Subcellular Localization of a Variable Surface Glycoprotein Phosphatidylinositol-specific Phospholipase-C in African Trypanosomes

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Abstract. African trypanosomes contain a membrane-bound enzyme capable of removing dimyristylglycerol from the membrane-attached form of the variable surface glycoprotein (mfVSG; Ferguson, M. A. J., K. Halder, and G. A. M. Cross, 1985, *J. Biol Chem.*, 260:4963–4968). Although mfVSG phospholipase-C has been implicated in the removal of the VSG from the trypanosome surface (Cardoso de Almeida, M. L., and M. J. Turner, 1983, *Nature (Lond.)*., 302:349–352; Ferguson, M. A. J., K. Halder, and G. A. M. Cross, 1985, *J. Biol Chem.*, 260:4963–4968), its precise function and subcellular location have not been determined. We have developed a procedure for the separation of the cell fractions and organelles of *Trypanosoma brucei brucei* (and other trypanosome species) by differential sucrose and isopycnic Percoll^R centrifugation. These fractions were tested for mfVSG phospholipase activity using *Trypanosoma brucei* mfVSG labeled with ^{3}H-myristic acid as substrate. The highest enzyme-specific activity was associated with the flagella and evidence is presented to suggest that it is localized in the flagellar pocket. Some activity was also associated with the Golgi complex. These results suggest that the mfVSG phospholipase is localized primarily in the membrane of the flagella pocket and possibly other membrane organelles derived from and associated with this structure, and may be part of the VSG–membrane recycling system in African trypanosomes.

The activity of mfVSG phospholipase amongst various trypanosome species was determined. We show that, in contrast to the bloodstream forms of *Trypanosoma brucei*, cultured procyclic *Trypanosoma brucei* and bloodstream *Trypanosoma vivax* had little or no mfVSG phospholipase activity. The activity found in bloodstream forms of *Trypanosoma congolense* was intermediate between *Trypanosoma vivax* and *Trypanosoma brucei*.

AFRICAN trypanosomes, which cause sleeping sickness in man or nagana in livestock, have a 12–15-nm-thick coat of a variable surface glycoprotein (VSG)[1] covering the entire surface of the organism (53). For *Trypanosoma brucei* and *T. congolense*, the generation of a new population of trypanosomes with a different surface coat has been shown to be the result of a clone-specific change in the expression of a particular VSG gene (14, 40). It appears clear that the remarkable ability of the trypanosome to alter its surface glycoprotein (14) may preclude the development of a conventional vaccine against trypanosomiasis. Since the integrity of the surface coat is known to be essential for parasite survival in the host, the purpose of this study was to investigate VSG synthesis, processing, and replacement on the cell surface. A clearer understanding of the cell biology of the VSG may suggest alternative approaches for the possible control of trypanosomiasis.

The VSGs from *T. brucei* are proteins that contain complex carbohydrate moeities of mannose, galactose, glucosamine, and myristilated phosphotidylinositol (17, 18, 39) conjugated to the protein through the alpha-carboxyl group of the carboxy-terminal amino acid via an ethanolamine residue (13, 28, 39). The biological importance to VSG of this carbohydrate side chain is evident since it is present on all variable antigens of *T. brucei* and *T. congolense* organisms so far studied and is probably responsible for cross-reactivity observed between soluble forms of VSGs (also known as the cross-reacting determinant [CRD]) (14). The carbohydrate side chain may be involved in anchoring the VSG to the plasma membrane (15, 17, 18, 39), and maintaining the integrity of VSG–VSG interactions within the coat (24).

This work was presented in part at the 6th International Congress of Parasitology (Brisbane, Australia) August 25–29, 1986. (Grab, D. J., S. Ito, J. D. Lonsdale-Eccles, P. Webster, and Y. Verjee, 1986, *ICOPA VI Handbook*, 140. [Abstr.])

1. *Abbreviations used in this paper*: CRD, cross-reacting determinant; GPDH, glycerol-3-phosphate dehydrogenase; LG fraction, large granule fraction; mfVSG, membrane-attached form of the variable surface glycoprotein; SG fraction, small granule fraction; sVSG, soluble form of the variable surface glycoprotein; VSG, variable surface glycoprotein.

It has been proposed that VSG is anchored to the plasma membrane by a hydrophobic domain within the CRD (16–18, 39). Although the precise structure of the CRD is not completely known, the presence of a covalently linked glycosyl-sn-1,2 dimyristoyl phosphatidylinositol in the VSG of trypanosomes has been revealed (18). The possibility that the VSG is anchored to the membrane via the myristyl residues is supported by the evidence that the membrane-attached form of the *T. brucei* VSG (mfVSG) contains glycerol and myristic acid whereas the soluble form of VSG (sVSG) lacks these residues (15, 17, 39).

The conversion of the mfVSG to sVSG may be mediated by the action of an endogenous phospholipase-C-like enzyme (also designated Enzyme-X; 9, 10, 15, 17, 18, 30, 39). It has been postulated that mfVSG is selectively released in vivo from the plasma membrane of trypanosomes as a result of the removal of the diacylglycerol domain from the phosphatidylinositol (17, 18, 39), and that the VSG-releasing enzyme is possibly located in the plasma membrane (10, 15, 17, 18, 39). However, the intracellular localization of the enzyme responsible for the conversion of mfVSG to sVSG is still uncertain. The present report is an attempt to address this problem.

Materials and Methods

Biochemicals

All reagents were of analytical grade and were obtained from the following sources. Uridine diphospho-D-[6-^{3}H]galactose (640 GBq/mmol), [9,10(*n*)]-^{3}H-myristic acid (2.04 TBq/mmol), ^{14}C-casein (1.1 MBq/mg protein), L-[^{35}S]methionine (30 TBq/mmol), and Hyperfilm MP were obtained from Radiochemical Centre Limited (Amersham, England). Aquasol and EN^{3}HANCE were obtained from New England Nuclear (Boston, MA). [*N*-(2-acetamido)]-2-Amino ethanesulfonic acid (ACES), 2-(*N*-morpholino)-ethanesulfonic acid (MES); Hepes; and [*N,N-bis*-(2-hydroxyethyl)] glycine (BICINE) were from Research Organics Inc. (Cleveland, OH). Aristar grade sucrose was from BDH Chemicals (Poole, England). RPMI-1640 and Iscove's culture media were from Flow Laboratories, Inc. (Irvine, Scotland). Zwittergent TM 3-14 was from Calbiochem-Behring Corp. (La Jolla, CA). Poly/Sep 47 IEF buffer was from Polysciences, Inc. (Warrington, PA). Sephacryl S-200 and Percoll were from Pharmacia Fine Chemicals (Uppsala, Sweden). DE-53 was from Whatman Ltd., (Maidstone, Kent, England). All phospholipids, and 4-methylumbelliferyl phosphate were from Koch-Light Ltd. (Haverhill, Suffolk, England); dimyristin was from Serva (Heidelberg, Federal Republic of Germany), and the protease inhibitors leupeptin, chymostatin, antipain, and E-64 were from Cambridge Research Biochemicals (Cambridgeshire, England). All solutions were prepared in a purification system developed by Christ (Aesch, Switzerland); namely, deionized water which had been filtered through a 5-μm coarse filter, an ultrafilter with an 80,000-D cutoff, and sterilized by ultraviolet radiation. The conductivity of the water was <15 megaohms.

Organisms

Trypanosoma brucei clones MITat 1.2, 1.52, ILTat 1.1, *T. congolense* clone ILNat 2.1, and *T. vivax* ILDat 1.2 (subclone 1392) were grown from cryopreserved stabilates in lethally irradiated rats (600–900 rad). The organisms were isolated from the infected blood using isopycnic Percoll gradients (21). Infected blood was mixed 1:2 with 90% Percoll containing 1% glucose, 0.73% NaCl (buffered to pH 7.4 with solid Hepes), and centrifuged at 15,000 rpm for 15 min in a JA20 rotor. The trypanosomes were collected, the pH was adjusted to 8.0 with 1 M Tris-base, and the trypanosomes immediately passed through a DE-53 column equilibrated in phosphate-buffered saline glucose (PSG), pH 8.0 (33) supplemented with nucleosides (0.1 mM adenosine, 0.05 mM hypoxanthine, 0.05 mM thymidine) as well as Baltz's additions; namely, 0.2 mM 2-mercaptoethanol and 2 mM pyruvate (2). After elution the pH was adjusted back to pH 7.4 with Hepes. Platelets and other blood cells are effectively removed by these isolation conditions. However, the ATP contents of the isolated parasites are several-fold higher than those isolated in PSG by previous workers (19, 35).

Subcellular Fractionation

For each experiment ~10^{11} trypanosomes were washed in SHK (250 mM sucrose, 50 mM Hepes, 25 mM KCl, pH 7.4, at 5°C) buffer containing known inhibitors of the major trypanosomal proteinases (36; 50–100 μg/ml of leupeptin, E-64, antipain, and chymostatin). The parasites were disrupted by passage through a French Pressure Cell under a chamber pressure of 2,500 psi.

Fractions containing lysosomes, glycosomes, and flagella were prepared at 0–5°C as follows: EDTA was added to the homogenate (to a final concentration of 1 mM; SHKE, pH 7.4), and unbroken cells, nuclei, and cell debris were sedimented at 705 g_{av} for 10 min in a JA-20 rotor (Beckman Instruments Inc., Palo Alto, CA). The resulting pellet was designated the crude nuclear pellet. The resulting supernatant was centrifuged at 2,800 g_{av} for 10 min to produce a large granule (LG) fraction; this was followed by a small granule (SG) fraction produced at 15,000 g_{av} for 10 min. Centrifugation of the (post-SG) supernatant at 123,000 g_{av} for 90 min in a 50.2 Ti rotor (Beckman Instruments Inc.) gave the crude microsomal pellet fraction and the soluble supernatant.

The LG and SG fractions were further fractionated by isopycnic centrifugation in Percoll. The LG and SG fractions were made 57.6% (vol/vol) with respect to Percoll in SHKE (pH 7.4) and layered under a discontinuous gradient consisting of 43.2, 28.8, and 20.3% Percoll in the same buffer. Centrifugation was for 30–35 min at 25,000 rpm in either an SW-41 or an SW-27 rotor. Fractions banding between the 28.8/43.3% and 43.3/57.6% Percoll interfaces were made 57.6% with regard to Percoll and centrifuged again in either the gradient described above or in continuous Percoll gradients in SHKE. Fractions on top of and within the 20.3% Percoll layer (crude flagella) were adjusted to 28.8% Percoll and layered under a discontinuous gradient containing 20.3, 17.3, and 14.4% Percoll in SHKE buffer, pH 7.4, and centrifuged again as above to obtain the lighter membrane fractions. Depending on yields, the fractions were washed with SHKE buffer and stored at −80°C. For some preparations, the entire fractionation was done in the presence of 5 mM magnesium instead of the EDTA.

To obtain endoplasmic reticulum and Golgi fractions, 5 mM $MgSO_4$ was added to the homogenate (in SHKM, pH 7.4) and processed using established procedures as described by Grab et al. (23) except that the separation

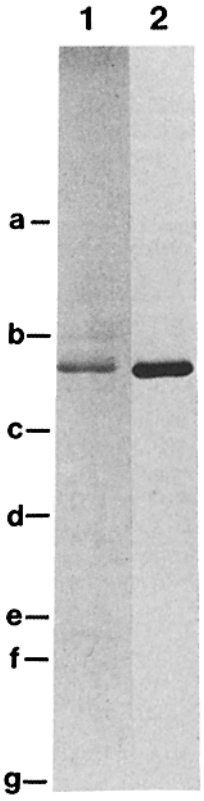

Figure 1. SDS-PAGE analysis of ^{3}H-myristic acid-labeled MITat 1.2 mfVSG. Radiolabeled MITat 1.2 mfVSG was subjected to electrophoresis on a 7.5–15% SDS-polyacrylamide gel. After staining in Coomassie Blue, the gel was soaked in EN^{3}HANCE and fluorography was done with preflashed Hyperfilm-MP (Amersham Corp.). (Lane *1*) Coomassie Blue-stained mfVSG; (lane 2) fluorograph of isolated mfVSG shown in lane *1*. The light band labeled with myristate above the mfVSG is serum albumin. Molecular weight standards were (*a*) Phosphorylase a, 94,000; (*b*) BSA, 67,000; (*c*) egg albumin, 45,000; (*d*) carbonic anhydrase, 30,000; (*e*) soybean trypsin inhibitor, 20,400; (*f*) lysozyme, 14,400; and (*g*) bromophenol blue (front).

of smooth endoplasmic reticulum from rough endoplasmic reticulum components was done in a vertical VTi50 rotor (50,000 rpm for 1 or 2 h) instead of an SW-27 swinging bucket rotor. The latter requires longer centrifugation times. These fractions were washed with SHKE buffer before further analysis or storage by freezing at −80°C. These fractionation procedures, developed for *T. brucei* bloodstream forms, may be used for *T. congolense* and *T. vivax* bloodstream forms as well as for cultured procyclic *T. brucei*.

Morphology

For electron microscopy, suspended fractions were fixed in a fixative containing picric acid, formaldehyde, and glutaraldehyde (29) in 100 mM sodium phosphate buffer, pH 7.2. After fixation at room temperature for 1 h, the fractions were centrifuged in an Eppendorf microfuge for 1–15 min to obtain a visible pellet. The samples were washed with 100 mM sodium cacodylate buffer, treated with 1% OsO_4 in sodium cacodylate buffer en bloc, stained with uranyl acetate, dehydrated, and embedded in Epon-Araldite. Thin sections were cut across the entire thickness of the pellet, stained with uranyl acetate and lead citrate, and examined in the electron microscope.

Biochemical Analysis

Acid phosphatase was assayed using as substrate either beta-glycerophosphate (52) or 4-methylumbelliferylphosphate (51). Acid proteinase was assayed using ^{14}C-methylated casein or benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin as substrate (37). Glycerol-3-phosphate dehydrogenase was assayed according the method of Rovis and Baekkeskov (50). The transfer of galactose from UDP-galactose to endogenous protein substrates (galactosyltransferase) and adenyl cyclase were assayed as described by Grab et al. (23). Malate dehydrogenase was measured according to the method of Opperdoes et al. (47). Protein determination was performed according to the method of Vincent and Nadeau (54) using BSA as a protein standard. SDS-PAGE was performed according to the method of Neville (43) except that a slab gel (2 mm thick by 200 mm long) containing a linear 10–20% polyacrylamide gradient was used.

Preparation of ^{3}H-Myristic-labeled MITat 1.2 mfVSG

^{3}H-Myristic acid (185 MBq) was dried in a rotary evaporator, washed several times with absolute ethanol/benzene 1:1 (vol/vol) to remove the toluene, and once with absolute ethanol. The labeled fatty acid (in ethanol) was coupled to defatted BSA (55), lyophilized, and added (3.7 MBq) to either Iscove's medium with transferrin (1 μg/ml), albumin (400 μg/ml), soybean lipid (100 μg/ml), or RPMI-1640. The media was also supplemented with 0.45% glucose, and nucleotides, and Baltz additions as described above. MITat 1.2 trypanosomes (5×10^7/ml) were incubated in the fatty acid containing medium for 2–3 h at 37°C. In some cases the trypanosomes were incubated with [^{35}S]methionine (3.7 MBq) instead of labeled lipid. After washing the organisms several times in radiolabel-free medium, the mfVSG was extracted (31). Analysis by SDS-PAGE showed that only mfVSG labeled with ^{3}H-myristic acid (Fig. 1).

mfVSG Phospholipase Assay

The assay mixture for VSG phospholipase contained, in a volume of 30 μl, 2.26 μg labeled mfVSG (8,500 cpm/μg VSG protein), and 7.5 ng membrane protein (in 15 μl SHKE) or Sephacryl S-200 enzyme protein (diluted in 15 μl SHKE), 50 mM Hepes, pH 7.4, 0.025% polyethylene glycol (PEG; M_r 6,000, wt/vol), 0.5% wt/vol Triton X-100, protease inhibitors (20 μg/ml each of E-64, leupeptin, chymostatin, and antipain). Under the conditions stated, the protein substrate-to-enzyme ratio was 300:1, while the ratios of substrate phosphatidylinositol to total lipid, glycolipid, total phospholipid, and membrane phosphatidylinositol were at least 11:1, 107:1, 16:1, and 160:1, respectively, based on the data of Baekkeskov et al. (1). After a 15–30 min incubation at 37°C the reaction was terminated by boiling in 100 μl of 0.3% (wt/vol) Zwittergent TM 3-14 after which 300 μl of 0.3 M NaCl/90% methanol (vol/vol) was added followed by 800–900 μl of *n*-hexane. The sample was vortexed until emulsification occurred and then centrifuged in an Eppendorf microfuge to separate the phases. The release of labeled myristic acid into the hexane phase was monitored by liquid scintillation spectrometry. The above assay was done in duplicate or triplicate and was found to be reproducible. No proteolysis occurred during incubation with the various fractions when [^{35}S]methionine-labeled mfVSG was the substrate.

TLC of the hexane-extractable product was done on heat-activated (100°C for 1 h) 0.25-mm-thick silica gel 60 TLC plates in a solvent system containing pentane, diethyl ether, glacial acetic acid (80:20:1; vol/vol/vol) (41). The plates were dried for 30 min at 60°C, sprayed with EN^{3}HANCE, dried for 1 h at 65°C, and exposed to Hyperfilm MP film at −80°C (Amersham).

Sephacryl S-200 Column Chromatography of mfVSG Phospholipase

Flagellar membrane fractions which banded on top of 14.4% Percoll (in SHKE) were solubilized in 2% 3-[(3)-cholamidopropyl]dimethyl-ammonio-1-propanesulfonate (CHAPS) (wt/vol), containing the protease inhibitors E-64 and leupeptin (20 μg/ml each) and centrifuged at 100,000 *g* for 1 h to remove undissolved cytoskeletal components. After dialysis against 0.1% CHAPS in 10 mM Hepes, 0.05% PEG, pH 7.0 (CHP buffer), the sample was applied on to a 1.6- × 95-cm Sephacryl S-200 column equilibrated in the same buffer. The active fractions were pooled. IEF was done on a Poly/Sep 47 column (6). Product analysis was conducted by TLC (41) as described above.

Results

Subcellular Fractionation: Morphological and Biochemical Analysis

Discontinuous Percoll gradients produced highly enriched preparations of lysosomes, glycosomes, and flagella. An important feature of the isolation procedure appears to be the hypertonicity of the 250 mM SHKE buffer (~410 mosmols) as more isotonic buffers resulted in fractions with poor purities (data not shown). The isolated lysosome-like organelles (Fig. 2 *A*) were spherical with a rim of high density substance and a clear center. These membrane-bounded structures

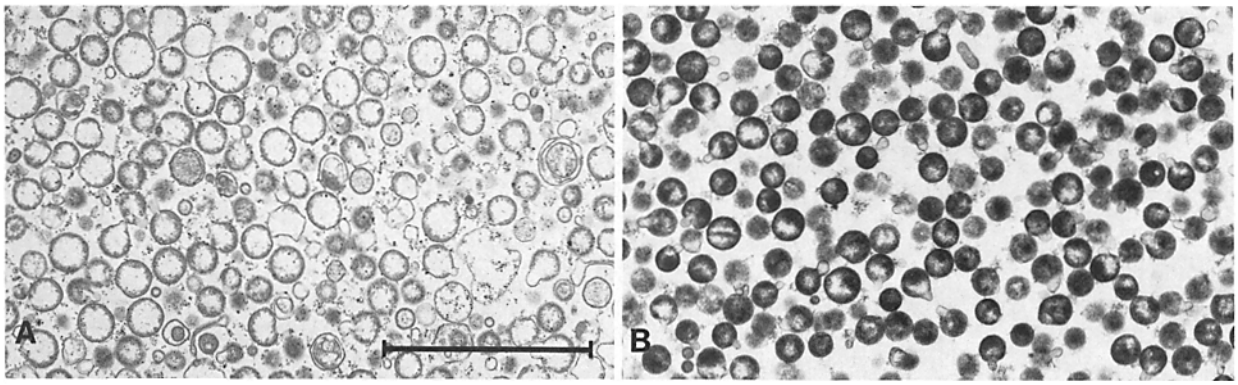

Figure 2. (*A*) Lysosome fraction from the 28.8/43.2% Percoll interface. (*B*) Glycosome fraction from the 43.2/57.6% Percoll interface. Bar, 2 μm.

Table I. Biochemical Analysis of MITat 1.2 Membrane Fractions

Fraction	Enzyme*					
	AC	AP‡	GT	APr§	GDPH	MDH
Membrane						
Homogenate	1.0	1.0	1.0	1.0	1.0	1.0
Crude nuclear pellet	1.4	1.0	1.0	0.6	1.2	0.3
LG fraction	2.3	2.1	1.0	1.0	1.0	0.1
SG fraction	3.4	2.0	1.0	1.9	1.3	0.1
Crude microsomal pellet	1.4	2.5	1.4	1.6	1.5	0.1
High-speed soluble supernatant	0.3	0.1	0.5	0.1	0.4	1.8
Rough endoplasmic reticulum	0.4	2.2	–	1.3	1.0	0.0
Smooth endoplasmic reticulum	1.0	0.3	–	1.3	0.8	0.0
Golgi fraction	2.2	14.7	5.7	0.5	0.3	0.1
Lysosome	1.1	5.3	–	110.9	3.7	0.1
Glycosome	0.4	1.4	–	11.9	7.3	0.0
Flagella‖						
I	1.9	1.4	1.2	11.8	0.7	0.0
II	2.8	2.2	1.0	0.7	0.2	0.1
III	2.1	2.2	0.5	0.4	–	–
IV	2.8	2.5	0.9	0.3	0.8	0.0
V	5.1	3.9	0.9	0.0	0.1	0.0

* Specific activities relative to the homogenate control. *AC*, adenyl cyclase; *AP*, acid phosphatase; *GT*, galactosyltransferase; *APr*, acid proteinase; *GPDH*, glycerol-3-phosphate dehydrogenase; *MDH*, malate dehydrogenase.
‡ Using 4-methylumbelliferylphosphate as substrate. Identical results were obtained with beta-glycerophosphate.
§ Determined in preparations made in the absence of protease inhibitors using benzyloxycarbonyl-Phe-Arg-amido-4-methylcoumarin (37); results were essentially identical to those obtained with radiolabeled casein.
‖ Flagella fraction I was from the 28.8/20.3% Percoll interface; II was from the 20.3/17.3% interface; III was within the 17.3% Percoll layer; IV was from the 17.7/14.4% interface; and V was from top of the 14.4% Percoll layer.

(density = 1.082 g/ml) were usually enriched in the SG fraction and consistently banded between the 28.8/43.2% Percoll interface. The sizes were similar to those of metrizamide-purified rat liver lysosomes (57). Biochemical characterization of these trypanosomal organelles is given by Lonsdale-Eccles et al. (37). Some Percoll particles (~25 nm in diameter) were identifiable in this and all other Percoll-derived fractions. In contrast, morphologically recognizable glycosomes (Fig. 2 *B*) were divided between the LG and SG fractions and could be obtained from the 43.2/57.6% Percoll interface. These spherical microbody-like organelles (density = 1.091 g/ml) contained densely staining crystalloid centers and were similar to those obtained by Opperdoes et al. (48). Little stratification was observed within the lysosome and glycosome fractions.

Biochemical analyses confirm the morphological observations. As can be seen in Table I, the fraction containing the highest specific activity of glycerol-3-phosphate dehydrogenase (GDPH), an enzyme marker for glycosomes (47, 48, 50), was found in the glycosomal fraction identified as such by electron microscopy (Fig. 2 *B*). The relative specific activity of GPDH as compared to the total homogenate was similar to the purified glycosomes obtained by Opperdoes et al. (48): 7.3 vs. 8.2, respectively (see Table I). However, some glycosomal enzyme activity was also observed in the lysosome fraction. Since GPDH activity was low in the high-speed soluble supernatant fraction, this suggested that the glycosomal contamination of the lysosome fraction is due to a nonsoluble glycosomal element. Some isolations were performed in the absence of protease inhibitors to establish the contribution of the lysosomal fractions to the other fractions. Little lysosomal protease/peptidase activity was associated with the glycosome fraction (Table I). The proteolytic activity against benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin, with the exception of the Golgi fraction, showed a slight lag phase (2–4 min) before full (linear) activity was observed. In the case of the Golgi fraction, however, the activity was unstable and rapidly diminished after the addition of the enzyme to the reaction mixture. When the proteolytic activity of the lysosomes was measured as described by Lonsdale-Eccles and Mpimbaza (36), it was observed to have an apparent molecular mass of 30,000 D, but upon the addition of a trace of serum (0.1%) additional bands were observed at ~100,000 D (38).

In addition to lysosomes and glycosomes, the LG subfraction was found to contain an extra-membrane band that consistently migrated slightly below the 20.3/28.8% interface, and which biochemical evidence suggested may contain remnants of mitochondrial membranes (data not shown). Occasionally, we found a very dense membrane fraction which resided deep within the 57.6% Percoll layer. This layer may contain morphologically intact mitochondria in low yield. However, it appears that most of the mitochondria were disrupted in the French Press since malate dehydrogenase, a known mitochondrial marker (47, 51), was found in the soluble fraction (Table I).

Flagella were found in both the SG and LG fractions. However, the flagella were readily separated from lysosomes and glycosomes by centrifugation in Percoll. In the presence of EDTA, the crude flagellar fractions containing microtubule-associated membranes, trypanosome skeletons, and other vesiculated membranes were found either within and/or on top of the 20.3% Percoll layer (Fig. 3 *A*). These could be separated from each other by a second centrifugation through a lighter Percoll gradient containing EDTA. The 17.3/14.4% Percoll interface (fraction IV) contained flagella with fewer trypanosome skeletons and more membrane than those seen in Fig. 3 *A* (see Fig. 3 *B*). The presence of tubular structures, with diameters of more than twice the diameter of microtubules, were found with the flagellar obtained from the fraction banding within the 17.3% layer (fraction III) just above the 20.3/17.3% interface (fraction II; Fig. 3, *C* and *D*). They are too large to be pellicular microtubules and are of the wrong shape to be the flattened cisternae of the Golgi apparatus. It is possible that they could be the collecting tubules described by Langreth and Balber (34) but seem not to be interconnected. They layered on the top of the fixed pellet. Also present were occasional glycosome-like structures (center, Fig. 3 *D*). The flagella fraction which was derived from the 20.3/17.3% Percoll interface (fraction II) contained axoneme profiles and much membrane (Fig. 4 *A*). However, the membranes did not seem closely associated with the flagella. Some pellicular microtubules were also present. Flagellar profiles with paraxial rods were abundant and membranes that were not directly attached to the flagella were present in part of the fixed pellet obtained from the fraction derived from the 20.3/28.8% Percoll interface (fraction I; Fig. 4 *B*). A different part of the same pellet also contained a few flagellar profiles and was rich in membranes (Fig. 4 *C*) which

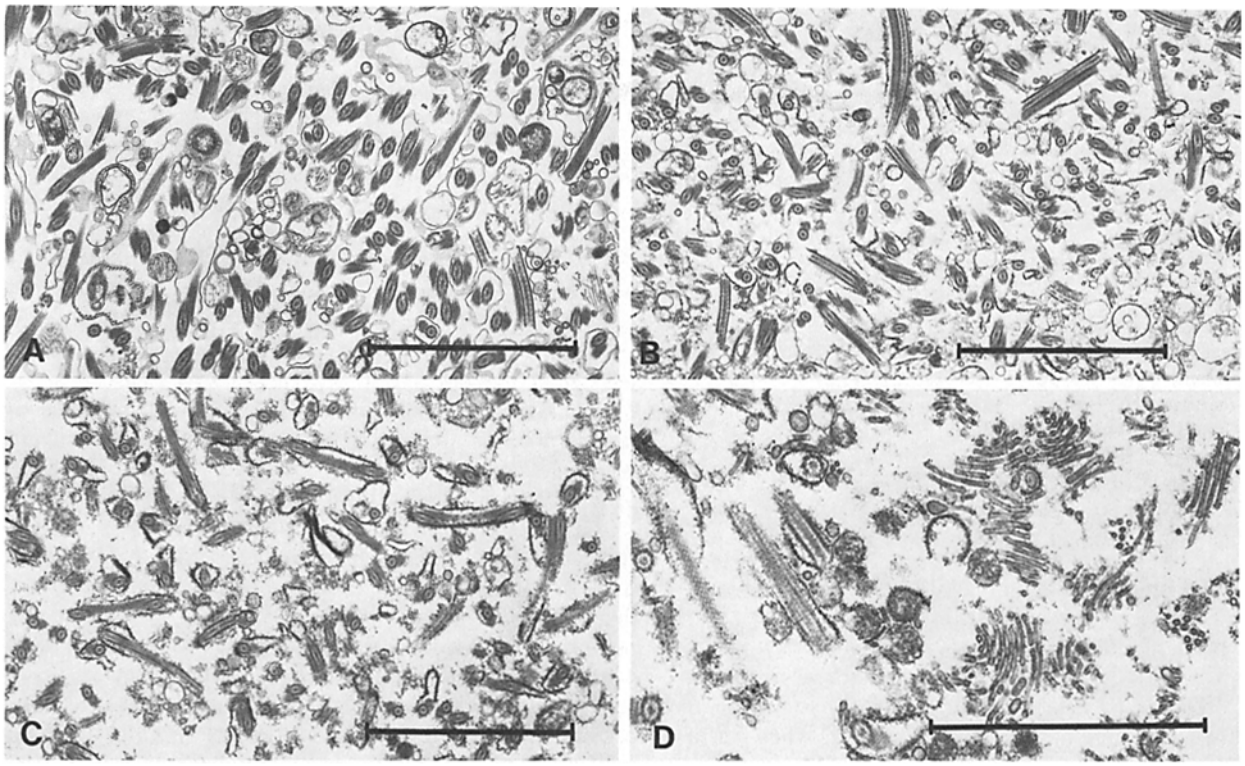

Figure 3. (*A*) Crude flagella fraction from the first Percoll gradient (entire 20.2% layer). Contains flagella with associated membranes as well as without. This fraction also contains other flagella-free membranes as well as trypanosome skeletons. (*B*) Fraction from 14.4/17.3% Percoll interface. Similar to Fig. 3 *A* but with fewer trypanosome skeletons and more membranes. Many flagellar profiles have membranes associated with them. (*C*) This fraction banded within the 17.3% Percoll layer above the 17.3/20.3% interface. It contains many flagellar profiles and, as shown in a higher magnification micrograph (*D*), tubular structures having more than twice the diameter of microtubules. Bars: (*A–C*) 4 μm; (*D*) 2 μm.

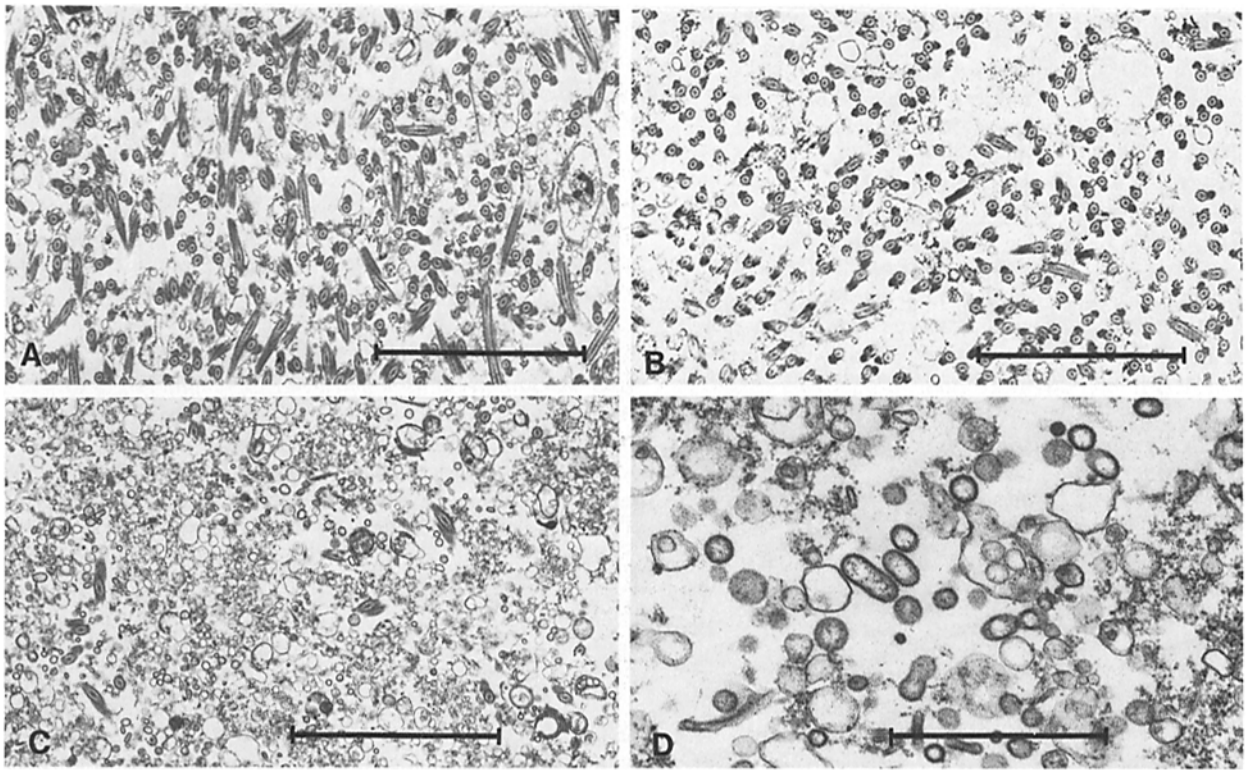

Figure 4. (*A*) Flagella fraction derived from the 17.3/20.2% Percoll interface. Contains axoneme profiles and membranes not associated with the flagella. Some pellicular microtubules are also present. (*B*) Flagella profiles with paraxial rods abundant are found in the fraction banding at the 20.2/28.8% Percoll interface. (*C*) Different part of the pellet seen in *B*. This fraction is rich in membranes many of which appear to be coated vesicles. A few flagellar profiles are present. (*D*) Higher magnification of *C*. Bars: (*A–C*) 4 μm; (*D*) 1 μm.

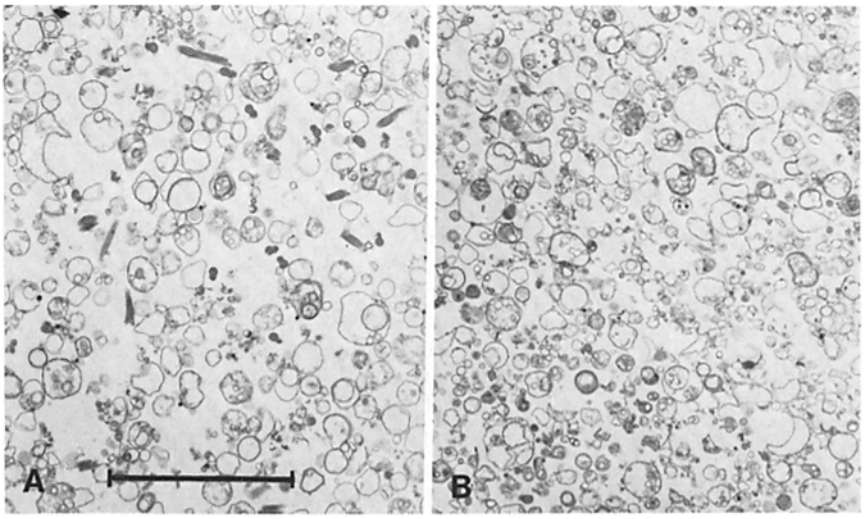

Figure 5. (*A*) Light membrane fractions derived on top of the 14.4% Percoll layer in the presence of EDTA. (*B*) Fraction derived on top of 20.2% Percoll layer in the presence of magnesium. Bar, 5 μm.

were recognizable under high magnification as coated vesicles (Fig. 4 *D*). Large vesiculated membrane profiles were found to band on top of the 14.4% Percoll layer (fraction V). These light Percoll membranes with diameters ranging from 500 to 1,000 nm (Fig. 5 *A*) were similar in size to the membrane surrounding the flagella (400–1,200 nm) when cut in cross section, as seen in Fig. 2, *A* and *B*. This fraction contained little or no GPDH, acid proteinase, galactosyltransferase, or malate dehydrogenase, but was rich in adenyl cyclase and acid phosphatase, which are enzymes reported to be in the flagella pocket (34, 51, 56).

mfVSG Phospholipase Distributions

Using ^{3}H-myristic acid–labeled MITat 1.2 mfVSG as the substrate, we have examined the distribution of mfVSG phospholipase in both intact trypanosomes and in the morphologically defined subcellular fractions. The amount of the enzyme activity was identical in slender or stumpy *T. brucei* bloodstream forms, while this activity was less in *T. congolense* and present in low amounts in both *T. vivax* bloodstream and *T. brucei* procyclic culture forms (Table II). This suggests that, unlike *T. brucei* and to a lesser degree *T. congolense*, *T. vivax* either lacks the enzyme or that its enzyme cannot use *T. brucei* mfVSG as substrate. The latter interpretation may be the more plausible because it appears that the CRD is immunologically cryptic in *T. vivax* compared with that in *T. brucei* and *T. congolense* (Fish, W. R., D. L. Grab, and G. W. N. Mpimbaza, unpublished observations).

The endogenous membrane-bound mfVSG phospholipase activity in *T. brucei* homogenates was independent of magnesium, manganese, or calcium cations, and did not need sulfhydryl reducing agents for activity. However, ~15–20% activation occurred with either 10–25 mM dithiothreitol (DTT) or with 5 mM EDTA; i.e., no further activation occurred when the membranes were incubated with DTT and EDTA together. This does not seem to be due to the method of trypanosome preparation or lysis as membranes we prepared as described by Hereld et al. (27) again showed only the minimal 15–20% activation by these agents.

Using a citric acid/MES/ACES/Hepes/BICINE (pH 3–9) mixed buffer, the enzyme showed optimal activity at pH 7.5 and little activity below pH 6.0. This differs from *T. brucei* phospholipase A_1 which, unlike VSG phospholipase, is mostly soluble with a pH optimum around 6.0. A particle-bound enzyme with a pH optimum of 5.2 is also present in low amounts (47). The reaction was dependent on both mfVSG concentration and time of incubation (data not shown) and was inhibited by phosphatidylinositol[2], by the detergent Zwittergent 3-12 and 3-14, and by unlabeled mfVSG (but not by sVSG). Although the mechanism of the Zwittergent inhibition is not obvious, it is clear that the detergent can effect VSG–VSG interactions and is an effective inhibitor of mfVSG phospholipase (22, 24–26). This Zwittergent effect also oc-

Table II. VSG Phospholipase Levels in Different Trypanosome Species

Trypanosome	Relative specific activity*
T. brucei MITat 1.2 bloodstream forms	1.0
T. brucei MITat 1.2 cultured procyclics	0.0
T. brucei ILTat 1.1 bloodstream forms (slenders)	1.1
T. brucei ILTat 1.1 bloodstream forms (stumpys)	1.1
T. congolense ILNat 2.1 bloodstream forms	0.6
T. vivax ILDat 1.2 (subclone 1392) bloodstream forms	0.1

* Relative to *T. brucei* MITat 1.2 bloodstream forms. Mean of two separate determinations.

2. It should be noted that high trypanosomal membrane concentrations can cause an adventitious release of ^{3}H-radioactivity from mfVSG into *n*-hexane. This release is independent of pH throughout the 3.0–9.0 range and the activity is found in the chloroform/methanol extract of the membranes. The reaction occurs after proteolysis of the substrate by papain and proteinase K and after boiling the extract in the presence of detergents. It can also be mimicked by relatively high concentrations of phospholipids: the best effectors being phosphatidylinositol, phosphatidylcholine, and sphingomyelin. We suspect that the effect may be due in part to a detergent-like effect on the labeled mfVSG leading to the release of some noncovalently bound polar lipid since the product(s) released does not migrate off the origin on TLC in a neutral solvent system (Fig. 6). However, we cannot completely rule out the possibility of some nonenzymatic covalent isotope exchange reactions nor that at higher membrane concentrations the effects of other phospholipases (e.g., D) may be of some consequence. However, the latter possibility seems unlikely in view of the broad pH range over which the effect is observed.

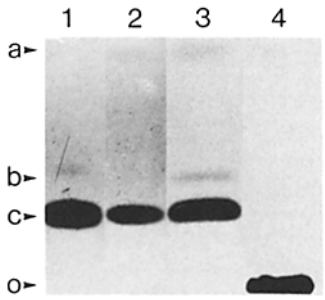

Figure 6. TLC of the reaction products released into *n*-hexane. Membranes, purified enzyme, or phosphatidylinositol were incubated in a volume of 500 μl until completion of the enzymatic reaction. The *n*-hexane phase was dried down under a flow of ($CaCl_2$-dried) nitrogen gas and the residue was washed several times in chloroform/methanol (2:1, vol/vol). TLC was done as described in Materials and Methods. (Lane *1*) Homogenate; (lane *2*) light Percoll-derived membrane fraction (14.4%); (lane *3*) partially purified VSG phospholipase; (lane *4*) mfVSG incubated with 4 μg phosphatidylinositol. The arrows refer to the migration of (*a*) free myristic acid; (*b*) an unknown lipid (possibly 1,3 diaclyglycerol); (*c*) 1,2-diacylglycerol; (*o*) origin.

curs in the presence of other detergents such as NP-40 or Triton X-100 (Grab, D. J., unpublished observations).

Since African trypanosomes are known to contain both phospholipase A_1 and A_2 (32, 42, 46), we used TLC to examine the products of mfVSG hydrolysis to determine the nature of the products released and the specificity of our assay system. The major hydrolysis product was found to be 1,2-diacylglycerol, with minor amounts of free myristic acid and some unknown lipid, possibly 1,3-diacylglycerol (Fig. 6). This confirms the phospholipase-C nature of the reaction assayed.

A comparison of Table I with Table III shows that the highest relative specific activity for *T. brucei* mfVSG phospholipase was associated with membranes presumably derived from the flagellar pocket. In Table III, column A shows data averaged from MITat 1.2, MITat 1.52, and ILTat 1.1; and column B shows data for MITat 1.2 which had a specific activity of the homogenate of 38 fmol myristate released per min/ng membrane protein. Although the yield was only 0.5%, little evidence for an endogenous activator or inhibitor was found under the assay conditions used (Table III). The activity from these flagellar fractions could be solubilized with detergent. Relatively high activity for the mfVSG phospholipase was also found in the Golgi fraction and in those flagella fractions where the membrane is associated with the flagella axoneme (fractions III and IV). Because any individual experiment must of necessity deal with a single clone and consist of parasites in different stages in development, it was necessary to determine whether the distributions shown in column B of Table III were representative of different clones and developmental stages. As can be seen from column A, which is a composite of three *T. brucei* clones, the same general pattern is internally consistent although detailed variations must exist because the standard errors are fairly large for some fractions. Similar distributions of activity were also obtained for both *T. brucei* ILTat 1.1 stumpy forms and *T. congolense* ILNat 2.1 (data not shown).

To confirm the localization of this VSG phospholipase within the flagellar pocket we attempted to manipulate the

Table III. Distribution of VSG Phospholipase in Different Subcellular Fractions from Trypanosoma brucei

Fraction	Yield	Relative specific activity* A	B	C
	%			
Homogenate	(100)	1.0	1.0	
Crude nuclear pellet	39.7	1.6 ± 0.6	1.5	
LG fraction	8.7	1.9 ± 0.5	2.2	
SG fraction	9.6	2.1 ± 0.5	2.1	
Crude microsomal pellet	18.5	1.6 ± 0.6	1.4	
High-speed soluble supernatant	11.0	0.2 ± 0.1	0.2	
% Recovery	87.5			
Rough endoplasmic reticulum	–	1.3 ± 0.3	1.3	
Smooth endoplasmic reticulum	–	1.6 ± 0.4	1.3	
Golgi fraction	–	2.6 ± 0.6	3.5	
1st Percoll gradient‡				
Top 20.3% (in magnesium)	–	–	–	3.1 (5 *B*)
Flagella (entire 20.3%)	14.2	2.4 ± 0.2	ND (3 *A*)	–
Flagella (within 28.8%)	–	–	–	2.5
Lysosomes	0.7	1.6 ± 0.4	1.3 (2 *A*)	1.7
Glycosomes	0.3	0.8 ± 0.2	1.1 (2 *B*)	1.1
Flagella subfractions				
I	–	1.9 ± 0.6	1.8 (4, *B–D*)	
II	–	1.9 ± 0.3	1.4 (4 *A*)	
III	–	2.5 ± 0.2	2.4 (3, *C* and *D*)	
IV	–	2.5 ± 0.6	2.6 (3 *B*)	
V	0.5	3.9 ± 0.8	3.7 (5 *A*)	

* Specific activity relative to the homogenate. (Column *A*) Fractionation of MITat 1.2, MITat 1.52, and ILTat 1.1 in EDTA (data from 3–5 determinations ± standard error). (Column *B*) Data for enzyme assay with EDTA prepared membranes for MITat 1.2 from which most electron micrographs in the text were taken (average of duplicate determinations). (Column *C*) Fractionation MITat 1.2 in magnesium (average of duplicate determinations).
‡ Flagella fraction I was from the 28.8/20.3% Percoll interface; II was from the 20.3/17.3% interface; III was within the 17.3% layer; IV was from the 17.3/14.4% interface; and V was from top of the 14.4% Percoll layer.
Note: % Yield = % total enzymatic activity. Terms in parenthesis refer to the electron micrograph figure in the text. ND, not determined.

densities of the various fractions and try to correlate these density changes with enzyme activity. Parallel changes in the enzyme activity and morphological structures were observed when the Percoll density centrifugation gradients were run in the presence of 5 mM magnesium instead of EDTA. The density of the flagellar fraction, morphologically similar to crude flagella (see Fig. 3 *A*), increased dramatically with the band migrating to within a few millimeters above the lysosome band within the 28.8% Percoll layer. The mfVSG phospholipase activity also shifted with this fraction (column C in Table III). Again this activity was solubilized by detergents. Although we do not understand the mechanism involved in the magnesium-induced transition, it is known that the concentration of magnesium in the isolation medium is critical when isolating flagella from other trypanosomatids (49). In addition, after centrifugation in magnesium, a membrane fraction was consistently observed on top of the 20.3% layer well away from the shifted flagella and other membranes, which had a high specific activity for mfVSG phospholipase (column C in Table III). Morphologically and biochemically this fraction resembles the EDTA-derived light Percoll flagella membranes (compare Fig. 5, *A* and *B*). This minor fraction banded on top of 14.4% Percoll. Collectively, the data suggest that the 14.4% Percoll layer (fraction V) and the 20.3% fraction obtained on Mg-Percoll gradients are identical.

Studies Using Partially Purified Lipase

Using the light density membranes derived from EDTA-containing Percoll gradients as starting material, we have partially purified the mfVSG phospholipase on Sephacryl S-200 columns in the presence of CHAPS (Fig. 7). The molecular weight of the enzyme, as assessed by gel filtration was ~43,000 which corresponds closely with the estimated value of 37,000–39,800 on SDS-PAGE by other laboratories (8, 20, 27). Sometimes the lipase tended to form higher molecular weight aggregates if the initial solubilization was done in 1% instead of 2% CHAPS. Like the crude enzyme, the partially purified mfVSG phospholipase did not require any metal ions for activity. However, it was stimulated somewhat better (~30%) than the enzyme in crude homogenates when incubated in the presence of DTT. Although Bulow and Overath (8) found that the enzyme was inhibited by CHAPS, no inhibition was observed under the assay conditions used here (<0.025% detergent in the assay). However, the presence of CHAPS may have affected the apparent pI of the enzyme which was 2.79 on IEF columns in Poly/Sep 47 buffer (data not shown).

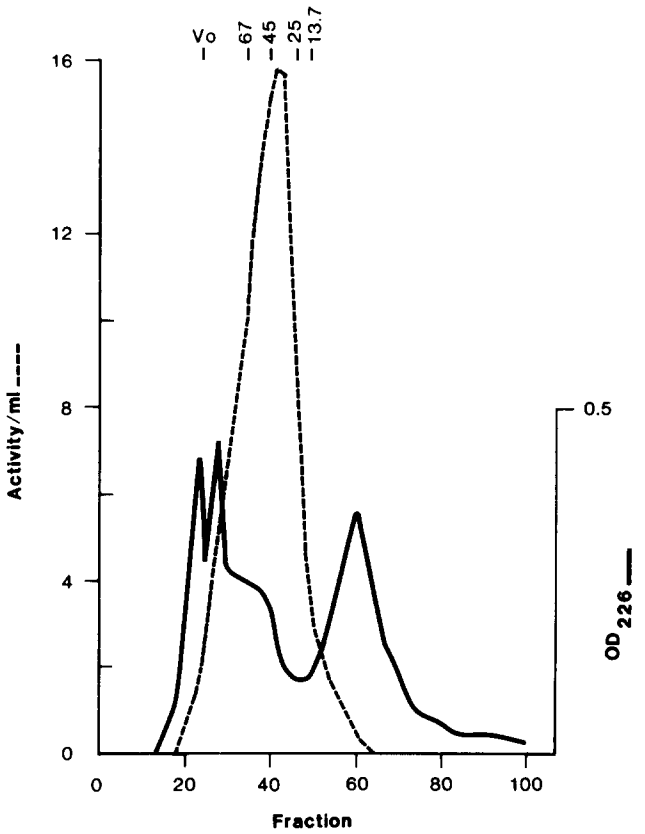

Figure 7. Sephacryl S-200 column chromatography of mfVSG phospholipase. MITat 1.2 membranes isolated on 14.4% Percoll were solubilized in 2% CHAPS with protease inhibitors as described in Materials and Methods. After centrifugation, the lysate in 2% CHAPS was applied onto a 1.6- × 95-cm Sephacryl S-200 column equilibrated in 0.1% CHAPS/Hepes/PEG buffer containing protease inhibitors. Protein was monitored at 226 nm and enzyme assayed as described. Protein standards (BSA, ovalbumin, chymotrypsinogen-A, ribonuclease-A; Pharmacia Fine Chemicals) were run in the absence of detergent.

Discussion

In this report we have presented biochemical evidence that a mfVSG-specific phospholipase-C activity is present within, or enclosed by, membranes of the flagella and flagellar pocket, as well as in the Golgi fraction. The principal evidence for the flagellar pocket localization of the enzyme is based on the finding that the fraction with the most mfVSG-specific phospholipase activity is high in two flagella pocket markers. Acid phosphatase is an enzyme activity found in the flagellar pocket, lysosomes, and in the Golgi apparatus of trypanosomes (23, 34, 51; Table I). Since this fraction is low in galactosyltransferase, a Golgi marker (23, 34), and in acid proteinase, a lysosome marker (35, 38, 51), the membrane fraction (which banded on top of 14.4% Percoll; fraction V) contains little or no lysosomes or Golgi fraction and must, therefore, be rich in flagellar pocket membranes. Furthermore, the high adenyl cyclase levels in these membranes, a postulated flagella pocket marker (56), support our conclusions. Other evidence for the flagellar pocket (and associated membranes derived from it) localization stem from the following arguments. Membranes isolated by Percoll centrifugation from fractions rich in flagella have the same dimensions as those membranes surrounding axonemes with attached paraxial rods. When the density of the flagella is changed with magnesium, part of the enzymatic activity is also seen to shift with the membrane-associated flagella components. In addition the association of the mfVSG-specific phospholipase with flagella pocket membranes is also supported by the preliminary finding (our unpublished results) that after incubation of membrane fractions (isolated from horseradish peroxidase–loaded trypanosomes) with diaminobenzidine and hydrogen peroxide, only the density of the flagella fractions increased in a manner similar to that de-

scribed for peroxidase-loaded endosomes (11). These shifts in band density were paralleled by shifts in the phospholipase activity which also shifted after incubation with hydrogen peroxide. Collectively, these data indicate the presence of the enzyme in the region of the flagella pocket and in the Golgi region. The possibility that the enzyme may also be found in coated vesicles, endosomes, or other prelysosomal compartments, membranes which are presumably derived from the flagellar pocket, cannot be excluded.

It has been reported that mfVSG phospholipase-C is a thiol-dependent enzyme. However, the role of thiols in mfVSG-lipase activation is not clear. Several laboratories report a 1.4–5.4-fold increase in enzyme activity with high concentrations of DTT (10–25 mM) (8, 20, 27). However, there have also been reports of enzyme activation by EDTA (8, 20, 27). Our data suggests that EDTA and DTT have similar activating effects and that these are not additive. Thus the DTT may act by either chelating heavy metal ions as suggested by Fox et al. (20) or by changing the tertiary structure of the substrate (27). The variations reported by several groups in enzyme stimulation by thiol reagents and chelators may simply reflect the purity of the water or other reagents used in the isolation buffers.

It is not clear what role the mfVSG phospholipase plays in trypanosomes. The enzyme is neither more active nor distributed differently between *T. brucei* slender or stumpy bloodstream forms of the parasites. Furthermore, the idea that the enzyme may be involved in the release of VSG during differentiation of bloodstream forms into uncoated procyclic forms is inconsistent with its disappearance before the release of the surface glycoprotein (7, 10). Thus, this release of VSG from the parasite may simply be due to dying parasites (5), or to some other complex interactions due to the use of inappropriate isolation/incubation conditions (35, 37). Consequently, if the VSG-specific phospholipase is involved in the release of VSG from the parasite surface as they are transformed to the supposedly VSG-free procyclic forms (14), then this release is not due to a simple increase in the total amount of enzyme. Our proposition is that the mfVSG phospholipase may be involved in surface coat recycling via an endocytotic mechanism. The involvement of phospholipase-C in VSG traffic within the parasite has also been postulated by Bülow and Overath (8). If the enzyme is in the same lipid bilayer as the VSG (8), activation of the lipase within the flagella pocket or endosomes would release the VSG from the membrane and be taken up by the endocytotic system. In addition to removal of VSG from the membrane, the enzyme, with the help of an acid proteinase, could release the CRD for subsequent recycling into the VSG synthetic pathway (Webster, P., and D. J. Grab, manuscript submitted for publication). The release of diacylglycerol from VSG could also regulate protein kinase C, if present, within the parasite (see references 4, 39). However, the release of the enzyme either actively or as the result of dying parasites could have some other far-reaching effects on the host. We have shown that under certain conditions, living trypanosomes can release a phospholipase-C which is capable of using mfVSG as substrate (35). Decay-accelerating factor, a membrane-bound glycoprotein that inhibits amplification of the complement system (44), as well as acetylcholinesterase, have been shown to contain a phosphatidylinositol membrane-binding domain similar, if not identical, to the CRD (12, 39). Anemia is a hallmark of African trypanosomiasis. It is thus tempting to speculate that if trypanosomes released mfVSG phospholipase, either actively, or from dying organisms, it could remove the decay-accelerating factor from the surface of cell membranes, such as erythrocytes. Erythrocyte membranes altered as a result of decay-accelerating factor removal would then be susceptable to hemolysis and/or removal from the circulation by the reticuloendothelial system as occurs in patients with paroxysmal nocturnal hemoglobinuria (45).

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