some higher plant walls may be xylose (1). Xylose may substitute for glucose as the primary sugar comprising the cell wall microfibrils in the cell walls of some algae (26), and xylose together with arabinose may comprise the principal hemicellulose polysaccharide of some tropical plants (36). Evidence also suggests that xylose may also be present in tubular mastigonemes along with other sugars.² Since xylan-de-

² Valaitis, A. and G. B. Bouck. Manuscript in preparation.

TABLE I

Flagellar length	20 μm , Diameter 1.5 μm
Tiers of mastigonemes	μm
Each mastigoneme unit	0.12 μm apart
Therefore units are half staggered.	0.26 μm long
No. of units per flagellar length	$20 \mu\text{m} \div 0.12 \mu\text{m} = 167$
No. of units per flagellar circumference	44 U
Total no. of units per flagellum	$44 \times 167 = 7,348 \text{ U}$

grading enzymes are also widely distributed in bacteria, fungi, insects, snails, etc. (10), it should be possible to identify the specific xylan-containing portions of nontubular mastigonemes by digestion with appropriate extracts in future studies.

The Euglena Surface is a Composite of Specific Domains

Structurally and biochemically, the *Euglena* surface can be separated into at least two and perhaps three distinct regions: the ridged pellicle, the smooth reservoir, and the flagellum. Despite the presence of a contiguous plasma membrane over all these surfaces, there is little or no lateral intermixing within the pellicle or between pellicle and flagella. Thus, pellicle replication requires intussusception into stable areas (14) which may in part be attributed to the semi-crystallinity of the pellicle membrane (22). The reservoir is an area of active exocytosis with smooth unridged organization bridging the gap between pellicle and flagella, and the flagellar surface is organized into a massive complex of mastigonemes. Such segregated areas pose unique questions in surface synthesis and maintenance. It is of particular interest that the primary carbohydrate sugar is glucose in pellicles and xylose in the flagellum. Such differences may facilitate the ultimate resolution of pathways of glycoprotein synthesis and insertion in different regions of the cell, either through the use of specific lectins or through the use of immunospecific markers.

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