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Trypanosoma brucei brucei: Inhibition of glycosylation of the major variable surface coat glycoprotein by tunicamycin

(parasite-directed synthesis/specific labeling via mannose/N-linked oligosaccharides of glycoproteins/antigenic variation)

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ABSTRACT *Trypanosoma brucei brucei* incorporates D-[^{3}H]mannose into protein *in vitro* in a medium we describe here. The label appears entirely in glycoproteins with approximately 90% in the major variable surface coat glycoprotein (VSCG). Incorporation is linear for 60 min and usually continues for an additional 30 min although at a decreased rate. In the same medium incorporation of L-[^{14}C]serine is linear for 90 min. Incorporation of [^{3}H]mannose is completely inhibited by tunicamycin at concentrations above 100 ng/ml, indicating that the label is being added as part of an N-linked oligosaccharide. This is reflected by a 5% decrease in the apparent molecular weight of VSCG on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Cycloheximide inhibits incorporation of both mannose and serine, although the rates and extent of inhibition differ. Based on the effects of tunicamycin or cycloheximide on incorporation of either precursor, we suggest that N-linked glycosylation occurs subsequent to synthesis of the VSCG polypeptide.

African trypanosomes, causative agents of sleeping sickness in man and nagana in cattle, produce chronic, relapsing infections. Each relapse population is serologically distinct from preceding populations, thereby rendering the immune response to these protozoa ineffectual. Alteration in serological identity is effected by production of antigens that are specific to a given population (1). These antigens lie in the 10-nm-thick surface coat overlying the cytoplasmic and flagellar membranes of the cell (2–4). The major component of this surface coat was purified individually from cloned variants of two strains of *Trypanosoma* (*Trypanozoon*) *brucei brucei*, and each isolate was shown to be variant specific (5, 6). The isolates are glycoproteins containing 6–17% (wt/wt) carbohydrate which is composed of glucose, mannose, galactose, and glucosamine (7).

These variable surface coat glycoproteins (VSCG) have been the subject of extensive study on their chemistry (5–8), surface organization (unpublished results), and role in immunopathology (9, 10). Taylor and Cross (11) described a defined, albeit complicated, medium with which they clearly demonstrated the parasite origin of VSCG by showing incorporation of [^{35}S]methionine into the protein; however, the labeling was not specific. For precise physiologic, regulatory, metabolic, biochemical, and cell biological experiments it is necessary to have parasite-directed labeling of the VSCG that is highly specific. The culture technique of Hirumi *et al.* (12), although yielding continuous propagation of the parasite, produces small numbers of cells at low concentrations and provides a large sink for any radiolabeled precursor—i.e., the monolayer of fibroblasts. In the present study we describe a relatively simple medium that allows the specific labeling of the carbohydrate portions of glycoproteins in *T. b. brucei*. The majority of the label appears in VSCG. In characterizing the system we provide data on the linkage of the carbohydrate to the VSCG.

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MATERIALS AND METHODS

Preparation of Cells. The triple-cloned V1 variant of *T. b. brucei* strain ETat3 (6) was used in all experiments. The parasites were grown in adult female Sprague–Dawley rats, collected by cardiac puncture into sodium citrate (final concentration, 5 mg/ml), and isolated from formed blood components by one differential centrifugation step followed by DEAE-cellulose chromatography (13) of the buffy coat layer. The trypanosomes, which pass through the column free of host cells, were collected into an equal volume of 50 mM sodium phosphate buffer, pH 8.0/100 mM NaCl/0.1% glucose/0.1% bovine serum albumin. The number of individuals in the resulting trypanosome suspension was counted and the appropriate number of cells for a given experiment was concentrated by centrifugation (12,100 × *g* for 15–30 sec) and resuspended in cold medium.

Labeling. We used Dulbecco's modified Eagle's medium, increasing both the number and concentration of amino acids and adding a Hepes buffer system. Cells were incubated at 37°C at a final concentration of 5×10^7/ml. Glycoproteins were labeled with D-[*N*-2-^{3}H]mannose (10–20 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) (New England Nuclear). Total protein synthesis was estimated from L-[*U*-^{14}C]serine incorporation (>135 mCi/mmol, New England Nuclear). [^{3}H]Mannose was used at 15 μCi/ml; [^{14}C]serine, at 5 μCi/ml.

Incorporation was measured by placing duplicate 100-μl aliquots of the suspension in tubes containing 2 ml of ice-cold 10% trichloroacetic acid at 10- or 15-min intervals. Precipitates were collected on mixed cellulose ester filters (0.45-μm pore size) (Millipore, type HA) and washed with 20 ml of 5% trichloroacetic acid and 5–10 ml of 95% ethanol. The filters were placed in scintillation vials, 5 ml of a water-holding scintillation cocktail (14) was added, and the filters were incubated for 16 hr at 4°C. Vials were assayed for radioactivity in a Beckman LS-330 liquid scintillation spectrometer.

Electrophoretic Analysis. Whole cells or VSCG isolated by lentil lectin-Sepharose 4B column (LL-column) chromatography (6) were analyzed on 6–20% linear polyacrylamide gradient slab gels in 0.1% sodium dodecyl sulfate (15). Gels were prepared for fluorography (16), dried onto Whatman 3MM chromatography paper, and placed in contact with x-ray film (Kodak X-omat R film XR 5) at −20°C. After fluorography, the dried gel was sliced into 1-mm segments. Slices were rehydrated in 25 μl of distilled water and digested at 37°C for 16 hr with 4 ml of 3% Protosol in a scintillation cocktail containing 0.1 g of 1,4-bis[2(5-phenyloxazolyl)]benzene and 4 g of 2,5-diphenyloxazole per liter of toluene.

Chemicals. Tunicamycin (lot no. 361-26E-117) was the kind gift of Robert Hamil of Eli Lilly. Cycloheximide and bovine

Abbreviations: VSCG, variable surface coat glycoprotein; MIM, mannose incorporation medium; LL-column, lentil lectin-Sepharose 4B column; LR, run-through fraction; LE, eluted fraction.

serum albumin were purchased from Sigma; electrophoresis chemicals were from Bio-Rad; Protosol, Triton X-100, and scintillation fluors were from New England Nuclear; and medium components were from GIBCO except the mannose incorporation medium (MIM) amino acids (see Table 1), which were from Schwarz/Mann.

RESULTS

Medium. We found that Hepes alone did not provide sufficient buffering capacity; hence, both Hepes and bicarbonate were included in the medium (Table 1), which was adjusted to pH 7.2 with 1 M KOH. During an experiment the pH of the medium rose to approximately 8.0 due to loss of CO_2 to the atmosphere. The cells appeared healthy on microscopic examination. To compensate for the increased molarity as a result of the addition of Hepes, KOH, and amino acids, the concentration of NaCl was decreased to 50 mM. The vitamin stock was divided into small portions and kept frozen until use. A stock that we did not divide killed the trypanosomes after about 1 year of freezing and thawing. Because we found that the ethanol in which the isotope is supplied killed the cells, the [^{3}H]-mannose was evaporated to dryness under a stream of N_2. This is in some way related to the vitamins, because MIM without vitamins does not show this effect. However the vitamins are required to achieve good incorporation. We completely recovered ^{3}H so treated.

Incorporation. In MIM, incorporation of ^{3}H into acid-precipitable material was essentially linear for 60–90 min (Fig. 1). Beyond 90 min incorporation leveled off and the amount of ^{3}H-labeled material declined due to cell death with subsequent liberation of intracellular proteases. The rate of incorporation was decreased by the addition of unlabeled mannose to 1 mM at 30 or 60 min (Fig. 1*a*). Supplementation at 30 min with glucose to give an additional 1 mg/ml caused a temporary decrease in the rate of incorporation, whereas at 60 min it had virtually no effect (Fig. 1*b*). NaDodSO$_4$ gel electrophoresis of cells isolated from the medium at 90 min showed a normal polypeptide profile with Coomassie blue staining. All of the counts applied to the gel were found to migrate at the same position as the VSCG (Fig. 2). To determine if these counts were actually present in the VSCG and to increase the specific activity (cpm/mg of protein), we isolated VSCG from cells following a standard incorporation.

Table 1. Composition of MIM

Component	mg/liter	Component	mg/liter
Salts		Amino acids	
$CaCl_2$	200	L-Alanine	160
KCl	400	L-Aspartic acid	32
$MgSO_4 \cdot 7H_2O$	200	L-Asparagine·H_2O	80
NaCl	2922	L-Arginine·HCl	168
$NaHCO_3$	3700	L-Cystine·2HCl	96
$NaH_2PO_4 \cdot H_2O$	125	L-Glutamic acid	288
Vitamins		L-Glutamine	205
Biotin	4.0	Glycine	144
Calcium pantothenate	4.0	L-Histidine·HCl·H_2O	192
Choline chloride	4.0	L-Hydroxyproline	128
Folic acid	4.0	L-Isoleucine	208
Inositol	7.2	L-Leucine	208
Niacinamide	4.0	L-Lysine	292
Pyridoxal·HCl	4.0	L-Methionine	60
Riboflavin	0.4	L-Phenylalanine	132
Thiamine·HCl	4.0	L-Proline	224
Other components		D,L-Serine	112
Hepes	5955	L-Threonine	352
Glucose	1000	L-Tryptophan	112
Bovine serum albumin	1000	L-Tyrosine	144
Phenol red	15	L-Valine	188

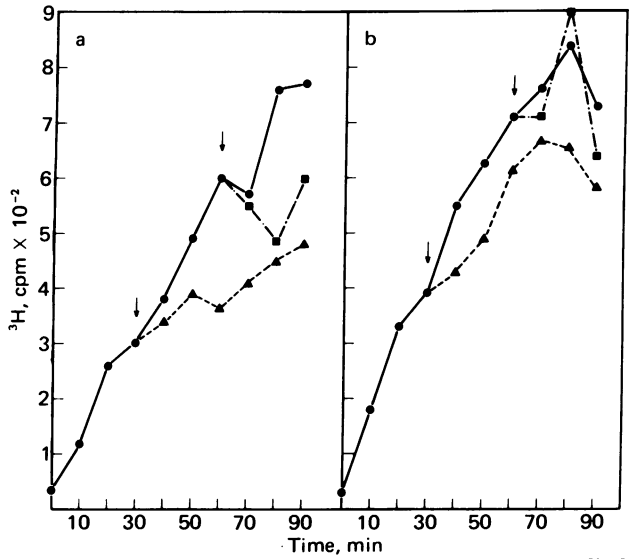

FIG. 1. Effect of glucose or mannose on incorporation of [^{3}H]-mannose into acid-precipitable material by *T. b. brucei in vitro*. Cells (5×10^8) were suspended in MIM (10 ml) and samples were taken every 10 min. At 30 min (▲) and 60 min (■), aliquots (3 ml) were placed in tubes containing 1/100 vol (30 μl) of either 100 mM mannose (*a*) or 100 mg of glucose per ml (*b*) and sampling was continued. The points represent the average of duplicates.

VSCG is the major protein isolated by specific elution of a LL-column after the application of a high-speed supernatant of sonicated trypanosomes (6). The LL-column eluate from ^{3}H-labeled cells had a normal polypeptide profile (Fig. 3*a*). Fluorography showed the majority of the label in VSCG (Fig. 3*b*). The sliced fluorogram confirmed this localization (Fig. 3*c*). The VSCG accounted for approximately 89% of the counts. The remaining counts were found in two minor bands at apparent M_rs of 86,000 (4%) and 76,000 (7%). Recovery of acid-precipitable ^{3}H-labeled material from the experiment described in Fig. 3 is shown in Table 2. Essentially no ^{3}H-labeled material was present in the protein that did not absorb to the LL-column (LR). In the absorbed fractions (LE) we recovered 28% of the

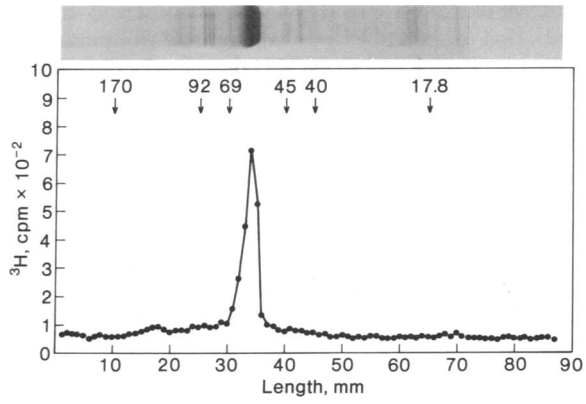

FIG. 2. Localization by NaDodSO$_4$ gel electrophoresis of ^{3}H-labeled material from *T. b. brucei* after a 90-min incubation in MIM containing [^{3}H]mannose. At the end of the 90 min, 2×10^7 cells were collected by centrifugation, dissociated in 100 μl of NaDodSO$_4$ sample buffer (3% NaDodSO$_4$/1% 2-mercaptoethanol/0.125 M Tris·HCl, pH 6.8/20% sucrose), placed in a boiling water bath for 3 min, and subjected to electrophoresis on a 6–20% linear polyacrylamide gradient slab gel. The positions of M_r ($\times 10^{-3}$) markers run in an adjacent well are shown above the profile.

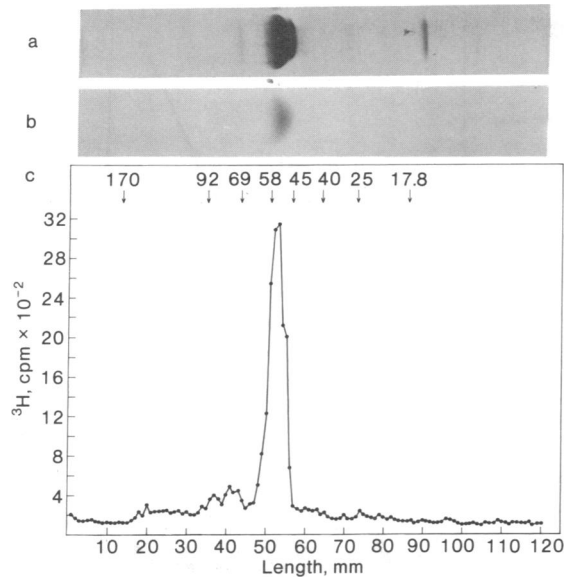

FIG. 3. Localization by NaDodSO$_4$ gel electrophoresis of ^{3}H-labeled polypeptides after purification of VSCG by LL-column chromatography. Cells (5×10^8) were suspended in MIM (10 ml) containing [^{3}H]mannose and incubated for 90 min. At 60 min 0.1 ml of glucose (100 mg/ml) was added. At 90 min the suspension was centrifuged at 12,100 × *g* for 20 sec, the supernatant was removed, and the cells were resuspended in 2 ml of 50 mM Tris·HCl, pH 7.4/50 mM KCl/5 mM MgAc. After sonication, the homogenate was centrifuged for 1 hr at 30,000 × *g*. Other than the substitution of the 30,000 × *g* centrifugation for two at 12,100 × *g* and one at 165,000 × g_{av}, the procedure was as described (6). The 30,000 × *g* supernatant was applied directly to the LL-column and run-through (LR) and specific elution (LE) peaks were collected. The LE, after concentration of 1 ml to 100 μl, was mixed with an equal volume of NaDodSO$_4$ sample buffer. This sample was prepared and subjected to electrophoresis as described in the legend to Fig. 2. The stained gel (*a*) was prepared for fluorography. After a 4-week exposure (*b*) the gel was cut into 1-mm slices and the slices were treated as described in the text (*c*).

counts. This is an underestimate for two reasons: (*i*) According to the acid-precipitated samples, we put 10,000 cpm on the gel shown in Fig. 3; however, when the gel was sliced we recovered 16,000 cpm. (*ii*) The protein concentration in the LE was 10–20 μg/ml; hence, the actual amount of protein we precipitated was 0.5–1 μg. This amount of protein is difficult to recover by acid precipitation without the addition of carrier protein. Based on the gel counts without correction for quenching, recovery in the LE can be increased to 45%.

Inhibitor Studies. We attempted to inhibit [^{3}H]mannose incorporation with tunicamycin or cycloheximide. At 100 ng/ml and after an initial 15-min equilibration period, tunicamycin dramatically decreased the rate of incorporation and by 30 min completely stopped it (Fig. 4*a*). Inhibition was no faster nor more complete at 100–2000 ng/ml (not shown). At 20 ng/ml, tunicamycin decreased the rate but did not stop incorporation. Below 4 ng/ml it had either no effect or a slight stimulatory effect. Cycloheximide, at all concentrations tested, inhibited incorporation of [^{3}H]mannose (Fig. 4*b*). At 2.5, 5, 10, or 20 μg/ml inhibition was complete by 45 min. A higher percentage of total incorporation was achieved by cells in 2.5 μg/ml. There was no difference in total incorporation by cells incubated in 5, 10, or 20 μg/ml at 45 min. The decrease in counts after 45 min at 20 μg/ml could be due to cell death. Unlike its effect on [^{3}H]mannose incorporation, tunicamycin at 100 ng/ml had no effect on the rate of incorporation of [^{14}C]serine for the first 45 min (Fig. 4*c*). After 45 min the rate decreased but did not stop. Total incorporation reached 80% of the level attained by controls. In 10 μg/ml cycloheximide the rate of incorporation was only 30% of the control level.

Table 2. Recovery of acid-precipitable ^{3}H-labeled material during VSCG isolation

Fraction	Acid-precipitable cpm	% recovery
Sonicated cells	12×10^4	(100)
30,000 × *g* pellet	4.9×10^4	40
30,000 × *g* supernatant	7.5×10^4	62.5
LR	13	—
LE	3.4×10^4	28

Fractions are defined in legend to Fig. 3.

NaDodSO$_4$ gel analysis of cells isolated at 90 min from the experiment in Fig. 4*c* exhibited no differences by Coomassie blue staining between control and either tunicamycin- or cycloheximide-treated cells (Fig. 5*a*). Fluorography, on the other hand, revealed a number of differences (Fig. 5*b*). With cycloheximide, all bands were less intense, indicating that there was an overall suppression of protein synthesis. With tunicamycin, the band at the position of VSCG (58,000) disappeared and a band of lower apparent M_r, 55,000, appeared. This represents a 5% shift in the M_r of the VSCG synthesized in the presence of tunicamycin. Three other new bands at apparent M_rs of 117,000, 46,000, and 40,000 were also visible. These result from the inhibition of glycosylation of as yet undefined glycoproteins. It should be noted that the staining pattern is derived from "old" protein because the M_r 55,000 VSCG synthesized with [^{14}C]-serine, and thus "new", was not apparent (Fig. 5*a*).

DISCUSSION

The major VSCGs of trypanosomes are of undoubted importance in their biology and of special importance in the host–parasite relationship. Until now, study of VSCG has been limited to the chemistry of the isolated protein or to serology. A medium allowing synthesis and specific labeling of VSCG by parasites *in vitro* would greatly facilitate a number of important studies on the biology of VSCG—e.g., synthesis and turnover rates, method of elaboration, and regulation of synthesis. Although the medium we describe here does not allow the long-term propagation of the parasite, it does support short-term incorporation of [^{3}H]mannose into protein of trypanosomes. Although VSCG, purified by LL-column chromatography, accounts for only 90% of the ^{3}H counts, when examined at the cellular level, all of the counts easily measurable represent ^{3}H incorporated into VSCG. The inhibition by cycloheximide indicates that the incorporation of [^{3}H]mannose is dependent on protein synthesis (Fig. 4*b*). Therefore, independent of the method of incorporation, the use of [^{3}H]mannose as a precursor provides a specific, parasite-directed *in vitro* labeling technique for VSCG.

Because of the high specificity of the label for glycoproteins (Fig. 3 and Table 2), we assumed that the label was being incorporated as carbohydrate. To test this assumption we attempted to prevent incorporation of [^{3}H]mannose with tunicamycin, an antibiotic that specifically inhibits the production of polyprenol-*N*-acetylglucosamine pyrophosphate, thus blocking synthesis and subsequent attachment of the N-linked core oligosaccharide of many glycoproteins (17). Hence, any inhibition we obtained would (*i*) confirm the covalent attachment of the label inferred from the cycloheximide results and the resistance of the label to dissociation in trichloroacetic acid or NaDodSO$_4$, (*ii*) show that at least some of the label was being incorporated as carbohydrate, and (*iii*) demonstrate that that label, confirmed as carbohydrate, was N-linked, probably to

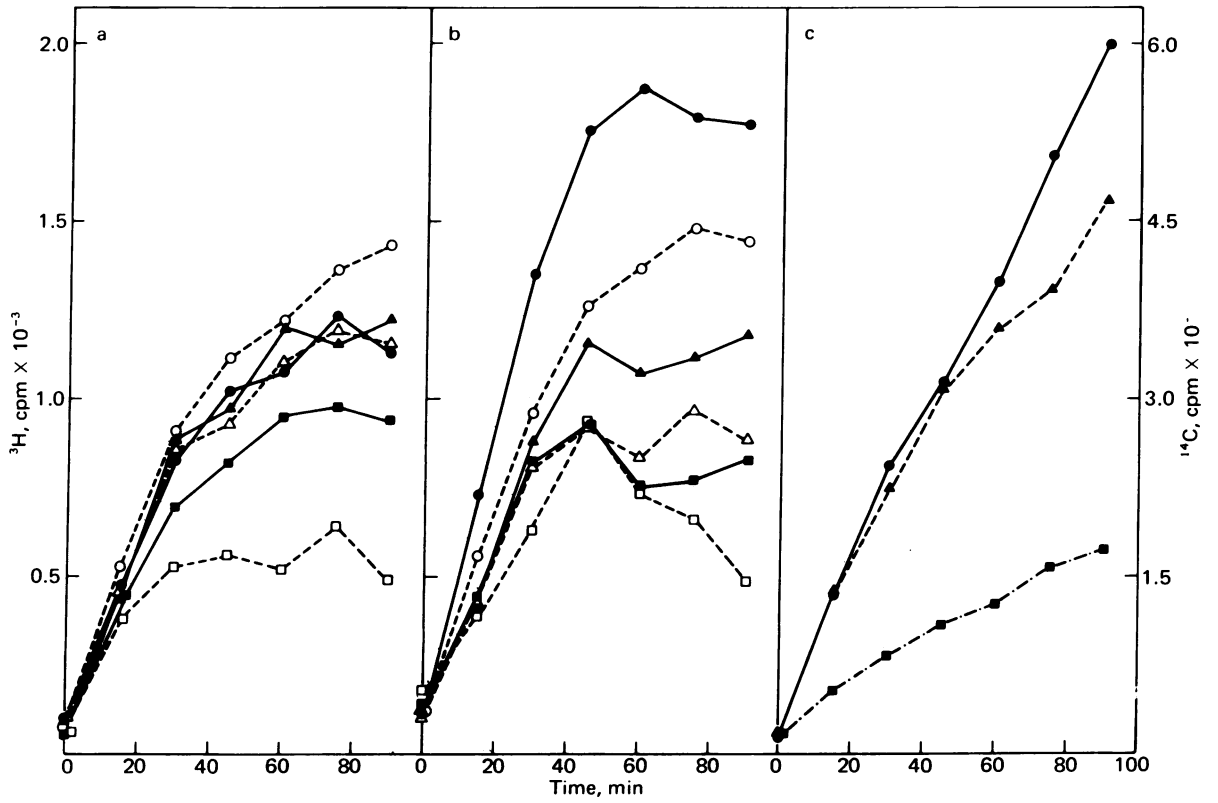

FIG. 4. Effect of inhibitors tunicamycin or cycloheximide on [^{3}H]mannose or [^{14}C]serine incorporation by *T. b. brucei in vitro*. In all cases cells were suspended at 5 × 10^7/ml in MIM containing either 15 μCi of [^{3}H]mannose per ml or 5 μCi of [^{14}C]serine per ml. At 30 min and 60 min 1/200 vol of glucose at 100 mg/ml was added to all tubes. Every 15 min duplicate 100-μl samples were taken. (*a*) Tunicamycin at 0.16 (○), 0.8 (▲), 4.0 (△), 20.0 (■), and 100.0 (□) ng/ml; ●, no inhibitor. (*b*) Cycloheximide at 1.25 (○), 2.5 (▲), 5.0 (△), 10.0 (■), and 20.0 (□) μg/ml; ●, no inhibitor. (*c*) Effect of tunicamycin at 100 ng/ml (▲) or cycloheximide at 10 μg/ml (■) on [^{14}C]serine incorporation is shown against controls (●). Each panel represents a separate isolation of cells. The points are averages of duplicates.

asparaginyl residues through GlcNAc (17, 18). At all concentrations of tunicamycin above 100 ng/ml, the shapes of the inhibition curves and the levels of inhibition were essentially identical. After an initial 15- to 30-min equilibration period, incorporation was completely inhibited (Fig. 4*a*). Because tunicamycin did not have this effect on [^{14}C]serine incorporation (Fig. 4*c*), we conclude that this result is due to the inhibition of addition of ^{3}H-labeled carbohydrates to VSCG. We believe that the initial 15–30 min represents an equilibration period for two reasons. First, tunicamycin inhibits the transfer of only the first GlcNAc to polyprenol phosphate (17). Therefore, incorporation of the labeled precursor could occur in the presence of tunicamycin on polyprenol-GlcNAc synthesized prior to the start of the experiment. Second, the completeness of the inhibition suggests that no further addition of carbohydrate occurs. If the incorporation seen during the initial 15–30 min represents attachment of O-linked carbohydrate, even partially, some incorporation should continue after 30 min.

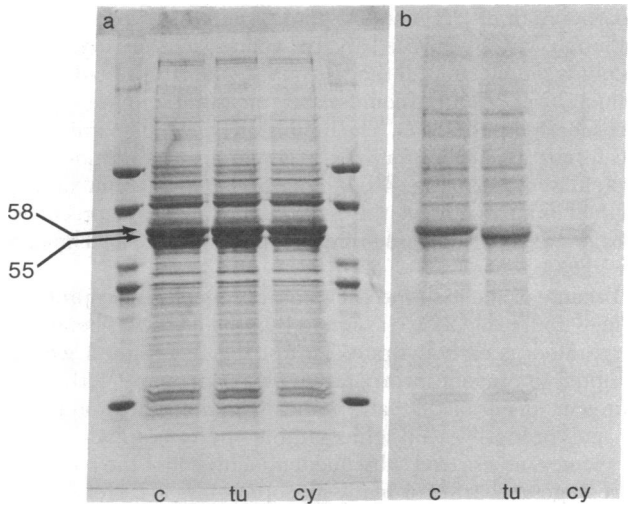

FIG. 5. NaDodSO$_4$ gel and fluorographic analysis of [^{14}C]-labeled polypeptides synthesized by *T. b. brucei in vitro*. Cells from Fig. 4*c* were separated from the medium and prepared for NaDodSO$_4$ gels as described in Fig. 2. After electrophoresis, the stained gel (*a*) was prepared for fluorography. The fluorogram (*b*) is a 2-week exposure. c, Control; tu, tunicamycin; cy, cycloheximide.

Furthermore, and based on the same rationale, assuming that the [^{3}H]mannose precursor can be converted to other carbohydrates found in VSCG, these results suggest that all carbohydrate incorporated into VSCG is N-linked. If conversion does not occur, the results indicate that the mannose present in VSCG is part of an N-linked oligosaccharide side chain. Bearing on this, the 5% change in apparent M_r observed in VSCG synthesized in the presence of tunicamycin (Fig. 5) is consistent with the range, in percent by weight, of carbohydrate found in other VSCGs (7). However, any interpretation of the degree of nonglycosylation of VSCG synthesized in the presence of tunicamycin must be considered inconclusive, because it is based on estimations of the M_rs of two glycoproteins by NaDodSO$_4$ gel electrophoresis, which may not be accurate for glycoproteins. Final resolution of the question of the sugar linkages awaits the demonstration of the presence or absence of the enzymes necessary for interconversion or carbohydrate sequencing of the oligosaccharides of VSCG.

Finally, we suggest that attachment of the N-linked oligo-

saccharide occurs subsequent to synthesis of the VSCG polypeptide chain. First, the extent and kinetics of [^{3}H]mannose incorporation varies with each experiment, but a trend of rapid incorporation for the first 60 min followed by a decrease in the rate is observed (Fig. 4). This decrease in rate cannot be explained by a decrease in the amount of mannose because only 0.1% of the label is incorporated. However, the synthesis of protein, as measured by [^{14}C]serine incorporation, occurs at a constant rate for the full 90 min (Fig. 4*c*) even though 15% of the label is incorporated. If glycosylation were concomitant with protein synthesis, incorporation of ^{3}H should also occur at a constant rate for 90 min. Second, cycloheximide at 10 μg/ml inhibits the rate of mannose incorporation by 30–40% over the first 45 min and completely stops incorporation after that. It inhibits the rate of serine incorporation by 70% for the entire 90 min of the experiment. We would expect equal inhibition of these rates, as well as equal duration of inhibition if VSCG were glycosylated as it was synthesized. Third, if glycosylation and synthesis were concomitant, we would expect to find [^{3}H]mannose-derived counts in low molecular weight bands on NaDodSO$_4$ gels of whole trypanosomes. Such bands would result from incompletely synthesized VSCG present at the end of the incubation. We can expect to see these bands, because VSCG is one of the major proteins synthesized by trypanosomes (Fig. 5*b*). No [^{3}H]mannose-containing polypeptides of apparent M_rs lower than VSCG are observed (Fig. 2). The fact that there is [^{14}C]serine in M_r 58,000 VSCG synthesized in tunicamycin (Fig. 5*b*) is not inconsistent with the above hypothesis. As tested, tunicamycin has no effect on either [^{14}C]serine or [^{3}H]mannose incorporation for the first 15–30 min of incubation. Therefore some [^{14}C]serine-containing protein synthesized during this period could also have been glycosylated.

Our results are consistent with a model of VSCG elaboration analogous to that for secretory protein synthesis and packaging (19). Under the model, VSCG would be synthesized on the rough endoplasmic reticulum and transported through the cisternae to *cis*-transitional elements and then to the Golgi apparatus where N-linked glycosylation would occur. From the Golgi the protein would be taken to the cytoplasmic membrane via small vesicles and "released" by exocytosis. Hence, glycosylation is dependent on transfer to the Golgi. The decline in rate of mannose incorporation that we observed after 60 min could reflect either the discontinuous nature of or an interruption in the en masse transfer to the Golgi. Consistent with this model, Steiger (3) found by thiocarbohydrazid/silver albumose staining in thin sections of *T. b. brucei* that carbohydrate is associated with the cytoplasmic membrane, Golgi-derived vesicles, and membranes of the Golgi complex, but not with the cisternae of the rough endoplasmic reticulum.

In conclusion, we describe a medium that supports incorporation of [^{3}H]mannose into N-linked oligosaccharides of VSCG. This labeling is highly specific, exquisitely sensitive to inhibition by tunicamycin, and somewhat less sensitive to cycloheximide. Furthermore, we suggest that N-linked glycosylation occurs subsequent to synthesis of the protein.

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