Synthesis and Mobilization of Flagellar Glycoproteins during Regeneration in *Euglena*

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ABSTRACT Flagellar glycoprotein synthesis and mobilization of flagellar glycoprotein pools have been followed during flagellar regeneration in *Euglena*. The glycosylation inhibitor tunicamycin has little effect on either regeneration kinetics or the complement of flagellar peptides as seen in SDS acrylamide gels, but tunicamycin totally inhibits incorporation of exogenously supplied [^{14}C]xylose into flagellar glycoproteins. Moreover, deflagellated cells pulsed with tunicamycin for 30 min or more, regenerated for 180 min, and then redeflagellated are completely or partially inhibited from undergoing a second regeneration even when tunicamycin is no longer present. These facts are interpreted as indicating that *Euglena* retains sufficient glycoprotein pool for one complete flagellar assembly. Some of this pool is present on the cell surface since [^{125}I]-labeled surface peptides can be chased into the regenerating flagellum. Glycosylation may also be taking place in the flagellum directly because [^{14}C]xylose has been found in three flagellar fractions: glycoprotein and two others, which are lipophilic and have properties similar to those described for lipid-carrier glycoprotein intermediates in other systems. Pulse-chase experiments also suggest a precursor-product relationship between the presumptive lipid carriers and flagellar glycoproteins. From these results a model is postulated in which *Euglena* is visualized as retaining sufficient pool of glycoprotein for one complete flagellar regeneration, but the pool is normally supplemented by active xylosylation *in situ* during regeneration.

During regeneration the flagellar membrane and its associated surface glycoproteins such as mastigonemes and scales are assembled in a relatively short period after flagellar withdrawal or excision. As with the well-studied flagellar axonemes, the regenerating flagellum can also be used to identify precursors and the mobilization of the components of the flagellar surface, and to determine how much, if any, control they exert on regeneration. In *Euglena* such analyses are facilitated by the distinctive properties of the flagellar surface when compared with those of the remainder of the cell. The predominance of xylose as the major saccharide of the abundant flagellar glycoproteins is particularly striking since other regions of the cell have no detectable quantities of this sugar (8). This unique biochemical marker suggested that flagellar glycoproteins might be selectively identified and their assembly and insertion followed in appropriate regeneration experiments. Evidence is presented in this report that exogenously added [^{14}C]xylose is incorporated into flagellar glycolipids and glycoproteins. This has permitted the tentative identification of putative lipid intermediates in the flagellum, of substantial glycoprotein pools, and has suggested that depletion of glycoprotein pools may be limiting to flagellar regeneration in this organism.

MATERIALS AND METHODS

Euglena gracilis "Z" was grown in batches of 500 ml in 1-liter Erlenmeyer flasks or in 16-liter cultures in 20-liter carboys under constant illumination and at room temperature in the acetate-containing medium of Cramer and Myers (10). Harvesting of cells, isolation of flagella, and extraction of xyloglycorien and mastigonemes were performed as previously described (31).

Radiolabeling

L-[^{35}S]Methionine (sp act, 400 Ci/mmol), glucose-D-2-[^{3}H] (N) (sp act, 10 Ci/mmol), Iodine-125 in 0.1 M NaOH (protein iodination grade, carrier free, sp act 17 Ci/mg) were purchased from New England Nuclear (Boston, MA). D-U-[^{14}C]xylose (sp act, 75 mCi/mmol) was obtained from Amersham Corporation (Arlington Heights, IL). Cycloheximide was supplied by Sigma Chemical Co. (St. Louis, MO). Tunicamycin initially was a gift kindly supplied by Dr. Robert Hamill, Lilly Research Laboratories, Indianapolis, IN, and later was purchased from Calbiochem-Behring Corp. (San Diego, CA). Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) was purchased from Pierce Chemical Co. (Rockford, IL).

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Vegetative cells were harvested from log phase cultures at a density of about 5×10^5 cells/ml. For each experiment cell number was calculated with a hemocytometer. Cells were deflagellated either by cold shock which gave cleaner preparations or by agitation in a fluted glass tube (33) when time was a factor. Flagella were removed from deflagellated bodies by centrifugation, and regeneration was accomplished in medium containing the appropriate radiolabel. [^{35}S]Methionine was generally applied in aliquots to achieve a final concentration of 20 μCi/ml, whereas [^{14}C]xylose was added to a concentration of 1–2 μCi/ml. Most experiments were carried out in 10 ml of medium.

Incorporation of radiolabel into proteins and glycoproteins was measured by removing duplicate 50-μl samples, spotting on Whatman 3 MM filter paper discs (Whatman, Inc., Clifton, NJ), and immediately immersing the discs into 10% trichloroacetic acid (TCA) at 0°C. The discs were further processed to determine incorporation into hot TCA-precipitable proteins by the method of Bollum as described by Mans and Novelli (24). Radioactivity was counted in a Packard Tricarb liquid scintillation spectrometer Model 3320 (Packard Instrument Co., Inc., Downers Grove, IL) at 86% efficiency for [^{14}C] and 25% for [^{3}H].

Iodination of the surface-exposed polypeptides of whole cells was performed by the method of Fraker and Speck (12). 10 μg of Iodogen was dissolved in 1 ml of chloroform and evaporated to dryness in glass scintillation vials under a stream of nitrogen at room temperature. Vials coated with Iodogen were used immediately or stored in a desiccator at room temperature. Whole cells or deflagellated cell bodies suspended in borate saline buffer, pH 8.2 (prepared by mixing 5 vol of 0.125 M sodium borate buffer with 95 vol of 0.145 M sodium chloride) were transferred into the Iodogen coated vials and the iodination was initiated by adding 500 μCi of carrier-free Na[^{125}I] and 3 μg of KI. The vials were gently shaken for 30 min at room temperature for whole cells or at 10°C to prevent flagellar regeneration. The reaction was stopped by removing the samples from the vials; the samples were further washed with ice-cold borate saline buffer, pH 8.2 containing 5 mM carrier NaI. Identical procedures without Iodogen were also performed simultaneously as a control. Iodinated samples were spotted on filter paper discs, processed as hot TCA-insoluble counts, and counted in a Searle Series 1175 Automatic Gamma counter (Searle Analytic Inc., Des Plaines, IL).

SDS PAGE

Whole cells or flagella were solubilized in a buffer containing 0.0625 M Tris-HCl, pH 6.8, 3% SDS and 5% 2-mercaptoethanol, briefly (60 s) heated at 100°C, and cooled. Samples were alkylated with iodoacetamide essentially by the procedure of Lane (19) and applied to 1-mm-thick slab polyacrylamide gels using a Bio-Rad (Richmond, CA) apparatus. Slab gel electrophoresis was conducted by using 4% stacking gel, 10% separating gel and discontinuous buffer system of Laemmli (18). After electrophoresis at 25 mA for ~4 h, the gels were fixed and stained for polypeptides with 0.2% Coomassie Brilliant Blue/25% (vol/vol) methanol/7% (vol/vol) acetic acid overnight and then destained in the same solution without dye. Gels were infiltrated into PPO (5) and dried with a Bio-Rad gel drier. Labeled polypeptides were visualized by fluorography using preflashed (20) Kodak Royal X-O mat XRP-1 x-ray film. Densitometry scans of fluorographs was carried out with a Zeineh soft laser scanning densitometer (Biomed Instrument, Inc., Chicago, IL). The following proteins (all from Sigma Chemical Co.) were used as molecular weight markers: lactate dehydrogenase (140,000 mol wt), phosphorylase a (94,000 mol wt), bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt), and myoglobin (17,000 mol wt).

Chromatographic Methods

Lipids extracted with chloroform-methanol (2:1) and chloroform-methanol-water (10:10:3) were fractionated by chromatography on DEAE-cellulose (acetate) prepared according to the procedure of Beevers and Mense (1). The chloroform-methanol soluble lipids (xylosyl-lipids) were applied to DEAE-cellulose columns (2.8 × 35 cm, Pharmacia Fine Chemicals, Inc., Piscataway, NJ) and eluted sequentially with 250 ml of chloroform-methanol (2:1) 200 ml of 99% methanol, followed by a 300-ml linear gradient of 0–0.2 M ammonium acetate in methanol. Gradient concentration was monitored with a conductivity meter (The London Company, West Lake, OH), and 8-ml fractions were collected, and 2-ml aliquots were placed in scintillation vials, evaporated to dryness, and the radioactivity was counted after the addition of the scintillation cocktail (16.5 g PPO, 0.5 g POPOP, 1 liter toluene, 1 liter Triton X-100) in a Packard Tricarb scintillation spectrometer (Packard Instruments). For chloroform-methanol-water soluble lipids, elution was carried out with 250 ml of 10:10:3, followed by a 500-ml linear gradient of 0–0.2 M ammonium formate in 10:10:3. 8-ml fractions were collected and the radioactivity was determined on 2-ml aliquots as described above. The peak radioactive fractions eluted from the column were pooled, evaporated to dryness, and subjected to thin-layer chromatography (TLC).

TLC was done on silica gel G (Analtech Inc., Pittsburgh, PA) or on cellulose plates (Brinkmann Instruments, Inc., Westbury, NY) in small glass tanks. Visualization was generally accomplished by autoradiography on x-ray film.

The following solvent systems have been used: (*A*) *n*-butanol/pyridine/water (60:40:30, by volume); (*B*) ethyl acetate/ pyridine/acetic acid/water (50:50:10:3); (*C*) *n*-propanol/acetic acid/water (30:30:20, by volume); (*D*) chloroform/methanol/acetic acid/water (50:30:8:4, by volume).

Mild Acid Hydrolysis

A solution of chloroform-methanol extracted (2:1), or chloroform-methanol-water extracted (10:10:3) radio-labeled fractions was evaporated to dryness in screw-capped tubes. 1 ml of *n*-propanol and 0.2 ml of 0.1 N HCl were added and the tube heated in a boiling water bath. At appropriate time intervals, tubes were removed, cooled, and 0.2 ml of 0.1 N NaOH and 2.5 ml of chloroform-methanol (2:1) were added. The contents of the tubes were homogenized with a Vortex genie (Scientific Industries, Springfield, MA) and centrifuged. The separated organic and aqueous phases were collected and dried before the determination of radioactivity.

Preparation of [^{14}C]Xylose-Labeled Lipids

Labeled flagella were sequentially extracted by lipid solvents (9) to obtain lipids soluble in chloroform-methanol (2:1), chloroform-methanol-water (10:10:3), and a lipid-free residue.

RESULTS

The pattern of flagellar regeneration has been established from numerous previous studies and is characterized in general by a short lag period followed by rapid growth. The extent of regeneration in *Euglena* is measured as the length of the flagellum beyond (distal to) its emergence from the canal. In fact, in this organism the flagellum remaining after mechanical or cold-shock amputation is a sizable stub extending from the reservoir into the canal and, unlike most other flagellates, flagellar detachment occurs at the canal opening to the cell exterior rather than at the basal body/flagellum junction (c.f. Discussion in reference 7). Despite this anomaly, regeneration kinetics appear to be identical to those of cells with basal body detachment (33). Regeneration in the presence of tunicamycin is essentially the same as in control cells (Fig. 1). However, cells treated with tunicamycin continuously during an initial regeneration are either completely or partially inhibited during a second regeneration, even though tunicamycin is no longer

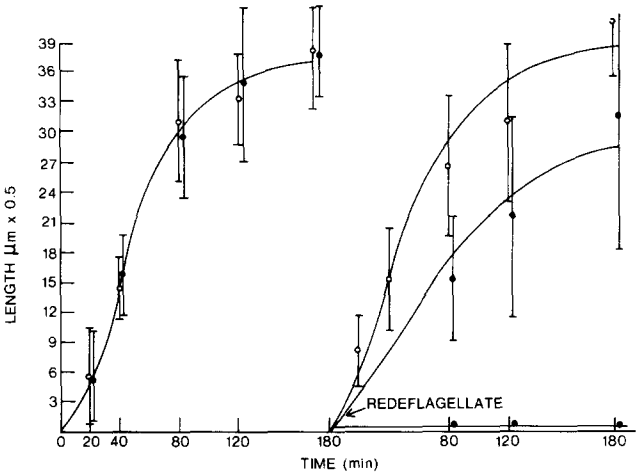


FIGURE 1 Flagellar regeneration in the presence (closed circles) and absence (open circles) of tunicamycin (2 μg/ml). After 180 min of regeneration, cells were redeflagellated and allowed to regenerate with no tunicamycin present. Tunicamycin has no effect on flagellar growth during the first regeneration but either completely (53% in this experiment) or partially inhibits regeneration during the second regeneration. Large standard errors (based on 50 measurements for each point) of the latter reflect varied response to the drug, probably because cells were not initially growing synchronously.

present (Fig. 1). To examine the possibility that this lack of visible effect during the first regeneration may be due to slow drug uptake, cells were exposed to tunicamycin for various lengths of time after deflagellation, were redeflagellated, and then were scored for regeneration. It is apparent that near-maximum effect can be achieved by a 30-min tunicamycin exposure (Fig. 2). The period of maximum tunicamycin sensitivity was also determined by deflagellating cells and pulsing with tunicamycin for different 30-min periods, allowing cells to regenerate for a total of 240 min. Such experiments indicated a decreasing effect on the second regeneration with later applications of tunicamycin during the first regeneration (Fig. 3). Thus, the general pattern appears to be one in which the periods of highest sensitivity during the first regeneration coincide with the periods of the fastest rate of growth.

Labeling of Flagellar Proteins and Glycoproteins

To further assess the effects of tunicamycin and the role of glycoproteins in flagellar growth, metabolic labeling using a peptide specific and a carbohydrate specific precursor was undertaken. [^{35}S]Methionine is rapidly incorporated into regenerating flagella and, although not limited to the flagellum, [^{35}S]methionine effectively labels flagellar peptides after a 30-min pulse. On the other hand, [^{14}C]xylose is taken up more slowly but exhibits a large preference (~80%) for incorporation into flagellar components during regeneration (Table I). [^{14}C]xylose is indeed incorporated into xylose-containing flagellar moieties as seen when flagella were labeled, isolated, and subjected to strong acid hydrolysis. Thin-layer chromatography of neutralized hydrolysates demonstrated that all the label migrated with the xylose standard and that no detectable sugar

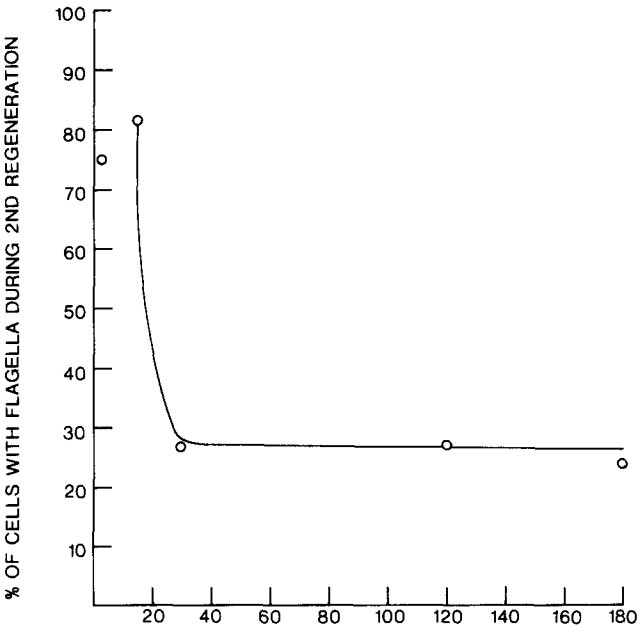


FIGURE 2 Effect of the length of time of tunicamycin treatment during the first regeneration on the subsequent ability to regenerate 180 min after a second deflagellation. Treatment for longer than 30 min had little additional effect on the capacity to regenerate. Thus, 30 min is considered to be minimal but adequate for maximal tunicamycin uptake and/or inhibition. Each point is the mean of 300 cells.

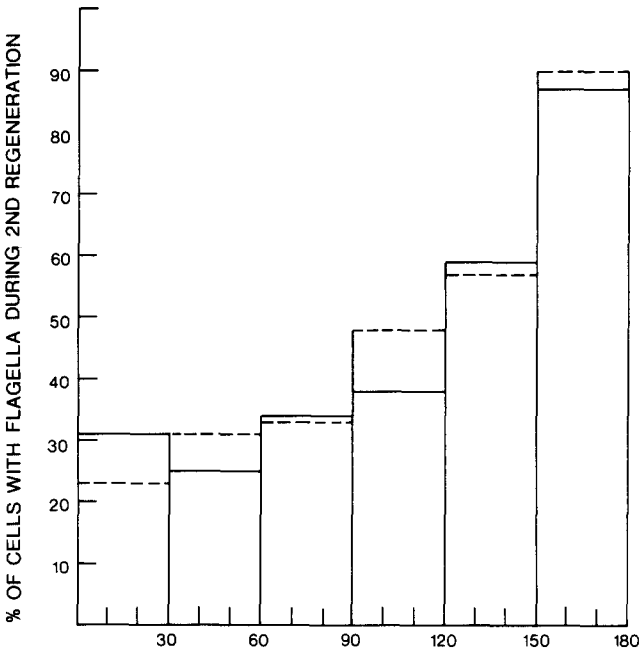


FIGURE 3 The maximal sensitivity of a second regeneration to a 30-min pulse of tunicamycin applied during various periods of the first regeneration is seen in this experiment. After 90 min of regeneration, cells become decreasingly sensitive to the drug. This sensitivity profile is roughly the converse of the growth-rate profile (Fig. 1) and the periods of maximum synthesis of protein and carbohydrate (Fig. 6), suggesting that periods of maximum drug sensitivity are the same periods of most rapid growth and pool utilization and that inability to replace this pool results in inhibition of the subsequent flagellar regeneration. *Solid line*, 180 min; *broken line*, 240 min of regeneration.

TABLE I

Distribution of [^{14}C]Xylose into Various Cell Fractions

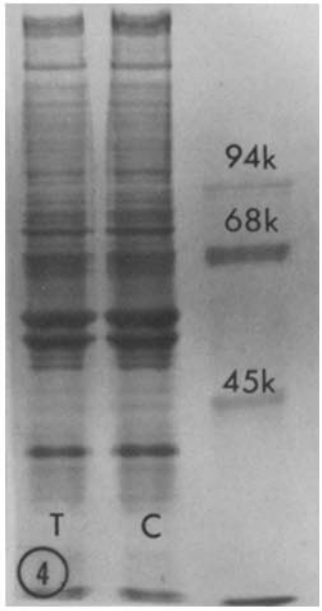
Fraction	CPM/10^5 cells	Percentage
Whole cells	8×10^4	100
Cell bodies	1.55×10^4	19.4
Flagella	6.3×10^4	78.7
Isolated cell surface	675	0.85

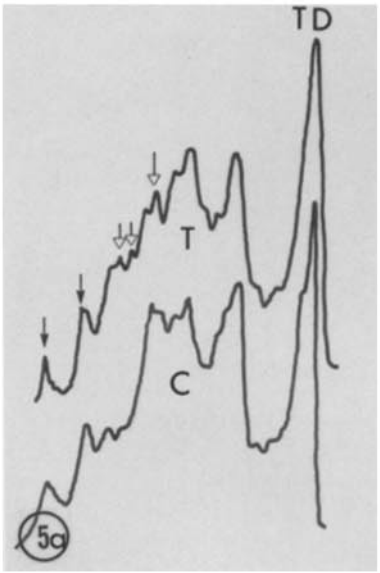
Cells were deflagellated and regenerated for 180 min in the continuous presence of [^{14}C]xylose. After rinsing five times in medium, aliquots were pipetted directly onto Whatman filter paper discs and hot TCA-insoluble radiolabel was counted as the whole cell fraction. Labeled cells were also mechanically deflagellated after washing and were separated by centrifugation into cell body and flagellar fractions. Aliquots were counted for radioactivity, and the cell bodies were further processed to obtain isolated cell surfaces as described by Hofmann and Bouck (13).

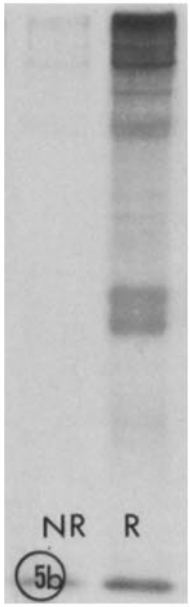
interconversions had taken place. By contrast, hydrolysates of regenerating flagella incubated with [^{3}H]glucosamine produced label coincident with both glucose and glucosamine standards (data not shown). It is therefore concluded that [^{14}C]xylose can be used as a specific label for xylosylated glycoproteins, particularly those of the flagellum.

Effects of Tunicamycin on Glycoprotein Synthesis

Dose-response data using increasing concentrations of tunicamycin indicate that at 2 μg/ml the drug inhibits 95% of







[^{14}C]xylose incorporation into TCA-precipitable flagellar glycoproteins Table II. At the same tunicamycin concentration, protein synthesis as measured by [^{35}S]methionine incorporation is reduced 10–15%. Higher concentrations of tunicamycin reduced [^{14}C]xylose incorporation by 100% but produced unacceptable levels of protein synthesis inhibition. Separate batches (Calbiochem and Lilly) gave twofold differences in effectiveness, probably due to various amounts of contaminating substances (23). All the experiments reported here were performed with the single batch of drug whose effects are tabulated in Table II at 2 μg/ml.

SDS acrylamide gels of solubilized flagella regenerated with and without tunicamycin in the medium produced similar banding patterns after Coomassie Blue staining (Fig. 4). However, fluorography of similar gels using flagella labeled with [^{35}S]methionine revealed quantitative and qualitative differences in the nascent proteins from treated and untreated sam-

TABLE II

Incorporation of [^{35}S]Methionine and [^{14}C]Xylose into TCA-precipitable Fractions of Euglena flagella

Tunicamycin concentration (μg/ml)	Incorporation	
	^{35}S	^{14}C
	%	%
0.1	100	100
0.2	99	93
0.3	99	80
0.5	98	66
1.0	95	28
2.0	86	4
5.0	72	0
10.0	55	0

Deflagellated cells were allowed to regenerate in the presence of various concentrations of tunicamycin and in the continuous presence of [^{14}C]xylose or [^{35}S]methionine. Whole cells were then washed, redeflagellated, and TCA-precipitable counts measured. 100% incorporation of [^{35}S]methionine equals 6.1×10^5 cpm; 100% incorporation of [^{14}C]xylose equals 2.3×10^4 cpm.

FIGURE 4 Coomassie Blue-stained polyacrylamide gels of isolated flagella after regeneration in the presence (*T*) or absence (*C*) of tunicamycin (2 μg/ml). No detectable differences in peptide migration is evident. Standards are 68,000 mol wt (bovine serum albumin), 94,000 mol wt (phosphorylase A), and 45,000 mol wt (ovalbumin).

FIGURE 5 (*A*) Densitometry scans of SDS gels of flagella regenerated in the continuous presence of [^{35}S]methionine with (*T*) or without (*C*) tunicamycin (2 μg/ml). Peaks marked with arrows are reduced, those with open arrows enhanced relative to corresponding region in control flagella gels during the first regeneration. Samples of treated and control flagella were obtained from the same number of cells. Gel origin is at left; *TD*, tracking dye. (*B*) Fluorogram of peptides obtained from flagella pulse-labeled for 30 min with [^{35}S]methionine undergoing (*R*) or not undergoing (*NR*) regeneration. Heavy labeling of high molecular weight components in regenerating flagella is seen, but only slight labeling of nonregenerating flagella suggests that little turnover is taking place. (*C*) Fluorogram of flagella labeled continuously during regeneration with [^{14}C]xylose in the absence or presence of tunicamycin. Label in high molecular weight region is completely abolished in the presence of tunicamycin. Xyloglycorien occupies the position of band *x*; mastigoneme components band at position *m* as well as at most of the high molecular bands seen in Fig. 5 *B*.

ples (Fig. 5 *A*). Bands in the high molecular weight range were reduced or enhanced in tunicamycin-treated flagella. That these enhanced or reduced bands are largely glycoproteins is evident from [^{14}C]xylose-labeled flagella in which most of the label is confined to the high molecular weight region of the gel (Fig. 5 *B* and *C*). The latter labeling is completely abolished in the presence of tunicamycin. The inability to detect these differences in Coomassie Blue-stained gels indicates that new peptides or glycopeptides are probably present in relatively small quantities and that a substantial amount (pool) of high molecular weight glycopeptides is available to the regenerating flagellum.

Most [^{14}C]Xylose is Incorporated Ultimately into Two Major Flagellar Glycoprotein Fractions

A membrane-associated glycoprotein (xyloglycorien) can be selectively extracted from flagella with buffered 0.1% Nonidet P-40 (31). Therefore, flagella were labeled for 3 h with [^{14}C]xylose, isolated, and extracted with Nonidet to release xyloglycorien. ~30% of the glycoprotein-bound (TCA-precipitable) label is solubilized in this manner (Table III). The remaining insoluble material was further treated with 1.5% Sarkosyl (sodium lauryl sarcosinate, ICN Pharmaceuticals, Inc., Plainview, NY) and pelleted. This mastigoneme fraction (8) retained ~53% of the original incorporated counts (Table III). These data indicate that ~84% of the [^{14}C]xylose incorporated into *Euglena* flagella glycoproteins is associated with two flagellar components: xyloglycorien and mastigonemes.

The time course of xylosylation of these two major flagellar glycoproteins was estimated by pulse labeling for different 30-min periods during 4 h of regeneration. Flagella were then isolated, xyloglycorien and mastigonemes were separately extracted, and the samples were counted for radioactivity. Fig. 6 illustrates the time course of general flagella labeling whereas Fig. 7 *a* illustrates that both major glycoproteins are xylosylated at about the same time and with similar kinetics during flagellar regeneration. Only low levels of incorporation are evident during the initial stages of regeneration, but maximal rates are obtained after ~90–120 min. This pattern was confirmed by collecting flagella after full regeneration that had been pulse-labeled at different times. These samples were then solubilized, electrophoresed, and fluorographed. Densitometry scans of the fluorograms (Fig. 7 *b*) clearly illustrate the accumulation of high molecular weight components with migrations in gels similar to those of mastigonemes (peak *b*) and xyloglycorien (peak *a*). These scans also demonstrate that maximal rates of incorporation are obtained only after ~90 min of regeneration.

Lipid-linked Saccharides and Their Relationship to Flagellar Glycoproteins

The incorporation of xylose into lipid-linked oligosaccharides was determined by their solubility in chloroform/methanol/ water (10:10:3) whereas pure lipids or lipids with few sugars were extracted with chloroform/methanol (2) (2:1). [^{14}C]Xylose uptake has similar kinetics in both fractions, but each fraction responds differently to inhibitors. Flagella regenerated in the presence of tunicamycin and [^{14}C]xylose yield greatly reduced levels of counts in the chloroform-methanol-water extracts when compared to flagella not treated with tunicamycin (Fig. 8). However, incorporation into chloroform-methanol-extractable components was not significantly reduced over a 180-min period. These results are consistent with

TABLE III

Distribution of [^{14}C]Xylose into Various Flagellar Subfractions

Fraction	CPM/10^5 Cells	Percentage	Identity (c.f. 31)
Flagella	6.3×10^4	100	
0.1% Nonidet P-40			
extract	1.87×10^4	29.7	xyloglycorien
pellet	4.03×10^4	63.8	
1.5% Sarkosyl			
extract	0.45×10^4	7.8	?
pellet	3.35×10^4	53.1	mastigonemes

Labeled flagella (see Table I) were further processed to obtain the two major flagellar glycoprotein fractions, xyloglycorien and mastigonemes, using methods described previously (31). Over 80% of the total incorporated label is associated with these two fractions.

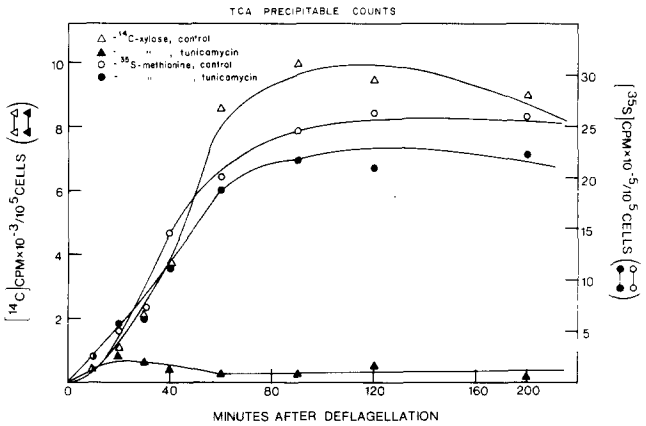


FIGURE 6 Kinetics of incorporation of [^{14}C]xylose into hot TCA-precipitable proteins during regeneration. In the presence of tunicamycin (2 μg/ml, closed symbols), methionine incorporation differs little from that of controls (open symbols) but xylose incorporation is almost completely inhibited.

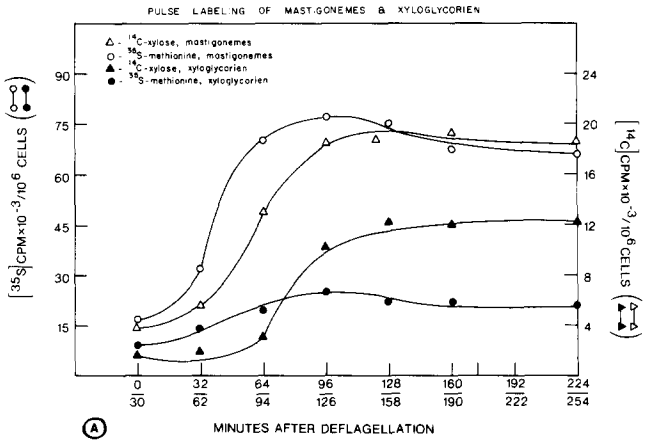
the expected behavior of oligosaccharide lipids (tunicamycin sensitive) and xylose$_{(1, 2, \text{ or } 3)}$ lipid (tunicamycin insensitive). To further test this possibility, pulse-chase experiments were carried out in the presence and absence of cycloheximide to determine the destination of the presumed oligosaccharide lipid. Cells were therefore deflagellated and allowed to regenerate for 90 min. [^{14}C]Xylose was added for an additional 60 min and then chased with unlabeled xylose. As seen in Fig. 9, radioactivity was rapidly lost in the chloroform-methanol-water extract, but this was not the case when the chase was performed in the presence of cycloheximide. The converse effect was evident in the TCA-precipitable protein fraction where the specific activity of this fraction increases rapidly and then levels off. In the presence of cycloheximide, incorporation into TCA-precipitable protein was completely inhibited (Fig. 9), presumably because no nascent polypeptide is available for xylosylation. These data are consistent with a precursor-product relationship between an oligosaccharide lipid and a newly synthesized protein(s).

Further Characterization of Flagellar Lipid-linked Saccharides

Chloroform-methanol extracts of flagella regenerated in the presence of [^{14}C]xylose were retained on DEAE-cellulose (acetate) columns even after elution with 99% methanol. However, a radiolabel fraction could be recovered when the column was

eluted with a salt gradient of ammonium acetate in methanol (Fig. 10). A similar retention was found when the chloroform-methanol-water-solubilized counts were applied to DEAE-cellulose columns, and radioactivity could also be recovered from these columns with a salt gradient (Fig. 11). Both eluted peaks were applied to silica gel type G plates, and each migrated as a single spot with R_f of 0.5 and 0.81 in solvent system D (see Materials and Methods). These data suggest that each of the extraction protocols is specific for a single component or a closely related class of lipid sugars.

The column-purified, labeled extracts also exhibited differ-



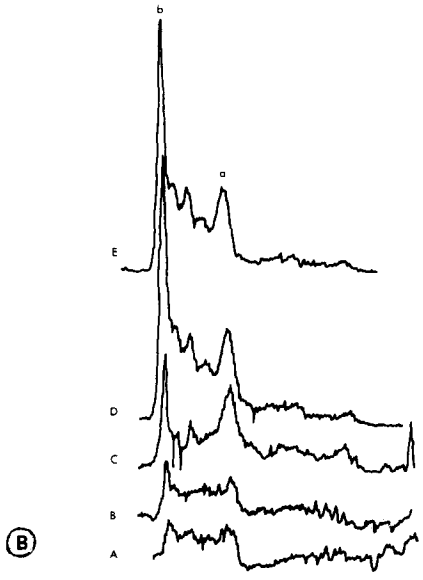


FIGURE 7 (*A*)Kinetics obtained from separate experiments of [^{14}C]xylose incorporation into flagellar mastigoneme (open symbols) or flagellar xyloglycorien (closed symbols) fractions. Cells were pulse-labeled for 30 min at the times indicated and then extracted for mastigonemes and xyloglycorien as described in Materials and Methods. Incorporation rates for both labels reaches a maximum at ~2 h of regeneration and then [^{35}S]methionine incorporation rates begin to decline. [^{14}C]xylose incorporation rates, however, remain about the same level throughout the later stages of regeneration. (*B*) Fractions from the [^{14}C]xylose-labeling experiment in *A* were electrophoresed in acrylamide gels, fluorographed, and scanned with a densitometer. Dramatic increase of label in high molecular weight components after 90 min is evident. Peak *a* corresponds to position of xyloglycorien peak *b* to that of mastigoneme components. Gel origin is at left. Scan *A* was taken from samples pulse-labeled from 0 to 30 min after deflagellation; *B*, 32–62; *C*, 96–126; *D*, 128–158; *E*, 160–190.

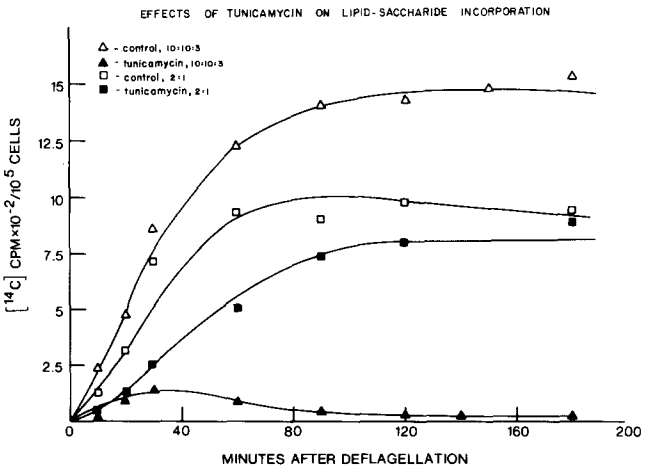


FIGURE 8 Time course of [^{14}C]xylose incorporation during regeneration into lipophilic material extracted from isolated flagella with chloroform-methanol-water (10:10:3) indicated by open triangles, and incorporation into lipophilic material extracted with chloroform-methanol (2:1) indicated with open squares. In the presence of tunicamycin (2 μg/ml), incorporation into the chloroform-methanol-water fraction is almost completely inhibited (closed triangles) whereas tunicamycin has relatively little effect on the chloroform-methanol-extracted material (closed squares). Labeling was present continuously from the time of deflagellation during this experiment, and fractions were obtained by sequential extraction as described in Materials and Methods.

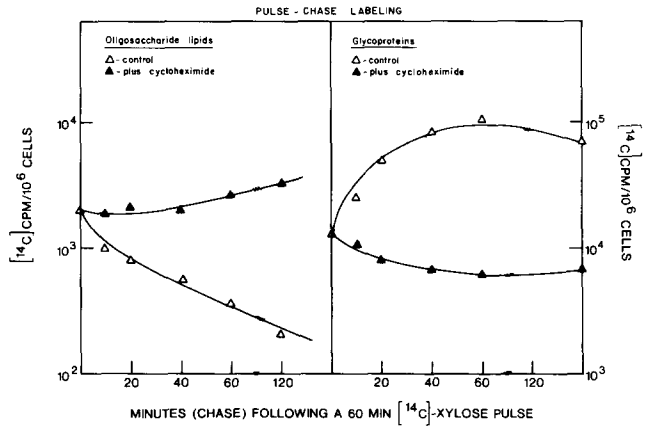


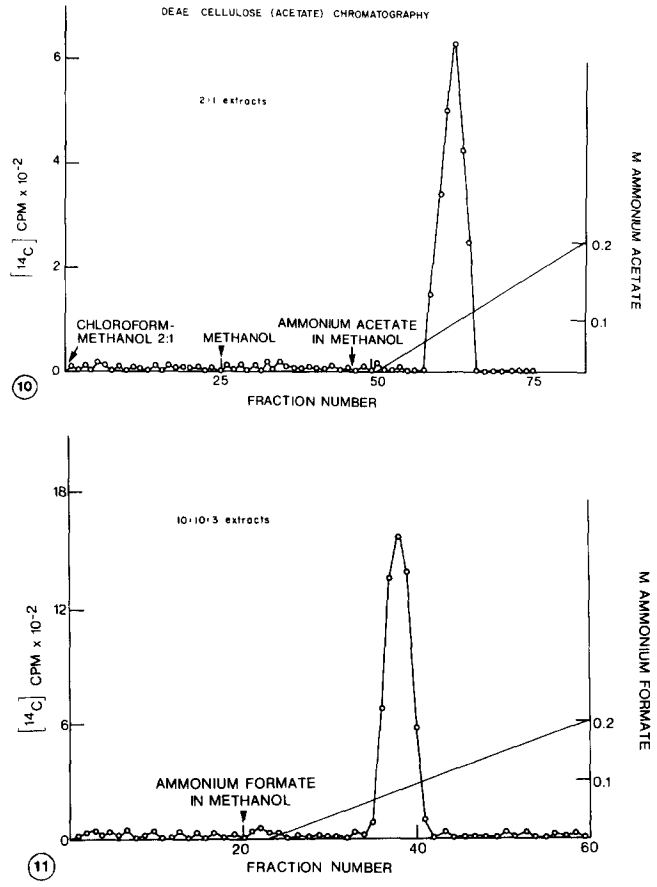
FIGURE 9 Pulse-chase experiment demonstrating the relationship between chloroform-methanol-water (10:10:3) extractable label (left panel) and flagellar glycoproteins (right panel). Closed symbols were chased in the presence of cycloheximide (10 μg/ml), open symbols, without cycloheximide. Counts in the lipophilic material declined tenfold over a 2-h period (left graph), whereas tenfold increase in incorporation into glycoproteins is evident during the same period (right graph). These results are consistent with a precursor-product relationship of [^{14}C]xylose-labeled components which is abolished in the presence of cycloheximide (closed symbols).

ing behavior under conditions of mild acid hydrolysis. Column-purified samples were hydrolyzed in 0.1 N HCl in propanol, and aliquots were removed at various periods, neutralized, and were made biphasic with the addition of chloroform-methanol. The suspension was then centrifuged and aqueous versus organic phases were assayed separately. Hydrolysis to water-soluble sugars occurred more rapidly and more completely with the chloroform/methanol/water extracts (Fig. 12 *A* and *B*). These observations are consistent with other reports that bonds between xylose and lipid residues in presumptive lipid

oligosaccharides are more easily hydrolyzed than the xylose-lipid linkages of monosaccharides-lipids (9).

External Iodinatable Peptides and Their Mobilization during Regeneration

Euglena cells contain at least 15 polypeptides accessible to external iodination. If cells are deflagellated, iodinated, and then flagella regenerated, much of the surface label is chased into the regenerating flagellum (Table IV). Redeflagellation of the same cells, however, produces newly regenerated flagella that receive virtually no additional contribution from the remaining iodinated surface proteins. These facts suggest that the mobile peptide surface pool destined for the flagellum is exhausted after one round of regeneration. Fluorograms of SDS gels of the labeled moieties that are transferred to the flagella indicate that it is predominantly high molecular weight (glyco)peptides that move into the flagellum. These labeled high molecular weight species are nearly entirely removed from the cell bodies during regeneration, but other lower molecular weight peptides do not appear to diminish from the cell surface during regeneration (Fig. 13).



FIGURES 10 and 11 DEAE-cellulose (acetate) chromatography of the chloroform-methanol counts (Fig. 10) and the chloroform-methanol-water counts (Fig. 11). Label remained bound to both columns until eluted with a salt gradient, indicating charged or acidic nature of the eluted material. In this experiment, [^{14}C]xylose was present continuously for 3 h of regeneration, and the flagella were isolated and sequentially extracted as outlined in Materials and Methods. Peak eluates from both columns were pooled, dried, and chromatographed on TLC plates using solvent system D (Materials and Methods), and migrated as a single radiolabeled peak with relative mobilities of 0.5 and 0.81.

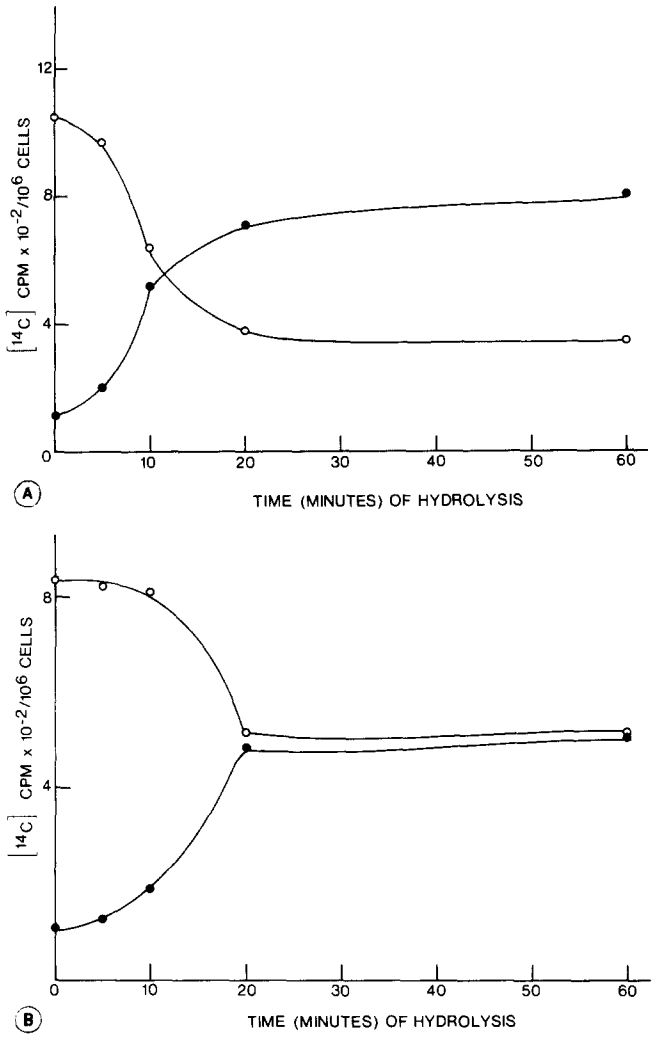


FIGURE 12 Time course of transfer of [^{14}C] label from the organic phase (open circles) to the aqueous phase during mild acid hydrolysis of the peak fractions obtained from DEAE cellulose columns. *A* shows rapid acquisition of counts by the aqueous phase from labeled flagella extracted with chloroform-methanol-water, whereas *B* illustrates slower and less complete transfer of counts derived from the chloroform-methanol extracts.

DISCUSSION

The use of flagellar glycoproteins during regeneration in *Euglena* appears to be a complex process involving pools of existing components as well as newly synthesized moieties. While many details of this process remain yet to be determined, the results of inhibitor and radiolabeling experiments suggest (*a*) that flagellar glycoproteins are present in substantial pools and are mobilized during regeneration, (*b*) that at least part of the flagellar glycoprotein pool is external, (*c*) that lipid-bound intermediates or carriers may be present in the flagellum (membrane) itself.

Glycoproteins are Mobilized during Regeneration

The major glycoproteins associated with the *Euglena* flagellum have been previously identified and detailed (31). These consist of a membrane-associated, xylose-rich surface component (xyloglycorien), and a largely extraflagellar assemblage of

TABLE IV

Distribution of [^{125}I] among Various Fractions After One and Two Deflagellations

Fractions	CPM/10^5 Cells	Percentage
Deflagellated cell bodies (CB 1)	1.13×10^6	100
Whole cells after one regeneration	1.14×10^6	114
cell bodies (CB 2)	6.5×10^5	57
flagella (F 1)	4.6×10^5	41
Whole cells after two regenerations	6.35×10^5	56.1
cell bodies (CB 3)	6.1×10^5	53.9
flagella (F 2)	0.2×10^5	1.8

Deflagellated cells were surface labeled for 30 min with Na[^{125}I] using Iodogen coated glass vials (12). After washing in medium five times, cells (CB 1) were allowed to regenerate for 3 h and then deflagellated and separated by centrifugation into cell bodies (CB 2) and flagella (F 1). Cells were then permitted to undergo a second regeneration for 3 h and were again deflagellated and separated into cell body (CB 3) and flagellar (F 2) fractions. >40% of the original surface label is transferred to the newly regenerated flagellum (F 1) but little additional label is transferred during a second regeneration (F 2).

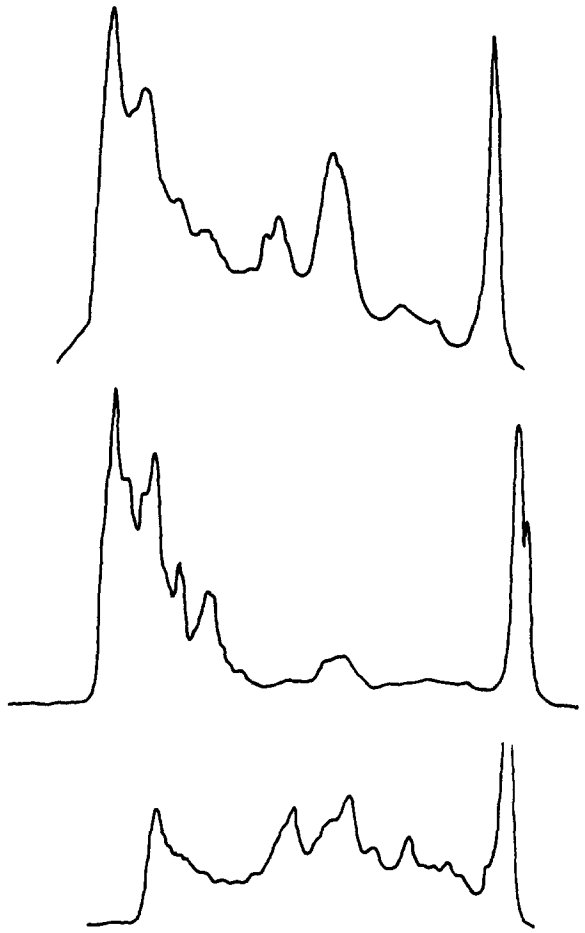


FIGURE 13 Scans of fluorograms demonstrating apparent transfer of [^{125}I]-labeled surface peptides to the flagellum during regeneration. Deflagellated cells were surface labeled for 30 min with [^{125}I], washed, and allowed to regenerate, and the cell body and flagella peptides were separated on SDS gels. In these scanned fluorograms of the gels, much of the initial surface label (top scan) is chased into the regenerating flagellum (middle scan). High molecular weight labeled components are efficiently transferred to the flagellum, leaving lower molecular weight peptides on the cell surface after regeneration (lower scan). Gel origin is at left.

regularly arranged mastigoneme units. Because mastigonemes and xyloglycorien together represent a significant portion of the total flagellar surface, it seemed likely that flagellar growth would depend on the availability and production of these glycoproteins. It was therefore unexpected that tunicamycin administered at concentrations which almost totally inhibit xylosylation of flagellar glycoproteins had no effect on regeneration. Thus, either (*a*) glycoproteins are not required for regeneration, or (*b*) unxylosylated glycoproteins are added to regenerating flagella, and/or (*c*) substantial pools of glycoproteins are available for new growth. The latter view is supported by direct evidence for such pools (see below) and by additional evidence which renders the first possibility less likely. For example, unglycosylated proteins should have an altered molecular weight, but SDS gels of flagella regenerated with or without tunicamycin are similar in the high molecular weight range (Fig. 4) when stained with Coomassie Blue and identical when stained with PAS (30). Treated cells swim normally and no changes are visible in the electron microscope. Thus, structurally and biochemically, tunicamycin appears to produce no detectable alterations in a newly regenerated flagellum. However, tunicamycin effects are clearly seen in a second round of regeneration, suggesting that the pool is exhausted and that further flagellar growth is dependent on the production of new glycoproteins. Similar expriments conducted with *Tetrahymena* (16) showed a lack of tunicamycin effect on regeneration of cilia despite inhibition of mannose incorporation. It would be of considerable interest to know whether a second deciliation of *Tetrahymena* results in retardation of regeneration comparable to that of *Euglena*. In *Chlamydomonas*, tunicamycin may impair flagellar-related functions, but these are fully reversible (29) and a single round of regeneration appears to be unaffected by the drug (4).

Evidence for Glycoprotein Pools

Euglena is characterized by an invagination at the anterior end of the cell. This invagination forms an expanded inner chamber, the reservoir, from the floor of which the two flagella (only one extends to the outside of the cell) arise. Cytochemical (26), immunofluorescence (32) and direct electron microscope observations (21) have suggested that flagellar components may be present along the reservoir lining. Further evidence that the reservoir may be a source of flagellar (glyco) peptides has been obtained in the present study, i.e. iodinated surface peptides can be chased into the regenerating flagellum, and these iodinated peptides have been identified as high molecular weight components similar in electrophoretic mobility to mastigonemes and xyloglycorien. These results do not distinguish between peptides that might have been present on the flagellar stub (still attached after deflagellation, c.f. Results) and those that are present in the reservoir, but it is clear that these iodinated peptides migrate to the newly assembled flagellar surface region during regeneration. Hence, high molecular weight flagellar components are present on the surface before flagellar growth. Experiments to determine whether the iodinated, migrating high molecular weight components were the same moieties xylosylated with [^{14}C]xylose produced [^{14}C]/I^{125} ratios of 0.22 for xyloglycorien and 0.162 for mastigoneme. This would seem to indicate that representatives of both individual flagellar glycoproteins were present in the pool and that some portion of both the flagellar glycoproteins were glycosylated during regeneration. Some of the pool is, however, xylosylated before regeneration, since tunicamycin-treated cells

produce flagella with the full complement of glycoproteins (see above). On the other hand, xylosylation can occur *de novo* on regenerating flagella because substantial amounts of [^{14}C]xylose are incorporated into glycoproteins of regenerating flagella.

Thus, from the available evidence it would appear that cells retain sufficient glycoprotein pools for one complete regeneration. This pool, under normal conditions, appears to intermix with newly xylosylated peptides during regeneration, and the old pool (peptide) is depleted after one regeneration (i.e., no iodinatable peptides are chased into a flagellum after a second deflagellation).

Possible Biochemical Pathways for the Synthesis of Flagellar Glycoproteins

The direct incorporation of exogenously added [^{14}C]xylose into flagellar glycoproteins provides a useful method of distinguishing flagellar glycoproteins from other cellular glycoproteins. Unlike exogenously added glucosamine, [^{14}C]xylose can be recovered from hydrolyzed glycoproteins as xylose and is not interconverted into other saccharides. This nearly selective use confirms previous findings (8) that xylose is the principal carbohydrate of the flagellum, whereas glucose predominates in *Euglena* cell-surface glycoproteins. It would also appear that [^{14}C]xylose supplied during regeneration is used for that round of regeneration only and that a relatively small proportion of exogenously supplied xylose enters the glycoprotein pool. These facts suggested that substantial glycosylation is occurring simultaneous with flagellar growth and that this glycosylation might be taking place in the flagellum some distance removed from the (cellular) source of nascent peptides.

The pronounced effect of tunicamycin on [^{14}C]xylose incorporation also indicated that the general pathway of flagellar glycoprotein synthesis might be similar to that described for peptide glycosylation in other systems (reviewed in references 11, 22, 27). For example, since tunicamycin inhibits the formation of *N*-acetyl glucosaminyl pyrrophosphoryl dolichol, the synthesis of diacetylchitobiose which forms the initial building block of the core oligosaccharide is prevented (17). Tunicamycin, when applied to regenerating *Euglena*, totally inhibits [^{14}C]xylose incorporation into both flagellar glycoproteins and into presumptive oligosaccharide lipids. As expected, tunicamycin has no effect on [^{14}C]xylose incorporation into a presumptive monosaccharide lipid which has no requirement for chitobiose synthesis (Fig. 8).

Additional evidence for lipid intermediates has been obtained by using extraction protocols similar to those previously used for isolating xylosyl oligosaccharide lipid and xylosyl lipid (36) from hen oviducts. These methods rely on the insolubility of the oligosaccharide lipid in pure organic solvents such as chloroform-methanol (2:1), but the addition of a small amount of water renders them soluble. The utility of the method has been repeatedly demonstrated (2, 27) as a procedure for distinguishing lipids with one to three saccharides (soluble in chloroform-methanol) from lipids with more complex sugar substituents (soluble in chloroform-methanol-water, 1:1:0.3). Although rigorous proof of the identity of similarly extracted lipid-bound carrier intermediates in the present study is not yet available, additional evidence supports these assumptions. For example, both extracts bind to DEAE-cellulose (acetate) columns and are released with salt gradients, properties which are consistent with those of an acidic (phosphorylated) lipid carrier such as a dilichol phosphate saccharide or oligosaccharide (2, 34). Moreover, [^{14}C]xylose incorporation levels are nearly threefold higher in the 1:1:0.3 fraction than in the chloroform-methanol (2:1) fraction, suggesting that more xylose residues are present in this presumed oligosaccharide lipid extract (Figs. 10 and 11). The DEAE purified 1:1:0.3 extract migrates with R_f similar to that obtained for lipid oligosaccharides extracted from rat mammary glands and chromatographed under identical conditions (35). Finally, pulse-chase experiments suggest a precursor-product relationship between the accumulation of glycoprotein label and the concomitant loss of label from the presumed oligosaccharide lipid fraction. This relationship is abolished in the presence of cycloheximide, possibly because new peptides are no longer available for xylosylation.

A particularly surprising finding is that these lipid-bound intermediates are present in the flagellum (membrane) where little or none of the machinery for peptide synthesis is located. It would thus appear that some peptides are transported to the flagellum and glycosylated post-translationally. While this is not the usual route (generally cotranslational and associated with the endoplasmic reticulum) for glycosylation, examples in other systems indicate that both post-translational glycosylation (3, 15) can occur and that plasma membrane-associated lipid intermediates (25, 28) can be isolated. A more puzzling question is why xylose is incorporated at all into lipid-intermediate oligosaccharides which usually have a well-defined core consisting only of glucosamine, mannose, and glucose (14). After this core is attached to a peptide, additional sugars are added or those present are processed. Nonetheless, xylose-lipid intermediates have been isolated from another system (36), and perhaps the xylose residues occupy a position so closely associated with the glucosamine residue (c.f. 37) that they are incorporated into the lipid oligosaccharide before peptide glycosylation.

Although major gaps in understanding the synthesis and mobilization of *Euglena* flagellar glycoproteins remain, a scheme summarizing the possible sequence of surface events

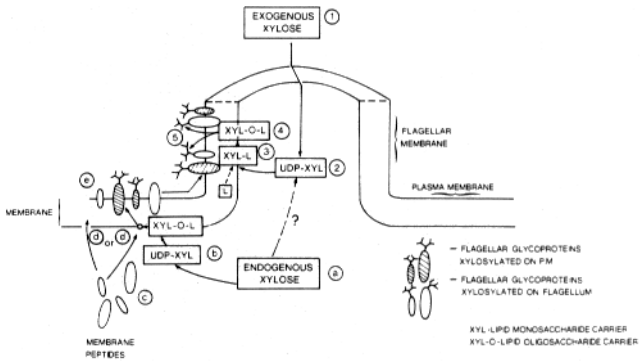


FIGURE 14 Diagram illustrating some of the possible steps in the synthesis and mobilization of flagellar peptides. Steps a–e are thought to occur in the cell body, whereas steps 1–5 are associated with the flagellum. In this report, evidence for steps 1, 3, 4, 5, and mobilization of material from the cell surface is presented; all other steps are conjecture based on similar events recorded in other systems. In this scheme, exogenous xylose (*1*) is activated with nucleotides (*2*) before transfer to lipid (*L*) to generate a xylosyl-lipid (*3*). Xylose from the latter compound is then transferred to a lipid oligosaccharide (*4*) before attachment to a surface peptide (*5*). Peptides from a cytoplasmic pool (*c*) are inserted into the membrane as glycosylated (*d*) or unglycosylated (*d*) proteins to comprise a surface pool of flagellar components (*e*). These components are then mobilized during regeneration.

during regeneration is presented in Fig. 14. Here, the synthesis of flagellar peptides may be stimulated by the act of deflagellation, and these peptides are inserted in the membrane adjacent to the flagellum. Some peptides may be xylosylated (possibly those present for longer periods in the pool) whereas other peptides remain unglycosylated. Both unxylosylated and xylosylated peptides migrate to the base of the flagellum (as probably occurs in the *Ochromonas* flagellum [6]) where they contribute to the expanding flagellar membrane. Unxylosylated flagellar peptides may be xylosylated *in situ* by means of flagellar-associated lipid carriers, which in turn are probably generated from UDP-xylose and dolichol-like lipids. Direct evidence for the latter two compounds has not yet been obtained. Exogenous xylose is preferentially used by the flagellum during *in situ* glycosylation.

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