Endogenous Glycosyltransferases Glucosylate Lipids in Flagella of *Euglena*

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ABSTRACT Flagella, intact deflagellated cells and isolated cell surfaces of the unicell, *Euglena* were separately assayed for glycosyltransferase activity by incubating these fractions with uridine diphosphate-[^{3}H]glucose and isolating radiolabeled products. Most of the label was incorporated into lipophilic products, soluble in chloroform/methanol, which could be separated via thin layer chromatography or LH-60 chromatography into four distinct classes. The most polar of these products was extracted from flagella and purified by column chromatography for use as an in vitro substrate to identify flagella-associated glycosyltransferases. After flagella were treated with the detergent CHAPS, a soluble fraction was removed that was capable of glycosylation in solution. The glycosyltransferase(s) responsible for this activity were further enriched on sucrose or fructose gradients and ultimately identified on acrylamide gels through the combined use of nondenaturing gels, dial-[^{3}H]uridine diphosphate binding, and fluorography. The enzyme had an apparent monomer molecular weight of 32,000 and consisted of four or fewer subunits. The occurrence of endogenous glycosyltransferase(s) in flagella suggests that modifications and/or assembly of the flagella surface can take place in situ in this organism.

Although the flagellar membrane is continuous with the plasma membrane in eucaryotic cells, the flagellar surface retains important biochemical (43) and physiological (c.f. references 6, 35) properties distinct from those of the cell surface. The existence of these regional differences in a single cell requires either (*a*) that glycoproteins and other molecules be in some way directed to the flagellum and/or (*b*) that assembly or synthesis take place directly on the flagellar surface. Since the flagellum apparently has none of the requisite machinery for synthesizing proteins, most flagellar components are imported from the cell proper (e.g. reference 44). Glycosylation of some proteins, however, may occur in situ. For example, lipid intermediates (dolichol-like) in protein glycosylation have been extracted from isolated flagella, and evidence from radiolabeling and inhibitor studies indicates that these intermediates are used in glycoprotein assembly (19). These findings suggest that flagella have some capacity to mediate the synthesis of their own specific surface domains. In further support of this view, previous studies have indicated that flagella might harbor enzymes (glycosyltransferases, GTs)[1] to effect glycosylation, since various sugar donors were incorporated into unidentified products (12, 33).

In the present report, GT activity has been documented in isolated flagella of *Euglena*. Products have been partially purified and compared with products from whole intact cells and with isolated cell surfaces. Surprisingly, the major acceptors for uridine diphosphate (UDP)-glucose are lipids that appear to be terminal acceptors and are not intermediates in glycoprotein synthesis. Four glycosylated products have been identified and a GT for a flagellar acceptor has been partially purified on sucrose or fructose gradients after detergent extraction. This GT has an apparent monomer molecular weight of ~32 kilodaltons (kd) and is inhibited by dial-UDP in an in vitro assay. These results indicate that *Euglena* flagella contain at least one of the enzymes required for assembly or modifi-

[1] *Abbreviations used in this paper:* BHT, butylated hydroxyl toluene, 2,6-ditert-butyl-*p*-cresol; GT, glycosyltransferase; kd, kilodaltons; PAS, periodic acid-Schiff; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; TLC, thin layer chromatography; UDP, uridine diphosphate.

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cation of the flagellar surface, and suggest that properties of the flagellar surface may be in part under local control.

MATERIALS AND METHODS

Culture Maintenance

Cultures of *Euglena gracilis* strain Z were grown axenically (13) in 1-liter flasks or 20-liter jugs under constant illumination. 3- or 4-d old cultures were harvested by centrifugation and resuspended in appropriate buffer or in fresh medium.

Cell Surface Isolation

Cell surfaces were isolated by a modification of published procedures (24). 500 ml of 3-d old cultures were harvested by centrifugation, and cells were rinsed with HEPES buffer (10 mM HEPES, 25 mM KCl, pH 7.0) 3 times. Resuspended cells were then deflagellated in a fluted glass tube by mechanical agitation with a Deluxe mixer (Scientific Products, Div. American Hospital Supply Corp., McGraw Park, IL) at maximum speed setting for 1 min. The cells were resuspended in 3 ml HEPES buffer containing 3 mg PMSF (phenylmethylsulfonylfluoride, Sigma Chemical Co., St. Louis, MO) and 1 g glass powder (25 μm; Heat Systems-Ultrasonics Inc., Plainview, NY), and then cavitated (Model W185, Heat Systems-Ultrasonics, Inc.) for 12 s. After removal of glass powder (by centrifugation), the disrupted cell bodies were layered over 70% sucrose in HEPES buffer in a 15-ml centrifuge tube, and centrifuged in a Sorvall RC-2B centrifuge (DuPont Instruments-Sorvall Biomedical Div., Wilmington, DE) with rotor SS-34 at 17,000 rpm for 15 min. The pellet was gently washed with HEPES buffer twice, suspended in 0.3 ml HEPES buffer, and layered over a fructose gradient (containing 1.2 ml each of 130%, 120%, 110%, and 95% fructose [wt/vol] in HEPES buffer) or a sucrose gradient (containing 1.2 ml each of 110%, 100%, 95%, and 85% sucrose [wt/vol] in HEPES buffer). After centrifugation in a Beckman L-2 65B ultracentrifuge with rotor SW65 at 50,000 rpm for 90 min, a white cell surface band could generally be recovered at the interface between 110% and 120% in the fructose gradient or between 85% and 95% in the sucrose gradient. These modifications of the original procedure (24) greatly increased the yield and speed for obtaining cell surfaces. The resulting preparations were electron microscopically similar to those of the earlier report. The purity of cell surfaces was monitored by light and electron microscopy and found to be essentially free of other cell components. These cell surface fractions consisted of the plasma membrane and a subtending layer of electron-dense material, as described earlier (24).

Flagella Isolation

5-d old cell cultures (in 16-liter jugs) were harvested by centrifugation in a Sorvall RC-2B centrifuge with rotor GSA at 2,000 rpm for 5 min. Cold shock deflagellation was performed by incubating cells at 4°C for 1.5 h. ~95% of the cells were deflagellated by this method, and the deflagellated cells were capable of regenerating new flagella, indicating no apparent cell damage. Deflagellated cells and cells with flagella still attached were removed by centrifugation. The supernatant was centrifuged in a Sorvall centrifuge with rotor SS-34 at 17,500 rpm for 30 min to pellet the flagella.

Enzyme and Acceptor Assays

Deflagellated cells, isolated surfaces, and isolated flagella were separately suspended in 10 mM HEPES or Tris buffer, at pH 7.0 containing 750 pmol of UDP-[^{3}H] glucose (3.26 Ci/mmol, New England Nuclear, Boston, MA) or 5.5 nmol of [^{3}H]glucose (18 Ci/mmol, New England Nuclear) or 1.81 nmol of UDP-[^{14}C]xylose (278.0 mCi/mmol, New England Nuclear) or 220 pmol of UDP-[^{3}H]galactose (11.6 Ci/mmol, New England Nuclear) in the presence of 5–10 mM $MgCl_2$. After incubation in radiolabel, samples were pelleted by centrifugation and resuspended in buffer. The pelleting and suspension were repeated three times and essentially all free label was removed. The samples were then divided into two parts: one part was applied to 2-cm discs of 3 MM Whatman filter paper (Whatman Co., Clifton, NJ) and then immersed two times (15 min each) in 10% trichloroacetic acid (TCA) at 0°C, two times in 5% TCA at 90°C (10 min each), two times in 100% ethanol at room temperature (5 min each). The other part was extracted with a mixture of chloroform/methanol/water (1:1:0.5, vol/vol/vol). After drying in air, the extracts were counted in a liquid scintillation spectrometer (Tri-Carb Packard, Model 3320, Packard Instrument Co., Inc., Downers Grove, IL). In some cases, chloroform/methanol extracts were dried under N_2 gas in the presence of BHT (butylated hydroxyl toluene, 2,6-ditert-butyl-*p*-cresol, Sigma Chemical Co.) to prevent oxidation of lipid acceptors.

Column Chromatography of Acceptors

SEPHADEX LH-60 COLUMN (FOR ISOLATING ACCEPTOR): Sephadex LH-60 (Pharmacia Co., Piscataway, NJ) was immersed in acetone overnight and the slurry carried from acetone to chloroform through acetone/chloroform (7:3, vol/vol and 3:7, vol/vol). After packing the slurry in a glass syringe (10 cm in height, 2.0 cm in diameter), glass beads were layered on the top of the Sephadex to prevent the gel bed from floating on the more dense chloroform. Samples were eluted with chloroform.

SEPHADEX G-100 COLUMN (FOR PURIFYING ENZYME): Sephadex G-100 (Pharmacia) was swollen in HEPES buffer and packed in a 5-ml plastic syringe. The column bed was saturated with 10 mM CHAPS (3-[(3-cholamidopropyl)-dimethyl ammonio)]-1-propane-sulfonate, Calbiochem-Behring Corp., La Jolla, CA) in 10 mM HEPES buffer. Samples then were applied to the top of the column and eluted with 10 mM HEPES buffer containing 10 mM CHAPS.

TLC of Acceptor

LIPID TLC: Deflagellated cell bodies, isolated cell surfaces, and flagella were extracted with 5 ml of a mixture of chloroform/methanol/water (1:1:0.5, vol/vol/vol). These lipid extracts were resuspended in 50–100 μl chloroform and applied to heat-activated silica gel G plates (250 μm in thickness, 20 cm square, Fisher Scientific Co., Pittsburgh, PA). The TLC plates were developed in one of the following solvent mixtures: (*a*) chloroform/methanol/water (50:21:3, vol/vol/vol); (*b*) chloroform/methanol/30% ammonia/water (60:35:5:2.5, vol/vol/vol) (11); (*c*) propanol:12% ammonia (4:1, v/v) (54), acetone/benzene/water (91:30:8, vol/vol/vol) (40). Lipids were detected with iodine vapors or by fluorography.

SUGAR TLC: Lipid extracts were dried under a N_2 vapor stream, resuspended in 1 N HCl and sealed in a 2-ml ampule. Hydrolysis was carried out at 100°C for 3 h. After neutralization with Dowex-1 resin (bicarbonate form) and deproteinization (52), the supernatant was applied to cellulose TLC plates (0.1 μm in thickness, 20 × 20 cm, Machery-Nagal Co. Postfach, Germany). The samples were then developed in a solvent mixture containing butanol/pyridine/water (6:4:3, vol/vol/vol) for 4–5 h (20). Sugars were detected by spraying with silver nitrate (55), and identified by comparing to authentic sugar standards.

Gel Electrophoresis

SDS GELS: Samples were resuspended in 50 μl of Tris buffer containing 25% mercaptoethanol, 1.25% glycerol, and bromophenol blue and boiled in a water bath at 100°C for 2 min. Alkylation of the reduced samples was carried out as described (29). Samples were either applied on gels immediately or stored at −20°C for longer periods. Gels were prepared according to Laemmli (28) (4% stacking gel and 10% separating gel) and cast in a slab gel apparatus (Model SE 500, Hoefer Scientific Intruments Co., San Francisco, CA). After electrophoresis the gel was fixed with 50% TCA overnight and stained with 0.1% Coomassie Blue for peptide detection or the periodic acid-Schiff (PAS) procedure (48) for identifying glycoproteins and glycolipids.

NONDENATURING GELS (NATIVE GELS): Nondenaturing gels were prepared with a 5% separating gel in 0.12 M Tris-HCl buffer, and with a 3.125% stacking gel in 0.02 M Tris-H_2PO_4 buffer. Whole CHAPS extracts of isolated flagella or extracts after centrifugation in sucrose gradients were applied to the gel and electrophoresed at 4°C. The gel was then fixed in 12.5% TCA overnight, stained with Coomassie Blue for 2 h, and destained in a mixture of 7% acetic acid and 5% methanol in water (23). Some lanes were removed before fixation in order to test for enzyme activity.

Autoradiography

TLCs of radiolabeled lipid separations were sprayed with [^{3}H]-Enhance (New England Nuclear, Boston, MA) and incubated with preflashed x-ray film at −80°C. Gels with radiolabeled protein were infiltrated with scintillator in DMSO (7), dried, and then incubated with preflashed x-ray film at −80°C (31). After incubation films were developed in Kodak x-ray developer, fixed, washed, and dried.

Partial Purification of GTs

Isolated flagella were treated with 10 mM CHAPS (c.f. reference 50) in 10 mM HEPES buffer for 1 h at room temperature. After incubation with detergent, the flagella were pelleted by centrifugation in a Sorvall centrifuge with rotor SS-34 at 17,500 rpm for 30 min. The supernatant was then applied to a linearized sucrose gradient (3/16 inch × 1 inch) that was initially constructed with 0.1 ml each of 60%, 20%, 15%, 10%, and 5% sucrose with 10 mM

CHAPS. After centrifugation in a Beckman L-2 65B centrifuge with rotor SW65 at 25,000 rpm for 16.5 h, the gradient was divided into eight fractions. Fractions 2 and 3 (~5–10% sucrose) usually contained the most enzyme activity.

Labeling of GTs

Either UDP (Sigma Chemical Co.) or [^{3}H]UDP (10.9 Ci/mmol, Amersham Corp, Arlington Heights, IL) were converted into dial-UDP according to the procedure of Powell and Brew (41) by incubating UDP with equal moles of $NaIO_4$ in the dark for 30 in. Samples were treated with dial-UDP for 1 h in the dark. Irreversible binding was achieved by subsequent reduction with $NaBH_4$ for 2 h in the dark. The labeled samples were either (*a*) precipitated with TCA in order to remove free dial-[^{3}H]UDP and then boiled for 2 min with 3% SDS in sample buffer or (*b*) directly boiled for 2 min in sample buffer and then electrophoresed. After staining with Coomassie Blue, gels were fluorographed as above to identify presumptive GTs.

RESULTS

Flagella Can Glycosylate a Lipid That Is a Terminal Product

To avoid confusion in terminology the following notations will be used to identify components of GT-mediated reactions: sugar donor (UDP-sugar) + acceptor (substrate) + cofactors (divalent cations) —→ product (glycosylated acceptor). Of three potential sugar donors that were incubated with isolated flagella, UDP-[^{3}H] glucose showed the highest levels of incorporation, UDP-[^{3}H] galactose about ½ of these levels, and UDP-[^{14}C] xylose only trace amounts of uptake. Various divalent metal cations were included in the assay mixture in order to optimize uptake conditions. Of the cations tested (calcium, cobalt, copper, magnesium, manganese, and zinc: each at 1–20 mM) magnesium was found to be the most effective and was used at 10 mM in most of the experiments reported here. None of the cations tested significantly improved the uptake of UDP-[^{14}C]xylose, which remained at least 10-fold less than UDP-[^{3}H]glucose uptake. These results were unexpected in view of the predominance of xylose in *Euglena* flagellar glycoproteins (8) and suggested that either, (*a*) glucose was converted to xylose or, (*b*) UDP-glucose was incorporated into glycolipids and not into glycoproteins. The first possibility was eliminated by examining fluorograms of the TLC-separated, acid hydrolysates of labeled acceptor. Label migrated only with the glucose standard, indicating the absence of sugar interconversions (data not shown). The second prediction, however, was verified by extracting flagella with various combinations of monophasic and biphasic solvents. Regardless of the extraction method used, >80% of the label partitioned into the organic phase, thereby indicating the lipophilic character of the acceptor. Incorporation is initially rapid, but essentially stops 30 min after addition of label (Fig. 1). It seems probable that the donor is subsaturating, since addition of fresh label results in increased levels of incorporation. Significantly, most of the label remains in the organic, chloroform/methanol-soluble fraction after a 2-h period, suggesting that the sugars are not transferred to proteins (i.e., TCA-precipitable counts, Fig. 1). The stability of the product with time and the absence of label in TCA precipitates suggest that the labeled lipids are terminal products and are not intermediates in glycoprotein synthesis. Incorporation also requires an activated sugar donor, as no uptake is evident in lipids or proteins when [^{3}H]glucose is added to isolated flagella (Fig. 1). Fluorography of SDS gels of whole labeled flagella (Fig. 2) further demonstrates that only low amounts of incorporation can be attributed to peptide bands with molecular weights >17,000. Most of the label

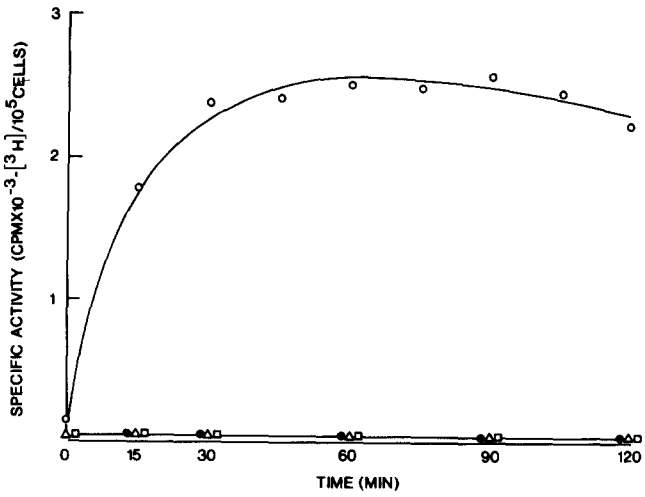


FIGURE 1 Isolated flagella were incubated in 100-μL volumes either with 750 pmol UDP-[^{3}H]glucose or with 5.5 nmol [^{3}H] glucose in the presence of 10 mM $MgCl_2$ and 10 mM HEPES at pH 7.0 for a total of 2 h. At the times indicated, aliquots were removed and assayed for radioactivity. (○), Flagella incubated with UDP-[^{3}H] glucose and extracted with chloroform methanol (CM) (●), Flagella incubated with [^{3}H]glucose and extracted with chloroform/methanol (CM). (△), Flagella incubated with UDP-[^{3}H]glucose and precipitated with 10% TCA. (□): Flagella incubated with [^{3}H]glucose and precipitated with 10% TCA. These experiments demonstrate that incorporation of UDP-[^{3}H]glucose can take place in isolated flagella, that free glucose cannot substitute for UDP-glucose and that the radiolabeled product is lipophilic.

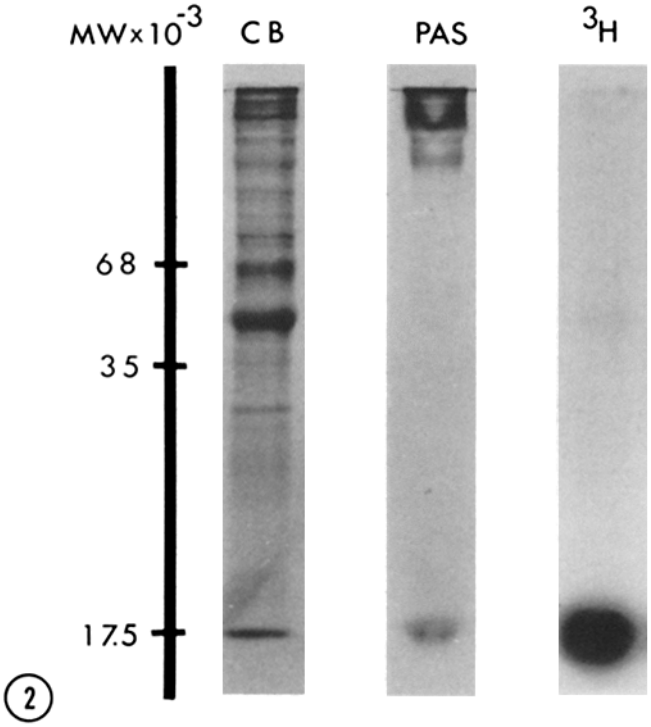


FIGURE 2 Isolated flagella were labeled for 1 h with UDP[^{3}H]-glucose, solubilized in SDS and then reduced and alkylated. Samples were distributed into two lanes of a SDS 10% gel with a 4% stacking gel. *CB*, Coomassie Blue staining; *PAS*, periodic acid-Schiff procedure (loaded with 2× proteins), *[^{3}H]*, fluorogram of PAS gel. Radiolabeling of a PAS-positive presumptive glycolipid band (arrow) at the dye front is evident. Polypeptides separated in the gel (>17,000 mol wt) are only slightly labeled.

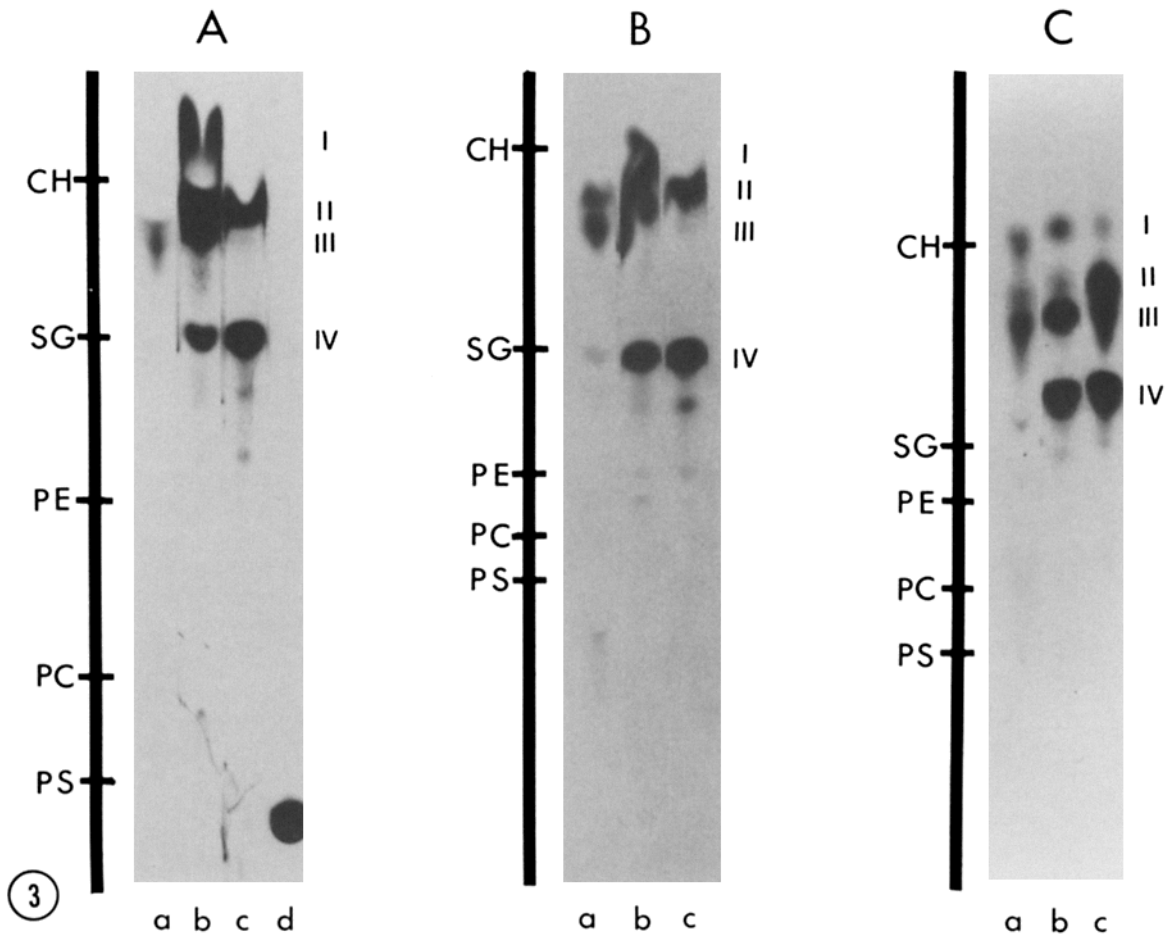


FIGURE 3 CM extracts of labeled (as in Fig. 1) cell bodies, isolated cell surfaces or isolated flagella were applied to separate lanes of a silica gel G TLC plate and developed either with; *A*, chloroform/methanol/water (50:21:3, vol/vol/vol), or, *B*, chloroform/methanol/30% ammonia/water (60:35:5:2.5, vol/vol/vol/vol), or *C*, propanol/12% ammonia (4:1, vol/vol). *PS*, phosphatidyl serine; *PC*, phosphatidyl choline; *PE*, phosphatidyl ethanolamine; *SG*, steryl glucoside; *CH*, cholesterol. Lane *a* = CM extracts of labeled cell bodies, lane *b* = CM extracts of labeled isolated cell surfaces, lane *c* = CM extracts of labeled flagella, lane *d* = UDP-[^{3}H]-glucose. These data suggest that products from all three sources are heterogeneous, are less polar than the phosphatidyl lipids and migrate to positions between those of a steryl glucoside and cholesterol in all the solvents tested. Lipid standards were run on separate lanes (not shown).

is associated with the PAS (+) dye front where small peptides, glycolipids, or possibly free UDP-[^{3}H]glucose would be expected to migrate.

Partial Characterization of the UDP-[^{3}H]Glucose Product

Products for UDP-[^{3}H]glucose were extracted with chloroform/methanol from flagella and separated on silica gel G TLC plates using several different solvent systems. The flagella TLC pattern was compared with products similarly labeled in intact, deflagellated cells and in isolated cell surfaces. Intact cells can presumably glycosylate only those acceptors exposed on the outer membrane surface, whereas isolated cell surfaces that consist of a plasma membrane and an underlying proteinaceous layer (24), can presumably glycosylate lipids on both sides of the membrane. The three solvent systems shown in Fig. 3 separate at least four labeled products. The most polar product (*IV*) co-migrates with an authenic steryl glucoside (plant derived; Stearaloids Inc., Wilton, NH) in two solvent systems, but is less polar in a third solvent (Fig. 3*c*). In decreasing order of polarity products III, II, and I approach the Rf of authentic cholesterol. In pulse/chase experiments, products II and IV from flagella do not seem to gain or lose label, and therefore are probably not interrelated. This assumption is further supported by the more or less distinct pattern of labeled products derived from the three cell fractions (whole cells, isolated surfaces, and flagella; Fig. 3), a pattern that is more clearly revealed after separation of products by LH-60 column chromatography (Fig. 4). The labeled products from isolated flagella are separated into two components, of which product IV retains substantially more label than product II. A fraction (*IV*) with similar column elution properties and TLC migration was obtained from isolated cell surfaces (Fig. 4*a*), but not from intact cells (Fig. 4*c*). On the other hand, the maximally labeled peak III from whole deflagellated cells is also present in isolated surfaces but not in isolated flagella. Peak I may be a contaminant from other cell fractions since it is green in color, variable in amount in different experiments, and is present in relatively small quantities. Cochromatography of labeled extracts from flagella and isolated surfaces produced the expected three peaks, thereby confirming their overlapping or separate identities. Cells lysed and then incubated with label yielded greatly reduced incorporation (10% of intact cells), possibly because of competition by endogenous sugar donors or by release of proteases. The

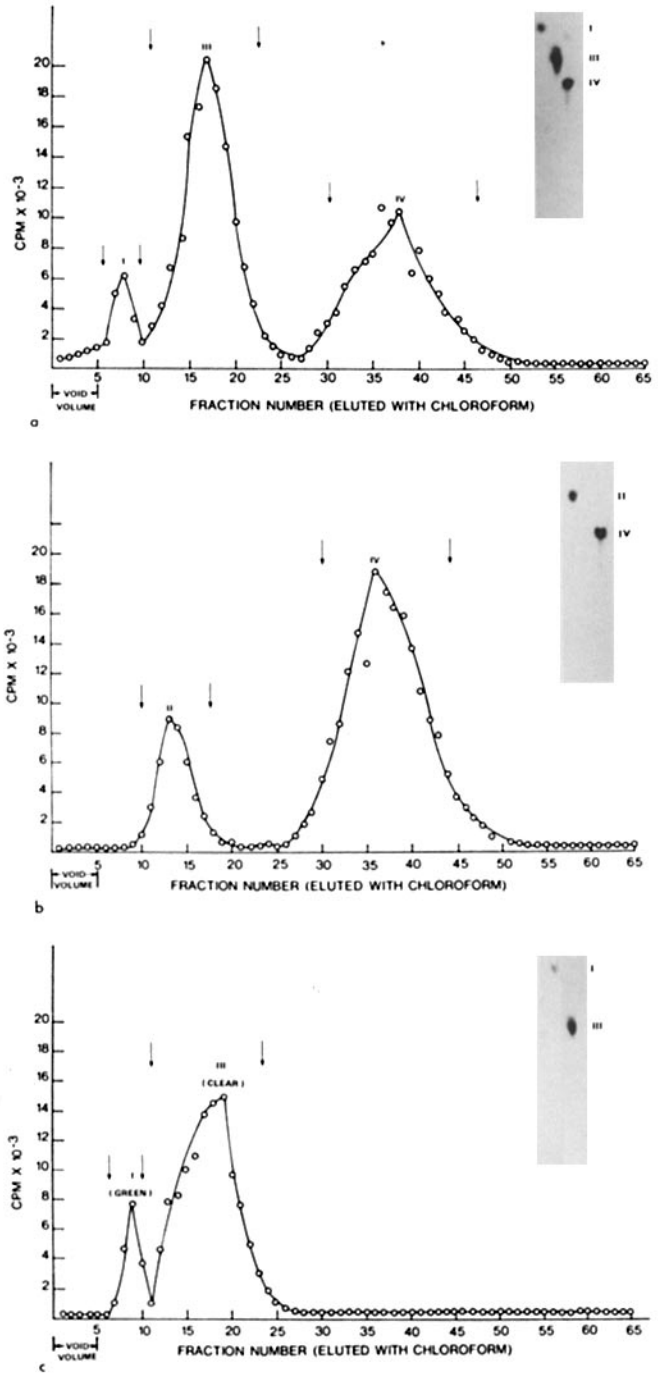


FIGURE 4 CM extracts of UDP-[^{3}H]glucose labeled (1 h) isolated cell surfaces (a), flagella (*b*), or deflagellated cell bodies (c) were applied to a Sephadex LH-60 column and eluted with chloroform as indicated in Materials and Methods. Fractions between the arrows from peaks I–IV were pooled separately, dried under a stream of N_2 gas, applied to separate lanes of a silica gel G TLC plate, and developed in chloroform/methanol/water (50:21:3, vol/vol/vol). A fluorogram of the TLC is shown in the inset. Flagella appear to contain a unique glycosylated product (*II*), as well as a product (*IV*) in common with isolated cell surfaces.

latter experiment does indicate, however, that contamination from broken cells is minimal in preparations of isolated flagella and isolated cell surfaces.

These data taken together suggest that (*a*) one product (II) may be unique to the flagellum, (*b*) one product (IV) is present in both flagella and plasma membranes, and (*c*) one product (III) is absent from flagella.

Identification and Partial Purification of the Product IV Glycosyltransferase

ASSAY AND EXTRACTION OF THE GLYCOSYLTRANSFERASE: Since product IV could be readily extracted from flagella and separated from other products by LH-60 column chromatography, this fraction was collected and efforts were made to generate an in vitro assay for further purification of the product IV glycosyltransferase(s). The rationale for this approach was the assumption that peak IV might also contain partially glycosylated acceptor that was still capable of further glycosylation. Column-purified acceptor IV in chloroform (fractions 30–45, Fig. 4 *b*) was dried under a stream of nitrogen in small glass tubes. These coated tubes were used to test the capacity of various detergent extracts from flagella to incorporate UDP-[^{3}H] glucose into fraction IV. After incubation with radiolabel and cofactors in the coated tube, the solution (100 μl) was extracted with chloroform/methanol and assayed for radioactivity. Fluorograms of TLCs of the extract were also run periodically to ensure that product IV was labeled.

Although a number of detergents were assayed (e.g. Nonidet P-40, Triton X-100, TPA [12-O-tetradecanoyl-phorbol-13-acetate]), only 5–10 mM CHAPS yielded extracts of flagella capable of significantly promoting incorporation into product IV. This activity was moderately stable providing that 10 mM CHAPS was continuously present. Preparations stored at 4°C overnight retained ⅔ of the activity that was present after immediate extraction. Frozen (−20°C overnight) samples lost ~50% of the initial ability to glycosylate product IV. Flagella extracted with CHAPS show little visible digestion of structural components (Fig. 5 *a*), but >30 polypeptides and several glycopeptides are solubilized (Figs. 5, *b* and *c*). Undoubtably some acceptor is also extracted by CHAPS, since some incorporation of UDP-[^{3}H]glucose into product IV is obtained when no peak IV is present. However, such lipids are present in too low a quantity to be detected by TLC and incorporation is always at least doubled when peak IV is included in the assay. Furthermore, incorporation seems to be exclusively into peak IV (Fig. 5 *d*), suggesting that it is the added peak IV lipids that are being glycosylated. The product glycosylated in vitro is identical after TLC chromatography to that glycosylated in situ (Fig. 5 *d*).

ENRICHMENT OF THE GT: In order to subfractionate the CHAPS supernatant, extracts were layered over 0.6 ml linear sucrose gradients and after centrifugation activity was measured in eight fractions (0.075 ml each) using the in vitro peak IV assay. As seen in Fig. 6 *b*, GT activity was found predominantly in fractions 2–4. When each of these fractions was applied to an acrylamide gel and electrophoresed, at least seven Coomassie Blue staining polypeptides ranging in molecular weight from ~30–70 kd, were obtained. Since the enzyme could be any one or none of these bands, efforts were made to identify those bands with GT activity. Therefore, two fractions from the sucrose gradient that showed the greatest activity (fractions 2 and 3, Fig. 6 *b*), were electrophoresed through a nondenaturing gel with a molecular pore size (5% polyacrylamide) empirically selected to localize maximum enzyme activity near the center of the gel (Fig. 7). Enzyme activity was measured after electrophoresis by slicing the two gels horizontally into 12 sections each, eluting the

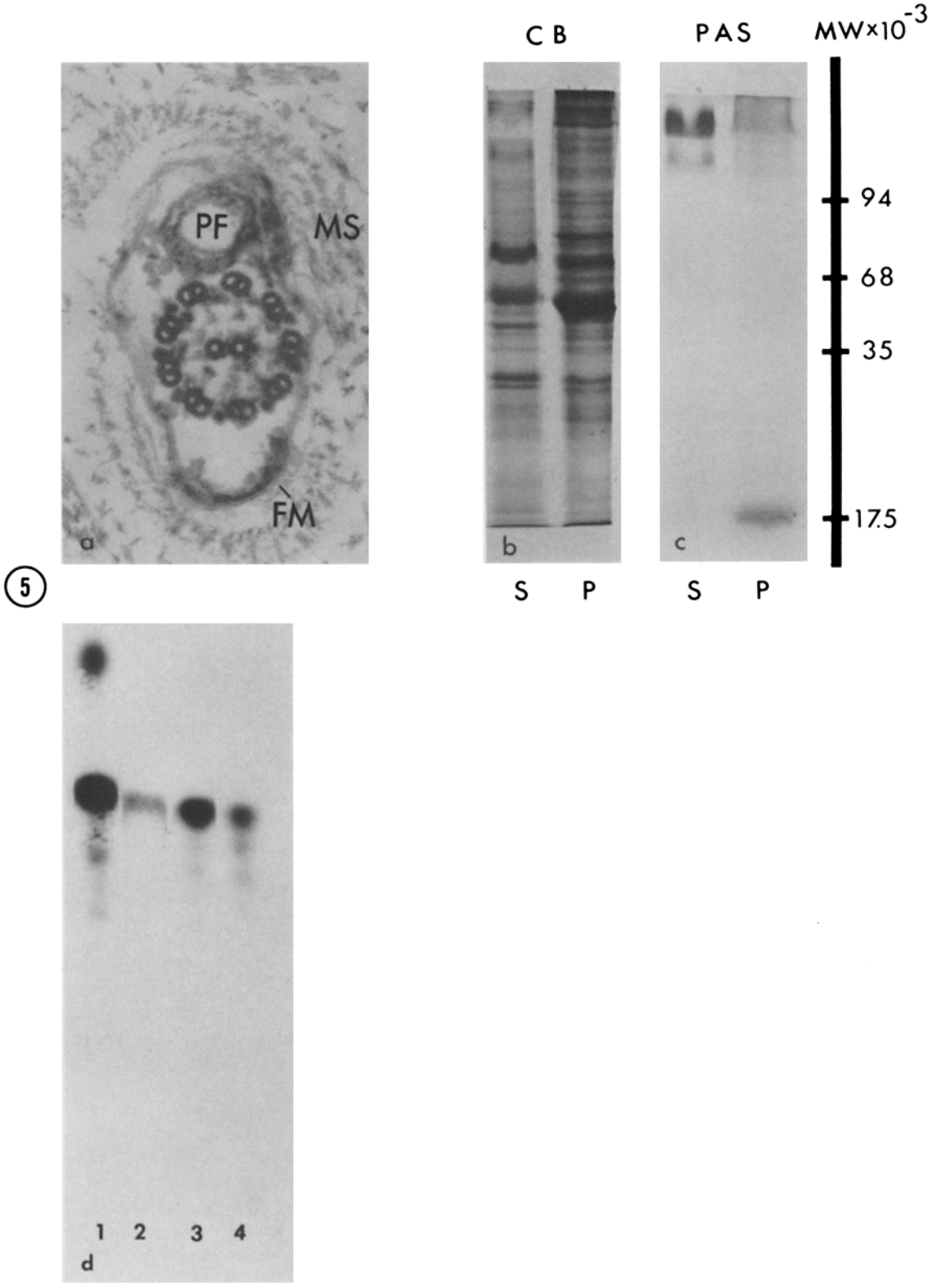
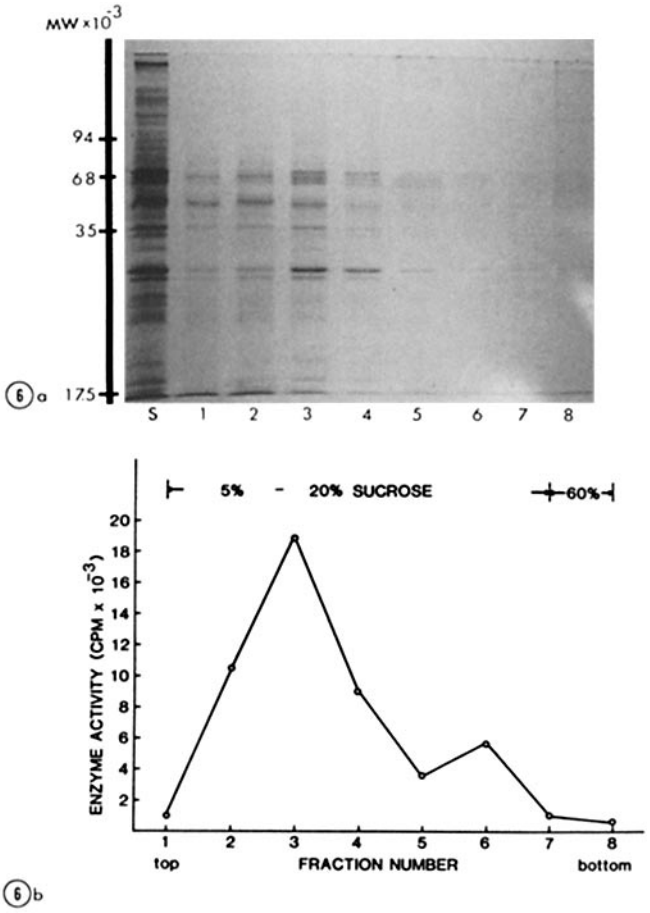


FIGURE 5 Isolated flagella were incubated with 10 mM CHAPS in 10 mM HEPES at pH 7.0 for 1 h at room temperature and then centrifuged at 17,000 rpm for 30 min. Both the pellet and the supernatant were reduced and alkylated and electrophoresed through a 10% separating gel. *S*, supernatant of CHAPS treated flagella; *P*, pellet of CHAPS treated flagella; *CB*, Coomassie Blue-stained gel, *PAS*, stained with the periodic acid Schiff procedure. In *d* a comparison of the CM-soluble radiolabeled product of whole intact flagella (lane *1*), CHAPS extract incubated with peak IV (lane *3*) and residual activity of flagella after CHAPS extraction (lane *4*). In all three cases the UDP-[^{3}H]glucose donor is incorporated into a product with an identical migration on the TLC plate. Plates were developed with chloroform/methanol/water (50:21:3). In lane *2* a small amount of product IV incorporated label can be removed with chloroform/methanol/water (1:1:0.5) after a preliminary extraction with chloroform/methanol. Although many flagellar peptides and glycopeptides are solubilized with CHAPS, the flagellar membrane (*FM*), axoneme, paraflagellar rod (*PF*), and mastigonemes (*MS*) remain visibly intact. The CHAPS extract is clearly able to incorporate UDP-glucose into a product identical to the endogenous product. *a* × 127,000.



(6)a (6)b

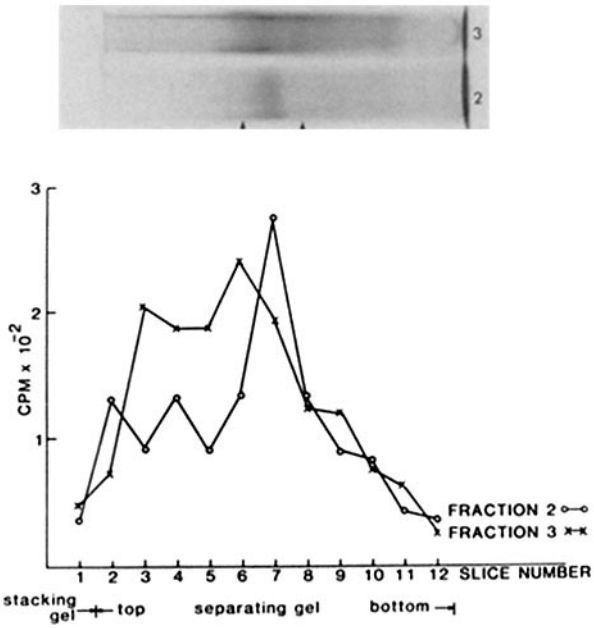
FIGURE 6 0.1 ml of CHAPS extracts (containing soluble enzyme) from 8 × 10^9 isolated flagella were applied to a linear sucrose gradient (5–20%) with 10 mM CHAPS and 10 mM HEPES buffer in a 0.6-ml cellulose centrifuge tube. After centrifugation at 25,000 rpm with a SW 65 rotor for 16.5 h, 0.075-ml fractions (*1–8*) were taken with a syringe from the top of the gradient to the bottom (fraction *8*) and each fraction was reduced, alkylated, and then electrophoresed through a 10% SDS gel. *a* is the Coomassie Blue–stained gel obtained for each fraction. Duplicate samples from the sucrose gradient were assayed for enzyme activity by incubation of each of the eight fractions in acceptor IV–coated glass tubes in the presence of 0.5 μCi UDP-[^{3}H]glucose and 10 mM magnesium chloride (total volume 100 μL). The CM-soluble fraction was then removed from the incubation mixture and counted for radioactivity (*b*). Most of the enzyme activity is found in fractions 2–4 of the sucrose gradient.

proteins from each section and incubating the eluate with UDP-[^{3}H]glucose, cofactors, and peak IV. Although the amount of glycosylation was relatively low after these manipulations, the regions of maximum activity were reproducible (two experiments).

CORRELATION OF THE GT WITH A 32-KD BAND USING DIAL-[^{3}H]UDP BINDING: Since GT activity could be localized near the center of a 5% nondenaturing gel, this region (0.8 cm) of the gel was removed and the proteins eluted. The eluate was incubated in dial-[^{3}H]UDP, followed by reduction in borohydrate and then electrophoresis in a denaturing 10% SDS acrylamide gel. These gels still exhibited a number of Coomassie Blue staining polypeptides (Fig. 8). If these gels are, however, sliced and measured for radioactivity, all dial-[^{3}H]UDP binding is confined to a region around 32 kd that corresponds closely with a Coomassie Blue staining band. Other prominantly stained bands do not bind significant amounts of label.

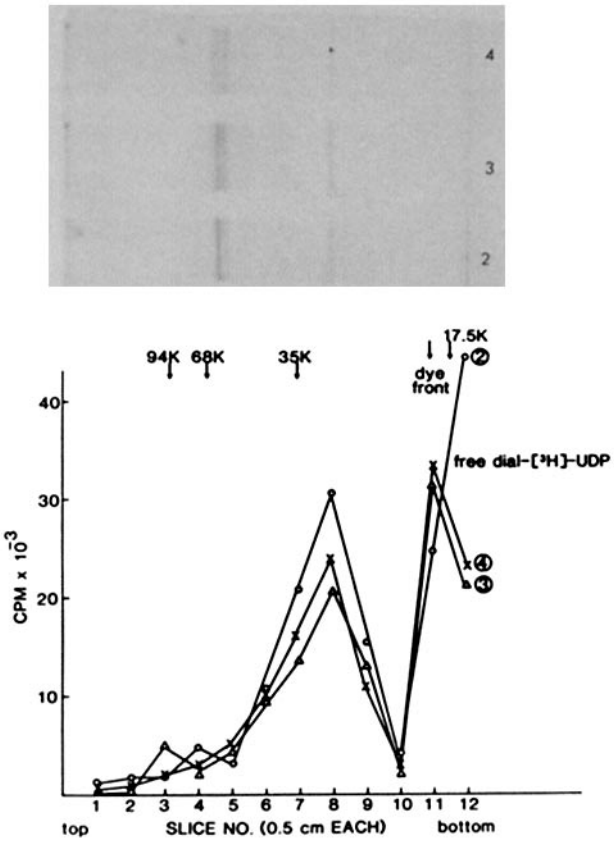
To demonstrate that dial-[^{3}H]UDP actually binds to product IV GT(s), CHAPS extracts from flagella were incubated with dial-UDP (unlabeled) under various conditions reported to reversibly or irreversibly inhibit enzyme activity. As seen in Fig. 9 (squares), extracts treated with dial-UDP followed by borohydrate reduction irreversibly lose GT activity, but over ⅔ of the activity can be recovered if reduction is omitted, and the dial-UDP is removed (Fig. 9, triangles). Borohydrate alone has only a slight reversible effect on this activity. These data suggest that dial-UDP does bind to the GT, although dial-UDP may bind to other moieties as well. An indication, however, of some selectivity is provided in Fig. 9, (*inset*) in which flagella incubated with dial-[^{3}H] UDP show a labeling pattern qualitatively and quantitatively different from the Coomassie Blue staining pattern. Some of the least stained bands bind the most dial-[^{3}H]UDP and, significantly, the large tubulin band stained with Coomassie Blue clearly binds no dial-UDP, as expected.

To ensure that manipulation with the nondenaturing gels did not influence the apparent molecular weight of the presumptive GTs, the proteins from the sucrose gradient were



(7)

FIGURE 7 Sucrose gradient fractions 2 (○) and 3 (×) of CHAPS extract of flagella were separately applied to a 5% nondenaturing gel. After electrophoresis, two lanes of the gel were sliced horizontally into 12 pieces and each slice of gel was then eluted with 50 μl of 10 mM CHAPS overnight at 4°C. Enzyme activity was assayed by separate incubation of each eluate with UDP-[^{3}H]glucose plus cofactors and the LH-60 column peak IV from flagella. CM-soluble counts were recorded. Similar lanes (unsliced) were stained with Coomassie Blue (upper figure). Maximum enzyme activity appear to be present in two adjacent peaks in fraction 3 and one major peak in fraction 2. Coomassie Blue staining shows a polypeptide pattern consistent with the enzyme activity; i.e., fraction 2 has a band at the middle region of the gel, whereas fraction 3 has a broader band around the middle region. The pattern of labeling was reproducible in two experiments.



(8)

FIGURE 8 CHAPS extracts from isolated flagella were centrifuged through sucrose gradients (5–20%) and fractions 2–4 were then collected and electrophoresed through a nondenaturing gel as in Fig. 7. The middle area of the gel (0.8 cm, indicated by arrowheads in Fig. 7) was excised, eluted with 10 mM CHAPS, and incubated with dial-[³H]UDP overnight in the dark. Samples were then reduced with borohydrate for 2 h in the dark, reduced with SDS, and alkylated with iodoacetate. After removing the acrylamide by centrifugation each supernatant was applied to two lanes of 10% SDS denaturing gel and electrophoresed. After electrophoresis, one set of lanes was stained with Coomassie Blue (upper photograph). The other set of lanes was sliced into 12 parts, dissolved in 30% hydrogen peroxide and 70% perchloric acid at 60°C and counted in a liquid scintillation spectrometer. (○), Sample from fraction 2 of the sucrose gradient; (△), sample from fraction 3 of the sucrose gradient; (×), sample from fraction 4 of the sucrose gradient. These results directly demonstrate that a protein(s) with GT activity on nondenaturing gel binds dial-[³H]UDP and migrates as a 32-kd polypeptide after reduction.

directly incubated with dial-[³H]UDP, reduced in borohydrate and then electrophoresed through a 20-cm long denaturing SDS gel. Two Coomassie Blue polypeptides appear in these gels around 32 kd (Fig. 10*a*). Each of these binds dial-[³H]UDP as seen in the accompanying fluorogram (Fig. 10*b*), and both are therefore likely candidates for the acceptor IV GT(s). This fluorogram was over developed (72 d) and showed some label in other bands. Nonetheless, a quantitative densitometry scan of this fluorogram showed that ~50% of the label was associated with the two 32-kd bands, whereas only ~20% of the Coomassie Blue staining was associated with the 32-kd bands.

DISCUSSION

Surface acceptors for UDP-[³H]glucose in *Euglena* include a number of lipophilic moieties that can be separated after glycosylation on the basis of size (Sephadex LH-60 chromatography) and polarity (TLC). Flagella can produce two of these glycosylated products. The latter are not intermediates in protein glycosylation (25) as judged from the following evidence: (*a*) radiolabel from UDP-[³H]glucose is not transferred from a chloroform/methanol-soluble fraction (glycolipid) to a TCA-precipitable fraction (glycoprotein) over a 2-h period (Fig. 1); (*b*) The labeled products can be eluted from a diethylaminoethyl cellulose column with chloroform/methanol indicating that the labeled moiety is not an acidic, phospho-dolichol-like lipid (3), data not shown; (*c*) incorporated label is not sensitive to trypsin digestion. Moreover, only slight amounts of label was extracted with chloroform/methanol/water (1:1:0.3) after a preliminary extraction with chloroform/methanol, indicating that an oligosaccharide ("core") lipid was not a major product (3). Nor is the incorporated glucose converted to other sugars, since glucose was the only labeled saccharide recovered in acid-hydrolyzed products. Thus, it seems likely that the lipophilic, glycosylated products of *Euglena* are terminal products, are relatively stable in flagella, are not interchangeable with each other, and are probably located in the flagellar and plasma membranes (since these are the only membranes present in isolated flagella [Fig. 5] and cell surfaces [24]).

The detailed biochemical nature of the lipophilic products has not been determined, although they migrate on TLC silica gel G plates in several solvent systems with Rfs close to those of an authentic steryl glucoside.

Interestingly, sterols and/or acylated sterols can be incorporated into cilia of *Tetrahymena* (34) and are present in cilia of *Paramecium* (26) and in flagella of *Chlamydomonas* (18). However, the possibility that the glycolipids in the present study are monoglucosyldiglycerides can not yet be ruled out (c.f. reference 14, for mobilities on TLCs). Moreover, steryl glucosides have not been previously reported in *Euglena*, although the principal sterol, ergosterol, of this organism (15), can be an effective acceptor for steryl GTs in other systems (16).

The four groups of glucosylated lipids of *Euglena* are distributed in a pattern that is characteristic for each of the fractions (flagella, whole cells, and isolated cell surfaces) tested. Since two products (I and III) are absent or present in small amounts in flagella, and product II is glycosylated in flagella but is present only in small amounts in cell surfaces, it would seem that each of the two surface regions has its own complement of glycolipids that is maintained independently of the neighboring membrane. The fact that product IV is found in isolated cell surfaces but not in whole deflagellated cells indicates that this product may be glycosylated on the inner side of the plasma membrane. Whether it occupies a similar position in the flagellum remains unknown, and whether the flagellar product IV is identical with IV from cell surfaces has not been determined. In fact metal cation requirements for glucosylation of flagella lipids are demonstratably different from those for glucosylation of cell surfaces (10), indicating that product IV may consist of a collection of similar but not identical glycolipids each of which may use a different GT. The possible occurrence of multiple GTs in flagella would also be consistent with the finding that multiple

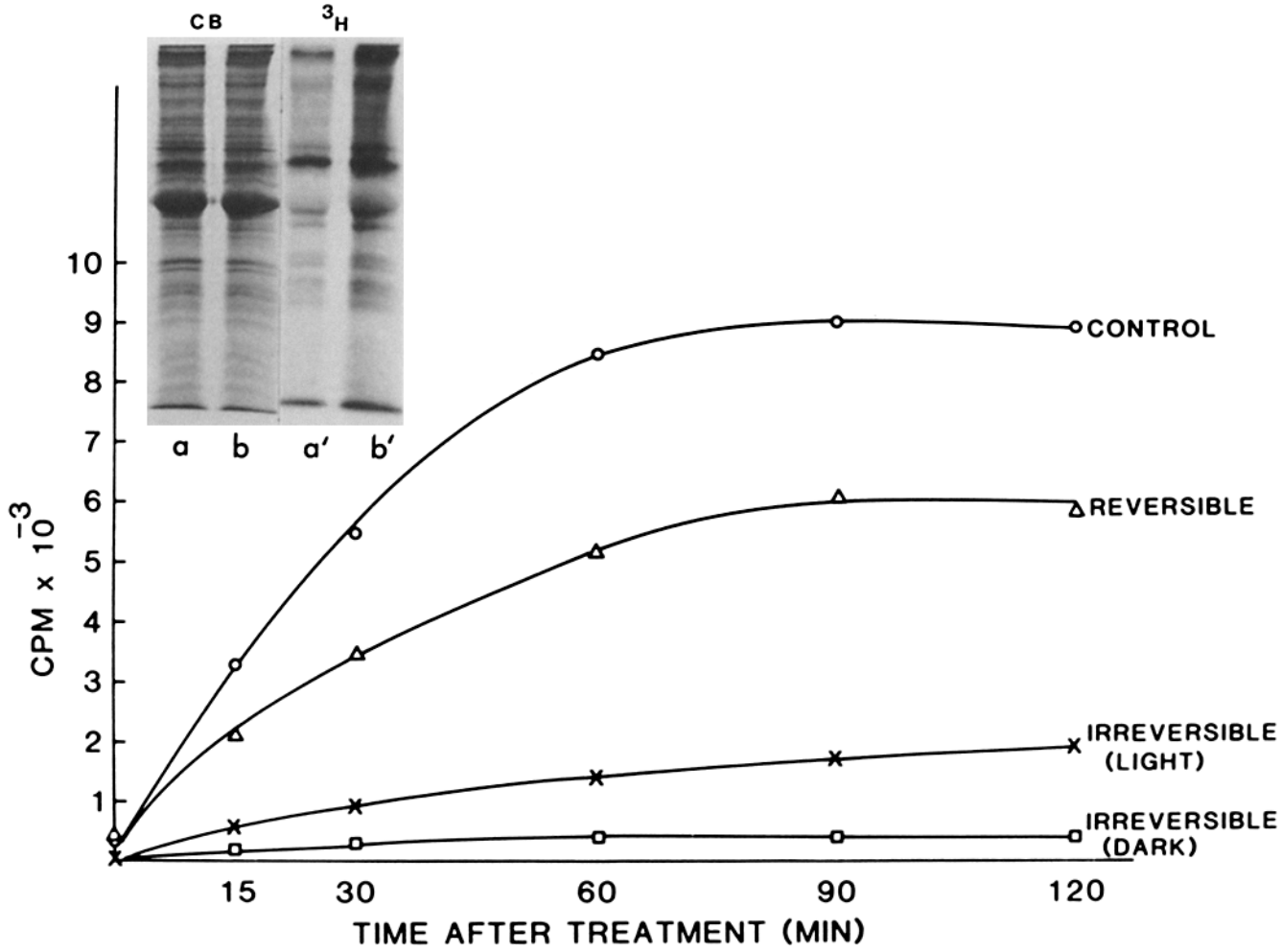


FIGURE 9 The effects of dial-UDP on flagellar GT activity. (○), Control, isolated flagella were assayed with UDP-[^{3}H]glucose at the times indicated. (△), Isolated flagella were treated with 2.5 mM dial-UDP for 1 h and then washed with buffer to remove dial-UDP. The pellets were then assayed for GT activity with UDP-[^{3}H]glucose after the times indicated. (×), Isolated flagella were treated with dial-UDP and 12.5 mM $NaBH_4$ simultaneously in the light, and then rinsed with buffer to remove dial-UDP. The pellets were then assayed for GT activity after the times indicated. (□): isolated flagella were pretreated with dial-UDP for 1 h and then incubated with $NaBH_4$ for 2 h in the dark, followed by rinsing in buffer. The pellets were then assayed for GT activity at the times indicated. It is evident that dial-UDP followed by reduction, particularly in the dark, results in irreversible inhibition of UDP[^{3}H]glucose incorporation, whereas dial-UDP followed by rinsing results in relatively little inhibition.

polypeptide bands seem to bind dial-UDP (Fig. 9) in acrylamide gels. Such conjectures, however, await confirmation by a more complete analysis of surface and flagella enzymes, acceptors, and products.

GTs of the Flagellum

UDP-[^{3}H]glucose incorporation by whole deflagellated cells is ~30-fold greater than that found in isolated flagella on a per-cell basis. However, an estimation of surface areas indicates that each cell possesses ~75-fold more surface area than its single emergent flagellum. These meaasurements assume for ease of computation that the cell is a smooth cylinder (10 μm diameter × 50 μm long) with no invaginations, and they are, therefore an underestimation of the true undulating surface with its anterior canal and reservoir. Flagellar lengths average ~16 μm and the asymmetric cross-sections have an average diameter of ~0.35 μm. Using these estimates it would appear that flagella on a unit area basis are enriched a minimum of twofold for GT activity. These approximations also indicate that a contamination rate of one whole cell per 15 flagella would be necessary to explain the observed level of incorporation in the flagellar fraction. In fact, flagellar preparations are rarely contaminated with whole cells (estimated at a frequency of less than one cell/5,000 flagella). Contamination from broken cells is also minor and consists mostly of starch grains with a frequency of less than one per 200 flagella. Starch grains themselves exhibit no GT activity. These data, together with the relatively gentle, non-destructive methods used to deflagellate cells, make it unlikely that cellular contamination contributes significantly to the recorded levels of flagellar incorporation.

Since labeled flagellar products could be extracted relatively easily and partially purified by LH-60 chromatography, an in vitro assay for the glycosylating enzyme was readily generated. Furthermore, the product was soluble in organic solvents and could be removed from radiolabeled donor and detergent extracts in a single step purification. These procedures facilitated the testing of a number of detergents that had been successfully used by others in isolating GTs in other systems.

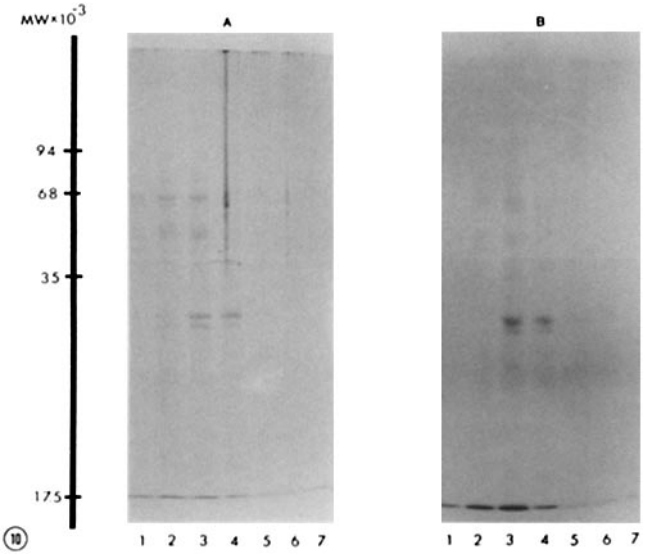


FIGURE 10 CHAPS extracts of isolated flagella were applied to a 0.8-ml sucrose gradient and after centrifugation, fractioned into 8 parts. Each part was incubated with dial-[^{3}H]UDP reduced in borohydrate, and applied to a 20-cm long SDS-polyacrylamide gel. Proteins were reduced and alkylated before SDS-gel electrophoresis. Left panel is Coomassie Blue staining, right panel is the fluorogram of left panel. Most of the dial-UDP binding is found in a pair of bands ~32 kd in fraction 3. In this preparation there is relatively little of the 32-kd polypeptide in fraction 2, and therefore little binding in the fraction 2 32-kd bands. The fluorogram was over exposed (72 d) to reveal minor binding. Quantitative densitometry shows ~50% of the binding is present in the 32-kd bands whereas these bands contain only about 20% of the Coomassie blue staining protein.

These included triton-x 100 (5, 17), Nonidet P-40 (53), and TPA (32), but none of these was found to be useful in the present study; only CHAPS of those detergents examined produced soluble, active fractions. Removal of CHAPS from these extracts by dialysis or Sephadex G-25 chromatography resulted in nearly complete loss of glycosylating activity. This would seem to suggest that the flagellar GT(s) had a requirement for a hydrophobic environment and was therefore partially buried in the flagellar membrane. GT activity extracted with CHAPS was retained on a Sephadex G-100 column indicating an upper molecular weight limit of 150 kd. Since SDS gels of a dial-[^{3}H]UDP bound peptide provided a subunit molecular weight of 32 kd, it would appear that the active enzyme must consist of four or fewer subunits. This is within the range of previously purified GTs (for glycoproteins and glycolipids) which may be 20–160 kd in molecular weight (1, 4, 9, 22, 27, 36, 37, 42, 47, 51, 56), and may consist of 2 (reference 1) or 4 (reference 46) subunits.

The correlation of enzyme activity with a 32-kd subunit depended ultimately in these studies on demonstrating that dial-UDP binds to the presumptive GT. The utility of this inhibitor of GTs and its limitations have been discussed elsewhere (39), but the principal limitation is that any UDP requiring enzyme can in theory bind the same inhibitor. Binding in the present study is inferred from the observation that dial-UDP inhibits the ability of CHAPS extracts from flagella to glycosylate product IV (Fig. 9). This inhibition can be made irreversible or reversible, depending on whether treatment is followed by reduction in borohydrate. Presumably, irreversible inhibition is due to the irreversible binding of dial-UDP to the GT. Thus it seemed reasonable to expect that radiolabeled dial-UDP would be a sensitive probe for identifying GTs, although it may bind other moieties as well. In the present study the number of potential dial-UDP binding proteins was reduced first by using detergent extraction (yielding ~30 polypeptides), secondly by fractionating the extracts on sucrose gradients, and thirdly by selecting fractions from the sucrose gradients and partially separating the components of two fractions on non-denaturing gels. Since a cut of the gel was found to have GT activity, this portion was eluted and incubated with radiolabeled dial-UDP under irreversible conditions. Reelectrophoresis of the enzymatically active fraction now labeled with dial-[^{3}H]UDP reduced the number of potential GT polypeptides to one. Electrophoresis of whole CHAPS extracts confirmed the presence of dial-UDP binding polypeptides with molecular weights around 32 kd.

Although it is by no means certain that every dial-UDP binding polypeptide (Fig. 9) is a GT, it seems that with appropriate fractionation and controls this can be a useful method for reducing the number of potential candidates to a relatively few bands on acrylamide gels. This general procedure provides an alternative to column (affinity) purification methods (45) in that small amounts of enzyme can be identified on gels and these potentially eluted and used as immunogens to generate antibodies for further enzyme purification and characterization. A novel colorometric assay using the reducing capacity of NADH has also been used to identify GTs on acrylamide gels (38). This assay precludes, however, measuring glucosyltransferase activity since UDP-glucose is added during the assay.

Possible Role of Glycosylated Lipids in the Flagellum

The *Euglena* flagellum has at leat two terminal lipid glycosylating systems (producing in the present report glycosylated products II and IV), and two kinds of lipid intermediates associated with protein glycosylation (19). The latter moieties contribute to the complex of glycoprotein surface appendages characteristic of this organism (8). The function of the membrane glycolipids examined here is uncertain. Complex glycolipids such as cerebosides, gangliosides, and blood group substances may function as receptors or antigenic determinants in many organisms (2). Glycosylated sterols, rare in animal cells, are widely found in plant tissues where their function is not well understood (21). Since sterols can, however, profoundly influence the general properties of membranes (e.g. reference 30), it seems likely that glycosylated sterols are involved with a similar function. With increasing evidence that sterols may be incorporated into and may possibly be selective constituents of flagellar and ciliary membranes, it seems probable that these lipids provide special properties to these organelles. The fact that *Euglena* flagella maintain an endogenous glycosylating system would seem to indicate that some final steps in maturation of glycolipids occurs during or after the flagella are assembled. Since dial-UDP can inhibit flagellar GTs, this may be an especially useful compound for testing the function of glycosylated lipids by adding it to regenerating flagella and examinating subsequent changes in stability or motility. A similar approach has already proven to be useful in understanding the possible biochemical basis for egg/sperm interactions in mice (49).

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