Characterization and Localization of a Flagellar-specific Membrane Glycoprotein in *Euglena*

ADRIENNE A. ROGALSKI and G. BENJAMIN BOUCK
Department of Biological Sciences, University of Illinois, Chicago, Illinois 60680

ABSTRACT   Purified flagella from *Euglena* yield a unique high molecular weight glycoprotein when treated with low concentrations of nonionic detergents. This glycoprotein termed "xyloglycorien" cannot be extracted from other regions of the cell, although a minor component that coextracts with xyloglycorien does have a counterpart in deflagellated cell bodies. Xyloglycorien is tentatively identified with a flagellar surface fuzzy layer that appears in negatively stained membrane vesicles of untreated flagella but not in similar vesicles after Nonidet P-40 extraction. The localization of xyloglycorien is further confirmed to be membrane associated by reciprocal extraction experiments using 12.5 mM lithium diiodosalicylate (LIS), which does not appreciably extract xyloglycorien, visibly solubilize membranes, or remove the fuzzy layer. Rabbit antibodies directed against the two major flagellar glycoproteins (xyloglycorien and mastigonemes) to some extent cross react, which may in part be caused by the large percentage of xylose found by thin-layer chromatography (TLC) analysis to be characteristic of both antigens. However, adsorption of anti-xyloglycorien sera with intact mastigonemes produced antibodies responding only to xyloglycorien, and vice versa, indicating the nonidentity of the two antigens. Antibodies or fragments of these antibodies used in immunofluorescence assays demonstrated that xyloglycorien is confined to the flagellum and possibly the adjacent reservoir and gullet. Binding could not be detected on the cell surface. The sum of these experiments suggests that, in addition to mastigonemes, at least one major membrane glycoprotein in *Euglena* is restricted to the flagellar domain and is not inserted into the contiguous cell surface region.

The continuous, wall-free plasma membrane of *Euglena* delineates three specialized regions of the total cell surface: (a) the helically organized, ridged pellicle complex, (b) the smooth invaginated reservoir, and (c) the emergent flagellum. Recent studies suggest that these three regions comprise separate and distinct surface domains which may be useful for examining patterns of surface growth, synthesis, and composition (14, 22). Regional differences in surface composition have been demonstrated by comparison of flagellar fractions with purified pellicle isolates using primarily sodium dodecyl sulfate (SDS) gels and thin-layer chromatography (TLC) analysis (3). The patterns of polypeptides, glycoproteins, and saccharides are strikingly different from these two sources and have prompted a further definition of the individual components and their specific localizations.

Among the flagellar components, two glycoproteins of high molecular weight have been recognized. The larger of these, that has been equated with the complex array of mastigonemes, is detergent resistent and appears to be polydisperse as judged

from its behavior in SDS agarose gels (3). The second flagellar glycoprotein, xyloglycorien, is considered in this report. Unlike mastigonemes, it migrates as a single band in SDS gels, and is easily extracted in nearly pure form with low concentrations of neutral detergents. Analyses are presented that define some of its properties and, by appropriate antibody labeling, localize it to the flagellum. Further experiments suggest that it is a unique flagellar membrane glycoprotein probably associated with the outer flagellar membrane surface. A preliminary account of these findings has been reported (29).

MATERIALS AND METHODS

Culture Maintenance and Flagellar Isolation

were pelleted by centrifugation at 1,000 g for 10 min in an International clinical centrifuge (International Scientific Instruments Inc., Santa Clara, Calif.), and the supernate containing flagella was subjected to two successive centrifugations at 1,000 g for 5 min to ensure complete removal of cell bodies. The supernate was then centrifuged at 17,500 rpm in a Sorvall SS-34 rotor for 30 min, yielding a white pellet of pure flagella as judged by light and electron microscopy.

Selective Solubilization of a Nonmastigoneme Flagellar Glycoprotein

Previous studies (3) have revealed that treatment of whole flagella with low concentrations of detergents selectively extracts one of the two major glycoproteins and leaves the other major glycoprotein(s) (mastigonemes) in a particulate fraction. The following method therefore provided soluble extracts of the one flagellar glycoprotein with minimal disruption of the axoneme, membrane, and mastigonemes. Whole flagellar pellets were suspended in 1.0–2.0 ml of 0.1% Nonidet P-40 (wt/vol) (Particle Data, Inc., Elmhurst, Ill.) or Triton X-100 (spectrographic grade, Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) in 0.1 M PIPES, 1 mM EGTA, and 10 mM TAME (p-tosyl-L-arginine methyl ester-HCl), pH 7.0, and incubated for 30 min at 0°–4°C. The flagellar suspension was then centrifuged at 17,500 rpm in a Sorvall SS-34 rotor for 30 min. To ensure complete removal of any contaminating flagella or mastigonemes, the supernate so obtained was then layered over 40% sucrose in the same buffer and centrifuged at 25,000 rpm for 20 min in an SW 50.1 rotor of a Beckman L2 65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) or alternatively at 40,000 rpm for 1 h without sucrose. The upper aqueous layer of the sucrose gradient was carefully removed and immediately frozen in 1-ml aliquots or lyophilized. Protein estimations were made using a modified Lowry method by the inclusion of the detergent SDS to prevent the formation of a water-immiscible nonionic detergent phosphomolybdate complex (2). Before chromatographic analysis, the extract was dialyzed against distilled water and 0.1% Na azide at 0°–4°C to remove PIPES buffer which will readily precipitate during acid hydrolysis. Attempts were also made to reduce detergent levels to ~0.01% before chromatography by a 2-h incubation with gentle agitation of 1 ml of extract with 0.8 g of wet, prepared SM-2 beads (Bio-Rad Laboratories, Richmond, Calif.). The beads were removed by pelleting at 1,000 g in an International clinical centrifuge.

Extraction of Flagellar Lipids and Selective Solubilization of Axonemal Proteins

CHLOROFORM-METHANOL: Lipid extraction was performed according to the method of Bligh and Dryer as described by Zahler and Niggli (42). Flagellar pellets were suspended in 0.4 ml of distilled water to which was added 0.5 ml of chloroform and 1 ml of methanol; the mixture was then homogenized for 2 min. The suspension was centrifuged at 17,500 rpm for 30 min which yielded a pellet of lipid-extracted flagella and a monophasic supernate. The supernate was carefully removed and an additional 0.5 ml of chloroform was added. Blending for 30 s followed by the addition of 0.5 ml of distilled water resulted in a biphasic mixture from which the upper aqueous phase was removed and prepared for electrophoresis along with the previously pelleted, lipid-extracted flagella.

LITHIUM DIIODOSALICYLATE: Flagellar pellets were suspended in 0.25 ml of 56 mM Na borate, pH 8.0, to which was added an equal volume of either 25 or 50 mM lithium diiodosalicylate (LIS) (Eastman Kodak Co.) was added (35). The suspensions were stirred for 10–15 min at 4°C, 0.5 ml of distilled water was added, and the final mixture was then centrifuged at 17,500 rpm for 30 min. The pellet and supernate were analyzed separately.

Isolation of Flagellar Mastigonemes

Flagellar mastigonemes were prepared by the general methods of Bouck et al. (3), with the following modification: isolated flagella were initially subjected to two successive extractions with 0.1% Nonidet P-40 in 0.1 M PET buffer (0.1 M PIPES, 1.0 mM EGTA, 10 mM TAME), pH 7.0, to remove any residual xyloglycorien. Flagellar pellets were then treated with 1.5% Sarkosyl in 0.1 M PET, pH 7.0, overnight at 0°–4°C to solubilize the remaining membrane and axonemal components, after which the Sarkosyl-resistant mastigonemes were pelleted by centrifugation at 40,000 rpm for 1 h in an SW 50.1 rotor of a Beckman L2 65B ultracentrifuge.

Thin-Layer Chromatography

Detergent extracts were exhaustively dialyzed against distilled water to remove PIPES buffer before acid hydrolysis. Hydrolysis of extract or flagella was carried out in 1 N HCl for 3 h at 100°C. The hydrolysates were subsequently neutralized with Dowex-1 resin (bicarbonate form) and deproteinized (32). Sugar standards were similarly prepared. Samples were applied to precoated cellulose MN 300 TLC plates (Brinkmann Instruments, Inc., Westbury, N. Y.) and developed in either butanol-pyridine-water (6:4:3) or ethyl-pyridine-acetic acid-water (5:5:1:2), and subsequently treated with either aniline phthalate or alkaline silver nitrate to detect sugars. To reduce the possibility of preparative artifacts, additional samples were prepared that had not been neither exposed to sucrose centrifugation or treated with SM-2 beads.

SDS-Acrylamide Gel Electrophoresis

All samples were reduced in 8 M urea and 1 mM mercaptoethanol (ME) and alkylated in iodoacetate (26). Samples were finally dialyzed against 0.1 M Na phosphate buffer, pH 7.2, 0.1% SDS, and 0.1% ME. 7.5% acrylamide was polymerized without stacking gel in 4-mm diameter glass tubes. Samples were electrophoresed (38) at 5 mA/tube with bromphenol blue as the tracking dye. Gels were fixed overnight in 50% TCA, stained in 0.1% Coomassie Blue, and finally destained in 7.5% acetic acid. For identifying glycoproteins, gels were fixed in 40% ethanol-5% acetic acid overnight and stained for carbohydrates by the periodic acid-Schiff procedure essentially as described by Segrest and Jackson (30).

Preparation of Anti-Flagellar Antibodies

Mature white New Zealand rabbits were immunized by weekly subcutaneous injections; the 1st wk with Freund's complete adjuvant and antigen followed by 3 wk with incomplete adjuvant with appropriate antigen. A booster injection was administered at the end of the 6th wk, and 5–10 d later the animals were bled. Each injection contained ~300 μg of Lowry-positive Nonidet P-40 (NP-40) extract (xyloglycorien) or 500 μg of Lowry-positive mastigonemes.

Preparation of Fab$_2$' and Fab' Fragments

Whole IgG obtained from rabbit antisera after passage through a Whatman DE-52 resin column was dialyzed against 0.1 M Na acetate, pH 4.5, at 0°–4°C overnight. After dialysis and centrifugation at 5,000 rpm, in a Sorvall SS-34 rotor for 10 min to remove any aggregated material, pepsin (Worthington Biochemical Corp., Freehold, N. J.) was added to 2.5% by weight of IgG and the entire mixture was then incubated for 16 h at 37°C to produce Fab$_2$' fragments. For Fab' fragments the pepsin digestion was followed by dialysis in the dark against iodoacetamide (Sigma Chemical Co., St. Louis, Mo.) 2.5 mg/ml in phosphate-buffered saline (PBS), pH 8.0, and finally overnight dialysis against PBS, pH 7.4. To ensure removal of intact IgG and Fab$_2$' fragments from Fab', 1 ml (14 mg/ml) of digest was incubated with Sepharose-protein A gel (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.), for 2 h at 37°C. The absorbent was removed by centrifugation at 1,000 g for 10 min in an International clinical centrifuge. The purity of Fab' fragments was checked by SDS-gel electrophoresis under nonreducing and reducing conditions.

Immunofluorescent Staining

Goat anti-rabbit antibodies (GAR, Miles Laboratories, Inc., Elkhart, Ind.) were passed through a Whatman DE-52 column and conjugated to fluorescein isothiocyanate (FITC) according to the method of Brandtzaeg (4) as modified by Louvard, Heggeness, and Ash (unpublished observations). Euglena were fixed in 3% paraformaldehyde in PBS or in 0.1 M Na phosphate, pH 7.4, for 20 min at room temperature. Cells were subsequently rinsed three times in PBS (10 min each rinse), with the second and third rinse supplemented with 0.2 M glycine to bind free aldehydes. Cells were then incubated with 1 mg/ml of primary rabbit antibody for 30 min at room temperature and rinsed an additional three times in PBS before incubation with 50 μg/ml GAR IgG-FITC conjugate (Fl-GAR) for 30 min at room temperature.

After three additional rinses in PBS, cells were examined by incident illumination in a Zeiss photomicroscope II or III equipped with Nomarski optics and a mercury vapor light source (HBO 100 W/4), an FITC excitation filter, a Zeiss 65 barrier filter to specifically absorb chloroplast autofluorescence, and generally, a 0.65-NA × 40 oil immersion Planapochromat objective. Chloroplast autofluorescence can also be reduced by pretreatment of cells with acetone.

To test for nonspecific staining by either primary or secondary antibodies, the following controls were performed: for nonspecific staining of rabbit serum, fixed cells were incubated with either preimmune serum or heterologous rabbit serum (100 μg-1 mg/ml) and then incubated with Fl-GAR; for nonspecific staining of secondary antibody, cells were incubated only in PBS and then treated with the Fl-GAR conjugate.

Preparation of Ferritin-labeled GAR Antibodies

Affinity-purified GAR IgG (kindly supplied by D. Louvard and R. Ash, University of California, San Diego, California) was conjugated to ferritin by the method of Kishida et al. (17). Optimally coupled fractions were separated from uncoupled ferritin and ferritin aggregates by passage through a 30- × 2-cm column of Bio-Gel A-5 (200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) equilibrated and eluted with Tris-glycine buffer.

Preparation of Ferritin-labeled Cells

Cells were prefixed for 15 min in 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4, rinsed in PBS-glycine three times, and treated with rabbit whole IgG, Fab', or Fab₂' against flagellar antigens. After incubation in antisera for 30 min, cells were rinsed in PBS-glycine and incubated in GAR-Fe^{++} for an additional 30 min. Two subsequent rinses in PBS-glycine and one rinse in 0.01 M Na PO$_4$ were followed by a second fixation of 4% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 0.1 M Na phosphate for 1 h at room temperature, and three rinses in 0.1 M Na PO$_4$. Whole mounts were prepared by applying a drop of sample on a carbon-coated copper grid, rinsing in distilled water, and then drying at room temperature.

Electron Microscopy

Pelleted flagellar preparations were fixed in 4% glutaraldehyde (vol/vol) in 0.1 M Na phosphate buffer, pH 7.0, for 1½ h at room temperature, rinsed three times with buffer, and postfixed in 1% OsO$_4$ in 0.1 M Na phosphate buffer, pH 7.0, overnight at 4°C. After an acetone dehydration series, preparations were embedded in Spurr's hardest resin mixture (34) and polymerized for 24 h at 60°C. Thin sections were stained with uranyl acetate and lead citrate (27) and examined in either a Philips 300 or Hitachi 125-A electron microscope. Negative staining was carried out in 1% aqueous uranyl acetate on Formvar-coated, carbon-stabilized copper grids.

RESULTS

The detailed organization of the *Euglena* flagellum was reported earlier (3). Of primary interest to the present study is the multiple tiers of mastigoneme glycoprotein units which *in toto* constitute a massive coating on the flagellar surface. Thus, the flagellar membrane is generally totally obscured by the mastigonemes but, after repeated passages of flagellar preparations through a 25-gauge hypodermic needle, mastigonemes are often displaced, exposing the underlying membrane. Negative staining of such preparations or of membrane vesicles detached by such treatments reveals a layer of fine 150-Å filaments oriented perpendicular to the membrane and closely associated with the outer membrane surface (Fig. 1a and b). Sections of flagella after fixation and embedding show no signs of this surface fuzz, probably because of the relative ease with which the layer is extracted with the organic solvents (see below) used during preparation for electron microscopy.

SDS-polyacrylamide gel electrophoresis gels of the entire flagellum after reduction and alkylation separate ~30 polypeptides (Fig. 2B) and two slowly migrating bands that stain heavily with the periodic acid-Schiff (PAS) procedure (Fig. 2A). The most prominent PAS band near the gel origin has been equated previously with mastigonemes (3) (Fig. 2G and H), whereas the faster migrating PAS band appears to be solubilized flagellar fuzz. Evidence for the latter assumption
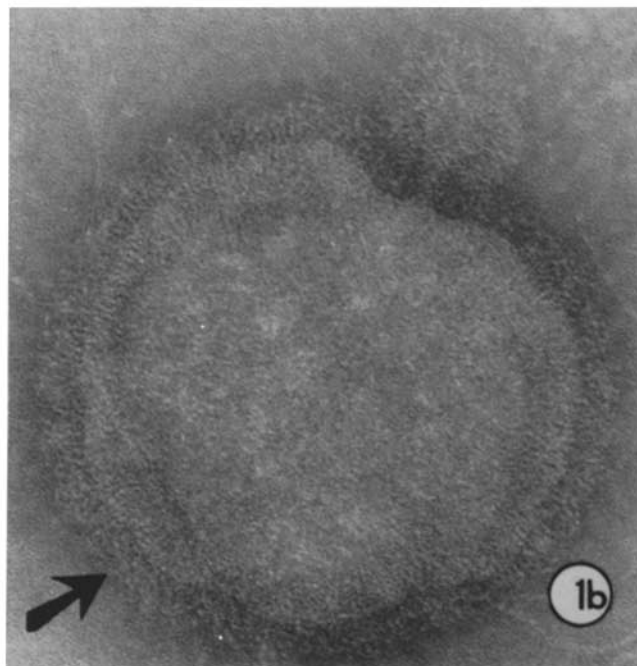
FIGURE 1 Negatively stained portions of *Euglena* flagella demonstrating (arrows) the presence of a surface fuzzy layer (xyloglycorien). Mastigonemes have been removed from the surface by successive passes of whole flagella through a 25-gauge hypodermic needle. Such shearing also releases flagellar membrane vesicles (b) in which the fuzzy layer can be seen to consist of 150-Å filamentous elements arranged perpendicular to the vesicle surface. a, × 62,000; b, × 357,000.
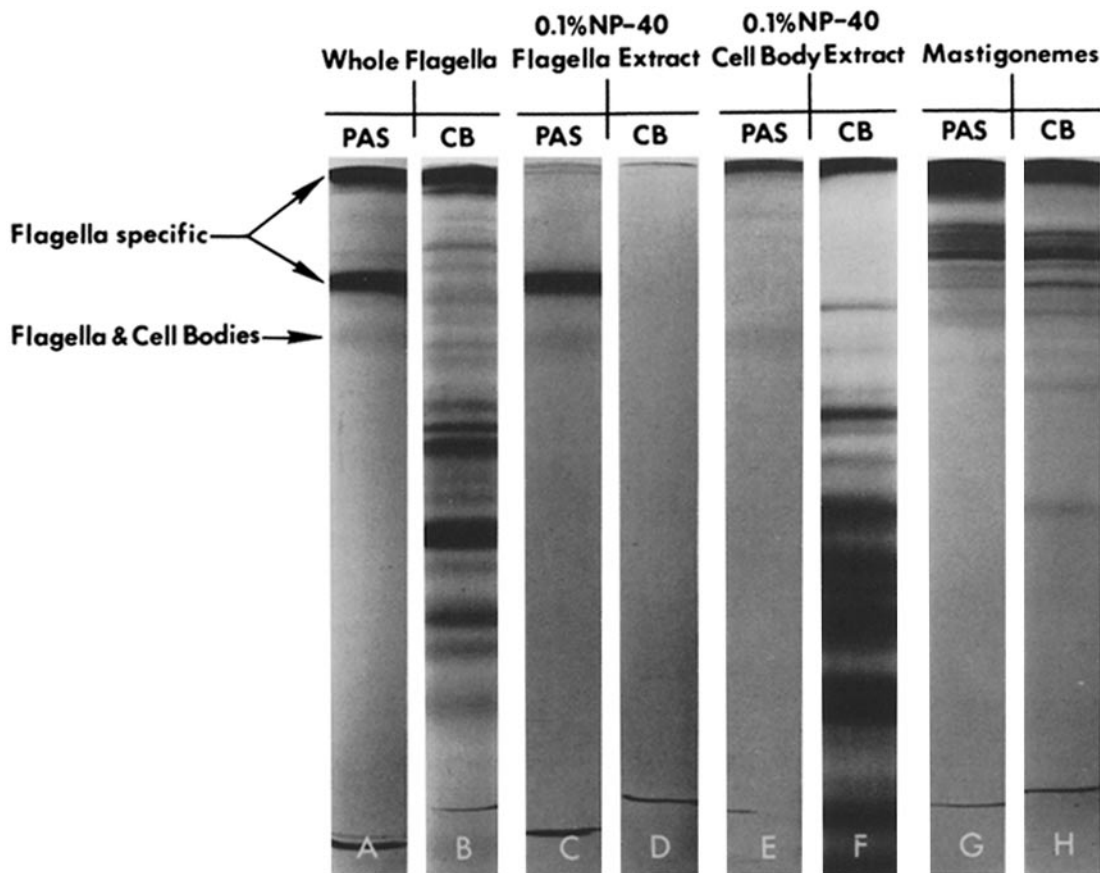
FIGURE 2 SDS-polyacrylamide gels of flagellar fractions stained for glycoproteins (*PAS*) or polypeptides (*CB*). Identical amounts of sample were applied to each PAS/CB gel pair, but different pairs are not quantitatively comparable. However, relative amounts of each component can be estimated in whole flagella gels A and B. Cell body extracts (E and F) were obtained from two to three times the amount of cells used in the flagellar gels. Xyloglycorien appears as a nearly pure band in the NP-40 flagellar extract (C) and is absent from whole body extracts (E). Mastigonemes isolated by resistance to Sarkosyl retain no xyloglycorien (G and H).

comes from the following: 0.1% NP-40 produces little apparent change in axonemes, paraflagellar rod, or sectioned membranes (Fig. 3 a and b). However, in SDS gels of extracted flagella, the faster migrating major PAS band can no longer be identified (not shown), but the NP-40 extract on similar gels produces only the faster migrating PAS band and two minor species (Fig. 2 C and D). Membrane vesicles derived from NP-40-extracted flagella consistently fail to show the fuzzy layer in negatively stained preparations. Such vesicles are smooth in outline and exhibit no surface granularity or filaments (Fig. 3 b). This correlation suggests that the PAS band and the flagellar fuzz are the same, and for convenience, the major PAS moiety removed from the flagellum by NP-40 will be referred to as "xyloglycorien."

To further test the structural identity of xyloglycorien, a reciprocal extraction procedure was sought in which the PAS band would remain insoluble and other flagellar components could be removed. The chaotropic reagent LIS as used in low concentrations by Yu et al. (41) proved to be excellent for this purpose. Flagella extracted with LIS yielded a pellet in which flagellar membranes and mastigonemes comprised the bulk of the unsolubilized material (Fig. 4 a). Membranes in thin section appeared to be structurally intact and of approximately the same width as in the controls (Fig. 4 b). Negatively stained, LIS-extracted membrane vesicles retain a granular surface (Fig. 4 c), but the thickness of the surface fuzz is somewhat reduced relative to untreated membranes. Conceivably, there is some

disruption of membrane integrity by LIS, resulting in partial withdrawal or flattening of the surface filaments after treatment. LIS-extracted vesicles subsequently treated with 0.1% NP-40 are entirely free of surface fuzz (Fig. 4 d).

SDS gels of LIS-extracted flagellar pellets produce two prominent PAS-positive bands but few Coomassie Blue-staining regions (Fig. 5 A and B). LIS extracts from such experiments separate into many polypeptides on SDS gels but only small quantities of glycoproteins as determined by PAS (Fig. 5 C and D). These results lend further support to the identity of xyloglycorien as membrane associated because only membranes and mastigonemes are present in the gel pellet preparations (see above). Moreover, isolated, untreated flagella extracted with chloroform-methanol, which should remove predominantly lipophilic molecules, lose nearly all the xyloglycorien (Fig. 5 G and H), leaving the majority of polypeptides and mastigonemes pellet associated (Fig. 5 E and F). These findings further suggest that xyloglycorien is membrane related.

To assess the extent to which xyloglycorien may be present on other regions of the cell, deflagellated cell bodies from an 8-liter culture (~4 × 10⁹ cells) were treated with 0.1% NP-40. A number of polypeptides and minor glycoproteins are solubilized in this manner, but only trace amounts of material migrating and staining as xyloglycorien could be identified (Fig. 2 E and F). One PAS band was found to be common as a minor component to both flagella and cell body extracts. To reduce the possibility that this common band might be a
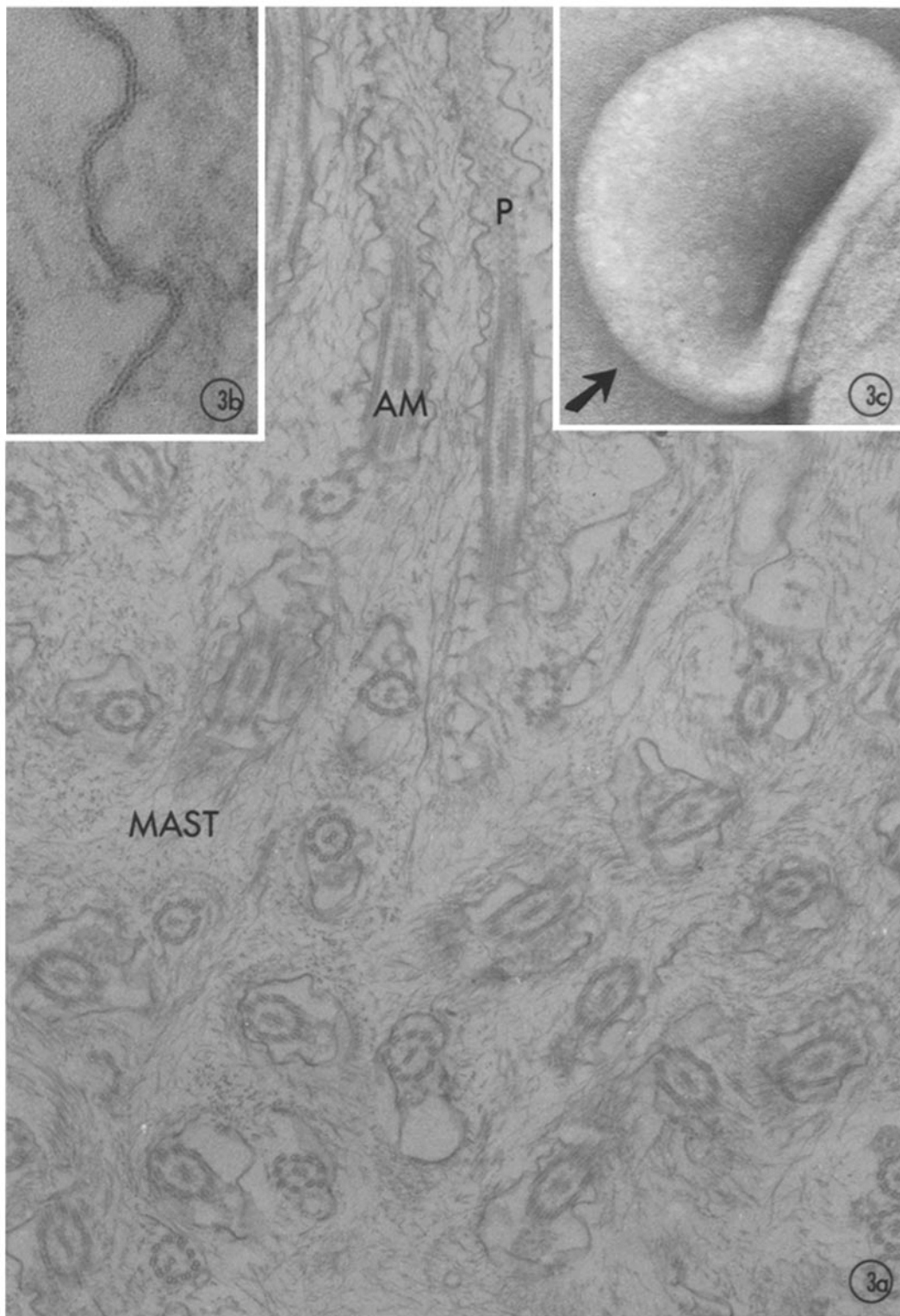
FIGURE 3 Section through a flagellar pellet that had been extracted with 0.1% NP-40 demonstrating the purity of preparations and the essentially undisrupted condition of flagellar components (a). Axonemal microtubules (AM), mastigonemes (MAST), para-flagellar rods (P), and membranes appear similar to untreated controls. In b, the details of a portion of an NP-40-extracted flagellar membrane are illustrated. No apparent alteration of the membrane relative to fixed and resin-embedded control membranes can be detected in this preparation. However, in negatively stained flagellar membrane vesicle (c) the filamentous surface layer can no longer be identified after NP-40 extraction a, × 48,000; b, × 275,000; c, × 275,000.

monomeric form of xyloglycorien, NP-40 extracts from flagella were subjected to the relatively extreme denaturing conditions of 2% SDS in 10 mM Tris-acetate followed by incubation at 100°C for 5 min and then electrophoresed using the gel system of Fairbanks et al. (9). No shift in the relative positions or apparent amounts of xyloglycorien or the minor band could be detected after these treatments. Thus, it is concluded that the minor bands are not monomeric or polymeric forms of xylo-glycorien.

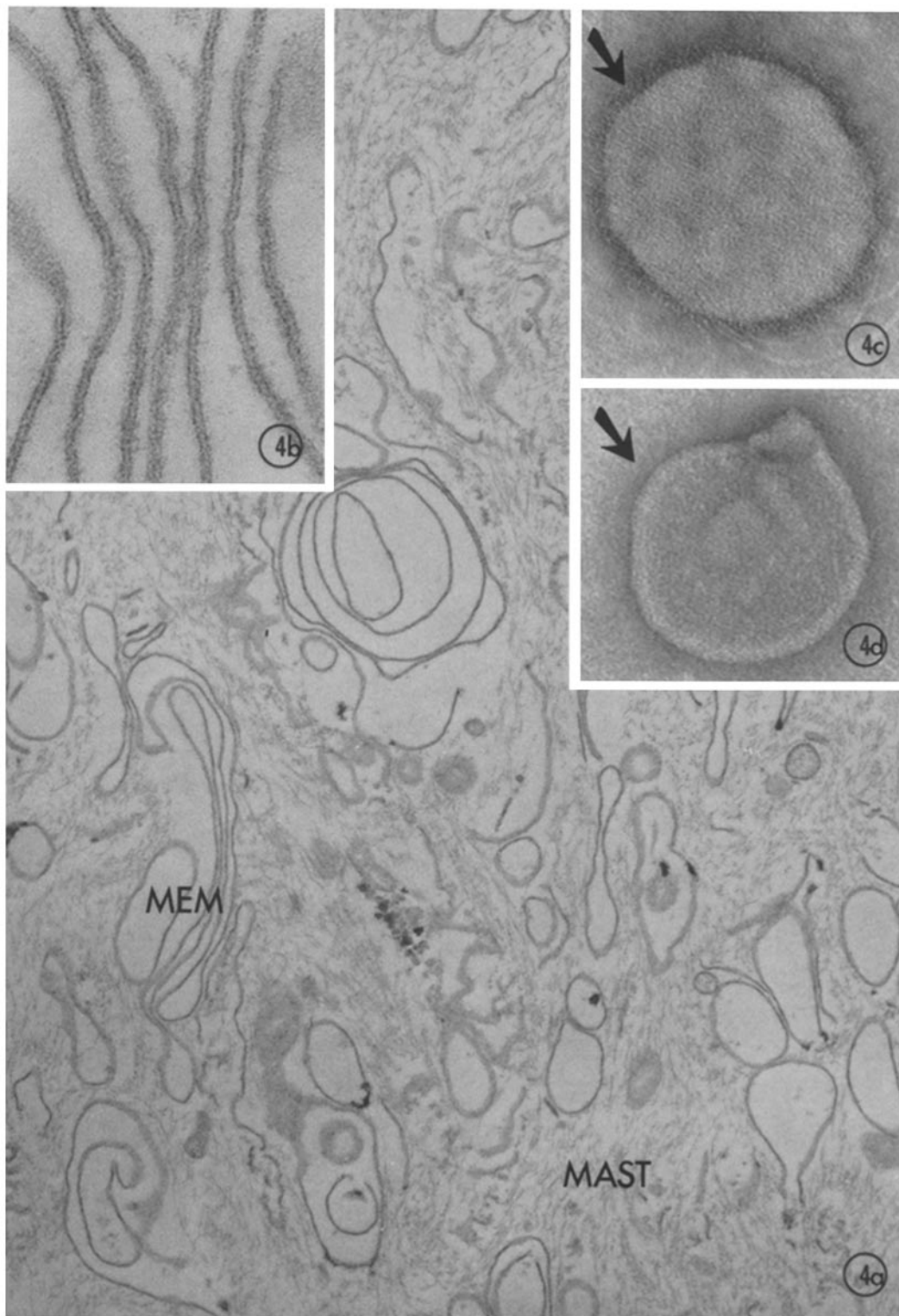The sugar composition of the NP-40 extract, whole flagella,

FIGURE 4   Flagellar pellet treated with 12.5 mM LIS (a). Membranes (MEM) and mastigonemes (MAST) are the only recognizable components, whereas axonemes and the paraflagellar rod have been solubilized. The thickness and organization of the flagellar membrane (b) appear to be similar to those of the NP-40-extracted flagellar membrane. No evidence of the surface fuzzy layer can be detected in this or other untreated preparations, suggesting that the layer is removed during embedding or that the layer exhibits low electron density. In c , a negatively stained membrane vesicle obtained through shearing as in Figs. 1 b and 2 C but treated with 12.5 mM LIS retains the filamentous surface layer, although it is somewhat less prominent than that of the controls. LIS-extracted vesicles subsequently treated with NP-40 (d) show no sign of the filamentous layer after negative staining. a, X 48,000; b–d, X 275,000.

and mastigonemes was determined by spotting samples (hydrolyzed under vacuum and neutralized) on cellulose TLC plates and developing in a butanol-pyridine-water mixture. After detection with alkaline silver nitrate, all three components

produce a prominent spot that migrates with authentic xylose (Fig. 6). Only the minor sugars vary in that glucose seems to be associated with the NP-40 extract whereas glucose and mannose appear to be components of whole flagella and mas-
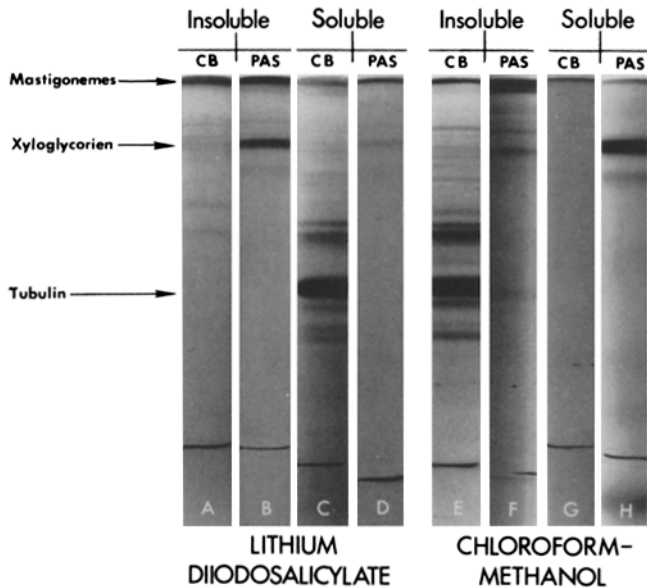
FIGURE 5   SDS gels of LIS- or chloroform/methanol-treated flagella identifying the principal polypeptides (CB) and glycoproteins (PAS). The four gels of each treatment were loaded with samples obtained from comparable quantities of flagella. LIS solubilizes most of the flagellar axonemal proteins (gel C) but almost none of the flagellar glycoproteins (gel D) that remain associated with the unsolubilized particulate fraction (gel B). On the other hand, xyloglycorien is the major glycoprotein extracted with chloroform-methanol (gel H), indicating the lipophilic or hydrophobic nature of this component. Most of the other flagellar constituents, including mastigonemes, are not solubilized by chloroform-methanol (gels E and F).

FIGURE 6   Chromatograms of acid hydrolysates of whole flagella (WF), mastigonemes (MAST), and xyloglycorien (XG) stained for carbohydrate with alkaline silver nitrate. The dominant sugar in all three preparations migrates with authentic xylose. Mannose appears along with trace amounts of glucose as a recognizable minor band in whole flagella and mastigonemes. The apparent two bands in xyloglycorien are in fact a single circular spot (compare with right-hand standard) of glucose. All these samples were prepared without using sucrose gradients or SM-2 beads.

tigonemes. These results were obtained from preparations that were not exposed to SM-2 beads or a sucrose gradient, thus excluding the possibility of glycoprotein removal or of contamination by nondialyzable glucose aggregates present in sucrose (cf. 15).

Antibody Characterization and Localization

Both flagellar mastigonemes and the NP-40 extract (xyloglycorien) readily elicit rabbit antibodies, but the resultant antisera exhibit varying degrees of cross-reactivity. For example, mastigoneme antiserum (anti-MAST) diffused against solubilized mastigonemes produces two prominent precipitin arcs, but the same serum also produces a precipitate with NP-40 extract. Solubilized whole flagella gave similar results, and antiserum against NP-40 extract (anti-XG) gives two precipitin arcs when reacted against whole flagella. However, preabsorption of anti-XG with native, intact mastigonemes eliminates two arcs attributable to reaction with mastigonemes but the sera retain the ability to react with xyloglycorien (Fig. 7 a). Anti-MAST serum absorbed against xyloglycorien gives a complex precipitin pattern consisting of at least two arcs, but no reaction occurs when challenged with xyloglycorien (Fig. 7 b). In all of these immunodiffusion tests, flagellar components were solubilized in a solution of 1% SDS and 0.1% ME at 100°C for 5 min, or in 8 M urea and 1% NP-40. In both procedures, samples were extensively dialyzed against PBS, and no significant differences in the precipitin reactions could be detected in urea or SDS. Solubilized pellicle isolates from Euglena (14) fail to produce a visible reaction when incubated against anti-MAST or anti-XG. Thus, suitably absorbed anti-XG and anti-MAST are judged to be specific for xyloglycorien and mastigonemes, respectively.
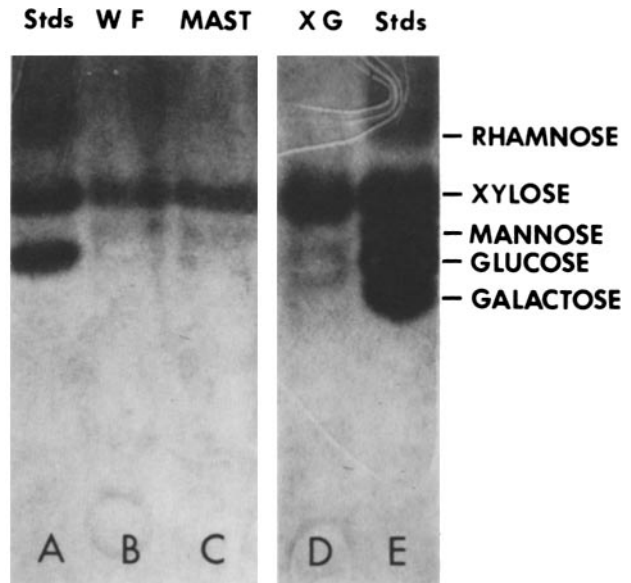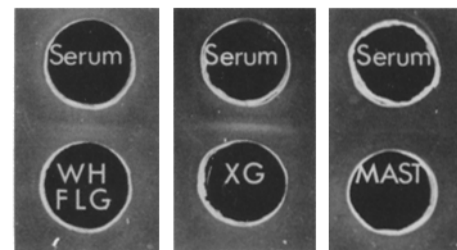

a.   Anti-Xyloglycorien Serum absorbed with Mastigonemes
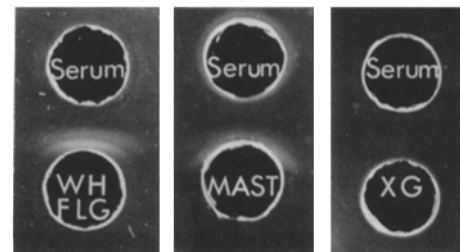
b.   Anti-Mastigoneme Serum absorbed with Xyloglycorien

FIGURE 7   Ouchterlony immunodiffusion plates verifying the absence of precipitin reaction in absorbed anti-XG and anti-MAST (right panels of a and b). Absorbed sera retain the ability to precipitate the homologous antigen (central panels) and whole flagella (left panels). Unabsorbed serum will precipitate the heterologous antigen (see text).

Application of absorbed rabbit anti-MAST to living or fixed cells followed by incubation in affinity-purified fl-GAR produces a dramatic and intense staining of the flagellum (Figs. 8A, B, and 9D). Anti-XG gives essentially the same staining pattern, except that the staining is less intense and the diameter of the flagellum appears to be substantially smaller (Figs. 8C, D, and 9C), as would be expected if the membrane and not the enveloping mastigonemes is labeled. Preimmune or heterologous serum followed by GAR gives no staining (Fig. 9A and B). Monovalent anti-XG (Fab') occasionally stains both gullet and reservoir as well as flagellum, perhaps because the smaller size of these fragments allows penetration past the gullet region which is generally occluded by the flagellum. The localization of anti-XG to the flagellum is further confirmed

from images of ferritin-GAR binding to partially disrupted flagella treated with anti-XG (Fig. 10). The dense mat of mastigonemes may physically prevent the large ferritin complex from making direct contact with the flagellar membrane in intact flagella. However, membrane vesicles partially free of the matted coat appear to be capable of binding the secondary antibody.

DISCUSSION

Although the flagellar surface has long been recognized as having special properties associated with mating, light reception, and surface movements, etc., relatively little information is available on the composition, development, and distribution
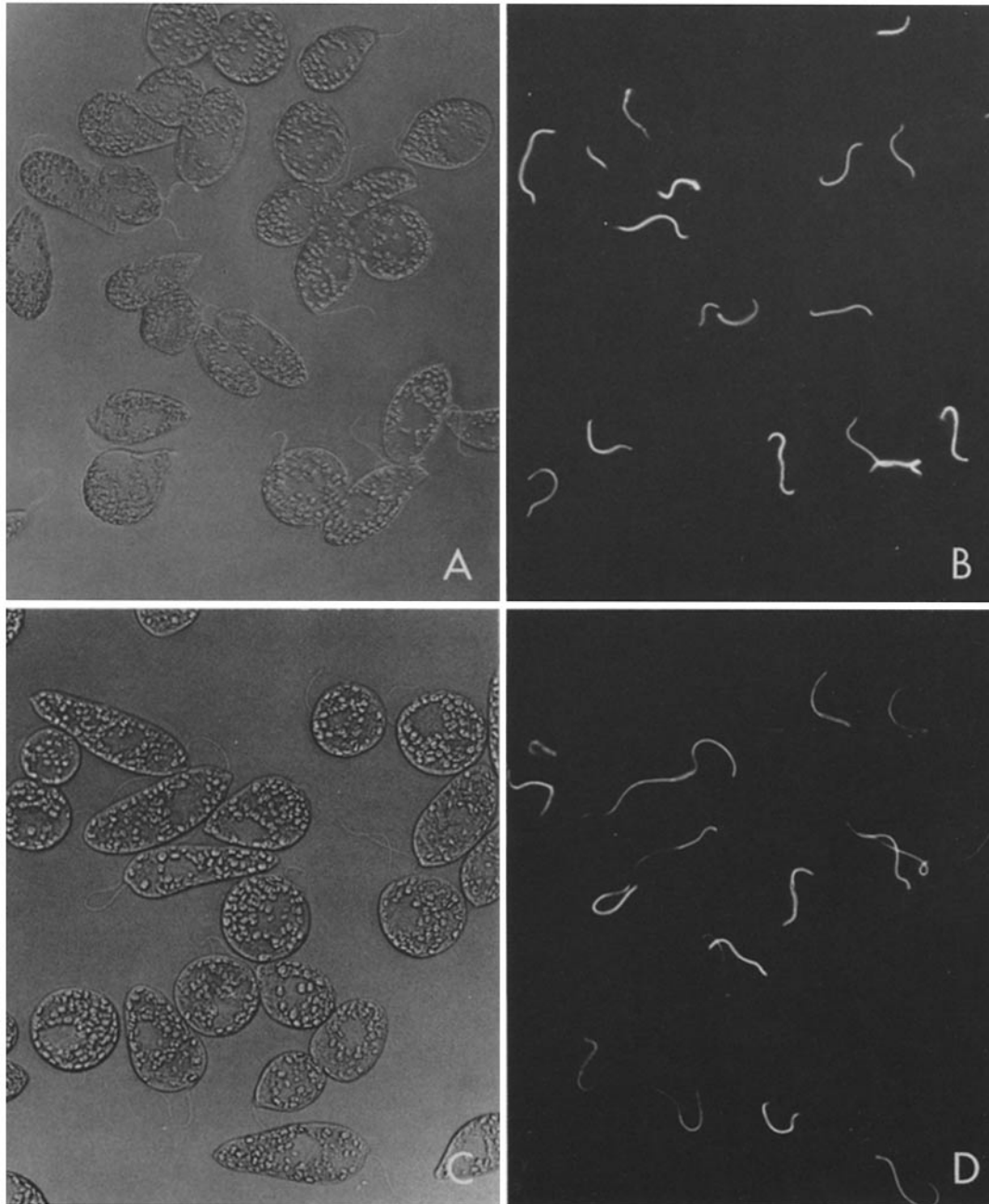

FIGURE 8   Fixed cells incubated with absorbed anti-MAST Fab$_2$' (A and B) or anti-XG (C and D) IgG and indirectly labeled with fl-GAR. Bright-field (A and C) and fluorescent images (B and D) from the same preparation demonstrate that only flagella bind anti-MAST and anti-XG antibodies. Chloroplast autofluorescence has been reduced with a red absorbing filter. X 400.
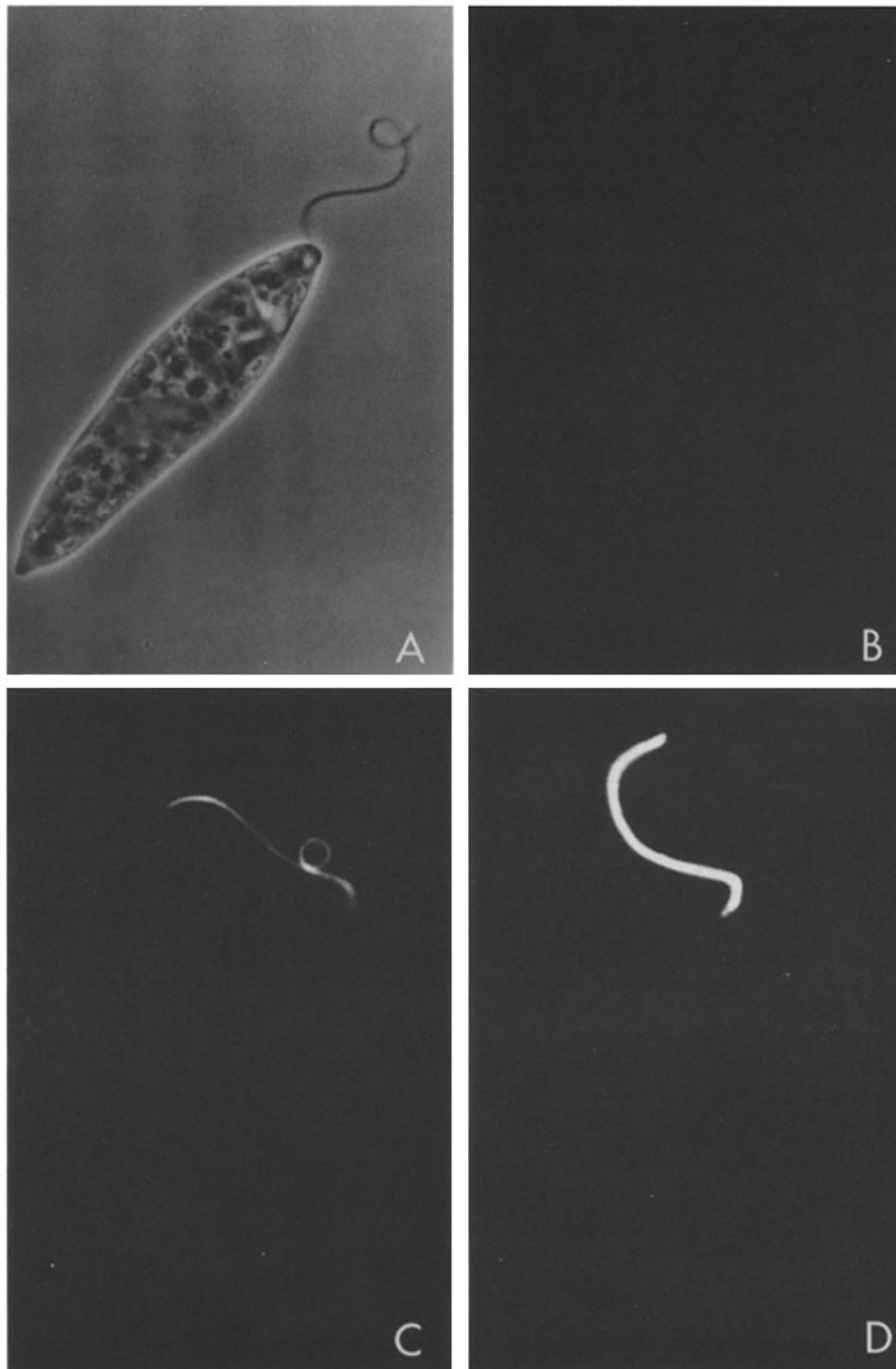
FIGURE 9   Cells incubated with preimmune serum (A and B), anti-XG IgG (C) and anti-MAST IgG (D) rabbit antiserum. All cells were postincubated with Fl-GAR. Preimmune serum exhibits no GAR binding (B). Anti-XG (C) shows a thinner line of fluorescence than anti-MAST (D), probably because xyloglycorien is confined to the flagellar membrane whereas mastigonemes form a thick enveloping meshwork on the flagellar surface. × 1,000.

of flagellar membrane components. *Euglena* is one of the flagellates that lends itself well to such analyses because it is easy to obtain large quantities of flagella for direct biochemical characterization and for antibody production. Thus, in this report, a major unique flagellar glycoprotein (xyloglycorien) has been extracted from purified flagella with mild neutral detergent, and has been localized to the flagellar but not body surface by means of immunofluorescent labeling. Xyloglycor-

ien is tentatively equated with a specific membrane filamentous surface fuzz from the following evidence: (a) Treatment of whole flagella with NP-40 removes only xyloglycorien and one or two minor PAS-positive components. Concomitantly, the fuzzy layer can no longer be demonstrated in negatively stained, detached, flagellar membrane vesicles. (b) After LIS extraction, xyloglycorien remains associated with the particulate fraction which consists only of membranes and mastigo-

FIGURE 10 Preparation of an isolated flagellum incubated with absorbed anti-XG followed by ferritin-conjugated GAR. Ferritin labeling is heaviest around vesiculated flagellar membrane, probably because the dense mat of mastigonemes hinders direct access of the relatively large ferritin molecule to the intact membrane. × 75,000.

nemes. The fuzzy layer is still visible by negative staining, although it is altered in appearance relative to untreated samples. (c) Xyloglycorien is located on the outside of the membrane because it is accessible to antibody binding in intact, living cells. (d) The extraction of xyloglycorien is best accomplished with reagents and under conditions in which hydrophobic interactions are perturbed (5, 35, 41): i.e. chloroform-methanol, neutral detergents; and xyloglycorien tends to form aggregates when detergent concentration is lowered by freezing or detergent is removed with SM-2 beads.

These latter facts are consistent with the notion that, in addition to the carbohydrate portion, xyloglycorien contains a hydrophobic, presumably membrane-associated, region.

Although there is a considerable immunological cross-reactivity between xyloglycorien and mastigonemes, at least some fraction of anti-XG will not react, i.e. cannot be adsorbed by intact mastigonemes. Cross-reactivity appears to be a real property of these sera, for, despite careful monitoring by SDS gels to assure antigen purity before inoculation, no serum was ever obtained that did cross react with the heterologous antigen.

The large amounts of xylose found in TLC analyses of both glycoproteins might may well account for a similar antibody response. That carefully adsorbed anti-XG serum is specific only for xyloglycorien is evidenced by the absence of binding to mastigonemes using ferritin label, and by the absence of visible precipitin reaction with mastigonemes in ring tests or immunodiffusion plates. Thus, the sum of these observations suggests that xyloglycorien is not part of the mastigoneme complex but is located on the flagellar membrane surface. Additionally, although several minor glycoproteins are coextracted with NP-40, it seems unlikely that they represent much if any of the antibody produced against xyloglycorien. Not only are they present as <10% of the total extract, but, in addition, one flagellar PAS-positive minor band is also present in the pellicle surface. Yet the cell surface produces negligible background staining when incubated with anti-XG IgG and fl-GAR. Furthermore, isolated pellicle will not form precipitin arcs with anti-XG. These facts suggest little or no contamination with antibodies to the minor bands.

Flagellar surface fuzz has also been clearly identified in flagella of Chlamydomonas (28) and in several other chlorophyceen (cf. 20) and haptophyceen (21) organisms, even after preparation for electron microscopy. In Euglena, fuzz is not visible after similar embedding and sectioning, possibly because of its removal by organic solvents during dehydration. This might suggest that surface fuzz is more stable in other flagellates, but, on the other hand, Chlamydomonas flagellar membranes are entirely solubilized (8, 40) at concentrations of neutral detergents less than one-half of those that remove only xyloglycorien from the Euglena flagella membrane. These observations make it difficult to generalize on the similarity of flagellar surfaces until a systematic examination of several systems under identical conditions has been undertaken. It is of interest, however, that a high molecular-weight glycoprotein has been found in the gill cilia and sperm flagella of the scallop Aequipectin irradians (36), and in whole Chlamydomonas flagella (24), although it is not yet known whether these glycoproteins are restricted to only those areas. In Tetrahymena cilia the major glycoproteins are of moderate size (45,000-55,000 mol wt), but a small amount of high molecular weight, PAS-positive material is present as well (7).

In contrast to this report on Euglena, previous efforts to identify a flagellar-specific antigen in other flagellates have found a more general distribution. For example, the immobilization, or "i", antigens of Paramecium that have been characterized as high molecular weight proteins ranging from 240,000 to 320,000 and comprising 30% of the protein content of cilia are also found on the cell surface (33). Antisera directed against whole flagella of Chlamydomonas appear to bind the major flagellar membrane glycopeptides (1). However, immunofluorescence studies with Chlamydomonas have shown antibody binding to the cell wall and to the flagellum (12).

The limited distribution of some membrane components in Euglena appears to be maintained through cell division (14) and flagellar regeneration, and in this respect the membrane domains appear to be unlike membrane domains in other cell types. In the mouse erythroblast (11) and in neonatal human erythrocytes and reticulocytes (37), there is a gradual restriction of total lectin mobility during maturation, presumably caused by the elaboration of an underlying spectrin network (13). The existence of "microdomains" of localized membrane components is evident in a number of systems: e.g. the lipopolysaccharides, or major somatic antigens (o-antigens) found on the

outer membrane of gram-negative bacteria appear to be organized into discrete domains in which metabolically older lipopolysaccharides do not freely intermix with newer components of differing composition (19); the H-Y (histocompatibility) antigen of mouse sperm appears to be restricted to only the acrosomal cap region, as has been demonstrated by use of hybrid antibody (anti-mouse/anti-tobacco mosaic virus (TMV) and TMV as a visual marker (18); a polar distribution of membrane components has also been reported over the surface of several mammalian spermatozoa using fluorescent lectin probes (23) or iodinated fluorescent markers (10). Furthermore, Papermaster et al. (25) have found that a large 290,000-mol wt intrinsic membrane component of rod segments (ROS) can be localized only to the incisures of the ROS disks. However, the more extensive regional (macrodomain) positioning of surface membrane components, such as that demonstrated here in Euglena flagella and previously in the pellicle surface, has been clearly shown elsewhere only in the case of the epithelial cell where marked cell surface polarity is evident at morphological, biochemical, and immunocytological levels (16), and in the intestinal villus cell membrane which can apparently be separated into three fractions each with distinct ATPase and glycosyltransferase activity (39). All the systems that possess micro- or macrodomains pose interesting questions as to the cellular control, insertion, and positional maintenance of surface antigens, and to the physiological significance of such specializations. It seems apparent that the characteristic lateral freedom of membrane proteins or glycoproteins of lymphocytes and other cells (31) is considerably modified and restricted to well-defined areas in at least some organisms.

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