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Release and Purification of Trypanosoma brucei Variant Surface Glycoprotein

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Conditions affecting the solubilization of variant surface glycoprotein (VSG) from Trypanosoma brucei have been investigated. The results obtained form the basis for a convenient and efficient method for VSG purification. VSG release from the cell surface was temperature-dependent, following osmotic lysis at 0°C, and was inhibited by low concentrations of Zn^{2+} but not by tosyl-lysine chloromethyl-ketone (TLCK), phenylmethylsulfonylfluoride (PMSF), or iodoacetamide. These and other results eliminated the possibility that release was due to proteolytic cleavage of the C-terminal hydrophobic tail present on newly synthesized VSG. Bolton and Hunter reagent reacted with several components on living cells.

Key words: Trypanosoma brucei, variant surface glycoprotein, purification, release

The original method described by Cross [1,2] for the purification of variant surface glycoproteins (VSGs) from T brucei is still used in some laboratories. Several alternative methods have been tried or proposed, but none of these seems entirely satisfactory. The use of monoclonal antibodies [3] provides potentially the most effective means for VSG purification but is too specific for general applicability. Considering the time-consuming nature of monoclonal antibody generation, it is unlikely that this could ever be an efficient approach to the general problem of VSG purification. Monospecific polyclonal antiserum produced by immunization of rabbits with purified VSGs may be a useful affinity ligand in some cases, although the general applicability of such a serum will also be restricted by its specificity. In addition, we have found the affinity of VSGs for such antisera to be so high as to require strongly denaturing eluants. This problem was not evident in the presence of detergents. A potentially useful immunoabsorbent method might be derived from the observation [4] that rabbits produce antibodies to a C-terminal epitope that is common to most isolated VSGs [5]. Although we have successfully used such antisera on an analytical scale, we have not tested the method for large-scale preparative use.

The use of lectin affinity chromatography is an obvious alternative approach, which has been used by Strickler [6] with T brucei (lentil lectin) and by Baltz and co-workers [7] with T equiperdum (concanavalin A), but is also likely to be limited by variability in the carbohydrate composition of individual VSGs [8]. Turner [9] was unable to bind several VSGs to lentil lectin, a result which we have also obtained for

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four out of four VSGs tested, despite the fact that the same batch of lectin was routinely used in the laboratory for purification of a major surface glycoprotein from T cruzi [10]. The same VSGs were also difficult or impossible to specifically elute from concanavalin A-Sepharose.

Reinwald and co-workers [11,12] reported two novel methods for isolation of VSGs from T congolense, using either preincubation at 0°C followed by mechanical shaking at 22°C or by promoting partial lysis of trypanosomes with dioxane. The principal objective of both methods was to obtain selective release of VSG without activating cellular proteases that would promote VSG degradation, to which the VSGs of T congolense appear to be susceptible. The use of the incubation and shaking method with T brucei was recently reported [13], but the yields of VSG were low.

We were prompted some time ago to seek a more convenient and totally reliable method for VSG purification. At the same time, during the intervening period when our knowledge of VSG structure has been increasing, we have repeatedly returned to the enigma of how the VSG attaches to the trypanosome in the form of a stable surface coat and yet is so readily released by several mechanical procedures. The rapid temperature-dependent release of soluble VSG, illustrated by the results that follow, which was observed following osmotic lysis of trypanosomes, suggested the possibility of enzymatic cleavage of the hydrophobic peptide tail that our nucleotide sequencing studies had predicted to be present at the C-terminus of VSGs as synthesized [14]. Although no protease inhibitors would prevent VSG release, it was not until the demonstration of the C-terminal carboxyl-linked ethanolamine residue [15] that we could be certain that proteolytic cleavage was not responsible for VSG release. The recent demonstration [16] that VSG can be isolated from trypanosomes under conditions where it retains a hydrophobic moiety has brought the subject of membrane attachment back into sharp focus. Recent work [17] has shown that membrane attachment is due to the presence of a covalently bound fatty acid at the C-terminus of the membrane-form VSG (mfVSG).

This paper describes some of the characteristics of VSG release from T brucei and the development of a simple method for the efficient purification of what should now perhaps strictly be called the soluble-form VSG (sVSG) [16]. The preparative method, which has been in routine use in our laboratory for some time, gives yields approaching 75% of cellular VSG.

MATERIALS AND METHODS

Trypanosomes

The experiments were performed with several previously described antigenic variants of T brucei [1].

Isolation of Trypanosomes and Purification of VSG

Blood was drawn by aortic puncture from rats (male Sprague-Dawley weighing about 450 g and obtained from Charles River Co) infected 68 hr previously and having a parasitaemia of $7-10 \times 10^8$/ml. The infected blood was cooled rapidly to 0°C then centrifuged at 1,200g for 10 min. The trypanosomes, which remained as a layer above the packed red cells, were resuspended in trypanosome dilution buffer (TDB) [1], resedimented at 1,000g for 8 min, resuspended in separation buffer [1],

and passed through a small diethylaminoethyl (DEAE)-cellulose column to remove residual blood cells. After sedimentation, cells were resuspended at 10^9/ml in distilled water containing 0.3 mM zinc acetate at 0°C. The presence of Zn^{2+} sometimes appeared to reduce to about half the 10% of VSG that was otherwise released at 0°C and inhibited proteolysis, which might otherwise have occurred to a very small extent. Zinc can probably be omitted, providing the lysis temperature is maintained strictly at 0°C. After 3–5 min the sample was centrifuged at 3,000 g for 5 min. The supernatant (S1) was discarded, except that a small sample was retained for subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to assess the efficacy of the purification. The loosely pelleted ghosts were resuspended to the original volume with 10 mM sodium phosphate buffer, pH 8.0, containing 0.1 mM TLCK at 40°C and held at 37°C for 4–5 min before cooling rapidly to 0°C and centrifuging at 10,000g for 15 min. The supernatant was centrifuged at 300,000g for 60 min at 3°C, then passed at a speed of 200 ml/hr at 20°C through a 30 × 120 mm column of DEAE-cellulose equilibrated with 10 mM phosphate, pH 8.0. The unretarded material was collected and consists of VSG of adequate purity for many purposes. Further purification could be accomplished by isoelectric focusing, in which case a brief diafiltration against water, using an Amicon PM10 membrane, was used to reduce the ionic strength of the sample. The isoelectric focusing column was run at the maximum recommended power (LKB Instruments) for 18 hr at 2,000-V maximum. The VSG peak was pooled and diafiltered against water to remove ampholines and sucrose. Normally we lyophilize the TSG then redissolve and store in solution at −70°C or −196°C.

VSG Release Experiments

Factors affecting the release of VSG from trypanosomes were investigated using small-scale incubations. Trypanosomes were grown and harvested as described above, then resuspended in TDB at a concentration of 5 × 10^8/ml. One-milliliter aliquots were distributed into 5-ml conical glass centrifuge tubes at 0°C, centrifuged at 1,000g for 10 min, and resuspended in 1 ml of the required solution. Following incubation as required by the individual experiment, the samples were rapidly cooled to 0°C and centrifuged at 2,500g for 15 min to sediment ghosts. The pellet was resuspended with 0.9 ml water. In many experiments the absorbance of the supernatant fractions at 280 nM was measured to provide a guide to the extent of lysis. TLCK, 0.1 mM, was added to supernatant and pellet fractions. Prior to electrophoresis, 10- or 20-μl samples of each fraction were mixed with an equal volume of SDS sample buffer and heated at 100°C for 5 min. In some experiments, trypanosomes were surface labeled with Bolton and Hunter reagent [18]. A total of 10^{10} purified trypanosomes was resuspended in 3 ml TDB adjusted to pH 8.6 and incubated with 200 μCi [^{125}I]-Bolton and Hunter reagent (Amersham International; specific activity about 2,000 Ci/mmol) for 15 min at 0°C. Labeled cells were washed three times with TDB at 0°C to remove unbound radioactivity.

Gel Electrophoresis

Polyacrylamide gel electrophoresis under reducing conditions in the presence of SDS was performed on 10% gels as described [19]. Protein standards were phosphorylase, albumin, aldolase, triose phosphate isomerase, and lysozyme, assuming mo-

lecular weights of 94,000, 67,000, 39,000, 26,600, and 14,300, respectively. Lysozyme ran at the front. Gels were stained, dried, photographed, and autoradiographed by standard methods.

RESULTS

VSG Release

Several methods have been applied to the breaking of trypanosomes, including sonication and freeze-thawing. The ballotini method [1] was a historical accident. The most obvious method, osmotic lysis, appears not to have been previously used. The surprising result was that VSG was not extensively released from cells that were osmotically lysed in distilled water at 0°C for periods up to 15 min (Fig. 1). However, 50% of the 280-nM-absorbing material which could be released by osmotic lysis at 37°C was released at 0°C. A 5-min incubation at 37°C apparently released >90% of the VSG (gels of residual cell pellet not shown, but see later). The proportion of VSG released could be estimated by showing that the intensity of Coomassie-stained

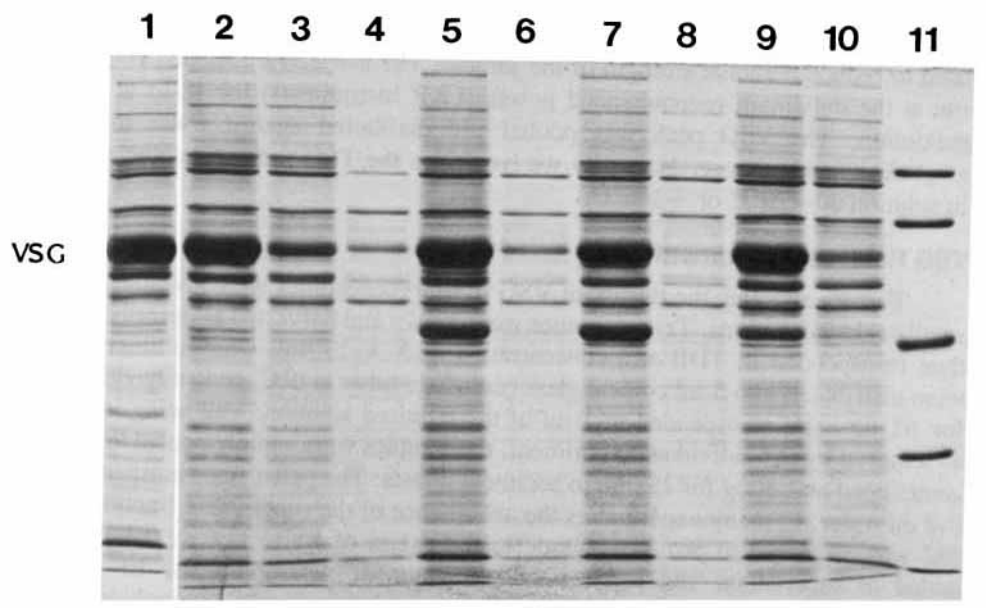

Fig. 1. Effect of temperature on cell lysis and VSG release in the presence and absence of 2 mM zinc acetate. Trypanosomes (variant 221) were resuspended in distilled water (pH 7.0) or 10 mM sodium phosphate pH 8.0 and incubated for 5 min at the indicated temperature. The A_{280} gives an indication of the extent of cell rupture. Supernatants from control cells incubated in TDB at 30°C and 37°C had A_{280} values of 0.14 and 0.20, respectively.

VSG band was equal when tracks containing one-tenth the volume of supernatant were run alongside the pellet fractions. Later experiments quantitated release by using surface-labeled trypanosomes. Two-millimolar zinc acetate almost totally prevented VSG release, even at 37°C. In this experiment, where incubations were performed in the absence of tosyl-lysine chloromethyl-ketone (TLCK), the characteristic, approximately 42,000 apparent molecular weight degradation product of VSG 221 [20] can be seen in the samples incubated in water. Cells which had been osmotically shocked at 0°C would release their remaining "soluble" contents (including VSG) on subsequent incubation at 37°C, even in isotonic medium (not shown).

The effect of zinc ions and TLCK was investigated for reasons related to a neutral protease [21], which appeared capable of degrading VSG rather specifically during purification, because it seemed possible, at the time, that VSG release from the membrane might also be mediated by proteolytic cleavage of the C-terminal hydrophobic tail predicted from C-terminal glycopeptide and DNA sequences [5,14]. As can be seen from the results illustrated in Figure 2, incubation of the trypanosome lysate at low ionic strength at 37°C resulted in extensive VSG degradation to

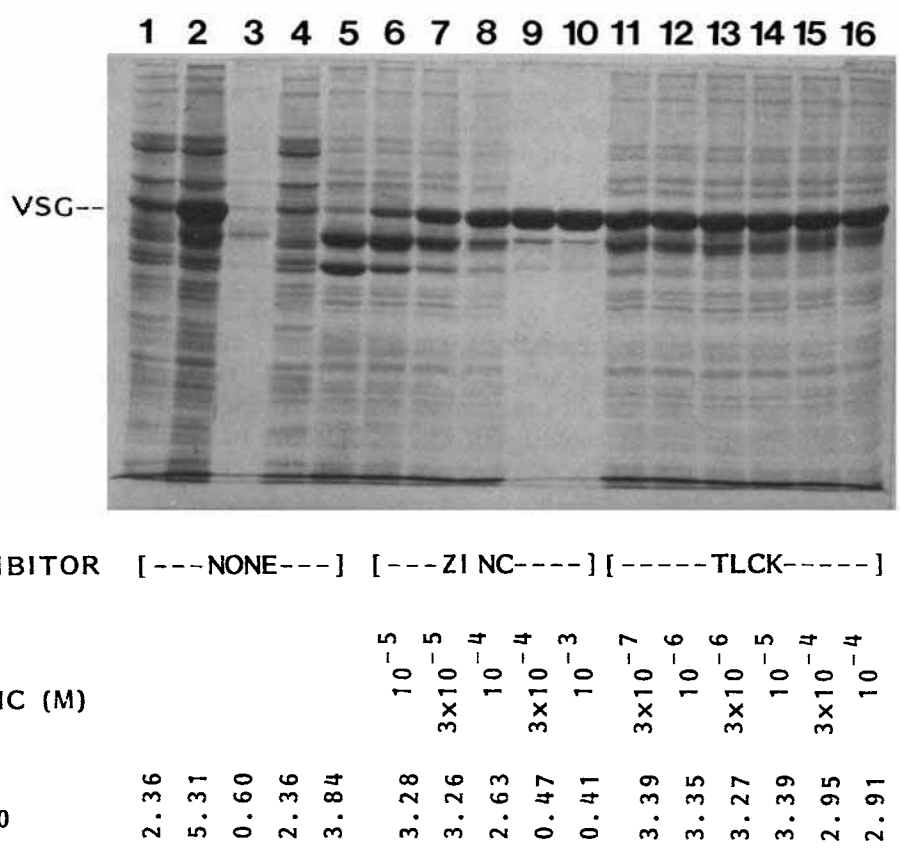

Fig. 2. Effect of Zn^{2+} and TLCK on VSG release and degradation. Trypanosomes (variant 117) were osmotically lysed by resuspension in distilled water at 0°C. After sedimentation, ghosts were resuspended in distilled water and incubated at 37°C for 10 min under the conditions shown on the figure. Tracks 1 and 4 show the proteins released in the first (0°C) lysis step. Track 2 shows the release in a single incubation at 37°C; track 3, the release in a second incubation at 0°C.

fragments of 55,000 and 45,000 molecular weight, in the case of this variant (equivalent to variant 49 in [20]). Neither Zn^{2+} nor TLCK appeared to inhibit VSG release at concentrations where they inhibited VSG degradation. Being 50% inhibited by 10^{-5} M Zn^{2+} or 3×10^{-7} M TLCK [21], the neutral protease activity had a similar level of sensitivity to Zn^{2+} and TLCK as did the fragmentation of VSG. Neither was inhibited by 2 mM phenylmethylsulphonylfluoride (PMSF). Although the current VSG purification method did not involve incubation in water at 37°C, the inclusion of 0.1 mM TLCK prevented the minimal degradation which otherwise occurred. Under the conditions of the experiment shown in Figure 2, there was some solubilization of a band migrating ahead of the VSG band, at 55,000 apparent molecular weight, which is probably tubulin.

The inhibitory effect of Zn^{2+} on VSG release appeared specific, in relation to similar concentrations of three other divalent cations used. Figure 3 shows the results of an experiment in which trypanosomes were surface labeled with [^{125}I]-Bolton and Hunter reagent then incubated at 37°C in water containing 1 mM or 0.2 mM zinc, calcium, magnesium or manganese. Several components were labeled in addition to VSG, including prominent bands of 30,000 and 40,000 apparent molecular weight which did not stain appreciably with Coomassie blue. Following autoradiography, the VSG bands were cut from the gels and the relative proportions of VSG label in the released (supernatant) and unreleased (pellet) fractions were determined by gamma counting (Table I). Although neither calcium, magnesium nor manganese ions inhibited VSG release at the maximum concentration used (1 mM), they did appreciably inhibit release of the other labeled components. The stronger inhibition of VSG release by Zn^{2+} in this experiment, compared to Figure 2, is attributed to the lower ionic strength in the previous experiment, resulting from the two-step incubation protocol.

In a subsequent experiment, a two-step lysis was again used. Cells were shocked at 0°C in either water or 0.2 mM or 1.0 mM Zn^{2+} for 5 min. Subsequent incubations at 40°C for 3 min were done under several conditions. The qualitative results (Fig. 4) showed that the presence of calcium in the second step, preceded by the presence of zinc in the first step, could result in very selective but incomplete (about 50%)

TABLE I. Release of VSG Quantitated by Surface Labeling (I)*

Track	Conditions		% VSG in		% 40k in	
			Super	Pellet	Super	Pellet
1	TDB	Control 37°C	9	91	2	98
2	1 mM	Zinc	7	93	1	99
3		Calcium	86	14	37	63
4		Magnesium	87	13	24	76
5		Manganese	88	12	22	78
6	0.2 mM	Zinc	51	49	8	92
7		Calcium	89	11	75	25
8		Magnesium	87	13	48	52
9		Manganese	89	11	44	56
10	TDB	Control 0°C	2	98	0	100
11	TDB	Control 0°C	3	97	0	100

*Following autoradiography (Fig. 3), the VSG and 40k protein bands were cut from the gels, and relative proportions of radioactivity in released (supernatant) and unreleased (pellet) fractions were determined by gamma counting.

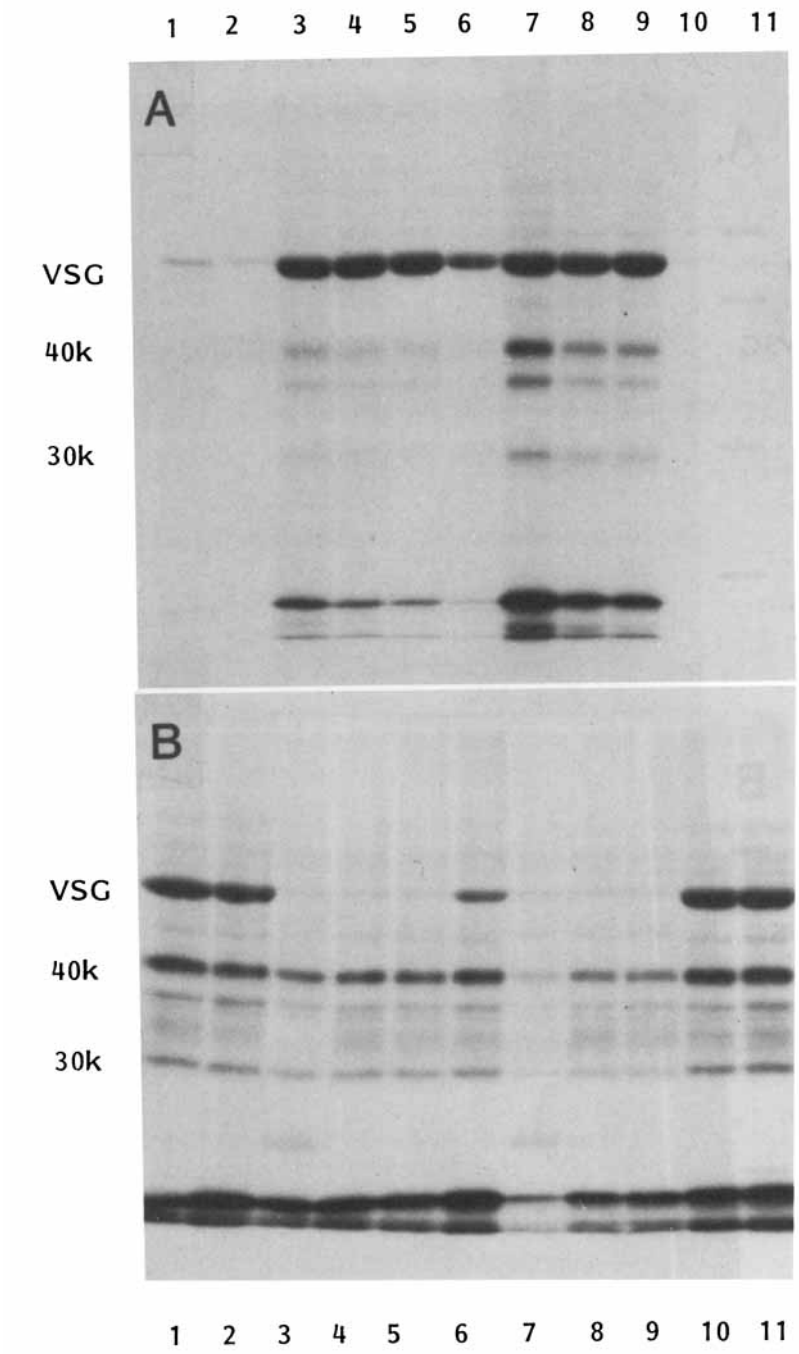

Fig. 3. Surface molecules labeled by Bolton and Hunter reagent and the effect of divalent cations on their solubilization during incubation in water at 37°C for 5 min. Trypanosome variant 121 was used in this experiment. Incubation conditions are given in Table I. Autoradiographs of released (A) and insoluble (B) fractions were exposed 5 days.

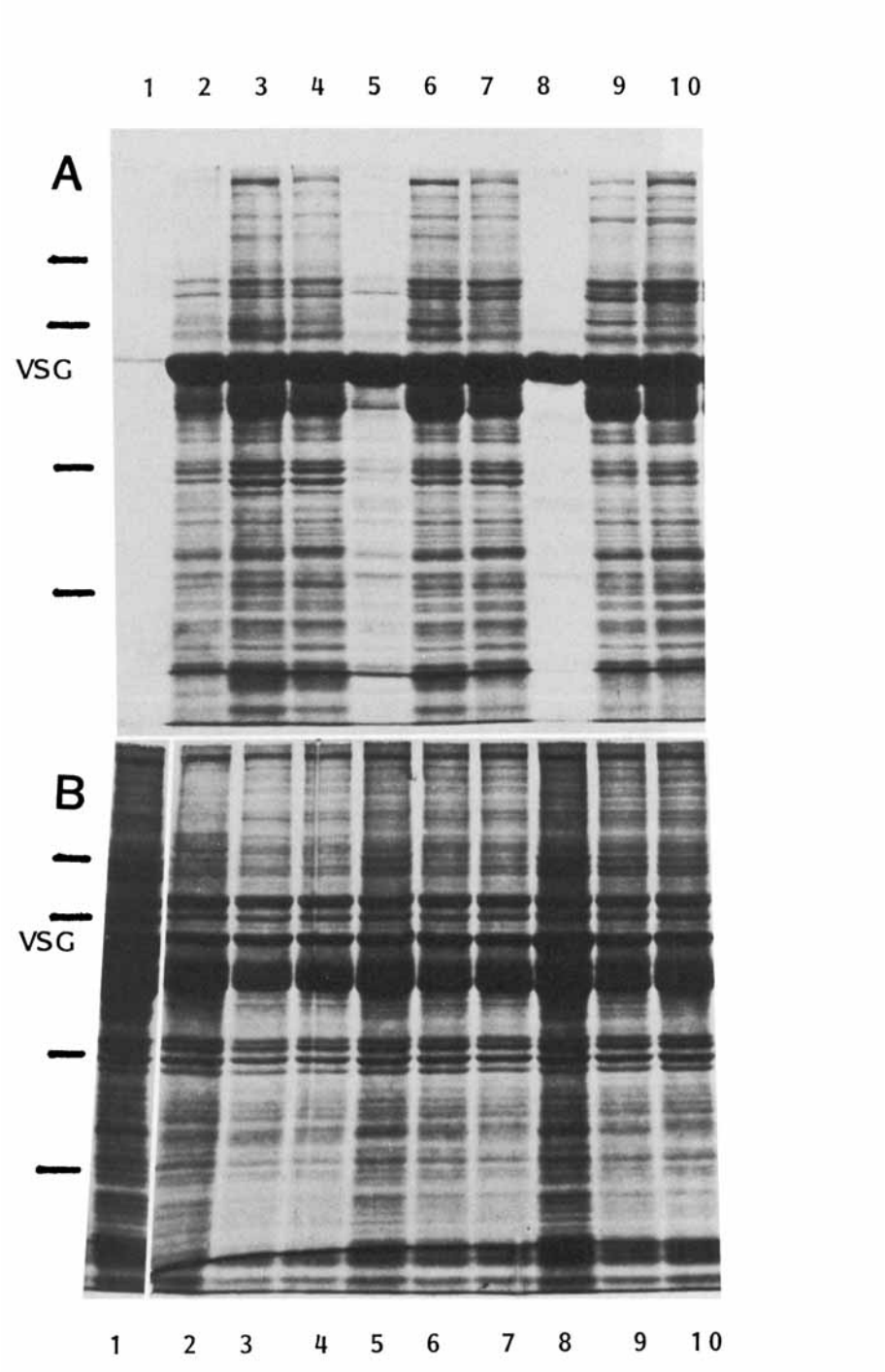

Fig. 4. Solubilization of VSG and other cellular proteins. Conditions are described in Table II. Bars indicate positions of protein standards.

TABLE II. Release of VSG Quantitated by Surface Labeling (II)*

Track	Condition 1	Condition 2	% VSG counts			% non-VSG counts		
			S1	S2	Pell	S1	S2	Pell
1	TDB control	TDB	2	12	86	2	2	96
2	Water	1 mM calcium	8	74	18	19	26	55
3		10 mM phosphate	8	77	15	20	48	32
4		10 mM citrate	8	76	16	22	41	37
5	0.2 mM zinc	1 mM calcium	10	71	19	28	8	64
6		10 mM phosphate	8	73	19	26	24	50
7		10 mM citrate	8	77	15	29	23	48
8	1.0 mM zinc	1 mM calcium	4	45	51	11	3	86
9		10 mM phosphate	4	76	20	14	27	59
10		10 mM citrate	4	76	20	12	25	63

*Trypanosomes (variant 121) were incubated for 5 min at 0°C in either water, 0.2 mM, or 1.0 mM zinc acetate. Following centrifugation the pellets were resuspended as shown (condition 2) and incubated 3 min at 40 °C. Samples of supernatants from the 0°C (S1) and 40°C (S2) incubations, together with the final pellet (Pell), were analyzed on gels (Fig. 4) and radioactivity quantitated as previously described.

VSG release. The results were again quantitated by counting bands cut from the gels (Table II). Solubilization of labeled non-VSG bands was reduced in the presence of calcium, but this was of no advantage since these components bound to DEAE-cellulose under the standard purification conditions where VSG was unretarded. VSG release in the presence of calcium, following lysis at 0°C in 1 mM zinc (as in Fig. 3), did not appear to increase above about 50% even when incubation was prolonged for 12 min at 37°C. The presence of 0.2 mM zinc during osmotic lysis of 0°C did not inhibit release of the VSG during the subsequent incubation at 37°C, whereas 1 mM zinc was inhibitory.

The final experiment in this series (data not shown) investigated the effects of pH on release of VSG. Cells were shocked at 0°C in water. The sedimented "ghosts" were resuspended in 0.02M HEPES-citrate buffers, ranging from pH 4 to pH 9, at 40°C for 6 min. VSG release was optimal at pH 7 and above and was severely inhibited at pH 5 and below, as was the release of all the proteins solubilised at pH 6 and above.

VSG Purification

The first point to emphasize in VSG purification is the importance of keeping the trypanosomes as close as possible to 0°C at all stages during their purification, to cool infected blood as rapidly as possible and to minimize centrifugation time and forces. Figure 5 shows the protein profiles at different stages of a representative purification, using the scheme described in Materials and Methods, except that TLCK was omitted, which accounts for the presence of a small amount of faster migrating VSG degradation products. In a large number of purifications from several variants, VSG yields have ranged from 30–60 mg/10^{11} cells.

DISCUSSION

We have failed to establish any treatment that consistently and specifically releases VSG from the surface of T brucei in high yield under conditions that do not

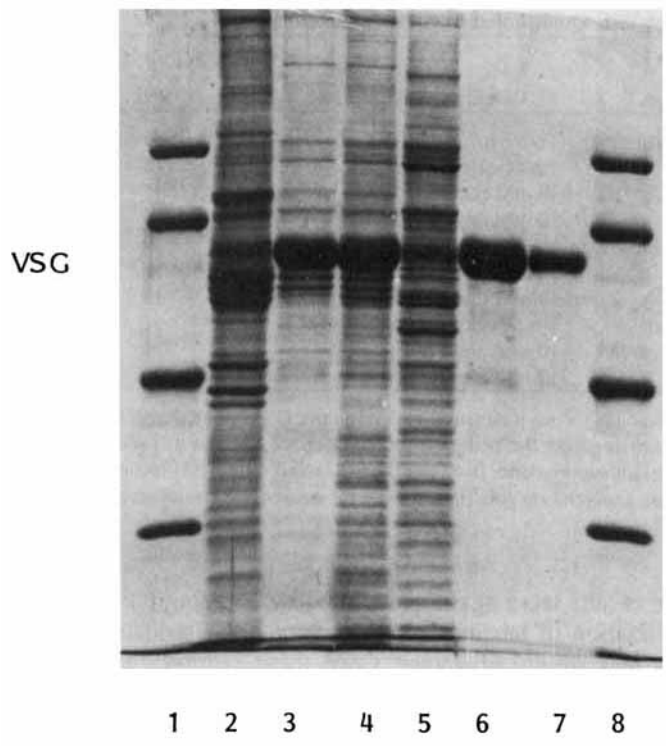

Fig. 5. Analysis of fractions during VSG purification. Equivalent volumes of pellet (track 2), 300,000g supernatant (3), 15,000g supernatant (4), and 0°C lysis supernatant (5) fractions were analyzed during bulk purification of VSG from variant 118. Tracks 5 and 6 contain 5 μg and 1 μg, respectively, of purified VSG after DEAE-cellulose chromatography.

allow extensive release of (presumably) cytoplasmic proteins. Other treatments which we have tried include incubation with sugars, di-iodosalicylate (a chaotropic agent), digitonin, saponin, heparin, sodium deoxycholate, choline, and inositol-specific phospholipase-Cs (which may have been unable to penetrate the surface coat), dibromoacetophenone (a phospholipase-A2 inhibitor), and dioxane. In all these experiments (except with the phospholipases) reagent concentrations ranging from no effect to cell lysis were used. In no case were we encouraged to seek a specific effect over a narrower concentration or an extended time range. However, Voorheis and his colleagues [22,23] showed that VSG was rapidly and selectively released from T brucei in the presence of 1 mM calcium together with the ionophore A23187 (5 μg/ml), although there was some leakage of cytoplasmic enzymes under the same conditions. These authors suggested that the effect they were observing was due to the activation of an as yet undefined enzyme.

In contrast to the negative results of many experiments, two significant facts emerged at an early stage of these studies: the temperature-dependence of VSG release from osmotically lysed cell ghosts and the inhibition of VSG release by Zn^{2+}. VSG release from ghosts was remarkably rapid and appeared to be complete within 3 min at 37°C, suggestive of an enzymatic cleavage. We were able to conclude that

VSG release was not attributable to proteolytic cleavage of the C-terminal hydrophobic peptide tail [14]. Cardoso de Almeida and Turner's recent work [16] has shown that some alternative hydrophobic moiety, now known to contain a fatty acid [17], is responsible for VSG attachment. The nature of the releasing enzyme and its activation remain to be elucidated.

The inhibition of VSG release by Zn^{2+} has been discovered, apparently independently, by two groups [16,23] in addition to ourselves. The mechanism of inhibition is unclear. It could be due to specific inhibition of a VSG-releasing enzyme but could also be due to nonspecific "fixation" or cross-linking of VSG or other membrane molecules. Prolonged exposure of trypanosomes to 1 mM Zn^{2+} during purification and lysis at 0°C led to an irreversible inhibition of VSG release.

Homage has often been paid to the apparent problems of purifying undegraded VSG, although the effective use of appropriate inhibitors has frequently been neglected. Inclusion of PMSF during purification would be an additional precaution against proteolytic degradation [1], but one which we have found unnecessary. Any degradation we have seen during purification appears to be due to a neutral protease that is exquisitely sensitive to inhibition by Zn^{2+} or TLCK. This protease was 90% released during the 0°C osmotic shock and was retained on DEAE-cellulose [21].

Diffley and Jayawardena [24] made a comparative analysis of various procedures used to isolate VSGs from T brucei. They also came to the conclusion that ion-exchange chromatography on DEAE-cellulose gave higher yields of VSG than either antibody or lentil-lectin–affinity methods. It would appear that the method described here for cell lysis and VSG release is more efficient than sonication or freeze-thawing used by Diffley and Jayawardena, although the best yield obtained by these authors was comparable. One aspect that is unclear is how previous solubilization methods [1], ostensibly performed at 0°C, could have resulted in good yields of VSG.

Reaction of living T brucei with Bolton and Hunter reagent labeled several components in addition to VSG. Although these are presumably surface molecules, that was not rigorously proved. The major 40,000 apparent molecular weight component did not represent the characteristic VSG degradation product, because it was seen in the presence of TLCK, it was less soluble than VSG, and it was retained on DEAE-cellulose (data not shown). The nature of these additional labeled components remains to be elucidated. They did not stain appreciably with Coomassie blue.

Our procedure for purification of sVSG represents a significant advance in convenience over previous methods. With the inclusion of 0.1 mM TCK, the product after DEAE-cellulose chromatography appears pure by SDS gel electrophoresis. The procedure takes about 6 hr from starting to bleed rats to having DEAE-purified material or 30 hr including isoelectric focusing and removal of ampholines and sucrose but excluding lyophilization. Preliminary results suggested that Chromatofocusing (Pharmacia) might have been a viable alternative to isoelectric focusing in VSG purification. However, although several VSGs could be resolved over the pH range 6.5 to 8.5 on columns of PBE 94 eluted with Polybuffer 96, recoveries of VSGs from the columns was only about 40%.

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