Flagellar Surface Antigens in *Euglena*: Immunological Evidence for an External Glycoprotein Pool and Its Transfer to the Regenerating Flagellum

ADRIENNE A. ROGALSKI and G. BENJAMIN BOUCK
Department of Biological Sciences, University of Illinois, Chicago, Illinois 60680. Dr. Rogalski's present address is Department of Biology, University of California at San Diego, La Jolla, California 92093.

ABSTRACT Antibodies raised against the Sarkosyl-insoluble, major flagellar glycoprotein fraction, mastigonemes, were used to determine the source of flagellar surface glycoproteins and to define the general properties of flagellar surface assembly in *Euglena*. After suitable absorption, mastigoneme antiserum reacts with several specific mastigoneme glycoproteins but does not bind either to the other major flagellar glycoprotein, xyloglycorien, or to other Sarkosyl-soluble flagellar components. When Fab' fragments of this mastigoneme-specific antiserum were used in combination with a biotin-avidin secondary label, antigen was localized not only on the flagellum as previously described but also in the contiguous reservoir region. If deflagellated cells are reservoir pulse-labeled with Fab' antibody, this antibody appears subsequently on the newly regenerated flagellum. This chased antibody is uniformly distributed throughout the length of the flagellum and shows no preferred growth zone after visualization with either fluorescein or ferritin-conjugated secondary label. From these and tunicamycin inhibition experiments it is concluded that (*a*) a surface pool of at least some flagellar surface antigens is present in the reservoir membrane adjacent to the flagellum and that (*b*) the reservoir antigen pool is transferred to the flagellar surface during regeneration.

Prominent among the surface features of the emergent flagellum of *Euglena* is the flagellar sheath consisting of nearly 30,000 precisely positioned mastigoneme filaments and their complex anchoring units (7), and a continuous 200-Å membrane fuzzy layer (33). These well-defined surface features are limited in their overall surface distribution and endow the flagellum with a distinct biochemical profile (7) not directly comparable with the rest of the *Euglena* cell surface (19) or to a number of other recently analyzed flagellar/ciliary membranes (1, 4, 5, 6, 10, 11, 16, 20, 25, 27, 28, 31, 36, 40, 41). These specialized membrane and surface properties exhibited by *Euglena* and other flagella raise interesting questions with respect to the mechanisms of synthesis, the pathways of mobilization from sites of synthesis, and the timing of surface assembly relative to axonemal growth. Some of these questions can be addressed in *Euglena* because two major flagellar glycoproteins have been identified and isolated and their structural counterparts in the flagellum confirmed (33).

For example, neutral detergent extraction of *Euglena* flagella yields "xyloglycorien" which comprises a 200 Å fuzzy surface layer, whereas flagella solubilized with Sarkosyl leave behind an insoluble particulate fraction consisting of surface "mastigonemes." While both of these glycoproteins share in common the presence of the pentose sugar xylose (7, 15), they can be readily distinguished by (*a*) their structure and association with the flagellar surface, (*b*) their molecular weights, and (*c*) their sensitivity to degradation by proteases (32). Results from the use of antibody probes specific to xyloglycorien and mastigonemes have led to the preliminary conclusion that both antigens are confined only to the flagellar surface (33).

For this study we further developed immunological labeling methods in order to map the origin and pathways of assembly of one of the two major *Euglena* flagellar surface antigens. Monovalent primary antibodies used in conjunction with various combinations of labeled secondary moieties have revealed a more extensive distribution of antigen than was previously apparent, and have indicated in suitable pulse-chase experiments that a substantial surface pool of glycoproteins is available to the regenerating flagellum. A preliminary account of this work has been reported (34).

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MATERIALS AND METHODS

Culture maintenance, flagella isolation, preparation of flagellar mastigonemes, preparation of antimastigoneme antibodies, and purification of IgG, Fab_2', and Fab' fragments were performed as previously described (33).

SDS Acrylamide Gel Electrophoresis and Immunological Labeling of Gels

Flagellar samples were solubilized by heating at 100°C for 5 min in 1% SDS/1% mercaptoethanol (ME) in 0.01 M sodium phosphate, pH 7.2, and were subsequently alkylated with iodoacetamide. Samples were then dialyzed for 24–48 h against several changes of 0.01 M sodium phosphate buffer, pH 7.2, 0.1% SDS, and 0.1% ME. They were then electrophoresed in 7.5% acrylamide (38) either in 4-mm diameter glass tubes (5 mA/tube) or in 1.5-mm thick slabs (15 mA). Gels were fixed either in 50% TCA or in 40% ethanol–5% acetic acid overnight and then stained for proteins with Coomassie Brilliant Blue or glycoproteins with periodic-acid-Schiff stain (PAS) as previously described (33).

Immunological labeling of slab gels was carried out essentially as described by Burridge (9), except that gels were generally fixed in methanol/H_2O/acetic acid (5:5:1) overnight, and rinsing of gels after overlaying with antibodies or iodinated Protein A was extended to 6 d. Protein A (Pharmacia Fine Chemicals, Piscataway, NJ) was iodinated by the Bolter and Hunter (24) procedure. Preparation of antigens for immunodiffusion was carried out as described previously (33).

Preparation of Fluorescein, Biotin, and Ferritin-labeled Secondary Markers

Goat anti-rabbit antibodies (GAR) directed against heavy and light chains or exclusively to light chains (Miles Laboratories, Elkhart, IN), were further purified by passage through a column of Whatman DE-52 resin (Whatman, Inc., Chemical Separation Div., Clifton, NJ). Flow-through fractions of IgG were then generally affinity purified by chromatography on Ultrogel AcA 22 (LKB Instruments, Inc., Rockville, MD) coupled with rabbit IgG (37). Eluted goat anti-rabbit IgG was then conjugated with fluorescein according to the method of Brandtzaeg (8).

Avidin (Sigma Chemical Co., St. Louis, MO) was conjugated to fluorescein using the methods of Heggeness and Ash (18). Biotination of goat anti-rabbit antibodies was preformed via biotinyl-N-hydroxy-succinimide ester (Calbiochem-Behring Corp., American Hoeschst Corp., La Jolla, CA) dissolved in dimethylformamide (2).

Horse spleen ferritin (Miles Laboratories) was purified before protein conjugation by column chromatography (2 × 30 cm) using Sepharose 4B (Pharmacia Fine Chemicals, Inc.) equilibrated with 0.1 M sodium phosphate buffer, pH 7.3 (39). One major peak was obtained. The front third of the peak which contained most of the ferritin oligomers was discarded. The remaining two-thirds of the peak was pooled and concentrated. This represented >95% monomeric ferritin. Affinity purified, goat anti-rabbit IgG and avidin were then conjugated to the purified ferritin as described by Kishida et al. (22).

Immunofluorescent and Immunoferritin Labeling of Cells

Euglena from 4-d-old cultures were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, for 20 min at room temperature. Under these conditions chloroplast integrity is maintained so that their autofluorescence can be clearly distinguished. Cells were subsequently rinsed three times in phosphate-buffered saline (PBS) (10 min each rinse with agitation): the second and third rinses were supplemented with 0.2 M glycine to quench free aldehydes. Cells were then incubated with 1 mg/ml of primary rabbit antibody (IgG, Fab_2', or Fab') for 30 min at room temperature and rinsed an additional three times in PBS before further incubation with 100–500 μg/ml of GAR-IgG-FITC, GAR-Fab'-FITC or biotinated GAR (B-GAR) for 30 min at room temperature. Cells were then rinsed an additional three times. If B-GAR was used, cells were further incubated with 500 μg/ml of avidin-FITC (Fl-avidin) for an additional 30 min and then subjected to the same rinsing protocol as described above. Cells were examined by incident illumination in a Zeiss photomicroscope II equipped with a mercury vapor light source (HBO 100 W/4), an FITC excitation filter, a Zeiss 65 barrier filter to specifically absorb chloroplast autofluorescence and a 0.65 NA × 40 oil immersion Planapochromat objective.

For ferritin labeling, cells were prefixed for 15 min in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, rinsed in PBS-glycine three times, and treated with either IgG, Fab_2', or Fab'. After incubation in antisera for 30 min, cells were rinsed in PBS–glycine and further incubated in either Fe^{++}-GAR or B-GAR for 30 min. Cells that were treated with B-GAR were rinsed three times and then treated with Fe^{++}-avidin for 30 min. For all preparations, completion of secondary labeling was followed by two rinses in PBS-glycine and a final rinse in 0.01 M sodium phosphate, pH 7.0. Cells were then subjected to a second fixation of 4% glutaraldehyde (Polysciences, Inc. Warrington, PA) in 0.01 M sodium phosphate, pH 7.0, for 1 h at room temperature. After three rinses in 0.1 M sodium phosphate buffer, preparations were processed for electron microscopy as previously described (33) and examined with a Hitachi 11 B electron microscope.

Antibody Labeling of Living Cells

Since living *Euglena* cannot tolerate PBS a low salt buffer was substituted. Cells from 4-d-old cultures were resuspended in 0.01 M sodium phosphate/0.01% NaCl, pH 6.5 and then deflagellated by 1-min agitation in a Vortex homogenizer (Vortex Genie, Scientific Products Div., American Hospital Supply Corp., Springfield, MA). After three rapid rinses in buffer to remove flagella, deflagellated cells were incubated in Fab' (1–2 mg/ml, dialyzed against buffer to remove azide) for either 1 h at room temperature or 2 h in the dark at 4°C. Cells were then rinsed three times in buffer (5 min for each rinse) and were chased in buffer for another 4 h at room temperature. In additional experiments, cells were incubated for 1 h at room temperature, rinsed three times in buffer, redeflagellated by 1-min vortexing, and then chased in buffer for another 4 h at room temperature. After completion of the 4-h chase, a period during which cells regenerate their flagella to nearly full length, all preparations were fixed in 4% paraformaldehyde in 0.1 M phosphate, pH 7.4, for 20 min, and rinsed one time in PBS and then two times in PBS–0.2 M glycine. Cells were then incubated with B-GAR (100 μg/ml) in PBS for 30 min at room temperature, rinsed three times and then subsequently incubated with either Fl-avidin (500 μg/ml) or Fe^{++}-avidin (500 μg/ml) for 30 min at room temperature. After three additional rinses in PBS, preparations were examined with a Zeiss photomicroscope using fluorescent excitation as described above. Ferritin-labeled preparations were generally examined by applying a drop of cells on a carbon-stabilized, Formvar-coated copper grid, rapidly rinsing in distilled water, and then drying at room temperature.

Double Deflagellation Experiments in the Presence of the Glycosylation Inhibitor, Tunicamycin

Euglena were subjected to two successive cycles of deflagellation and a 4-h period of flagella regeneration in the absence or presence of 2 μg/ml of the glycosylation inhibitor (35) tunicamycin (Calbiochem-Behring). Tunicamycin was initially prepared as a stock solution of 1 mg/ml in 0.02 N NaOH and then diluted to 2 μg/ml in *Euglena* media (cf, reference 15). These experiments were performed either on small scale using 1–2 ml of cells for subsequent fluorescent staining of fixed cells as described above, or on a macroscale of 48 l of cells, concentrated to 500 ml for subsequent biochemical analysis by SDS gel electrophoresis.

RESULTS

Antisera Specificity to the Flagellar Mastigonemes

As shown previously (33), *Euglena* flagellar mastigonemes isolated by their resistance to two successive extractions of whole flagella with 0.1% Nonidet P-40 (NP-40) and 1.5% Sarkosyl are both structurally complex and biochemically heterogeneous. Solubilized, reduced, and alkylated samples of mastigonemes can be separated into 12 polypeptides when electrophoresed in 7.5% acrylamide SDS gels: (*a*) two major high molecular weight (Fig. 1 *A*, *a* and *e*) Coomassie Blue-staining bands with estimated molecular weight of >250,000 and 200,000, both of which are PAS-positive (Fig. 1 *B*); (*b*) six minor Coomassie Blue-staining bands with estimated molecular weight ranging from 250,000 to 100,000 which are also PAS-positive (Fig. 1, *b*, *c*, *d*, *f*, *g*, and *h*); and (*c*) four minor bands (Fig. 1, *i-l*) with estimated molecular weight of 100,000 to 50,000 which are only weakly stained with Coomassie Blue and which are not PAS-positive (Fig. 1 *A* and *B*). The mastigoneme glycoproteins are sensitive to protease digestion and are either partially digested with 1% trypsin or completely digested with 1% pronase, suggesting that the peptide portion

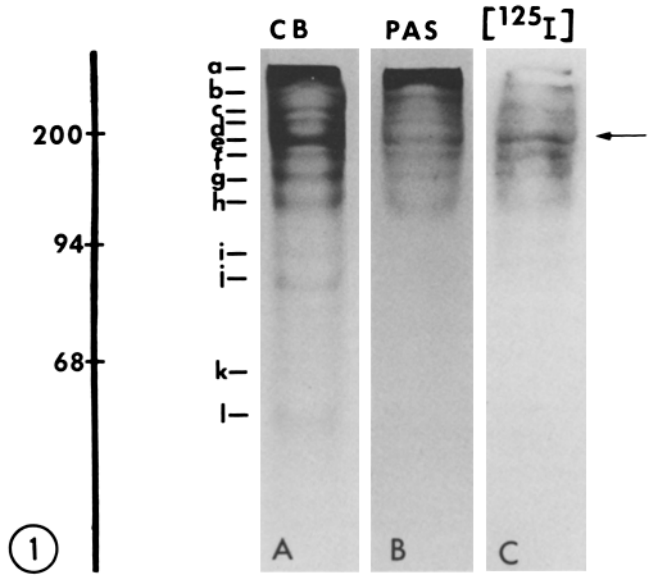
of these glycoproteins is externally exposed and not protected by carbohydrate residues. Proteolytic digestion of mastigonemes results in their fragmentation into several low molecular weight PAS-positive bands, thus precluding separate analysis of carbohydrate and peptide portions of these glycoproteins by this means. Commercial (Sigma Chemical Co.) xylosidase had no detectable effect on PAS staining or on apparent molecular weights.

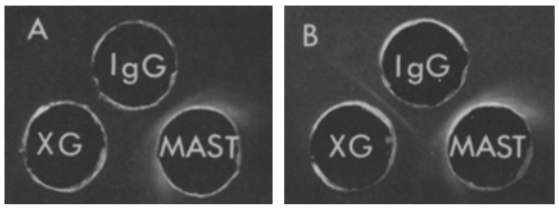
Previous studies (33) indicated the specificity of antisera to the flagellar mastigonemes, providing the preabsorption of the antisera with xyloglycorien due to its inherent cross-reactivity with this antigen was carried out. To determine which of the multiple mastigoneme bands were immunogenic, absorbed antisera to mastigonemes were tested in the present study by gel immuno-overlay procedures. One major mastigoneme polypeptide (~200,000 mol wt) and five minor polypeptides appear to be reactive (Fig. 1 *C*). Unabsorbed antisera yield similar results except that the major polypeptide (>250,000 mol wt) barely penetrating 7.5% acrylamide gels is also recognized.

As indicated earlier (33), complete absence of reaction between mastigonemes and xyloglycorien can be achieved only by absorption of antisera with xyloglycorien (Fig. 2 *A* and *B*). This absorbed antiserum will form immunoprecipitin arcs with whole Sarkosyl-solubilized flagella and with mastigonemes but shows no reaction with Sarkosyl-solubilized flagella from which the mastigonemes have been removed (Fig. 2 *C*) by centrifugation. These results indicate that absorbed antiserum is specific for a few Sarkosyl-insoluble mastigoneme components. Blot transfer experiments (R. Dubreuil, personnel communication) using nitrocellulose sheets have confirmed this specificity although the very high molecular weight glycoproteins are not readily transferred with this method. Since mastigonemes are xylosylated (33), efforts were also made to determine whether the antiserum recognized the carbohydrate (xylose) or the peptide portion of the glycoprotein. Therefore unabsorbed antiserum was incubated with either 1% xylose or 1% xylan(B-1,4 linked xylopyranose; Sigma Chemical Co.) for 72 h at room temperature. Although this treatment had little effect on the precipitin arc subsequently produced when the serum was diffused against mastigonemes, a clear reduction but not elimination of the precipitin arc against xyloglycorien was evident (data not shown). Arabinose did not mimic this effect. These results, while not conclusive, are consistent with the assumption that absorbed mastigoneme antiserum may be at least partially directed towards the peptide portion of the glycoprotein.

Evidence for a Surface Pool of Flagellar Antigens

The basal portion of the *Euglena* flagellum is not anchored at the anterior tip of the cell but arises in the reservoir, an invaginated portion of the anterior cell surface. Application of fluorescent antibody using either divalent IgG against mastigonemes or against xyloglycorien suggested that these antigens were present only on that portion of the flagellum visible outside the reservoir (33). However, in this study the use of Fab'$_2$, monovalent Fab', and a number of single and double secondary labels, demonstrated a more extensive labeling pattern (Table I). In general it has been found that monovalent Fab' and an affinity-purified biotinated secondary label followed by fluorescent avidin consistently revealed antibody binding in the area adjacent to the flagellum as well as the flagellum itself. A comparison of several combinations of immunolabeling using primary antibody with the same specificity is illustrated in Figs. 3 *A–D*. Intense fluorescense of the reservoir lining was evident in all developmental stages examined by the procedure demonstrated in Fig. 3 *D*. Therefore this procedure was adopted for all subsequent experiments.





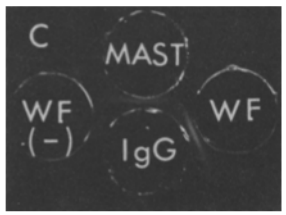


FIGURE 1 SDS gels of *Euglena* mastigonemes stained for polypeptides (*CB*) or carbohydrates (*PAS*). Most of the high molecular weight polypeptides are also PAS-positive. In gel *C* which was electrophoresed in the same slab gel as lane *A* and *B*, the unstained gel was overlaid with xyloglycorien absorbed antimastigoneme IgG. After removing excess antibody, the gel was further incubated with iodinated protein A and fluorographed. Note that the predominant glycoprotein (band *a*) does not interact with the absorbed serum. Band *e* appears to be the primary antigenic component, but band *f* and several other minor constituents also bind antiserum. Molecular weight standards (× 10^{-3}) are myosin (200,000), phosphorylase a (94,000), and bovine serum albumin (68,000).

FIGURE 2 Immunodiffusion plates of antimastigoneme IgG reacted against xyloglycorien (*XG*) and mastigonemes (*MAST*). In *A*, IgG was first absorbed with xyloglycorien and shows no precipitin arc with XG. In *B*, unabsorbed IgG clearly cross-reacts with xyloglycorien despite the electrophoretic purity of the original injected antigen. In *C*, absorbed IgG reacts with whole flagella solubilized in Sarkosyl (*WF*) but not with Sarkosyl-solubilized flagella from which the mastigonemes have been removed by centrifugation [*WF*(−)]. The Sarkosyl-resistant mastigonemes from these latter flagella strongly react (*MAST*) with the absorbed serum.

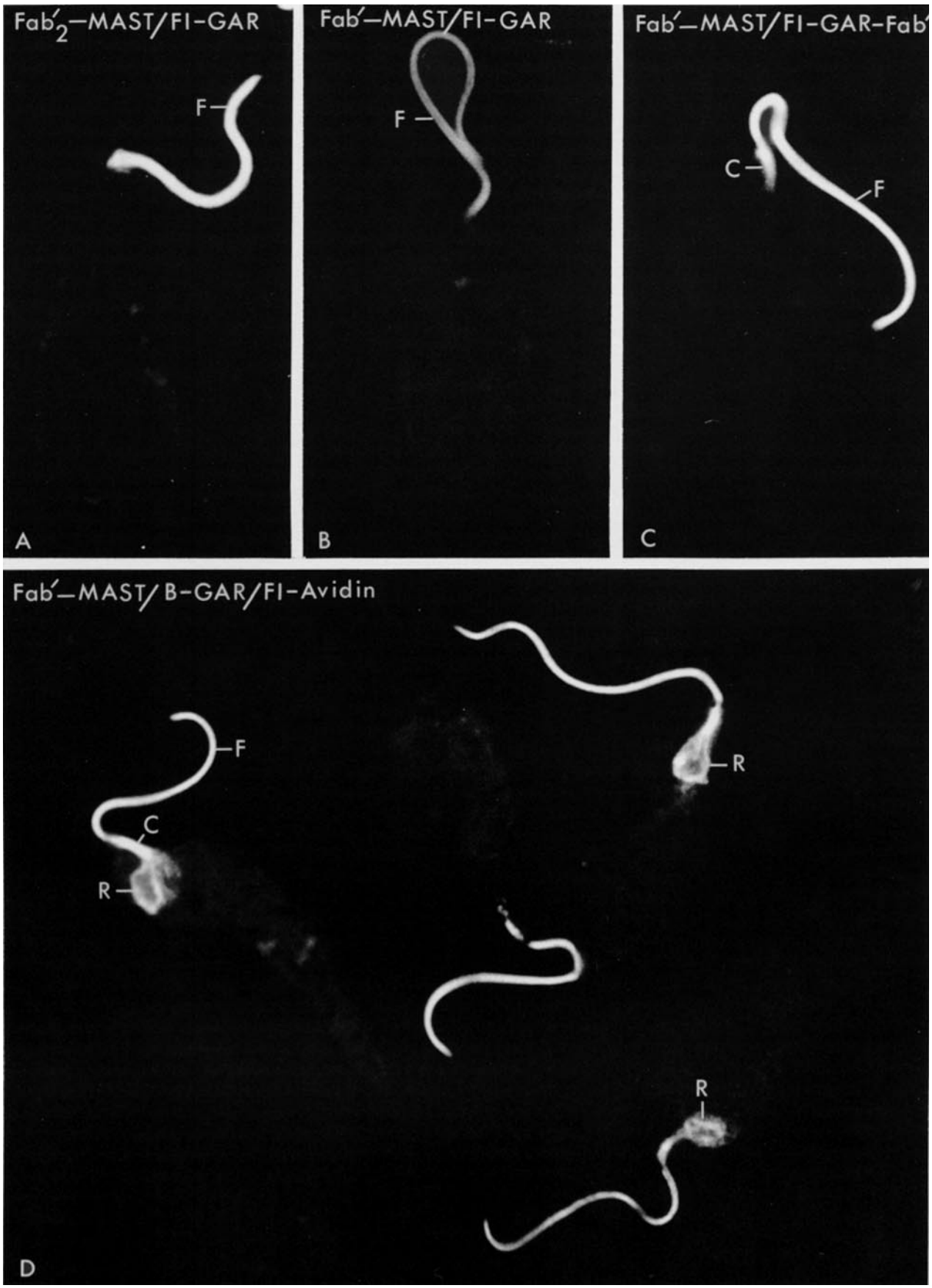


FIGURE 3 Immunofluorescent detection of mastigoneme antigens using four different primary and secondary labeling protocols. Cells were fixed in aldehyde and then reacted with the combinations indicated. Divalent Fab′$_2$ (*A*) binds strongly to the exposed flagellum but does not penetrate canal (label *C*) or reservoir. Monovalent Fab′ produces less intense staining (*B*) but with appropriate secondary label (*C*) reveals antigens in the canal. Only with a three-step incubation procedure (*D*) is the reservoir (*R*) consistently labeled. *A–C*, × 1,600. *D*, × 1,000.

TABLE I

Summary of Fluorescent Antibody Labeling Techniques

Labeling condition	Location and intensity of immunofluorescent label		
	Emergent flagellum	Canal region	Reservoir region
IgG + Fl-GAR	+++	–	–
Fab_2' + Fl-GAR	+++	–	–
Fab' + Fl-GAR	++	–	–
Fab' + Fl-GAR-Fab'	++	+	+
B-Fab' + Fl-avidin	++	+	+
B-Fab' + avidin/Fl-anti-avidin	++	+	+
Fab' + B-GAR/Fl-avidin	++	++	++

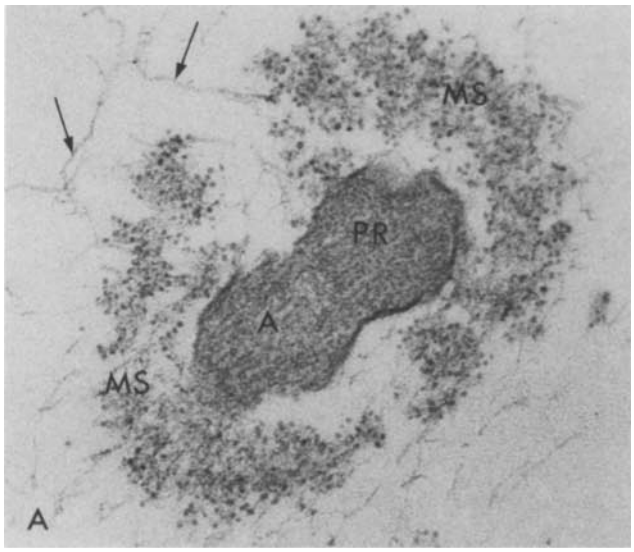
+++, Intense staining. ++, Moderate staining. +, Light staining. –, Absence of staining.

By replacement of Fl-avidin with Fe^{++}-avidin in both whole-cell mounts and thin-section electron microscope preparations (Fig. 4 *A* and *B*), Fab' to mastigonemes was found to label exclusively the mastigoneme sheath. No labeling of the 1.5 μm or 3.0 μm mastigoneme filaments was ever detected in these preparations. Nor was ferritin found in the reservoir, suggesting that the large size of the ferritin conjugates prevents penetration past the canal, unlike the counterpart fluorescent labeling procedures.

Use of the Surface Glycoprotein Pool during Flagellar Regrowth

Pulse-chase of immunlogical label was accomplished with three different protocols: (*a*) pulse labeling in the cold (nonregenerating conditions); (*b*) pulse labeling at room temperature followed by regeneration at room temperature; and (*c*) pulse labeling at room temperature, redeflagellation, followed by regeneration at room temperature.

(*a*) Deflagellated cells which were pulse labeled at 4°C for 2 h (nonregenerating conditions) with Fab' to mastigonemes and then chased under regenerating conditions (22°C) provided the most direct evidence for transfer of labeled antigens from the reservoir to the emerging flagellum (Fig. 5 A–D). When a sample of these pulsed cells was rinsed in buffer, fixed, and subsequently incubated with B-GAR/Fl-avidin, fluorescence of the reservoir could be detected in 90% of the cells (Fig. 5 *A* and *B*). Thus, pulse-labeled living cells were rinsed thoroughly and allowed to regenerate flagella at room temperature in buffer only. After a 1-h chase followed by fixation and fluorescence detection, label in the reservoir was significantly diminished as a result of its apparent transfer into the newly forming emergent flagellum (Fig. 5 *C*). After a 4-h chase, label in the reservoir was barely detectable, whereas the emergent flagellum was uniformly and faintly fluorescent (Fig. 5 *D*). Random labeling of flagella after a 4-h chase was confirmed with ferritin labeling (Fig. 6 *A–B*). Under these conditions staining of the emergent flagellum is significantly less intense



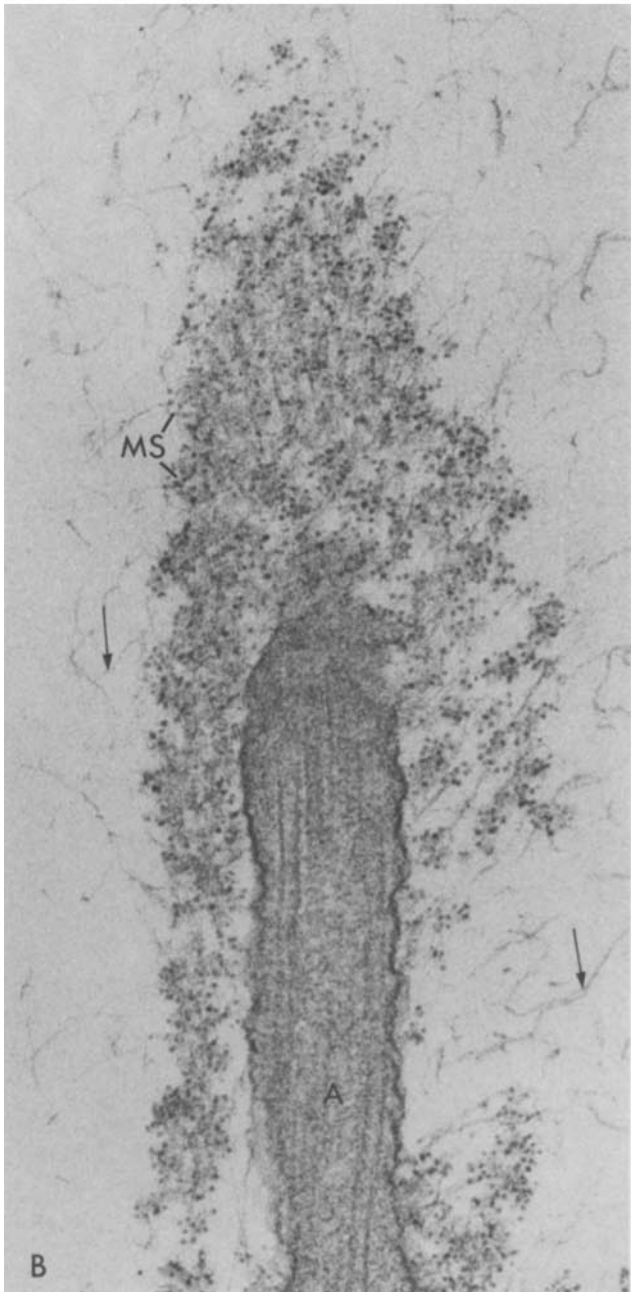


FIGURE 4 In these preparations flagella were fixed and then incubated in xyloglycorien absorbed, anti-mastigoneme Fab' followed by B-GAR and then Fe^{++}-avidin. After embedding and sectioning it is apparent that ferritin label is restricted to the mastigoneme sheath (*MS*) and not to the longer mastigoneme elements (arrows). *A*, axoneme. *PR*, paraxial rod. *A*, × 162,000. *B*, × 108,000.

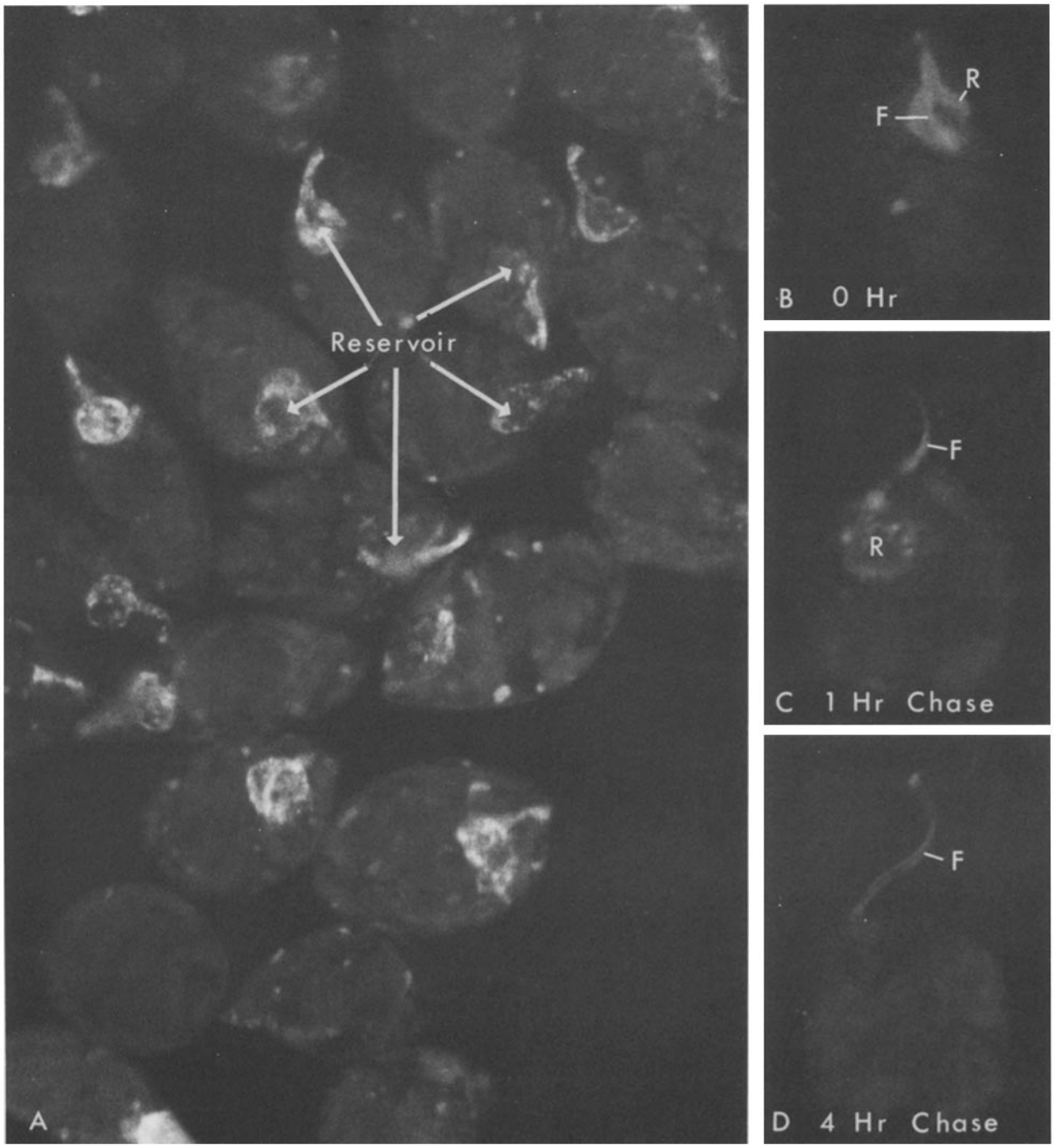


FIGURE 5 Immunolabeling of living deflagellated cells under conditions in which the flagella do not regenerate (0–4°C). Antimastigoneme Fab' readily binds to the reservoir (*A* and *B*), although the binding is not so intense as in fixed cells (c.f., Fig. 3). In *C* and *D*, cells were labeled as in *A* and *B* but were washed free of excess antibody and allowed to regenerate for 1 h or 4 h before fixation and secondary antibody application. Immunolabel present in the reservoir at 0 h is apparently transferred or chased onto the flagellum during regeneration. Flagellar labeling is diffuse and shows no well-defined growth zone. *F*, flagellum. *R*, reservoir. *A*, × 1,800. *B–D*, × 1,500.

than whole cells fixed and reacted for antigens because in the chase experiment all label is derived from the reservoir and flagellar stub. (*b*) If deflagellated cells were allowed to regenerate in the presence of antibody for 1 h at room temperature, binding could be detected in the reservoir as well as on the emerging flagellum. An additional chase period of 4 h, i.e., continued flagellar regeneration in buffer alone, resulted in a depletion of label from the reservoir and a uniform, continuous labeling of the fully developed flagellum. (*c*) If cells were similarly labeled with antibody for 1 h under regenerating conditions, but then were rinsed and subjected to a second round of deflagellation with a 4-h regeneration period in buffer only, uniform but very weak staining of the emergent flagellum was obtained (data not shown).

Evidence for Intracellular Supplementation of Pools of Flagellar Glycoproteins during Regeneration

In addition to the evidence for the reservoir as an external

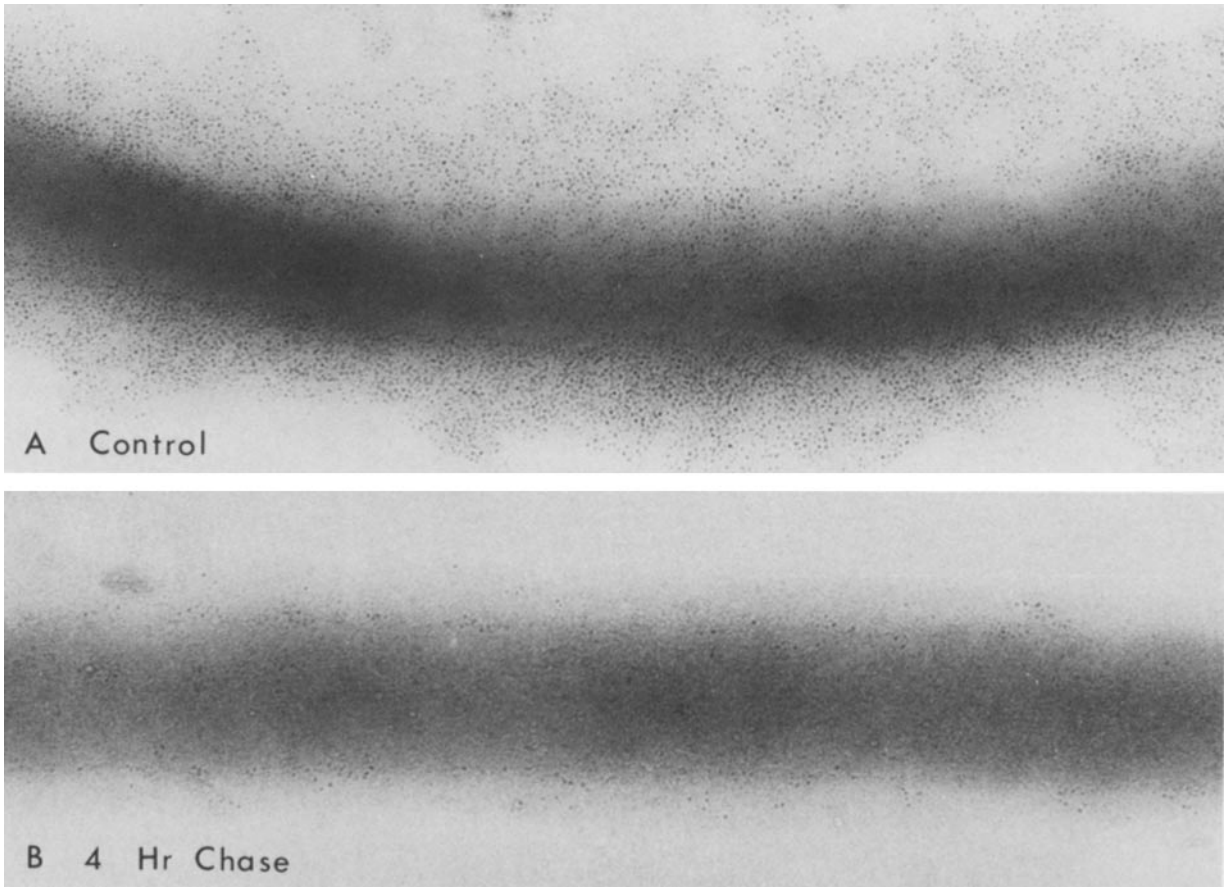


FIGURE 6 Ferritin-label on whole fixed flagellum (*A*) or on flagellum after immunolabeling followed by a 4-h chase as in Fig. 5 *D*. Ferritin is randomly dispersed over the flagellar surface (*B*), confirming the apparent diffuse labeling seen in the fluorescent images. Control (*A*) was fixed and immunolabeled directly. Uniform distribution of antigenic sites in this mature flagellum is evident. *A*, × 120,000. *B*, × 135,000.

pool of flagellar surface antigens, evidence was sought for supplementation of pools. These data were obtained indirectly when flagella regenerated under conditions where glycoprotein synthesis is blocked with tunicamycin (monitored by inhibition of [^{14}C]xylose incorporation by tunicamycin at 2 μg/ml, reference 15). After the first deflagellation/4-h regeneration cycle (scheme *a* above), there was essentially no difference between control and tunicamycin-treated cells as monitored by the intensity of antibody staining of the emergent flagellum and the reservoir. However, after a second deflagellation–4-h regeneration cycle, a significant difference between control and tunicamycin-treated cells was evident. In such preparations there was a general reduction of antibody binding in the reservoir, the majority of the cells retained antibody only in the canal region (Fig. 7 *D*) in contrast to control samples (Fig. 7 *B*) which display antigens in reservoir, canal, and flagellum.

DISCUSSION

In all eucaryotic flagella most, if not all, of the proteins and glycoproteins associated with the flagellar surface must be imported from cellular sites of synthesis and then assembled on the flagellar surface. In *Euglena*, this assembly process ultimately results in an ordered array of two major flagellar glycoproteins, xyloglycorien and mastigonemes (33). The latter consist of a number of subsets of glycoproteins whose heterogeneity is undoubtably correlated with the structural complexity of the mastigoneme units (the basic repeating element of the flagellar sheath). The immediate source of the mastigonemes glycoproteins appears, from results presented in this study, to be a surface pool in the reservoir adjacent to the flagellar base. This has been demonstrated by direct antibody labeling, and by pulse-chase experiments using antibodies which suggest that reservoir antigens are transferred to the flagellum during regeneration. This interpretation is also supported by the results of surface iodination experiments indicating that the external pool of labeled surface peptides is transferred to the flagellum during the first regeneration (15). However, the external reservoir pool appears to be supplemented during flagellar regeneration, as evidenced from the tunicamycin results of this study, i.e., reservoir antigens can still be detected in the reservoir until a second regeneration is initiated; but these experiments do not distinguish newly inserted vs. surface pool antigens. These results contrast with the loss of surface pool mastigoneme label from the reservoir in immunopulse labeled regenerating cells and suggest that new antigens must be added to the reservoir at the same time that other antigens are transported from the reservoir to the flagellar surface.

Critical to these summarized conclusions is the specificity of the mastigoneme antiserum, and the presumed ability of the antiserum to recognize the peptide portion of the mastigoneme glycoproteins. Evidence for specificity has been provided by gel overlay experiments in which antimast IgG absorbed with the other major flagellar glycoprotein, xyloglycorien, binds to one major mastigoneme glycoprotein (~200,000 mol wt) and

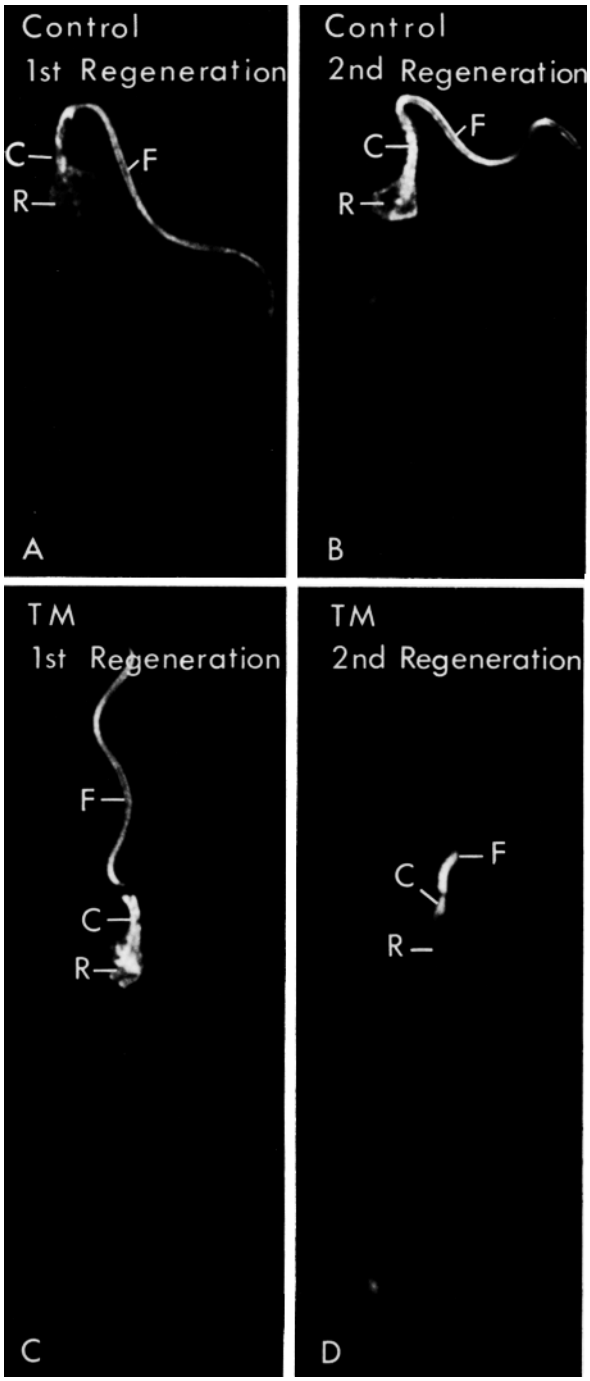


FIGURE 7 Cells that have undergone one and two deflagellation/regeneration cycles in the presence (*TM*) or absence (control) of the glycosylation inhibitor, tunicamycin (2 μg/ml). No obvious difference in fluorescence-staining labeling patterns is apparent in treated or untreated cells after the first regeneration cycle. After a second successive deflagellation, most of the TM-treated cells (*D*) fail to regenerate flagella and exhibit a general loss of antibody binding in the reservoir. In contrast to the procedures used in Figs. 5 and 6, cells were immunolabeled here after fixation. Thus antibodies bind both to the original pool of antigens and to antigen which might have been inserted in the reservoir during the first regeneration. *C*, canal. *F*, flagellum. *R*, reservoir. × 1,200.

several minor bands. Similarly absorbed serum no longer reacts with xyloglycorien or other Sarkosyl-soluble components, and it seems probable that absorbed serum loses much of the IgG which recognizes the carbohydrate portion of the mastigoneme. This latter conclusion is suggested from the following indirect evidence: (*a*) xyloglycorien and mastigonemes share the pentose sugar xylose as their principal carbohydrate moiety. The extensive cross-reactivity between xyloglycorien and antiserum to mastigonemes is probably the result of this carbohydrate similarity, and it seems reasonable to assume that xyloglycorien-absorbed mastigoneme antiserum has lost much of the xylose-recognizing antibody. (*b*) Gel overlay experiments using xyloglycorien-absorbed anti-mastigoneme serum show no binding of antibody to the major, slow migrating, strongly PAS-positive (xylose rich) band. However, the latter is recognized by antimastigoneme serum which is not preadsorbed with xyloglycorien. These results are consistent with the interpretation that xylose-recognizing antibodies are removed during absorption. (*c*) Competition experiments (data not shown) with a commercially available xylan or pure xylose demonstrate that xyloglycorien binding to unabsorbed mastogoneme antiserum can be at least partially inhibited, suggesting that the cross reactions of unabsorbed serum may be related to this sugar or similar polymers. If these assumptions are correct, then absorption of antimastigoneme serum with xyloglycorien should remove much of the xylan-recognizing antibodies and leave those specific for nonxylan (e.g., peptide) regions of the glycoprotein.

The specific structural mastigoneme component recognized by the xyloglycorien-absorbed mastigoneme antiserum appears from ferritin-labeling experiments, to be some portion of the mastigoneme units (7) which lie parallel (similar to a picket fence) to the surface of the flagella. Thus, the behavior of labeled pools in the reservoir and their subsequent migration to the flagella are restricted, in this study, only to the mastigoneme units, or precursors to mastigoneme units.

Incubation of deflagellated cells with absorbed serum resulted in clear labeling of the reservoir, providing the appropriate combination of primary antibody and secondary marker was selected. During regeneration, the reservoir immunolabel diminishes and the newly synthesized flagellum acquires a uniform antibody distribution. The conclusion inferred from these experiments is that reservoir antigens are transferred to the flagellum during regeneration. Somewhat more difficult to explain is the even, diffuse distribution of antibody over the entire regenerated flagellum rather than concentration in a basal growth zone. This could be interpreted as random incorporation of mastigoneme units (or precursors) throughout the entire regenerating flagellum, or basal addition of mastigoneme units masked by label present on the flagellar stub remaining after deflagellation, or intermixing of newly synthesized (unlabeled) glycoproteins with those of the labeled surface pool during regeneration. At present it is not possible to distinguish among these possibilities, although addition of antigen to the flagellar tip appears to be ruled out. The antagonistic growth zone model for control of flagellar growth still remains a viable possibility (12) to explain the characteristic flagellar growth patterns of these cells.

Cells regenerated in tunicamycin at concentrations which inhibit 95% of flagellar xylosylation (15) produce normal flagella—presumably because of the presence of a substantial glycoprotein pool. This latter prediction has been directly demonstrated in this study. However, it was not anticipated that the reservoir would continue to retain mastigoneme antigens after one round of regeneration in tunicamycin which should exhaust the pool and inhibit glyosylation of any new antigens introduced into the reservoir. A possible explanation for this observation is that the mastigoneme antibody is rec-

ognizing the peptide portion of the glycoprotein (see above) and that the unxylosylated antigens continue to be inserted in the reservoir from intracellular sources. It may well be that these unxylosylated moieties cannot replace the function of the normal glycoprotein and that as a result, further flagellar regeneration is inhibited.

Of particular interest in *Euglena* is the apparent absolute unidirectionality of antigen migration from the reservoir to the flagellum. Despite continuity of the reservoir membrane with the outer cell surface, neither mastigoneme nor xyloglycorien antiserum binds to regions other than the reservoir and flagellum. These latter regions thus constitute surface domains similar to those found in a wide range of other systems (14, 21, 26), using a variety of probes, such as monoclonal (29) or rabbit antibodies (23), lectins (23), antibiotics (3, 30), viruses (17), and iodinated reagents (13). Flagella-specific antigens are probably more widespread than reported thus far since mastigoneme-bearing flagella are not uncommon (6). In *Euglena*, both flagella-specific and cell body-specific (19) antigens have been identified, and such surface regionalization poses interesting and as yet unanswered questions as to how different portions of the same cell are separately assembled and maintained during development and how they are duplicated during cell division.

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