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DISTRIBUTION AND CHARACTERIZATION  
OF ANTIGENS FOUND IN  
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DISTRIBUTION AND CHARACTERIZATION OF ANTIGENS FOUND  
IN SUBCELLULAR FRACTIONS OF AFRICAN TRYPANOSOMES

John McLaughlin

August 1983

For the Period August 1982 to August 1983

(ANNUAL REPORT)

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## GENERAL INFORMATION

### 1) Duration of Support and Requested Starting Date

Initial support is being sought for a period of 12 calendar months. In view of the scope of the proposal it would be hoped that subsequent renewal would be granted.

### 2) Personnel to be Funded

#### a) Principal Investigator: Dr. John McLaughlin

Present address: Dept. Microbiology and Immunology R-138  
University of Miami Medical Center, P.O. Box 016960  
Miami, Florida 33101

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Position: Research Assistant Professor

PII Redacted

Commitment: During the period for which funding is being sought, no other major commitments or extended periods of leave are anticipated.

b) Other Personnel: Mrs. Gladys Guerra, Research Assistant. One professional assistant position is requested to be filled at the time of approval.

c) Consultant: Dr. Arba Ager, The Rane Laboratory, University of Miami

### 3) Other Sponsors

No other support is being sought at this time for this particular proposal from any private or government agency. Support is being requested from W.R.A.I.R. for a companion project.

### 4) Location of Research Institutions Involved

All research connected with this proposal will be undertaken at the University of Miami locations listed below:

1) The Department of Microbiology and Immunology  
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University of Miami  
P.O. Box 016960  
Miami, FL 33101

2) The Rane Laboratory  
University of Miami  
5750 N.W. 32nd Ave.  
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## INTRODUCTION

The previous report included details of experiments to investigate the membrane association of Trypanosoma rhodesiense flagella pocket membrane (FPM).

For the period of this report (Aug. 1982-Aug. 1983) these investigations have been continued with a number of significant refinements. Firstly antisera to FPM has been raised in rabbits and used as a source of antibody. Secondly we have developed an immuno-blotting technique that permits a much more sensitive means of detecting separated antigenic membrane components.

Additional experiments have involved the use of phospholipase A2 and sphingomyelinase as probes for studying the release of membrane antigens. Both induce pronounced release of two major antigenic components in addition to 3-4 more minor components, all of these being glycoprotein. There would seem good reason to implicate hydrophobic interaction between FPM protein and acyl side groups of membrane phospholipid. In addition a more specific role for sphingomyelin, which we have detected by thin layer chromatography in the FPM, appears probable. Such results are discussed in the context of recent evidence of hydrophobic residues being involved in the attachment of various specific antigen to the surface membrane of Trypanosoma brucei.

Studies have commenced, in collaboration with Dr. John G. Olenick and Dr. Ruth Geller at WRAIR, to further elucidate the nature and degree of clonal specificity of FPM antigens. Results to date demonstrate that such identical antigens are present in two T. rhodesiense clones. However using monoclonal antibody to VSA of two cloned isolates no cross reaction can be demonstrated if incubated with blot transfers of electrophoretically separated FPM proteins.

## METHODS

The isolation of trypanosomes and their subcellular fractionation was as previously described (13). For some experiments (see Table 1) cells were surface labelled using the fluorescamine- $\beta$ -cyclodextrin complex (16). A 15mg amount of fluorescamine (Floram) was used to prepare the above complex and dissolved at 45°C in 75ml 100mM glucose, 150mM PO<sub>4</sub>, pH 8.0, then equilibrated to 30°C. At this time 5.0ml of loosely packed trypanosomes were added, then after 10 min 75ml PO<sub>4</sub>-glucose containing 0.5% bovine serum albumin (to consume excess fluorescamine) was added and the trypanosomes sedimented and twice

washed at 3000 r.p.m.

Exposure of the FPM fraction to phospholipase A2 and sphingomyelinase. To further investigate the nature of the interaction between FPM antigens and lipid membrane, preparations were exposed to phospholipase A2 (porcine pancreas-Sigma) or sphingomyelinase (umbilical cord-Sigma). A FPM preparation (approximately 3.0mg protein) was resuspended in 300 $\lambda$  5mM calcium acetate; 225mM sucrose, 15mM HEPES, pH 7.0 and to this was added 7.5 $\lambda$  phospholipase A2 (1200u/ml) and 30 $\lambda$  10% bovine serum albumin. The BSA was included to bind membrane active products (1) such as lysophospholipids that could have non-specifically solubilized membrane antigens. After 30 min at 28°C phospholipase activity was stopped by adding 30 $\lambda$  37mM EGTA and residual membrane removed by centrifugation at 33,000g using a Sorvall SS34 rotor.

To approximately 3mg FPM protein in 300 $\lambda$  50mM acetate, pH 5.0, was added 7.5 $\lambda$  sphingomyelinase (5000 U/ml). After incubation as for phospholipase A2, 50 $\lambda$  of a 600mM Tris-HCl pH 9.0 was added to stop the reaction. Residual membrane was removed by centrifugation.

Production of rabbit anti-FPM antibodies. Approximately 8-10mg FPM protein, representing the yield from two cell fractionation experiments, was resuspended in 0.75ml 250mM sucrose and mixed 1:1 with Freund's complete adjuvant (Calbiochem-Hoechst). A New Zealand white rabbit was then injected subcutaneously with this preparation with two further injections at 3 week intervals followed by a final injection after a further 4 weeks. After two weeks the animal was bled by cardiac puncture. The serum obtained was used for immunoblotting whilst protein A-sepharose was employed to isolate the IgG fraction for immunoelectrophoresis.

Preparation of samples for SDS-polyacrylamide gel electrophoresis. For SDS-PAGE aliquots of fraction P<sub>b</sub> or a FPM fraction were extracted with 0.4% Triton X-100/0.15% zwittergent 3-12 or exposed to phospholipase A2 or sphingomyelinase, as described above. After centrifugation at 33x10<sup>3</sup>g for 1.0 h (Sorvall SS-34 rotor) the soluble components were removed and 20% SDS added to a final concentration of 2.0%. In some instances the insoluble residue was also solubilized using 20% SDS, as were aliquots of the untreated FPM and P<sub>b</sub> fractions. All samples were then heated for 10 minutes at 55°C with 7.5mM dithiothreitol and sucrose added to a final concentration of 5%.

Dansylation of membrane proteins. In some instances, FPM proteins were dansylated prior to electrophoresis, using a modified version of a previously

described method (14). To 100 $\lambda$  FPM fraction (5 mg protein/ml) added 15  $\lambda$  20% SDS, 20  $\lambda$  500mM Tris-HCl, pH 7.8, and 10  $\lambda$  dansyl chloride in 2-chloroethanol. After heating for 15 min at 50°C 15  $\lambda$  50% sucrose and 5  $\lambda$  2-mercaptoethanol were added with further heating at 50°C for 15 minutes. For electrophoresis 12.5  $\lambda$  of the dansylated FPM protein was applied to each sample well of a 7% gel prepared as below.

Preparation of polyacrylamide gels and electrophoresis of membrane proteins. Gel slabs for SDS-PAGE were routinely prepared using 13.3g acrylamide (Bio-Rad); 0-475g bis; 0.95ml 20% SDS; 142.5  $\lambda$  TEMED and Tris-HCl, pH 8.8 added to a final volume of 190ml. After adding 0.95ml ammonium persulphate solution (0.16g/ml) the gel solution was introduced into a GSC-8 cassette holder (Pharmacia) containing 4 empty glass cassettes (14x8x0.27cm) and sample slot former. Following polymerization two of the gel cassettes were placed in a electrophoresis tank containing circulating buffer (25mM Tris-192mM glycine, pH 8.3). The samples, prepared as above, were layered into each sample slot and electrophoresed into the gel for 20 min using a constant voltage of 75 V. The voltage was then increased to 200V, and electrophoresis allowed to proceed until the bromophenol tracking dye was 1cm from the lower edge of the gel.

For staining, one of the gel slabs was soaked in a solution containing 0.25g Coomassie Brilliant Blue G250 in 210ml methanol + 40ml glacial acetic acid and 250ml saturated HgCl<sub>2</sub>. After 30-40 minutes the gel was destained using repeated washes in 250ml ethanol, 650ml water and 80ml glacial acetic acid. Stained gels were then immersed in 10% acetic acid containing 1% glycerol for 1.0 h and dried for storage using a gel drier. The remaining gel was used for immunoblotting as described below.

For certain applications the silver staining procedure of Morrissey (15) was used where greater sensitivity was necessary.

In situ cleavage of electrophoretically separated FPM proteins. Dansylated FPM proteins were separated by disc gel electrophoresis using the same gel and buffer as described above with 140x7.5mm gel rods. After electrophoresis the gels were visualized using a Black Ray u.v. lamp (305 mm) and gel slices containing fluorescently labelled proteins removed. The separated proteins were then cleaved by incubating each gel slice in a solution of N-chlorosuccinimide as described by (12). The gel slices were then placed on top of a 12.5% acrylamide gel (1:30 cross-linked) and electrophoresed for 4.5 h at 225V (375mA).

The separated cleaved fragments were then transferred to nitrocellulose and those possessing antigenic sequences or sugar residues compared using peroxidase conjugated lectins or protein A as described below.

Iso-electric focussing of FPM proteins. Iso-electric focussing gels were prepared using 1.25g acrylamide 0.125g bisacrylamide; 2.5ml glycerol; 5.0ml 2% Triton X-100 1.185ml 2% zwittergent 3-12; 0.5ml Polybuffer (5, Pharmacia); 15.5ml water; 12.5 TEMED and 0.5ml % ammonium persulphate. Gels were cast as 140x5mm rods, samples applied in zwittergent/Triton X-100 and focussed at 400 V for 5 hs at constant power.

Immuno-blotting procedures. Samples subjected to SDS-PAGE were electrophoretically transferred to either nitrocellulose or DPT paper (Bio-Rad Laboratories) using a Trans-Blot Cell (Bio-Rad). Where transfer was to nitrocellulose, 25mM Tris/192mM glycine, containing 20% methanol, pH 8.3 (22) was used with a current of 0.17A (60 V) for 4.0 h. Before transferring separated proteins from gel to DPT paper, the gel was soaked in 3 changes (20 minutes each) of 200ml 25mM  $PO_4$ , pH 6.5, to remove reactive buffer components. The gel slab was then placed in the Trans-Blot cell containing 25mM  $PO_4$ , pH 6.5, sandwiched next to freshly activated DPT paper (prepared according to manufacturer's instructions). A current of 0.46A (27 V) was used and after 3.0 h the paper removed for subsequent treatment.

Post transfer treatment of blots. Following transfer to nitrocellulose any remaining binding sites were blocked by exposure for 1.0 h to three changes of 25mM Tris, 500mM NaCl, pH 7.4, (TNB buffer) containing 3% gelatin. The strip was then quickly blotted dry and placed in a clean plastic food bag (13.5x15.5cm). To 9.5ml TNB buffer containing 0.3% gelatin was add 0.2ml FPM anti-sera and the solution careful poured over the nitrocellulose strip. The plastic bag containing the strip was then placed flat on a glass plate and the open end rolled up as far as the flattened strip which was now covered by a thin layer of buffered anti-serum.

After at least 2-3 hs at room temperature the strip was removed, dried with filter paper and washed five times in TNB with the first wash containing 0.05% Nonidet. Those components binding FPM antibodies were detected using horseradish peroxidases (HRP) conjugated goat anti-rabbit IgG (Boehringer) or HRP conjugated protein A (Sigma). The former was used diluted 1:2000 in 50ml TNB with the latter diluted 1:2500 in the same buffer. After a 1.0 h incubation the nitrocellulose strips were dried with filter paper and washed with 5

changes of TNB as above.

To visualize bands of peroxidase activity the nitrocellulose strips were placed in a solution containing 100ml TNB buffer plus 20ml freshly prepared 0.3% 4-chloronaphthol (Bio-Rad) in ice cold methanol to which was added 60  $\mu$ l 30% hydrogen peroxide. Blue bands appeared with 2-3 minutes and staining was complete within 5-10 minutes at which time the strips were rinsed with de-ionized water and dried for storage (8).

The DPT-paper blots were blocked following a previously described method (17) then processed as above to detect antibody or lectin binding components.

TNBS labelling of trypanosomal membranes. Approximately 5-7ml packed, washed trypanosomes were resuspended in 75ml 5.0mM trinitrobenzene sulfonate (TNBS), 40mM  $PO_4$ , 30mM NaCl, 55mM glucose, pH 7.8, and incubated at 0°C for 20 mins. The cell suspension was then centrifuged at 3000 r.p.m. for 10 minutes (Sorvall SS34 rotor) after adding an equal volume of 750mM glycine. The trypanosomes were washed twice more as above, to remove unreacted TNBS, and processed as indicated.

Production of antibodies to DNP conjugated key hole limpet hemocyanin. Large breeder mice were injected intraperitoneally with DNP-KLH prepared according to (11) using 0.2ml of a 1:1 solution of Freund's complete adjuvant (Calbiochem) and DNP-KLH (20mg/ml). Injections were repeated at monthly intervals for 3 months at which time it was possible to withdraw fluid from the ascites tumors which formed. Such fluid was then used as a source of anti DNP-KLH antibodies which were recovered using protein A-Sepharose affinity chromatography.

## RESULTS

1. The use of trinitrobenzenesulfonate to surface label *T. rhodesiense*. The rationale for these experiments was to label *T. rhodesiense* with the hapten TNBS and to use antibodies raised to a keyhole limpet-DNP (dinitrophenol) conjugate, to affinity purify trypanosomal membrane. Fig. 1a shows a FR1 antigenic profile of a HSP fraction obtained from TNB labelled trypanosomes against anti-DNP antibodies after isopycnic gradient centrifugation (see reference 13 for cell fractionation procedures). It is quite evident that labelling has occurred throughout the cell with no specific association, in contrast to what was found using fluorescamine (13). Fig. 1b shows the single precipitin line obtained after CIEP of pooled low density fractions (FPM) from

the gradient. Despite using various buffers and temperatures to label cells, exclusive labelling of either the surface or flagella pocket membrane did not occur. There was no option but to discontinue these experiments in view of the lack of progress obtained.

2. Further investigation of the nature of the membrane association of FPM antigens. Before extending this aspect of the investigation, the nature of the FPM fraction (see last year's annual report for isolation procedures) with reference to marker enzyme content and fluorescamine labelling, was more thoroughly established.

The activities of various marker enzymes assayed in those fractions recovered after sucrose gradient centrifugation of fraction P<sub>b</sub> are shown in Table 1. The FPM fraction is clearly enriched in both arylamidase and phosphatase 1, as expected from our previous investigation (13), with slightly less enrichment in phosphatase 2. This latter activity, as compared to phosphatase 1 is present to a greater extent in the SM fraction. The low levels of  $\alpha$ -glycerophosphate dehydrogenase, both DCPIP and NAD linked, and proteinase indicate at most only slight contamination with mitochondrial, glycosomal and lysosomal elements respectively. Compared to free fluorescamine, the fluorescamine- $\beta$ -cyclodextrin complex resulted in less label being recovered in the cytosol, 9% as compared to 15% and, as can be seen from Table 1, reduced labelling of FPM as compared to SM. As stated previously (13) the unexpectedly high recovery of label in the cytosol is probably due to release of VSA during cell breakage.

As a means of monitoring the effect of exposure of FPM to phospholipase, the release of protein and three enzyme activities were measured as shown in Table 2. Noticeable is that whilst almost one-third of the protein was released in the presence of hypotonic buffer, virtually all of the arylamidase and phosphatase II remained membrane associated. Of the FPM components not solubilized, phospholipase A<sub>2</sub> and C induced the release of almost half the remaining protein. However, little of either the arylamidase or phosphatase II enzyme activities were now soluble, but very low recoveries were obtained suggesting that the products of phospholipase activity were exerting an inhibitory effect. This was confirmed, as can be seen in Table 2, where in the presence of BSA, phospholipase induced release of FPM protein was greatly reduced and enzyme recoveries much higher.

The release of antigenic material from FPM as detected using antibodies to fraction  $P_b$  was reported in the last annual report, these plus some additional experiments utilizing phospholipase have been conditionally accepted for publication to Infection Immunity (see Appendix). In addition to these results it is now possible to use antibodies prepared from anti-serum to the FPM fraction. Also the results using CIEP can now be compared with those using an immune blotting procedure.

1. Crossed Immunoelectrophoresis of antigens released from FPM. Fig. 2 shows the precipitin patterns obtained after CIEP of the products released from an FPM fraction after exposure to phospholipase A2 or sphingomyelinase, compared to those removed by extraction with zwittergent 3-12/Triton X-100. These in turn can be compared with components released from a  $P_b$  fraction, also electrophoresed into a gel containing anti-FPM antibodies.

The use of zwittergent/Triton resulted in an antigenic profile (Fig. 2a) using antibodies to FPM consisting of three cross reacting antigens ( $a^1$ - $a^3$ ) as well as antigens b, c and d, the latter being much fainter than the others. The incomplete nature of peaks b-d suggests some degradation has occurred. As was found earlier (see Appendix) phospholipase A2 exposure resulted in the release of antigen a, though using anti-FPM antibodies resulted in a much more prominent precipitin line. There was evidence of only antigens  $a^2$  and  $a^3$  with the latter being less pronounced, whilst antigen b was absent. Antigen d appeared more pronounced than after zwittergent/Triton extraction, in keeping with our previous finding (see Appendix). Sphingomyelinase (Fig. 2e) released material giving a similar antigenic profile to that seen with phospholipase A2, including the increased release of antigen d as compared to the use of detergent. Of some interest is the notable increase in precipitin peak d observed if the FPM fraction is allowed to remain at room temperature overnight prior to detergent extraction (Fig. 2g). This suggests some endogenous release factor is operating. The reduction in precipitin peak a might indicate degradation with release of antigen, d, though it is difficult to discern any obvious cross reactivity between these two components. It is noticeable that the FPM anti-serum recognizes no additional components when a  $P_b$  fraction, submitted to the same treatments (Fig. 2b, d, f), is compared to the FPM fraction (Fig. 2a, c, e).

For more recent experiments cobra venom phospholipase A2 (Calbiochem-Hoechst) as opposed to that from porcine pancreas has been used. Most interestingly we have on repeated occasions been unable to discern any release of antigenic material (Fig. 2h) as compared to pancreatic phospholipase A.

11. The development of an immune blotting procedure to detect FPM antigens after separation using SDS-PAGE. Before considering the results obtained using immune-blotting it is first necessary to examine the results obtained after SDS-PAGE of FPM. Fig. 3 shows the separation of FPM components after SDS PAGE using 40 mM Tris/Borate, pH 8.3 as compared to a 40 mM Tris 175 mM glycine buffer, pH 8.5. Both gave similar separation, though resolution was superior using with the latter buffer. At least 11 components ranging from a m.w. of  $80-20 \times 10^3$  have been discerned using CBB staining with a further 5-7 components after silver staining (figure not shown). Exposure to phospholipase A released components seen in lane 3, whilst the residue solubilized in SDS gave the pattern observed in lane 2. Apart from the absence of a range of lower m.w. components, cobra venom phospholipase A also released the two principal components of m.w.  $\approx 66$  and  $60 \times 10^3$ . Lane 6 shows those bands contributed by the two phospholipases as well as the bovine serum albumen. The latter as will be seen migrates very close to an authentic FPM component which is obscured for that reason. In lane 5 components released after three freeze/thaw cycles are shown. Clearly all three components were in part released though none of the slower moving antigen (m.w.  $\approx 66 \times 10^3$ ). Subsequent extraction with zwittergent/triton (lane 1), as previously stated released a variety of additional components. Phospholipase A exposure of a similar freeze/thawed FPM fraction also released these same antigens though the bands are obscured by the BSA that had to be added during incubation.

Fig. 4a shows the separation of FPM protein, derived from whole cells labeled with the  $\beta$ -cyclodextrin-fluorescamine complex, liberated after various treatments. Exposure to either sphingomyelinase (lane 3) or phospholipase A (lane 4) or extraction with zwittergent/Triton (lane 2) can be compared with a control incubation in 50mM acetate, pH 5.0 (used for sphingomyelinase) plus 5mM mercuribenzoate to inhibit autolysis. Extraction with zwittergent-Triton produced the most complete removal of FPM proteins, especially the  $60 \times 10^3$  m.w. component plus other minor proteins. Interestingly only slight extraction of the  $66 \times 10^3$  m.w. protein is obtained with detergent, compared to the acetate buffer. Both phospholipase and sphingomyelinase

effected excellent release of the two major membrane proteins, though the presence of BSA obscures the details of those two bands. After blotting to nitrocellulose and viewing under long wave u.v. light it was possible to discern labelling by fluorescamine, of the two principal antigens. There was some faint streaking of fluorescence where the higher m.w. proteins migrated. It could be tentatively concluded that both of the principal FPM antigens possess sequences disposed toward the lumen of the flagella pocket and therefore accessible for labelling.

By immune-blotting to nitrocellulose (Fig. 4b) it was possible to detect components reactive toward anti-FPM antibodies. The 60 and  $66 \times 10^3$  m.w. components were liberated, to various degrees, by all of the treatments, the acetate buffer (lane 1) being the least effective. Phospholipase A and sphingomyelinase were especially effective in releasing the  $66 \times 10^3$  m.w. antigen, as compared to detergent extraction. The effectiveness of sphingomyelinase is well illustrated by Fig. 4d where protein A conjugated HRPO was used to detect antibody rather than goat anti-rabbit IgG conjugated HRPO (Fig. 4b). The increased sensitivity resulted in poorer resolution but served to illustrate the efficacy of sphingomyelin in liberating the  $66 \times 10^3$  m.w. membrane protein.

By use of concanavalin A conjugated (Fig. 4c) HRPO it was possible to identify FPM components containing carbohydrate. Not only are the two principal antigens glycoprotein in nature, but most of the other low m.w. proteins are also highly reactive toward the lectin. A noticeable feature is the apparent lack of released lectin positive material after extraction with the acetate buffer.

This is evidence of a range of higher m.w. antigens (m.w.  $\approx 80-100 \times 10^3$ ) which may be more reactive than indicated by the immune blots obtained. Even with overnight transfer of proteins, as used in the present study, the decrease efficiency in transfer of high m.w. proteins can still be a problem (6).

From a comparison of the CIEP and immune blotting procedures it can be seen that both have detected two principal FPM antigens. The latter technique is clearly more sensitive, disclosing certain minor antigens not revealed by CIEP. Although not capable of directly revealing cross-reactivity between separated antigens, immune blotting has the advantage of detecting soluble immune complexes since the antigen is immobilized by virtue of its tight binding to the nitrocellulose.

In some earlier experiments fraction  $P_b$  was extracted with 4% SDS electrophoresed using the PAGE-SDS system and blotted to nitrocellulose. Fig. 5 shows the results obtained after incubating such a blot in anti-FPM serum (1:20 dilution) and using Protein A-HRPO to reveal immune complexes. Those minor components (below m.w.  $50 \times 10^3$ ) that were present using FPM are more evident suggesting that they may not be authentic FPM proteins. Both of the major antigens observed using an FPM extract are present as would be expected.

A comparison was made between FPM antigens and other subcellular fractions--lysosomes (LYS) and surface membrane (SM)--removed from the 1,112/1,87g/cm<sup>3</sup> and H87/1.252g/cm<sup>3</sup> interfaces respectively of a discontinuous sucrose gradient (see Appendix). Fig. 6a is a sketch of a blot obtained after electrophoresis and immunoblotting, using goat-anti rabbit IgG-HRPO as for Fig. 4b. Lane a is the profile obtained for SM, two faint bands corresponding to the 60 and 66x10<sup>3</sup> dalton glycoprotein antigen are present. By contrast the LYS fraction exhibited a more intense staining, not unexpected in view of the inter-related nature of the flagella pocket membrane and the adjacent membrane system, which includes lysosome like elements. In Fig. 6b concanavalin A-HRPO was used to detect glycoprotein--the absence of a prominent band for the sm suggests that much of the USA is released during cell fractionation.

The reactivity of FPM extracts with anti-FPM antibodies was compared with a series of VSA monoclonal antibodies obtained by Dr. K. Esser (WRAIR). In this instance a detergent extract, phospholipase A released material and the insoluble residue were separated using SDS PAGE (100mM PO<sub>4</sub> pH 7.2) and transferred to a DPT paper with blocking as described above. The paper blot was then incubated overnight with a monoclonal antibody (1:20 dilution) obtained from 2.1B7.1 and any immune complexes present detected using fluorescein isothiocyanate (FITC) labelled protein A. No reaction could be discerned on viewing the blot under a high intensity long wave v.s. source. The blot was then quickly exposed to 50ml 100mM glycine-HCl pH 3.0 at 0°C for 2 minutes removed blotted dry and rinsed in Tris buffered saline. The blot was then exposed to a second monoclonal antibody (50.20.103B) as above and again no reaction was observed. After a final exposure to the glycine-HCl buffer the blot was incubated overnight with the anti-FPM serum (1:50 dilution) and immune complexes visualized using protein A-HRPO (Fig. 7). It is evident that FPM antigen did not react with the VSA monoclonal antibodies. It is worth reporting at this point some pertinent studies by Dr. Ruth Geller at WRAIR to

whom samples of rabbit anti-FPM serum were sent. They have found that mRNA from the Wellcome CT-strain CP3B4 of T. rhodesiense and a serially related organism V13 when introduced into the message dependent reticulocyte lysate system synthesize products that can be precipitated by antibodies present in the anti-FPM serum. On SDS-PAGE the precipitated components were found to be identical for both organisms, indicating that they were not variant specific.

Recently in this laboratory we have commenced using a two dimensional separation of FPM antigens using Iso-electric focussing followed by SDS PAGE, see Fig. 8 for sketch which may in the future be able to discern any subtle differences between T. rhodesiense strains.

Possible binding of lipid to FPM antigens. As discussed in the paper attached as an appendix, the presence of covalently bound lipid could explain the apparent greater efficacy of phospholipase A, as compared to mild detergent treatment, in releasing the 60 x 10<sup>3</sup> dalton antigen. There is evidence for tightly bound hydrophobic residues, probably not amino acids, on the C-terminal end of VSA (3).

In effort to dissociate any proteolipid a P<sub>b</sub> fraction was extracted in 90% ethanol at 80°C for 15 minutes. The residue was removed by centrifugation, and the supernate reduced in volume by rotary evaporation at 45°C. Aliquots of the concentrated extract were then spotted onto washed blotted nitrocellulose strips which after blocking were incubated with anti-FPM serum (1:20 dilution). The use of protein A-HRPO revealed no binding of anti-FPM antibodies.

This negative finding does not eliminate there being some tightly bound lipid, but it seems unlikely that proteolipid antigens as found in Schistosoma mansoni (19) or the glycopeptide conjugated sphingolipid of Trypanosoma cruzi (20) are present.

#### Presence of sphingomyelin in FPM

In view of the effectiveness of sphingomyelinase in releasing FPM antigen, it would seem fairly obvious that sphingomyelin should be a constituent of these membrane. Pooled FPM fractions, approximately 20mg protein, were extracted with chloroform/methanol using the Bligh and Dyer method (see 9). The extract was evaporated under nitrogen using a rotary evaporator and samples spotted on to sheets of Silica Gel H (Brinkman). The sheets were then developed in a solvent system of chloroform/methanol/ammonia, 65:25:5 and after drying visualized using sprays of chromic acid or benzidine/Clorox (21),

the latter to detect sphingosine containing compounds. Shown as Fig. 9 in the separation of lipids obtained, where the most polar appeared to be lysophosphalids choline, a somewhat less polar spot that co-migrated with authentic sphingomyelin and the most conspicuous co-migrated with lecithin. The finding of sphingomyelin is therefore compatible with the efficacy of sphingomyelinase in releasing antigen.

At present work is in progress to evaluate a technique we have developed for purifying antibody to specific FPM antigens. The procedure is a modification of a recently published method (17). The first modification is the dansylation of FPM proteins (see Methods) which are then separated using one of the SDS-PAGE systems described in this report with 100mM  $\text{PO}_4$  pH 7.2 as buffer. Separated material can then be visualized using u.v. illumination and removed in appropriate gel slices. It is advantageous to apply sample to almost the whole width of the gel (8x14cm gel). The gel slices removed are then placed onto activated strips of DPT paper and electroblotting commenced for at least 4 hrs using a current of 0.27A. The strips of DPT paper are now blocked, as previously described, and incubated with anti-FPM serum diluted 1:10. Since we usually ran 4 gels the four DPT strips obtained per separated component are incubated together. Bound antibody is then eluted using a 100mM glycine-HCl pH 3.0 buffer at 0°C for 2-3 minutes. The pH is then immediately adjusted to pH 7.4 and the solution of purified antibody concentrated by ultrafiltration. The strips of DPT bound antigen can be used over at least 4 times, and can be stored at -20°C if not required immediately. By three repeated incubations with anti sera, using strips derived from four gels it has been possible to purify almost 1.5mg antibody to the  $66 \times 10^3$  m.w. antigen.

We believe this method will be most useful in the work involving targeted liposomes that we have proposed. It would in addition be of much interest to use this purified antibody to localize the  $66 \times 10^3$  antigen by immune-electron microscopy.

In situ cleavage of FPM proteins. Other work currently in progress is an evaluation of the in situ N-chlorosuccinimide cleavage of the two major FPM antigens. It has been possible, using a silver staining procedure to identify 5-6 components after a 30 min incubation of gel slices with NCS. Fig. 10 shows a sketch of such a separation, there is as yet no estimation of the molecular weight of these fragments available. Blot transfers using nitrocellulose and Protein A conjugated HRPO as probe, have failed to reveal so far

any binding of FPM antibodies to any of the cleaved products. It is intended to use the purified antibody obtained as described in the previous section, for further work.

It should be stated that one aspect of this study that awaits further investigation concerns an evaluation of the protective potential of FPM antigens against infection. In the previous annual report encouraging results were obtained using fractions obtained after isopycnic centrifugation of a T. rhodesiense high speed pellet. It had been hoped to perform further experiments during Spring of this year. Unfortunately the death of two rabbits in the medical school animal facility that were being used to raise anti-FPM sera forced the use of further FPM preparations for producing anti-sera at the expense of the vaccination experiments. This compounded a delay caused by paperwork problems in receiving a shipment of cloned T. rhodesiense isolates from Dr. K. Esser. The vaccination experiments have now been scheduled for October of this year.

#### CONCLUSIONS

As well as the indications of the importance of hydrophobic interaction between FPM protein and phospholipid (see Appendix for discussion), there is now more specific evidence that implicates binding between the two principal FPM antigens, more especially the  $60 \times 10^3$  dalton glycoprotein, and sphingomyelin. This is in part not surprising in view of the importance of sphingomyelin as a constituent of African trypanosomes (7) and its presence, as demonstrated in this investigation in FPM. It should be noted that whilst phospholipase C and sphingomyelinase both cleave from their substrates, Bacillus cereus phospholipase, as used in this investigation, will not split sphingomyelin. This could explain the latter enzyme's reduced effectiveness in liberating FPM antigens. The relevance of these findings to those of others regarding, for instance, the peptidoglycophosphosphingolipid complex in T. cruzi and, of more immediate interest, the hydrophobic attachment of VSA to the surface membrane of T. brucei, must await further investigation (3,20).

Our own findings of a lack of reaction between FPM antigens and monoclonal sera to USA were to be expected. They assume more significance when reviewed in the context of the clear evidence, presented by Dr. Geller, of identical components present in two CT Wellcome isolates that react with FPM antibodies. There is now a clear impetus to further elucidate the structural

features of FPM antibodies. It is hoped in conjunction with Dr. Olenick (WRAIR) to precisely localize the site of subcellular attachment of the antigens using immune-electron microscopy. For this purpose the specific antibody to the  $66 \times 10^3$  dalton antigen should be most useful.

Ever since the initial description of the surface coat of African trypanosomes (23), it has been assumed to cover the flagella pocket membrane. However this is a membrane which from the few studies available (10,18) appears quite specialized and actively involved at least in the pinocytotic uptake of protein. It would not therefore be surprising if the composition of the FPM was different from the more fixed surface membrane.

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The technical assistance of Mrs. Gladys Guerra is noted with thanks.

## FIGURE LEGENDS

Fig. 1. Immunelectrophoresis of TNB labelled *T. rhodesiense* fractions obtained after isopycnic centrifugation of a HSP.

- (a) Fused rocket immunelectrophoresis of gradient fractions against anti-DNP mouse antibodies.
- (b) Crossed immunelectrophoresis of pooled fractions 3-6 from (a) using the same anti-DNP antibodies.

Fig. 2. Release of antigens from an FPM fraction after detergent, phospholipase or sphingomyelinase treatment.

- (a) 0.12% Zwittergent 3-12/0.4% Triton X-100 (detergent) extract of FPM
- (b) Detergent extract of  $P_b$  fraction
- (c) Components released after incubation of FPM with porcine pancreas phospholipase  $A_2$
- (d) As for c but using  $P_b$  fraction
- (e) Sphingomyelinase (umbilical cord) released components from FPM
- (f) As for e but using  $P_b$  fraction
- (g) Overnight incubation of FPM fraction in the presence of detergent
- (h) Components released after exposure to cobra venom phospholipase  $A_2$  (Calbiochem-Hoechst).

For all experiments anti-FPM rabbit serum was used as a source of antibodies.

Fig. 3. SDS-Polyacrylamide gel electrophoresis of FPM proteins.

- (a) mM Tris Borate, pH 8.3
- (b) mM Tris- mM glycine, pH 8.5, both using 7% acrylamide, 1:30 cross-linked.

Fig. 4. Electrophoretic separation of FPM proteins and subsequent immune blotting to nitrocellulose.

- (a) SDS-PAGE using the discontinuous buffer system (see Methods) and a 7% gel 1:30 cross-linked Gel stained with  $HgCl_2$ /Coomassie Brilliant Blue.
- (b) Immune blot obtained using gel as in (a) incubated with anti-FPM serum and goat-anti rabbit IgG-HRPO as probe.
- (c) Immune blot to nitrocellulose with protein A-HRPO as probe.
- (d) Nitrocellulose blot of gel as in (a) using concanavalin A-HRPO as probe. For (a) (b) (d) lane 1 = 50 mM acetate extract, 2 = detergent extract (as for Fig. 2a), 3 = sphingomyelinase liberated antigens, 4 = phospholipase  $A_2$  released components. For (c) treatments were 1 = phospholipase  $A_2$ ; 2 = detergent; 3 = sphingomyelinase; 4 = acetate buffer.

Fig. 5. Immune blotting of electrophoresed samples of fraction  $P_b$  obtained from *T. rhodesiense*.

- (a) Residue remaining after phospholipase treatment of fraction  $P_b$  was solubilized using 4% SDS and submitted to PAGE-SDS as for 4a. Separated components were electroblotted to nitrocellulose, incubated with anti-FPM sera (1:20) dilution) and protein A-HRPO used as a probe.
- (b)  $P_b$  material released after phospholipase treatment.
- (c) Detergent soluble material released from fraction  $P_b$ .

Fig. 6. Comparison of antigens present in FPM, lysosome (LYS) and surface membrane (SM) fractions obtained from *T. rhodesiense*.

- A - components reactive toward anti-FPM antibodies
- B - concanavalin A reactive components using goat anti-rabbit IgG-HRPO

and concanavalin A-HRPO respectively. Lane a = FPM; lane b = LYS; lane c = SM.

Fig. 7. Immunoblotting of electrophoresed FPM samples to diazophenylthio-ether (DPT) paper.

Blot was sequentially exposed to monoclonal antibodies 2.1B7.1 and 50.20.103B using protein A-FITC as a probe (see text). No antibody binding was detected to any of the components, blot was then incubated with anti-FPM serum (1:50 dilution) and protein A-HRPO used as a probe. Lane 1 = SDS soluble residue after exposure of FPM to phospholipase A; 2 = detergent extract of FPM; 3 = phospholipase A released antigens from FPM.

Fig. 8. Two dimensional separation of FPM antigens using Iso-electric focussing and SDS-PAGE.

See Methods for details.

(A) Coomassie Brilliant Blue/HgCl<sub>2</sub> staining

(B) Blot transfer to nitrocellulose, incubated with anti-FPM serum with antigen-antibody complexes detected with protein A-HRPO.

Fig. 9. Thin layer chromatograph of a chloroform/methanol extract of pooled FPM fractions.

Methods described in the text; plate sprayed with the chromic acid spray. Spot b also gave a faint but positive reaction with Clorox-benzidine spray for sphingosine, on a separate plate.

Fig. 10. Separation of cleavage products obtained after treatment of the FPM  $60 \times 10^3$  m.w. antigen with N-chlorosuccinimide (NCS).

A dansylated sample of FPM was subjected to disc gel electrophoresis and the appropriate gel slice removed, and after NCS cleavage, electrophoresed into a 12.5% polyacrylamide gel containing 0.1% SDS. Separated antigen fragments detected using silver staining (15).

APPENDIX

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(Please note: due to cost prints have not been supplied. When published, reprints will be forwarded).

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Evidence for Lipid-Protein Interactions in the  
Attachment of Antigens to a Low Density Membrane Fraction  
Isolated from *Trypanosoma rhodesiense*.

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## SUMMARY

A putative *Trypanosoma rhodesiense* flagella pocket membrane fraction (FPM) was previously found to possess a range of antigenic components that were released after exposure to a detergent mixture containing 0.1% Zwittergent 3-12/0.4% Triton X-100. In the present investigation an effort was made to determine the role of membrane lipid in binding FPM antigens, using phospholipases A<sub>2</sub> and C as membrane probes. Exposure to the former was notable for the release of one antigen in particular that was only poorly extracted using the above detergents. Evidence was obtained suggesting that this release was not due to the detergent action of degradation products formed by the action of phospholipase A<sub>2</sub> on membrane phospholipids. This phospholipase released antigen, as were most other FPM antigens, was a glycoprotein though the carbohydrate sequences do not appear to influence antigenicity. It was also possible to demonstrate the presence of a group of three cross reacting FPM antigens that partitioned as hydrophobic membrane proteins using Triton X-114 extraction. This was in contrast to the predominantly hydrophilic nature of most other FPM antigens.

The principal antigen described for bloodstream forms of African trypanosomes is a surface glycoprotein notable for undergoing repeated changes in antigenic specificity as a result of alterations in the amino acid sequence of the externally exposed N-terminal portion. Details of the structure and genomic control of antigen expression have been comprehensively reviewed (6,9).

In a previous investigation of *Trypanosoma rhodesiense* (15) it was established that, as well as a high equilibrium density surface membrane fraction, a much lower density membrane fraction, predominantly of flagella pocket membrane (FPM) origin, also possessed a range of antigenic components. Although the surface coat was originally described associated with the flagella pocket membrane (26) there was no evidence of any extensive cross reaction between surface and flagella pocket membrane antigens (15). Also reported in this latter study was the efficacy of various extraction procedures in removing antigens: the combination of Zwittergent 3-12 and Triton X-100 was found to give optimum results. This indicated a potential role for lipid-protein interactions in stabilizing the association of antigens to FPM.

The relative importance of lipid in the membrane association of other protozoan antigens is uncertain, though prominent surface antigens have been described for *Leishmania* (8), *Trypanosoma cruzi* (1) and for both the sporozoite (19) and merozoite stages (11) of the malaria parasite. In *T. brucei*, VSA is apparently synthesized with a C-terminal hydrophobic amino acid tail which is absent from VSA extracted or released from the parasite (4).

Most recently evidence has been obtained that VSA is present in situ as an amphiphilic integral membrane protein (7) though the hydrophobic determinants involved are not believed synonymous with the above hydrophobic amino acid tail segment.

The present study has compared the release of *T. rhodesiense* FPM antigens using procedures aimed specifically at disrupting phospholipid-protein interactions and identifying hydrophobic membrane antigens. This has revealed in particular the probable involvement of phospholipid in binding a limited number of FPM antigens with tentative indirect evidence for one antigen possessing very tightly bound lipid.

#### **METHODS**

The Wellcome CT strain of *T. rhodesiense* was isolated from infected rat blood and the cells washed and fluorescamine labeled as described previously (15). In some instances trypanosomes were surface labelled using the fluorescamine- $\beta$  cyclodextrin complex (12) as follows. A 15 mg amount of fluorescamine (Fluram, Roche Diagnostics) was used to prepare the above complex (12) and dissolved at 40°C in 75 ml 100 mM glucose 150 mM PO<sub>4</sub>, pH 8.0, then equilibrated to 30°. At this time 5.0 ml of loosely packed trypanosomes were added, then after 10 min. 75 ml PO<sub>4</sub>-glucose buffer added and the trypanosomes sedimented at 3,000 r.p.m. (Sorvall SS-34 rotor) for 10 minutes and twice washed.

Isolation of Flagella Pocket Membrane. The washed trypanosomes were disrupted by grinding with glass beads and two particle fractions, P<sub>a</sub> and P<sub>b</sub> respectively, sedimenting at 7720 g (10 min.), and 48,200 g (60 min.) obtained (see reference 15). Frac-

tion  $P_b$  was resuspended in no more than 3.0 ml buffered sucrose (250 mM sucrose, 1.5 mM EDTA; 1 mM KCl, 5.0 mM HEPES pH 7.5) and layered over a discontinuous sucrose gradient of the same pH and  $K^+$ /EDTA concentrations as above. The gradient consisted of sucrose solutions of the following densities: 1.252 g/cm<sup>3</sup> (6.0 ml, 54%); 1.187 g/cm<sup>3</sup> (8.0 ml, 42%); 1.112 g/cm<sup>3</sup> (10.0 ml, 35%) and 1.094 g/cm<sup>3</sup> (8.0 ml, 23%), the figures in parentheses are the volumes used and w/w percent composition respectively. These solutions were chosen based upon the median equilibrium densities of flagella pocket membrane (1.118) lysosome (1.175) and surface membrane (1.22) as previously determined (15).

The gradient was centrifuged for 75 min. at 18,000 rpm (33,000 g) using a Sorvall SV288 vertical rotor, after which the material banding at each density interface was withdrawn by tube puncture. The 1.112/1.094 interface material was diluted to 30 ml with 250 mM buffered sucrose and after centrifugation at 18,000 rpm the resulting FPM pellet resuspended in an appropriate buffer.

The distribution of relative fluorescence (in fluorescamine labelled cells) and various marker enzymes was followed using previous methods (15). In addition, carboxypeptidase activity was also assayed using 17.5 mM cbz-glu-tyr in 50 mM acetate pH 5.0 containing 0.075% Triton X-100 to a final volume of 500  $\lambda$ . After a 30 minute incubation 1.0 ml 0.5 M trichloroacetic acid was added, precipitated material was sedimented by centrifugation at 3000 r.p.m. for 10 min. (Sorvall HB4 rotor), a 100  $\lambda$  aliquot

of the supernatant was removed, and the released tyrosine assayed fluorometrically (21).

Limited trypsinization of the FPM fraction. In an effort to remove exposed FPM components susceptible to trypsin cleavage, a membrane fraction (containing 4-8 mg protein) was resuspended in 2.0 ml 250 mM sucrose, 5 mM calcium acetate, 20 mM Tris-HCl, pH 7.5. Trypsin (Worthington TRTPCK, 198U/mg) was added to a final concentration of 30 µg/ml and followed by incubation at 30°C for 15 min. The cleaved products were then recovered in the supernatant following centrifugation for 1.0h at 18,000 r.p.m. (Sorvall SS-34 rotor).

Exposure of the FPM fraction to phospholipases A<sub>2</sub> and C. In order to assess the importance of interactions between FPM antigens and phospholipid, membrane preparations were exposed to phospholipases A<sub>2</sub> and C (16). A FPM preparation (approximately 3.0 mg protein) was resuspended in 300 µl 5 mM calcium acetate, 225 mM sucrose, 15 mM HEPES, pH 7.0, and to this was added either 12 µl *Bacillus cereus* phospholipase C (Sigma 833 U/ml) or 7.5 µl porcine pancreatic phospholipase A<sub>2</sub> (Sigma 1200 U/ml) and 30 µl 10% bovine serum albumin (BSA-Sigma type, fatty acid free). The BSA was included to bind membrane active products (2), such as lysophospholipids, that could have non-specifically solubilized membrane antigens. After a 30 min. incubation at 28°C, phospholipase activity was stopped using 30 µl 37 mM EGTA and residual membrane removed by centrifugation, as for trypsinization.

In certain experiments the FPM fraction was either subjected to 3 freeze/thaw cycles or resuspended in 0.45 ml hypotonic buffer (2 mM calcium acetate, 15 mM HEPES, pH 7.0) left overnight

on ice, and the membrane pellet obtained after centrifugation then exposed to phospholipase A<sub>2</sub> or C. For other experiments the FPM fraction was subjected to mild trypsinization as described above prior to phospholipase treatment.

The use of Triton X-114 to separate hydrophobic and hydrophilic membrane antigens. The use of aqueous solutions of the non-ionic detergent Triton X-114, which undergoes a temperature dependent phase separation, to partition hydrophobic from hydrophilic membrane proteins was modified from the original method (5). In the present instance, an FPM pellet (6-9 mg protein) was resuspended in 10 ml 25 mM Tris-HCl, pH 7.2, containing 300 ng/ml leupeptin (Sigma) to which was added 500  $\lambda$  ice cold 10% Triton X-114. Incubation at 30°C for 10 mins. resulted in the formation of minute detergent enriched droplets which could be separated from the bulk aqueous phase by centrifugation at 3,000 r.p.m. for 10 minutes (Sorvall, HB4 swinging bucket rotor). The upper aqueous phase, containing hydrophilic membrane protein, was removed and concentrated by ultrafiltration (Amicon UM-10 filter) to approximately 0.5 ml. The lower detergent enriched phase, where hydrophobic components had separated, was then removed, care being taken not to disturb any overlying flocculent material.

Crossed Immunoelectrophoresis (CIEP) and affini-immunoelectrophoresis (CAEP) of FPM antigens. The FPM antigens released by the above treatments were compared with those solubilized using a mixture of 0.1% Zwittergent 3-12 and 0.4% Triton X-100 using crossed immunoelectrophoresis (CIEP) and crossed affini-immuno-

very low recoveries were obtained suggesting that the products of phospholipase activity were exerting an inhibitory effect. This was confirmed, as can be seen in Table 2, where in the presence of BSA, phospholipase induced release of FPM protein was greatly reduced and enzyme recoveries much higher.

After solubilizing the FPM fraction with 0.4% Triton X-100/0.1% Zwittergent 3-12, analysis by CIEP produced the antigenic profile seen in Fig. 1a, which is representative of repeated experiments. Precipitin peaks a-e were all constant features, especially a, c and e with f and g being more variable in occurrence. The profile obtained after this detergent extraction was used as a reference to compare other treatments of the FPM. Figure 1b shows the much reduced release of antigens after overnight extraction of FPM in hypotonic buffer (as used in Table 2). No increase in the number or intensity of the precipitin peaks was observed after 3 repeated freeze/thaw cycles of the FPM fraction (figure not shown). Noticeable are the clear-cut indications of proteolytic degradation (3) of antigens b (flying precipitin line) and c (now present as a doublet) absent from the detergent extract. Possibly the reduced intensity of the precipitin lines, especially band c, is due to a loss of antigenic sequences.

Exposure of hypotonic buffer washed FPM to phospholipase A<sub>2</sub> resulted in release of antigen a with an especially conspicuous precipitin peak corresponding to antigen d. Measurement of peak area d, for Fig. 1a as compared to Fig. 1c by planimetry, revealed a 17-fold increase in antigen d after phospholipase A<sub>2</sub>

exposure as compared to detergent extraction. There was no indication of substantial release of any other antigenic components other than a and d.

Phospholipase C exposure (Fig. 1d) also released antigen a with much less removal of antigen d. A number of other less prominent antigens ( $A^1-A^3$ ), which were not immediately comparable with detergent released components, were also in evidence.

Trypsin treatment of FPM (Fig. 2a) released a reactive fragment of antigen a (note incomplete precipitin peak and increased mobility) and what appeared to be a poorly reactive peak d. Subsequent phospholipase exposure of trypsin treated FPM (Fig. 2c, d), particularly phospholipase  $A_2$ , resulted in the appearance of antigen d. Pre-treatment with trypsin did not cause an increase in the amount of antigen d released but the reduced intensity of the precipitin peak suggests trypsin was cleaving exposed antigenic groups.

Inclusion of an intermediate gel containing concanavalin A as in Fig. 3 removed both antigens a and d released by the action of either phospholipase  $A_2$  or C. Some minor antigens released by phospholipase C were not affected. If trypsin pre-treated FPM was exposed to phospholipase  $A_2$ , there was less retardation of antigen d by concanavalin A (Fig. 3c). This indicates that although exposure to trypsin apparently removed glycopeptide, this did not appear to affect antigenicity (compare precipitin peak d of Fig. 3c with that of Fig. 2b).

Phase separation of Triton X-114 was used in an attempt to partition the predominantly hydrophobic from the more hydrophilic FPM antigens. From Fig. 4a it is evident that a range of less

hydrophobic antigens were recovered in the upper phase including an incomplete peak that appears synonymous with antigen a (Fig. 1). Both for the upper and lower phases it was not possible to precisely correlate the antigenic profiles with those seen in Fig. 1a. However, a most conspicuous feature of the lower phase (hydrophobic) antigen profile (Fig. 1b) was the presence of peak a' and cross reacting antigens a<sup>2</sup> and a<sup>3</sup>, none of which were present in the upper phase nor discernible after Zwittergent-Triton extraction (Fig. 1a). Antigen a was also present in the lower phase and partially cross reacted with a'.

The presence of a concanavalin A intermediate gel (Fig. 4c) removed hydrophobic antigens a, a' and a<sup>2</sup> but not antigen a<sup>3</sup>, which suggests the latter to be a non-glycosylated form of a'/a<sup>2</sup>. It is not known whether these cross reacting antigens are authentic FPM components or the products of proteolytic degradation during incubation with Triton X-114. In the upper phase, antigen c' was the only component not reactive toward concanavalin A.

#### DISCUSSION

The present investigation has produced evidence implicating interactions between a limited number of FPM antigens in T. rhodesiense and both the hydrophobic fatty acyl side chains and more polar head groups of membrane phospholipids. For antigen d at least the involvement of phospholipid fatty acid residues would appear especially important for membrane binding. The inclusion of serum albumin should have eliminated non-specific release due to any detergent-like action attributable to phospho-

lipase reaction products. Certainly the presence of BSA during phospholipase treatment had an obvious effect in reducing the release of FPM protein and relieving apparent inhibition of FPM enzyme activities.

Evidence from Triton X-114 phase separation of FPM indicates at least one antigen (a' and cross reactants a<sup>2</sup> and a<sup>3</sup>) is present as a hydrophobic integral membrane glycoprotein. At this time it is not known whether the cross reacting components, a<sup>2</sup> and a<sup>3</sup> are authentic membrane components, or degradation products formed during detergent extraction. If authentic, it is of interest that antigen a' is present in a non-glycosylated form that is still immunoreactive; carbohydrate sequences also appear non-essential for the antigenicity of component d. While the inclusion of leupeptin would have inhibited a major *T. rhodesiense* proteinase (15) there could be more specific proteinases, not affected by this inhibitor, able to cause limited degradation.

The most extensively studied protozoan membrane associated antigen is the glycoprotein VSA of African trypanosomes (6,9). For *T. brucei* at least a recent review (6) concluded that there is probably no insertion of any VSA segment into the surface membrane lipid bi-layer. Since then evidence has been presented (7) to indicate that *in situ* VSA occurs as an integral membrane protein and that an as yet uncharacterized hydrophobic segment is susceptible to cleavage by some trypanosomal "release factor" (enzyme?). Discounted in membrane binding is a C-terminal hydrophobic amino acid tail segment not present on extracted VSA, but which has been predicted from C-DNA sequences (4) to occur on

newly synthesized VSA. It is quite possible, as the above authors (7) surmise, that tightly bound lipid could be involved in the attachment of VSA to surface membrane. The existence of tightly bound phospholipid, if proven, could explain why in the present study FPM antigen d was more effectively released by phospholipase A<sub>2</sub> as opposed to detergent. There are precedents for implying the presence of tightly bound lipid: a number of membrane glycoproteins have been described possessing what appears to be covalently bound fatty acid including various virally induced glycoproteins (22,23) and a transferrin receptor (18). Covalently bound sphingolipid has been characterized as part of the lipopeptidoglycan synthesized by *T. cruzi* (14) and *Leishmania donovani* (23). At least for the former there is clear evidence for a surface membrane localization for this component (1). In view of these, and the present findings, the potential role of phospholipid in influencing the immunogenicity of trypanosome antigens could be of interest, since antibodies to liposomal phospholipids have been detected in experimental *T. rhodesiense* infections (20).

At present, evidence for endogenous phospholipases affecting the release of FPM components *in vivo* is not available. It is worth noting, however, that in *T. brucei* at least, an extremely active phospholipase A<sub>2</sub> is present (10).

There is no reason to suppose that antibodies once synthesized against FPM components could not gain access to externally disposed FPM antigens, since there is good evidence for the FPM being of importance in the pinocytotic uptake of proteins

(13,25). The stimulation of antibody production, during trypanosomiasis, to FPM components is undoubtedly compromised partly by the overwhelming response to VSA and also the pronounced immunosuppression associated with the infection (12). FPM antigens, however, would seem of potential interest for further investigation for either vaccine use or for developing antibodies for use in targetting liposome encapsulated drugs. The flagella pocket is known to be a prominent site for drug uptake (25). The present investigation has at the very least revealed the usefulness of phospholipase A<sub>2</sub> as a means of selectively releasing a major antigen of the flagella pocket membrane. Efforts to isolate antibody specific to antigen d are now in progress using DPT electroblotting.

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## FIGURE LEGENDS

### Fig. 1

Crossed immunoelectrophoresis of a *T. rhodesiense* FPM fraction. Components released after: (a) extraction with 0.1% Zwittergent 3-12/0.4% Triton X-100; (b) overnight extraction at 0°C in 5 mM Ca acetate, 20 mM HEPES, pH 7.5; (c), (d) exposure of FPM, after extraction b, to phospholipases A<sub>2</sub> and C respectively. In all instances an antibody containing agarose gel was used that contained anti-P<sub>b</sub> IgG (15) at a final concentration of 7.5%.

### Fig. 2

Crossed immunoelectrophoresis of a *T. rhodesiense* FPM fraction after mild trypsinization. Precipitin patterns obtained for material released after: (a) trypsinization alone; (b) trypsinization followed by exposure to phospholipase A<sub>2</sub>; (c) trypsinization followed by exposure to phospholipase C. Agarose gel contained detergent and anti-P<sub>b</sub> IgG as for Fig. 1.

### Fig. 3

Crossed affini-immunoelectrophoresis of a *T. rhodesiense* FPM fraction with an intermediate gel containing concanavalin A. Precipitin patterns obtained for components released after: (a), (b) exposure to phospholipases A<sub>2</sub> and C respectively; (c) mild trypsinization followed by exposure of residual FPM fraction to phospholipase A<sub>2</sub>. Intermediate lectin containing gel prepared with 80 µg concanavalin A/cm<sup>2</sup>. Detergent and antibody incorporation as for Fig. 1.

### Fig. 4

Antigenic profiles of *T. rhodesiense* FPM hydrophilic and more hydrophobic membrane components partitioned by extraction with Triton X-114.

(a) CIEP of hydrophilic membrane components recovered in the upper bulk aqueous phase after Triton X-114 extraction; (b) CIEP of more hydrophobic membrane components partitioned into the detergent enriched lower phase; (c) as for b with an intermediate gel containing concanavalin A as used in Fig. 3. Detergent and antibody incorporated as detailed for Fig. 1.

Table 1. Distribution of marker enzymes and relative fluorescence after discontinuous gradient centrifugation of a *T. rhodesiense* light particle ( $P_b$ ) fraction.

Enzyme	Specific Activities* for fractions:			
	$P_2$	FPM	LYS	SM
Proteinase	5.8 $\pm$ 1.2	1.7 $\pm$ 0.5	13.2 $\pm$ 1.8	0
Carboxy peptidase	12.0 $\pm$ 2.1	32 $\pm$ 1.9	48.1 $\pm$ 3.4	0
Arylamidase		28 $\pm$ 2.0	6.8 $\pm$ 1.2	0
Phosphatase I <sup>a</sup>		134 $\pm$ 11	20 $\pm$ 2.5	5 $\pm$ 0.7
Phosphatase II <sup>a</sup>	63 $\pm$ 4.0	155 $\pm$ 13	38.5 $\pm$ 3.1	26 $\pm$ 2.9
$\alpha$ -glycerophosphate dehydrog. (DCPIP-linked)	220 $\pm$ 12.5	150 $\pm$ 10.1	1150 $\pm$ 95	75 $\pm$ 8.3
$\alpha$ -glycerophosphate dehydrog. (NAD-linked)	500 $\pm$ 37	0	0	4500 $\pm$ 107
Relative Fluorescence (a)	15 $\pm$ 3.1	50 $\pm$ 6.3	4.0 $\pm$ 1.2	10 $\pm$ 2.5
Relative Fluorescence (b)	12 $\pm$ 2.1	42 $\pm$ 5.4	7.0 $\pm$ 2.0	14 $\pm$ 3.1

\* Enzyme activities expressed as mU/mg protein where 1 Unit  $\equiv$  1  $\mu$ mole product formed per minute, with standard deviations based on five determinations. Relative fluorescence for (a) free and (b)  $\beta$ -cyclodextrin complexed fluorescamine.

<sup>a</sup> Phosphatase I assayed using 10 mM  $\alpha$ -glycerophosphate and phosphatase II with 7.5 mM  $p$ -nitrophenylphosphate; both using 50 mM acetate pH 5.5 (see 15,16).

Table 2. Release of Enzyme Activities from a FPM fraction after exposure to the treatments indicated

Treatment	% Enzyme Release from FPM*			
	Protein	Arylamidase	Phosphatase I	Phosphatase II
Hypotonic buffer	35 (95)	0(90)	14 (91)	1.7 (94)
Phospholipase A <sub>2</sub>	42 (104)	1.7(57)	11 (87)	1.7 (74)
" " + BSA	9.0 (98)	4.0(94)	8.0(90)	5.8(101)
Phospholipase C	48 (98)	9.0(64)	25 (128)	0 (68)
" " + BSA	7.0(101)	5.0(92)	10 (92)	2.3(103)

\* Percent recovery (bound + released) shown in parentheses, with results based upon five determinations with  $\pm$  S.D. no greater than 5.6.

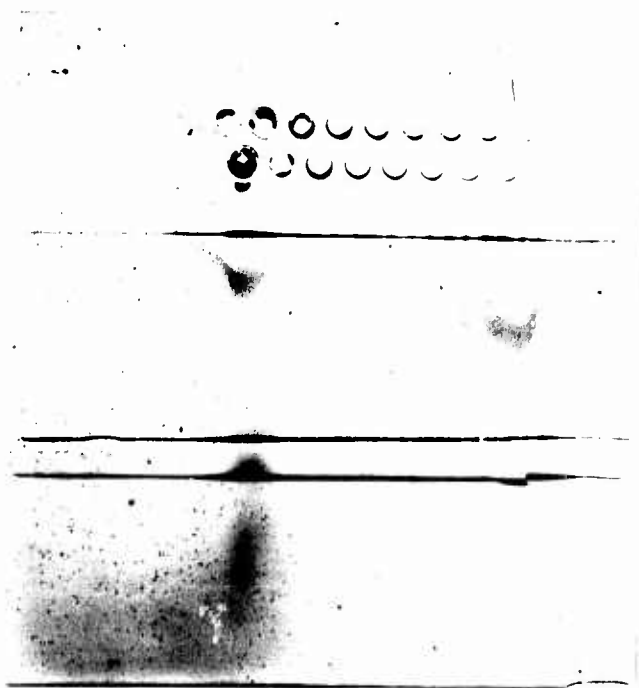


Figure 1a



Figure 1b

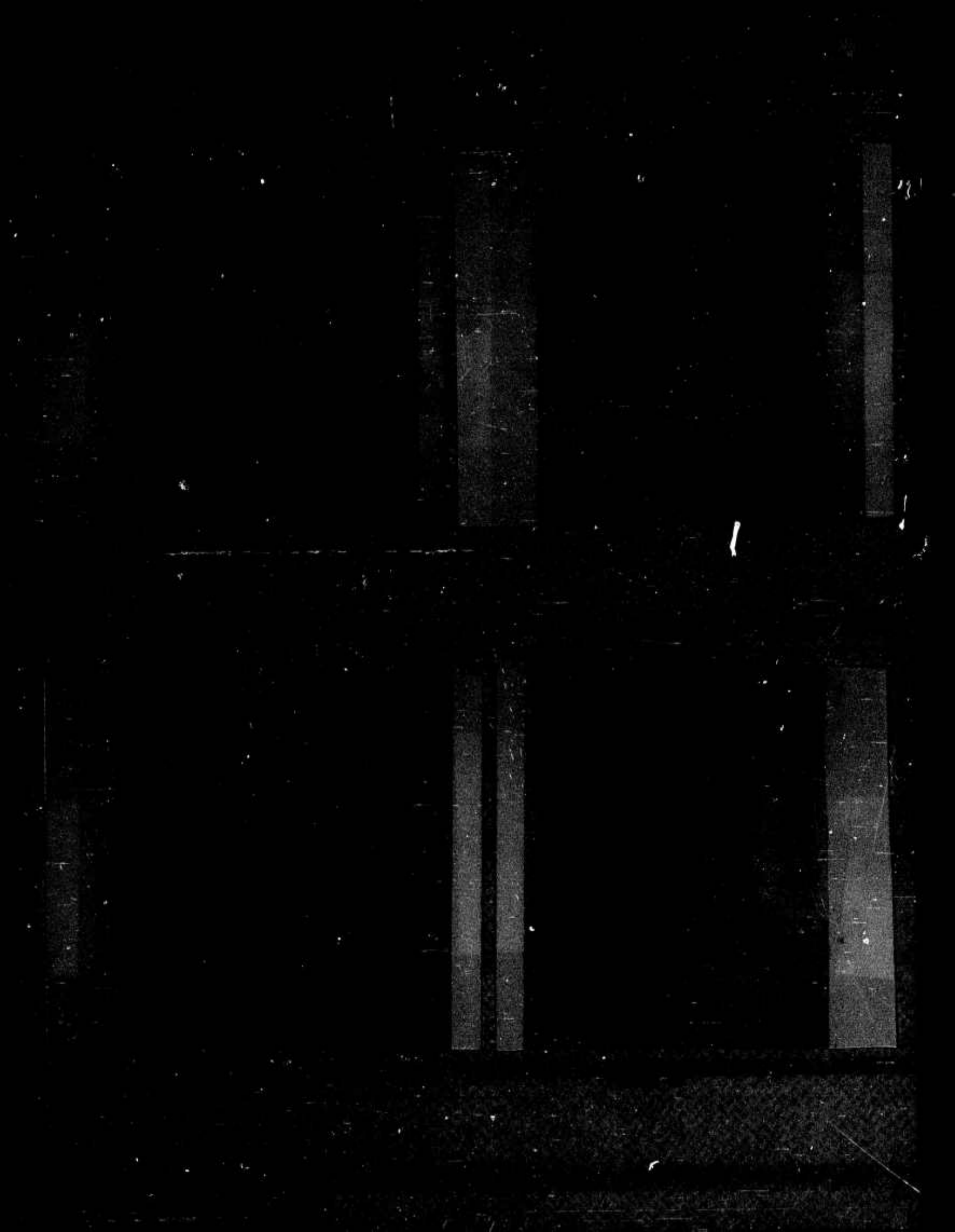




Figure 2e



Figure 2f

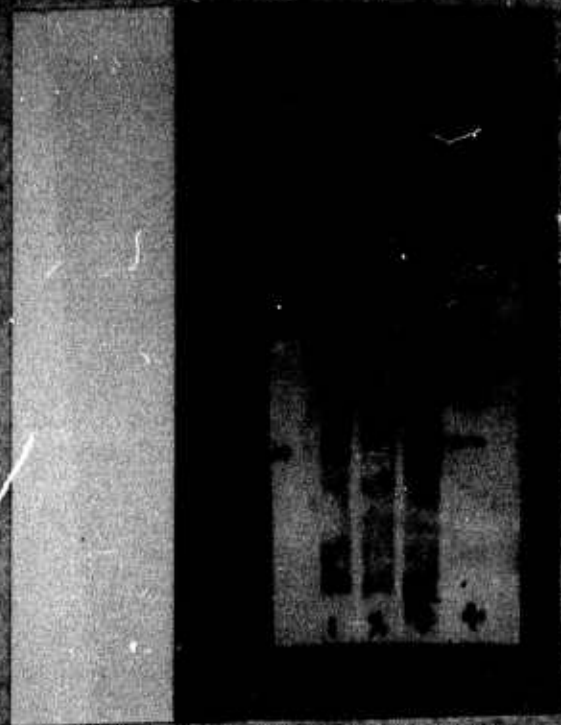
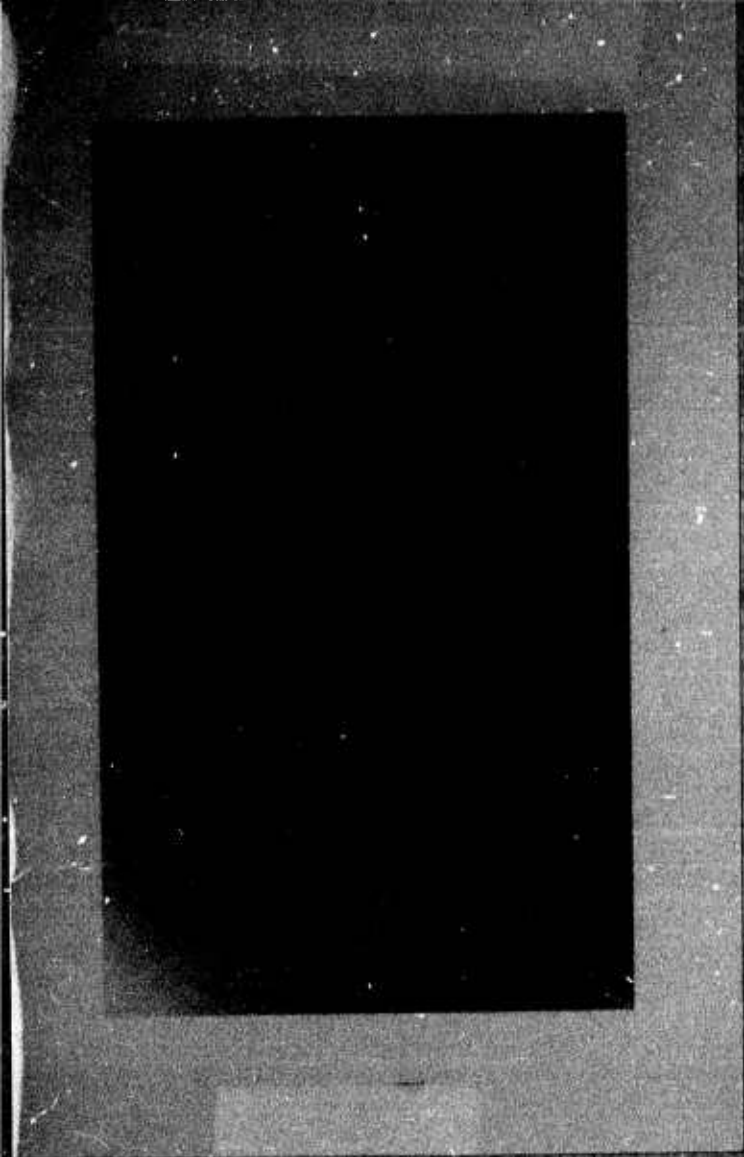


Figure 2g



Figure 2h





Figures 4b and 4d



Figure 5

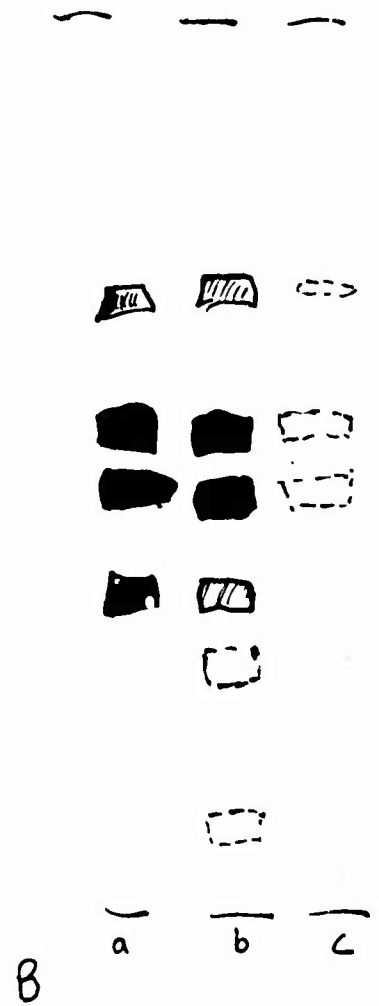
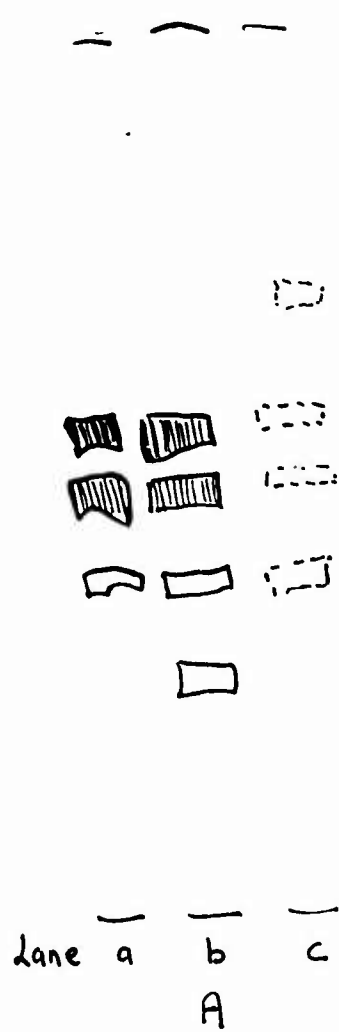


Fig. 6



1 2 3

Figure 7

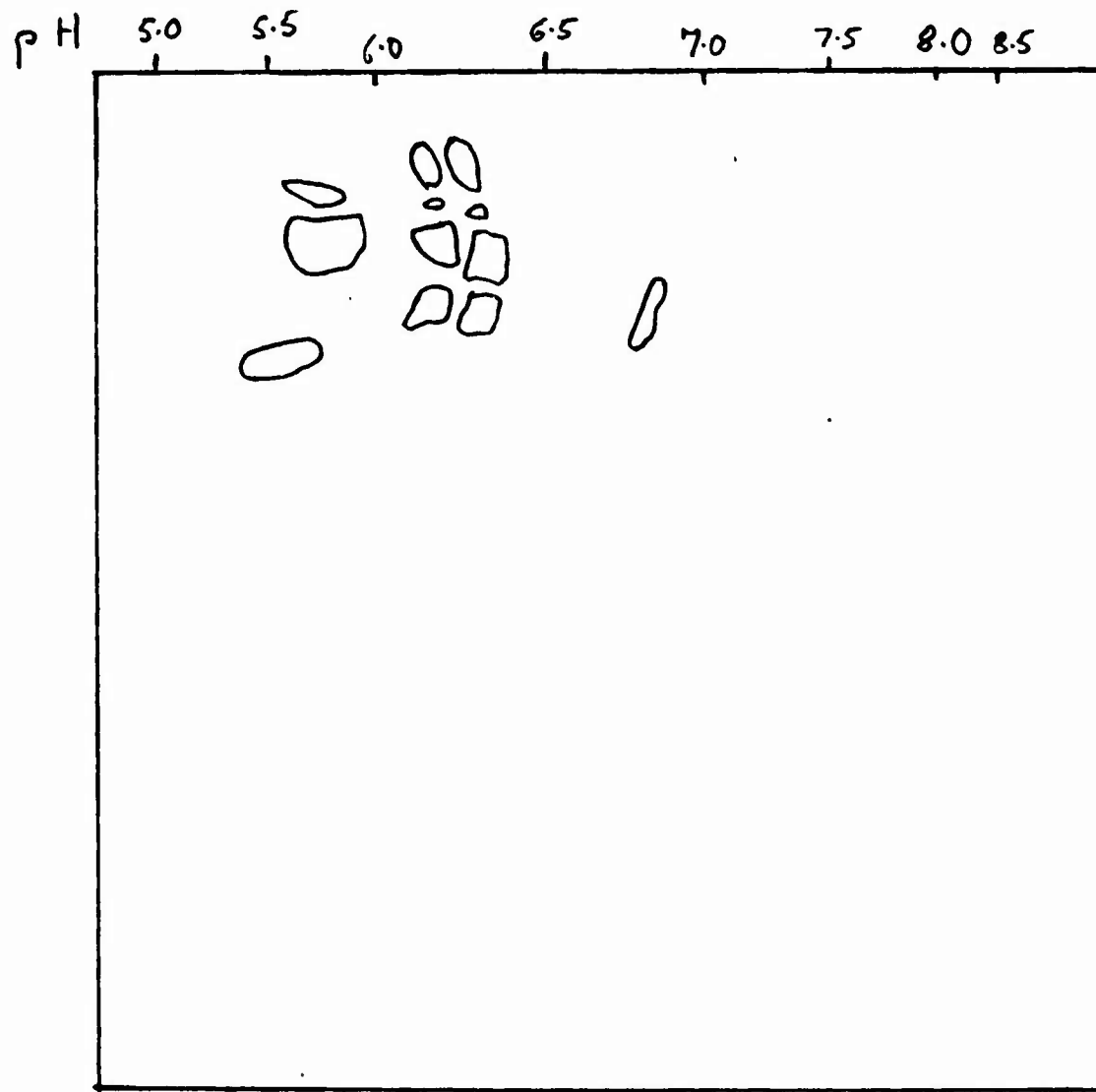


Fig BA

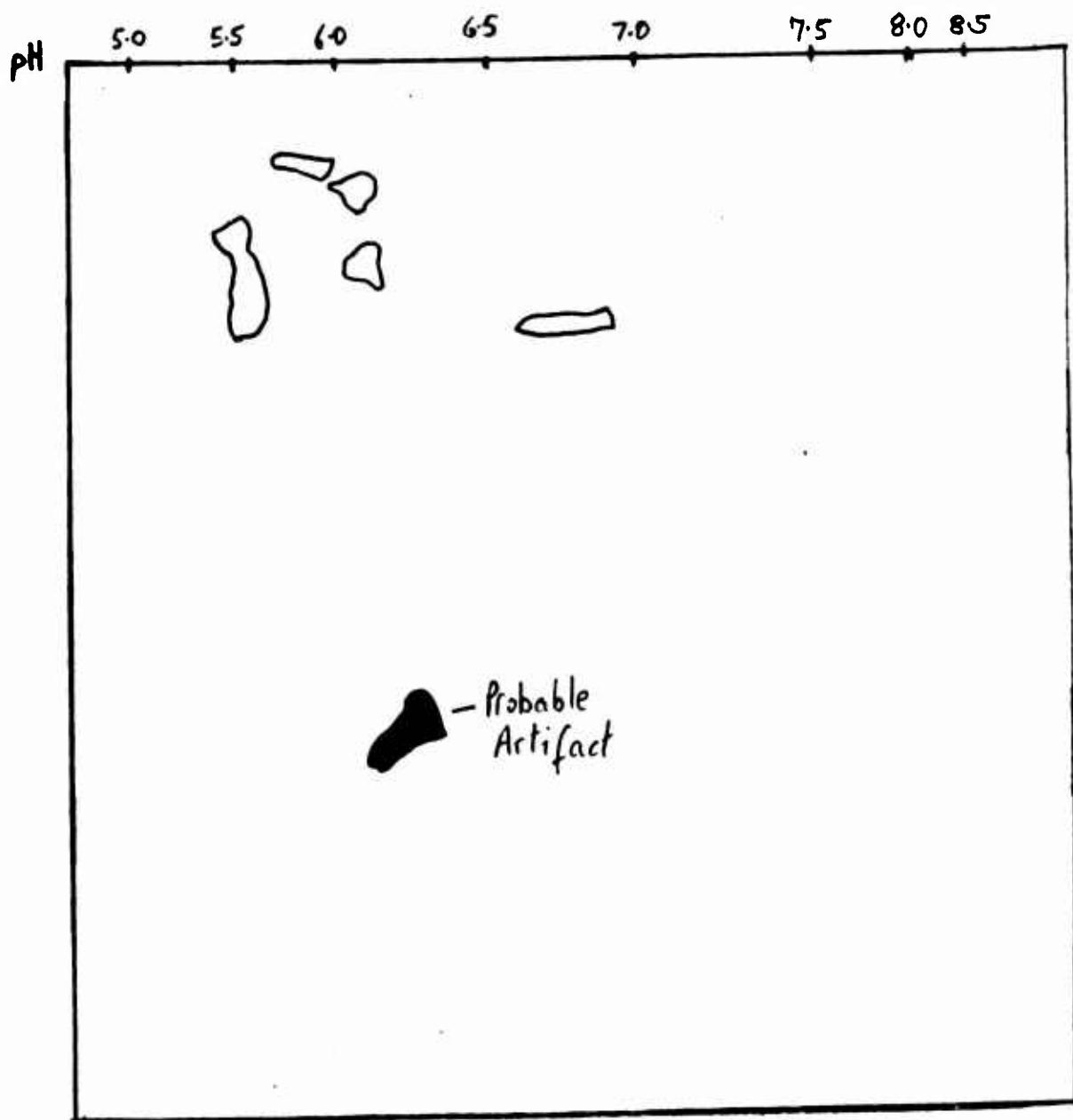
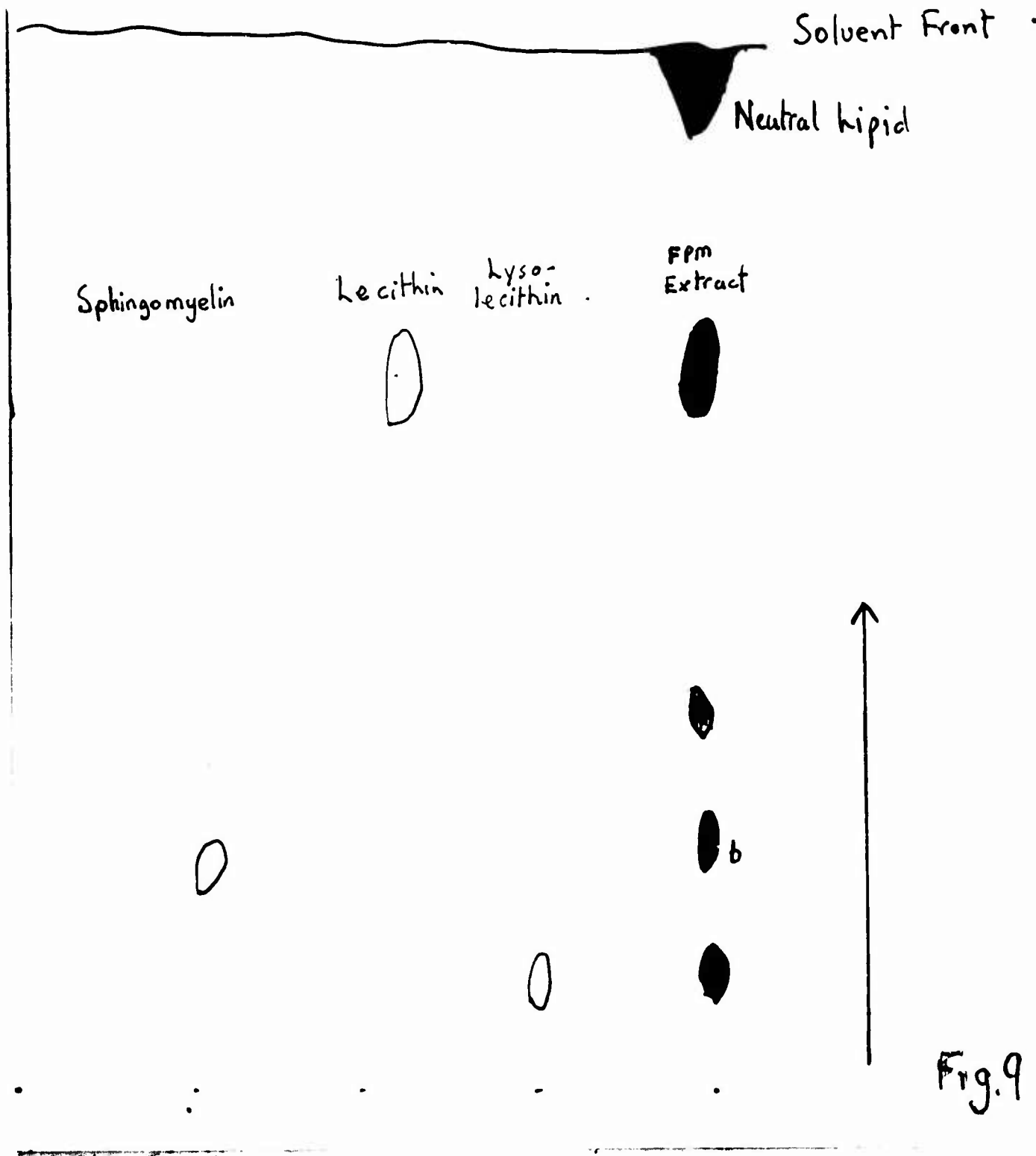


Fig 8B



FPM Antigen ( $66 \times 10^3$ )

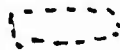
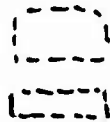
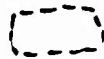


Fig 10

