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The intracellular pathway and assembly of newly formed variable surface glycoprotein of *Trypanosoma brucei*

(cross-reacting determinant/immunocytochemistry/trypanosomes)

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ABSTRACT **Pulse–chase experiments using L-[^{35}S]methionine suggest that *Trypanosoma brucei* MITat 1.2 variable surface glycoprotein (VSG) synthesized in the rough endoplasmic reticulum, a process that takes 6–8 min, is shuttled to the Golgi complex 8 min later. Labeling of ultrathin frozen sections with affinity-purified anti-cross-reacting determinant (CRD) IgG followed by protein A–colloidal gold shows that the CRD is localized in the *trans*-Golgi region. *cis*-Golgi is not labeled. VSG, when solubilized by treatment with the detergent Nonidet P-40, behaves on sucrose density gradients as a non-membrane protein with a sedimentation value of 5 S. In contrast, VSG solubilized in the presence of Zwittergent TM 3-14 yielded several VSG-containing fractions >5 S, and only the 5S fraction contained the CRD. Lack of the CRD in VSG complexes with sedimentation values >5 S suggests that this determinant is either masked from antibody, perhaps by involvement in polymer formation, or represents the membrane form of VSG recently described by Cardoso de Almeida and Turner [Cardoso de Almeida, M. L. & Turner, M. J. (1983) *Nature (London)* 302, 349–352].**

African trypanosomes have on their surface a continuous 12- to 15-nm-thick glycoprotein coat (VSG) (1) that has been shown to vary both immunochemically (2–5) and biochemically (1, 5–8) during different stages of host infection. It is known that the VSGs of both *Trypanosoma brucei* and *Trypanosoma congolense* contain at least two classes of carbohydrate side chains (9). One, referred to as the internal side chain, is located in the COOH-terminal third of the glycoprotein, contains mannose and *N*-acetylglucosamine, and can be removed by endo-β-*N*-acetylglucosaminidase (10). Its incorporation into VSG is mediated by a dolichol monophosphate intermediate (9) and is inhibited by the antibiotic tunicamycin (11–14). Recently, using highly enriched Golgi and endoplasmic reticulum (ER) fractions isolated from African trypanosomes (15, 16), we tentatively concluded that inhibition of *N*-acetylglucosaminyl transferase by tunicamycin A_1 occurred only in the smooth ER subfraction, suggesting that core glycosylation of the VSG may occur in this organelle (16) and not in the rough ER, as previously assumed (10, 11, 13). As with any centrifugally prepared smooth ER subfraction, it is difficult to ascertain the ratio of ER to other vesiculated fragments of other membranous organelles. Studies showing the *in vivo* incorporation of mannose into VSG also suggest that *N*-glycosylation occurs subsequent to VSG synthesis (14). The existence of a dolichol-dependent mechanism in the smooth ER of trypanosomes is not at all unlikely, especially in light of recent evidence by Rip *et al.* (17), which shows that in rat liver significant quantities of dolichol phosphate are found in both smooth ER and Golgi. Of more potential pharmacological interest is the second class of carbohydrate side chain, which contains mannose, *N*-acetylglucosamine, and galactose (9), and which is located at the COOH-terminal end of the protein conjugated through an ethanolamine linkage to the α-carboxyl group (18), possibly via phosphatidylethanolamine (19). The addition of this carbohydrate is not inhibited by tunicamycin (12). The biological importance of this carbohydrate side chain to VSG function is evident from the observations that it remains constant on all the variable antigens of *T. brucei* and *T. congolense* organisms so far studied and is probably responsible for cross-reactivity between VSGs (cross-reacting determinant: CRD) (9, 20–22, *). In addition, workers in several laboratories have shown that immunological cross-reactivity between solubilized VSG is attributed with the carbohydrate (9, 10, 21).

It is assumed that CRD transfer to VSG protein occurs after the synthesis of the internal carbohydrate side chain (10, 11, 13). One might postulate that the transfer of the CRD carbohydrate side chain occurs in Golgi complex because of its high galactose content. Alternatively, it has been suggested that the CRD is put on at the level of the plasma membrane (14) or shortly after synthesis (11). To clarify the intracellular site for CRD addition, we have labeled ultrathin frozen sections of *T. brucei* with affinity-purified anti-CRD IgG followed by protein A–colloidal gold. The time of addition, as well as the possible role of the CRD, is also discussed.

METHODS

Biochemicals. All reagents were of analytical grade or better. Most biochemicals were purchased from either Sigma, Boehringer Mannheim, or Serva. L-[^{35}S]Methionine (40.7 TBq/mmol) was obtained from Amersham. Aquasol was obtained from New England Nuclear and methionine-free RPMI 1640 culture medium was from GIBCO. Zwittergent TM 3-14 was from Calbiochem–Behring. Nonidet P-40 (NP-40) was from Bender and Hobein (Switzerland).

Organisms. *T. brucei* MITat 1.2, MITat 1.52, MITat 1.6, and ILTat 1.1 were grown from cryopreserved stabilates in lethally irradiated (600–900 rad; 1 rad = 1.0×10^{-2} gray) rats and isolated from infected blood isopycnically on Percoll gradients as described (23).

***In Vivo* Incorporation of L-[^{35}S]Methionine and Subcellular Fractionation of *T. brucei* MITat 1.2.** *T. brucei* MITat 1.2 (10^8 per ml) was preincubated for 30 min in methionine-free RPMI 1640 medium supplemented with 20% fetal bovine serum. L-[^{35}S]Methionine was added (0.4 mCi/ml; 1 Ci = 37 GBq), and after a 3-min pulse, unlabeled methionine was introduced to give a final methionine concentration of 10 mM. At various times after chase, an aliquot was removed and added to 1/10th vol of inhibitor solution [containing per ml: 1

Abbreviations: VSG, variable surface glycoprotein; ER, endoplasmic reticulum; CRD, cross-reacting determinant; NP-40, Nonidet P-40; Tos-LysCH_2Cl, *N*-α-tosyllysine chloromethyl ketone.

*Barbet, A. F., Musoke, A. J., Mpimbaza, G. & Rovis, L., 54th Annual Meeting of the American Society of Parasitology, 1979, Minneapolis, MN (abstr.).

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mg of cycloheximide, 0.5 mg of heparin, 100×10^{12} international units (IU) of aprotinin, and 10 μmol of N-α-tosyllysine chloromethyl ketone (Tos-LysCH$_2$Cl)]. The samples were then immediately chilled in an ice-water bath. Homogenization was carried out in a French Press under 2000 psi chamber pressure (1 psi = 6.89 kPa). Carrier crude microsomes ($15,000 \times g$ supernatant fraction) from 2×10^{10} organisms in 250 mM sucrose/50 mM Hepes/25 mM KCl/5 mM MgCl$_2$/10 μM MnCl$_2$, pH 7.4, were then added to the homogenate. The homogenate was centrifuged at $15,000 \times g$ for 10 min in a JA-20 rotor. Golgi and rough ER fractions were isolated essentially as described by Grab *et al.* (15, 16) with the exception that an SW-41 rotor was used during the sucrose density fractionation steps. Protein was determined in NaOH-digested membrane fractions according to the method of Whitaker and Granum (24).

Preparation of Anti-VSG Antisera or Affinity-Purified IgG. All surface glycoproteins used in this study for antisera production were isolated as described by Cross (6). The clone-specific VSGs isolated appeared to be homogenous by analysis on NaDodSO$_4$/PAGE and isoelectric focusing gels. Anti-VSG antiserum was raised in rabbits as described (20), using purified VSG, and it was shown to be monospecific for VSG by immunoprecipitation and by immunoblotting. In some experiments, affinity-purified IgGs were used and prepared as follows: MITat 1.2 or MITat 1.6 VSG was slowly added at room temperature to cyanogen bromide-activated Sepharose purchased from Pharmacia, and VSG-Sepharose columns were prepared according to the manufacturer's suggestions. Monospecific anti-MITat 1.2 antiserum was chromatographed on a column containing MITat 1.2 VSG coupled to Sepharose. Anti-CRD-IgG was prepared by chromatography of monospecific anti-ILTat 1.1 antiserum on a MITat 1.6 VSG Sepharose column. After incubation (1 hr at room temperature) of the antisera with the appropriate VSG-Sepharose column, the columns were extensively washed with Dulbecco's phosphate-buffered saline (P$_i$/NaCl) and the specific IgGs were eluted with 0.1 M glycine buffer, pH 3, and immediately neutralized with 1 M Tris base. The isolated IgGs were then extensively dialyzed against P$_i$/NaCl. It is well known that antiserum raised against a purified VSG will only react with living trypanosomes that have the same VSG type on their surface (6, 20). Antiserum raised against the CRD fails to react, demonstrating that this determinant is internalized in the coat and is not accessible to antibody. Using indirect immunofluorescence (20), affinity-purified anti-MITat 1.2 VSG IgG intensely stained live *T. brucei* MITat 1.2 organisms and not *T. brucei* clones MITat 1.52 or ILTat 1.1. In addition, immunoblotting on whole organisms showed that only the VSG was labeled. Anti-CRD IgG failed to stain all three clones of living trypanosomes; however, the antibody does recognize only the VSGs of *T. brucei* on immunoblots (data not shown).

Immunoprecipitation of VSG from Rough ER and Golgi Membranes. Affinity-purified anti-MITat 1.2 VSG IgG was diluted in carrier normal rabbit serum. P$_i$/NaCl diluted in normal rabbit serum was used as control. The rough ER and Golgi membrane fractions (100–200 μg of protein) were boiled in 2% NaDodSO$_4$, and NaDodSO$_4$/Triton X-100 mixed micelles were prepared (25). VSG was immunoprecipitated according to the method of McConnell *et al.* (25) except that sheep anti-normal rabbit antiserum was used instead of *Staphylococcus* protein A to precipitate IgG–VSG complexes. The final precipitates were boiled in 2% NaDodSO$_4$ and aliquots were counted in Aquasol in a Packard TriCarb scintillation counter. Background radioactivity was subtracted and the data are expressed as cpm of L-[^{35}S]methionine incorporated per mg of membrane protein.

Immunoelectron Microscopy. Thin frozen sections for immunoelectron microscopy were obtained using the technique introduced by Tokuyasu (26) and Tokuyasu and Singer (27) with the modifications suggested by Griffiths *et al.* (28, 29). Bloodstream form *T. brucei* from lethally irradiated rats isolated as described above were fixed in 0.5% gluteraldehyde in 100 mM Pipes [piperazine N,N'-bis(2-ethane sulfuric acid)] buffer (pH 7.0) for 1 hr at 23°C. They were then embedded in gelatin (30) trimmed to 1-mm^2 blocks and fixed for a further 30 min in the same concentration of gluteraldehyde. These blocks were infused in 2.3 M sucrose in 100 mM phosphate buffer, mounted on copper stubs, and frozen by immersion in liquid nitrogen. Frozen sections, cut on a Sorvall MT-2B ultramicrotome with FTS cryochamber (Sorvall), were mounted on formvar/carbon-coated nickel grids, labeled with affinity-purified anti-CRD IgG, and then incubated with protein A–gold of 5-nm particle size (31). Sections were stained with uranyl acetate and embedded in 1.5% methyl cellulose (Tylose MH 300, Fluka). Electron microscopy was carried out on a Zeiss EM 10 A microscope.

Treatment of Trypanosomes with Detergent, and Sucrose Density Analysis of MITat 1.52 VSG. *T. brucei* MITat 1.52 was isolated from infected rodent blood on Percoll gradients (23) and the VSG was solubilized either by freeze-thawing (6) in 20 mM Hepes/1 mM phenylmethylsulfonyl fluoride/1 mM Tos-LysCH$_2$Cl, pH 7.4 at 5°C (buffer A), or by detergent treatment for 20 min at 5°C in either 1% NP-40 or 1% Zwittergent TM 3-14 in buffer A. After centrifugation to remove undissolved material, the solubilized proteins were applied to a 15%–40% sucrose gradient in buffer A and centrifuged at 60,000 rpm for 16 hr at 5°C in a Beckman SW-60 rotor, and the VSG-containing fractions were detected by RIA as described. The sedimentation values ($s_{20,w}$) were estimated according to the method of McEwen (32).

RIA. RIA against VSG MITat 1.52 was performed exactly as described by Barbet and McGuire (20), using monospecific anti-MITat 1.52 antiserum. To determine the CRD, anti-MITat 1.2 antiserum was used. The RIAs were performed by selecting anti-VSG antiserum dilution precipitating 50%–80% of the ^{125}I-labeled MITat 1.52 VSG added in the assay. VSGs in this study were all iodinated using the chloramine-T method of Hunter (33), using 5–10 μg of protein.

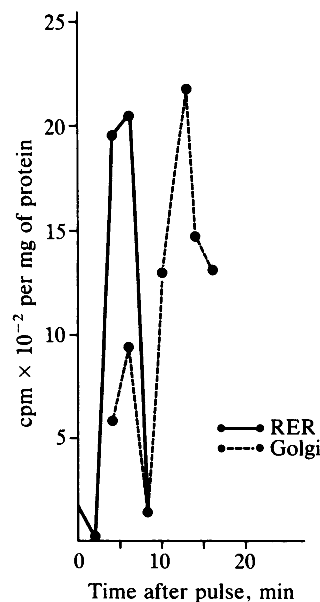

FIG. 1. Distribution of newly formed *T. brucei* VSG. *T. brucei* MITat 1.2 was pulse-labeled for 3 min with [^{35}S]methionine and the cells were fractionated into rough ER (RER) and Golgi membranes, and the radioactive VSG was recovered antigenically as described in *Methods*. Specific activity was obtained by dividing the radioactivity by the total membrane protein. Each point represents the mean of duplicate determinations.

RESULTS AND DISCUSSION

It is known that some of the biochemical mechanisms operating in the production of *T. brucei* VSG share similarities to biosynthetic processes known to occur in other eukaryotic cells. As with other membrane-associated proteins, pre-VSG is synthesized with a rather long 30–40 amino acid hydrophobic NH_2-terminal signal peptide, which is subsequently cleaved in the rough ER, where the molecule is synthesized (25). However, unlike eukaryotes, core glycosylation in trypanosomes appears to take place not in the rough ER but rather in some other organelle (tentatively assumed to be the smooth ER) (16). From pulse–chase experiments with L-[^{35}S]methionine (Fig. 1) we find that MITat 1.2 VSG is synthesized in 6–8 min, a value in agreement with McConnell *et al.* (11). It can also be seen from the data in Fig. 1 that it takes ≈8 min for VSG to be transported from the rough ER compartment into the Golgi complex. The small peak at 6 min seen in the Golgi fraction is most likely due to some cross-contamination with ER elements. It should be noted that for the experiment to be reproducible it was essential to include Tos-LysCH_2Cl after homogenization of the organisms to prevent VSG degradation. Apparently, trypanosomes contain a Tos-LysCH_2Cl-sensitive protease. Addition of the internal carbohydrate side chain(s) presumably takes place before entry into the Golgi, although final maturation of these side chains may still take place in the Golgi as it does in other organisms.

A most exciting area in the field of VSG biochemistry concerns the CRD. Where and when this carbohydrate determinant is added to the VSG molecule has been an important question. It has been suggested that CRD is put on the plasma membrane (14) or shortly after synthesis (11). Using affinity-purified anti-CRD IgG, we have attempted to clarify

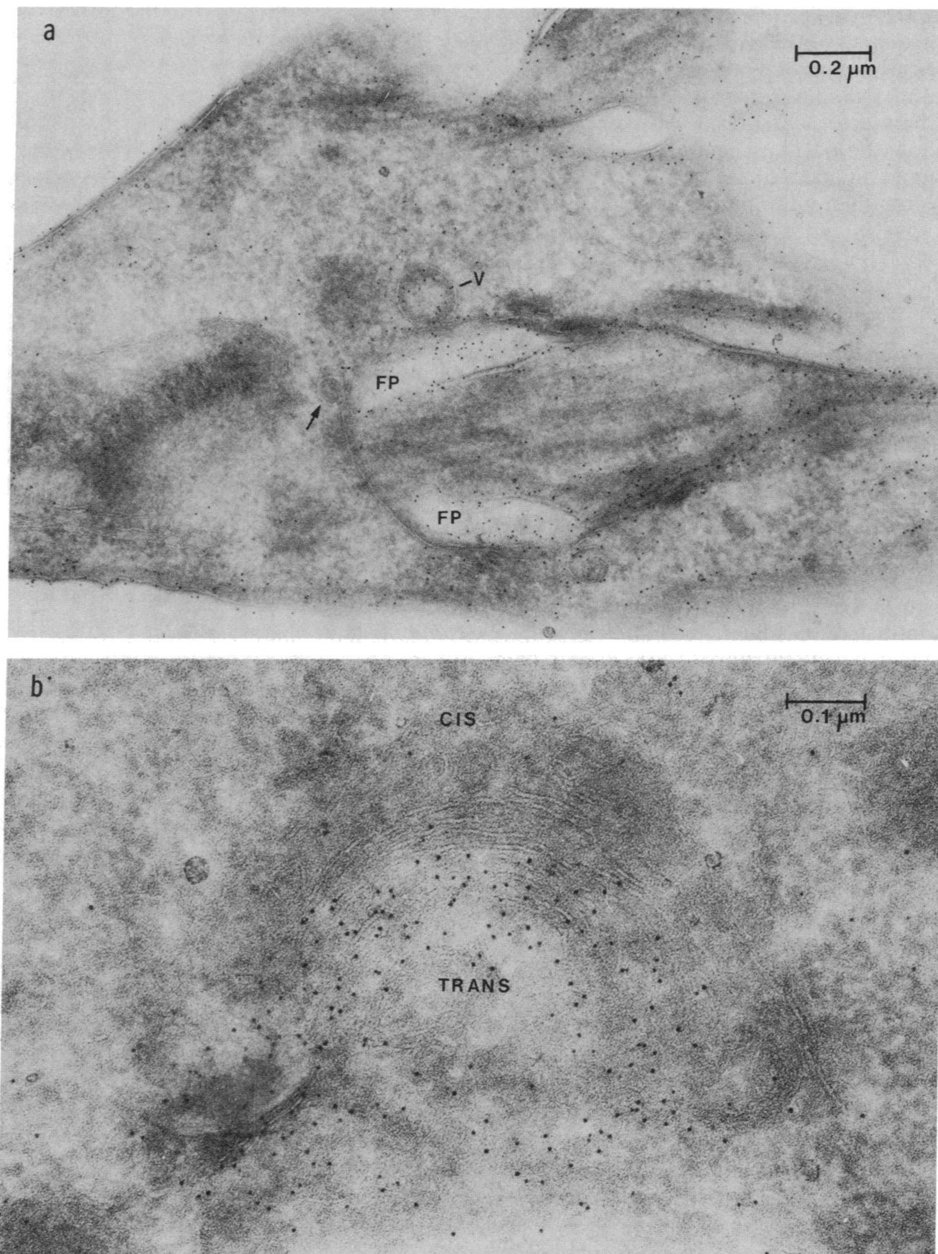

FIG. 2. (*a*) Frozen section of a trypanosome through the flagella pocket region, labeled with affinity-purified anti-CRD IgG and protein A–colloidal gold (5-nm particle size). Gold particles (representing specific anti-CRD binding) are located in the flagellar pocket (FP), on the surface of the trypanosome, and in an intercellular vesicle (V). An unlabeled vesicle (arrow) is also present. (×49,000.) (*b*) Frozen section through the Golgi region of a trypanosome labeled as in *a*. Only the *trans* side of the Golgi is labeled with the anti-CRD IgG. (×105,000.)

the intracellular site for CRD addition on ultrathin frozen sections of *T. brucei*. Immunoelectron microscopy reveals that the anti-CRD antibody binds to the surface of the trypanosome, the flagellar pocket, and to some cytoplasmic vesicles; ER was unlabeled, and nonspecific (background) labeling was low (Fig. 2*a*). Fig. 2 also shows that the addition of the CRD, as detected by anti-CRD IgG, occurs in the *trans*-Golgi region of the cell facing the flagellar pocket (Fig. 2*b*). This would also put the time for CRD addition on the VSG molecule to be ≈8 min after synthesis in the ER. McConnell *et al.* (11) suggested that CRD addition occurred shortly after synthesis but because of the long pulse period used in their study (8 min), they were unable to accurately measure the transfer time of *de novo*-synthesized VSG. One final indirect outcome of this study would suggest that the COOH-terminal hydrophobic tail, whose existence has been inferred from DNA sequence analysis (34–36) but never seen in isolated VSGs, is most likely cleaved in the Golgi complex or in some other organelle earlier in the pathway, because this hydrophobic moiety must be cleaved before the CRD can be added to the α-carboxyl group of the COOH-terminal amino acid. A better understanding underlying the biochemical mechanism(s) of how the COOH-terminal hydrophobic tail is removed and how the CRD is incorporated onto the VSG, tentatively assumed to occur in the Golgi region of the trypanosome, clearly has inherent importance as a potential target for chemotherapy.

Auffret and Turner (37) showed by gel filtration and by $NaDodSO_4$ electrophoresis that purified *T. brucei* VSG exists in solution as dimers and, occasionally, as higher oligomers after treatment with bifunctional cross-linking reagents. This work has also been confirmed by Strickler and Patton (38). We find that VSG on *T. brucei* may exist naturally in the form of higher oligomers.† *T. brucei* MITat 1.52 was isolated on Percoll gradients and the VSG was solubilized either by freeze-thawing or by detergent treatment and then analyzed by density gradient centrifugation on sucrose gradients as described in *Methods*. As determined by RIA, VSG solubilized by either freeze-thawing or NP-40 treatment behaved as a non-membrane protein with a sedimentation value of ≈5 S (Fig. 3). Essentially all the molecules contained the CRD. In contrast, VSG solubilized in the presence of 1% Zwittergent yielded several major VSG-containing fractions >5 S, and only the 5S species contained the majority of the CRD (Fig. 4). This has also been observed for *T. brucei* MITat 1.2 and ILTat 1.4 (data not shown). When ^{125}I-labeled MITat 1.52 VSG was added to the trypanosome preparation prior to Zwittergent treatment, the labeled VSG still behaved as a 5S species, indicating that there was no Zwittergent-induced aggregation of the molecule (Fig. 5). Lack of CRD in VSG complexes with sedimentation values >5 S suggests that this determinant either is masked so that antibody binding does not occur, perhaps by involvement in polymer formation, or represents the membrane form of VSG (mfVSG) recently described by Cardoso de Almeida and Turner (39). Immunoprecipitation of *in vivo* L-[^{35}S]methionine-labeled VSG from sucrose gradients after Zwittergent treatment of trypanosomes showed only the VSG to be immunoprecipitated. No other proteins were associated with VSG (data not shown). However, there is strong evidence that what we observed is indeed the membrane form of the VSG. Like mfVSG, VSG in the form of higher Zwittergent complexes appears to lack the CRD as determined immunologically. Preliminary data also show loss of cross-reactivity on immunoblots. MITat 1.52 trypanosomes, after detergent treatment or freeze-thawing, were subjected first to $NaDodSO_4$/PAGE and the proteins were transferred unto cellulose nitrate membranes according to the method of Burnette (40). Immunoblots were done on the total transferred proteins (40) using monospecific anti-MITat 1.52 VSG or anti-CRD IgG and scans done on the autoradiograms. After freeze-thawing, essentially 100% of the VSG molecules contained the CRD, whereas 85% and 55% of the molecules contained this determinant after 1% NP-40 or 1% Zwittergent TM 3-14

†Grab, D. J. & Vergee, Y., 10th International Conference of the World Association for the Advancement of Veterinary Parasitology, Aug. 18–20, 1983, Perth, Australia (abstr.).

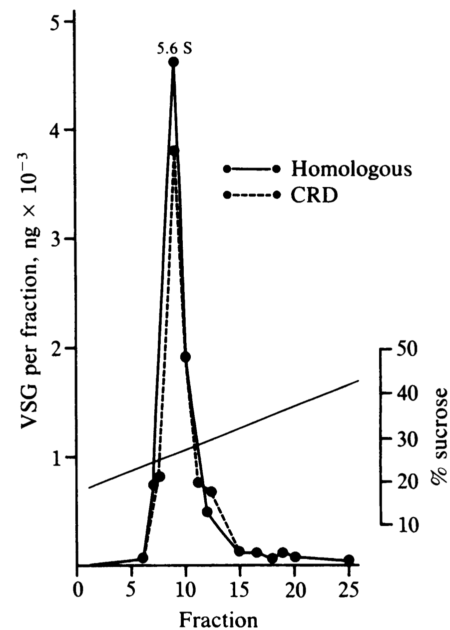

FIG. 3. Sucrose density analysis of VSG after solubilization with NP-40. *T. brucei* MITat 1.52 (10^8 cells) was solubilized in 0.2 ml of 1% NP-40 in buffer A and subjected to sucrose density analysis; VSG content per fraction was determined by RIA using either anti-MITat 1.52 VSG serum (homologous serum) or anti-CRD antiserum. Data are expressed as ng of VSG per 0.16 ml of fraction. Sucrose concentration was determined using a Zeiss refractometer. Results obtained after exposing trypanosomes to three cycles of freeze-thawing as described by Cross (6) were identical to those obtained after NP-40 treatment.

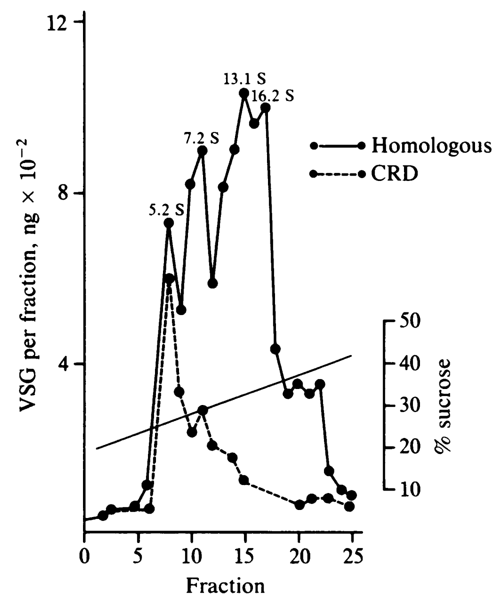

FIG. 4. Sucrose density analysis of MITat 1.52 VSG after solubilization of trypanosomes with Zwittergent TM 3-14. Experiment was conducted exactly as described in Fig. 3, except that 1% Zwittergent was used as the solubilization agent.

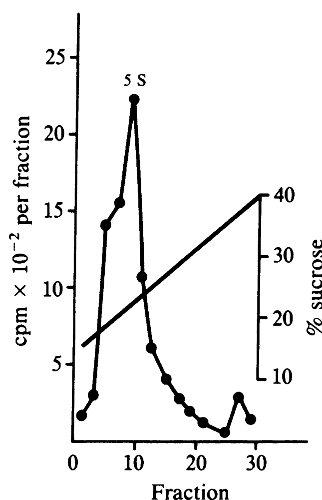

FIG. 5. Effect of Zwittergent TM 3-14 on 5S MITat 1.52 VSG. Iodinated 5S VSG (≈5000 cpm per 20 μl) was added to 2 × 10^6 trypanosomes. After incubation with 200 μl of 1% Zwittergent, the cells were subjected to sucrose density analysis. Each fraction (130 μl) was counted in a Packard γ scintillation spectrophotometer. Sucrose concentrations were determined using a Zeiss refractometer.

treatment, respectively. In addition, other than boiling in $NaDodSO_4$, no detergents other than Zwittergent TM 3-08 to TM 3-16 are able to prevent conversion of the mfVSG to the soluble form (M. L. Cardoso de Almeida and M. J. Turner, personal communication). From these data, it appears that Zwittergent treatment is probably a more gentle method for maintaining the intramolecular forces between mfVSG molecules than boiling in $NaDodSO_4$, which yields only monomeric species.

Since the CRD is common to VSGs, at least in *T. brucei* and *T. congolense*, it can be assumed to have a common function. Perhaps it is involved in zipping up the surface coat. It may alternatively be involved in binding the VSG to the membrane or to some lipid or protein membrane element. Laminin, a large adhesive glycoprotein that occurs primarily in basement membranes, has been described in *T. cruzi* trypomastigotes (41). Preliminary findings suggest a laminin-like protein in African trypanosomes, as assayed by RIA (unpublished observations). Only coated bloodstream trypomastigotes, but not the uncoated fly midgut trypomastigotes, contained this laminin-like moiety. This suggests that a laminin-like protein may be involved, in part, in anchoring the VSG to the parasite's cell membrane.

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