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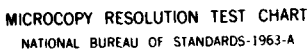
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DISTRIBUTION AND CHARACTERIZATION OF ANTIGENS FOUND
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Annual Report

John McLaughlin

August 1982

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A. INTRODUCTION

In the previous report, the results of cell fractionation experiments of bloodstage forms of the African trypanosome Trypanosoma rhodesiense together with various forms of immuno-electrophoresis, revealed the principal antigenic sites to be associated with the surface and flagella pocket membranes. These findings have since been published and copies of the resulting paper are attached to the appendix of this present report.

For the period of this report (Aug. 1981-Aug. 1982) the investigations described were firstly concerned with utilizing the information referenced above to permit the preparative isolation of both these membrane fractions. Having achieved that objective it was then possible to assess various procedures for the isolation of antigenic components from both membrane fractions with a comparison of their properties. For this purpose attempts were made both at dissociating intact antigenic components from labelled membranes, through the use of detergents, or releasing exposed moieties by means of limited peptidolysis. The usefulness of various chromatographic procedures could then be appraised as a means of separating and characterizing specific membrane antigens.

The other aspect that has been studied is the importance of membrane lipid in the binding of antigens and whether any of these antigens are associated with integral membrane protein. This was of interest in view of the published evidence for a hydrophobic c terminal portion on the variable surface antigen that is believed to penetrate the surface membrane lipid bilayer.

B. METHODS

Procedures for recovering T. rhodesiense (Welcome CT strain) from infected rat blood have been described in previous reports.

1. Preparative Fractionation of T. rhodesiense

a. Isolation of Flagella Pocket Membrane. A total cell homogenate was prepared using glassbeads and centrifuged to yield the two particle fractions P_a and P_b , sedimenting at 7720g (10 mins.) and 48,200g (60 min.) respectively, as described by McLaughlin (1982). Each fraction was resuspended in no more than 3.0 ml buffered sucrose (250 mM sucrose, 1.5mM EDTA. 1.0 mM KCl 5.0 mM HEPES. pH 7.5) and was then layered over a discontinuous sucrose gradient of the same pH and K^+ /EDTA concentration. After some initial trial experiments sucrose solutions of the following densities were finally used (the numbers in parenthesis show the volume used and W/W percent composition) 1.2519 g/cm³ (6.0 ml, 54%); 1.1868 g/cm³ (8.0 ml, 42%), 1.1115 g/cm³ (10.0 ml, 35%) and 1.0944 g/cm³ (8.0 ml, 23%). The above sucrose 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.

The gradient samples were centrifuged for 75 min. at 18,000 rpm ($33 \times 10^3 g$) using a Sorvall SV 288 rotor after which the material banding at each of the sucrose density interfaces was carefully withdrawn using a 5.0 ml syringe with a L shaped needle. The flagella pocket membrane fraction (FPR) was diluted with buffered sucrose to a volume of 30 ml, centrifuged at 18,000 rpm ($30 \times 10^3 g$) for 1.0 h and the resulting pellet resuspended in an appropriate buffer.

b. Isolation of Surface Membrane. The fraction removed from the 54/42% interface after centrifugation of the P_a fraction as described above, was mixed with 7.5 ml Percol (Pharmacia); 2.5 ml 1.25 M sucrose and made up to 30 ml with buffered sucrose. After centrifugation at 15,000 rpm ($21 \times 10^3 g$) for 40 mins. in an SS-34 rotor the surface membrane was recovered as a loose flocculent layer near the top of the gradient. Contaminating glycosomes sedimented to the bottom of the gradient as judged by the distribution of NAD^+ linked α -glycerophosphate dehydrogenase.

2. Chromatographic Analysis of Membrane Antigens

a. Affinity Chromatography

i. Protein-A Affinity Chromatography. A 4 x 0.8 column of Protein A -Sepharose CL-4B (Pharmacia) was prepared by swelling 0.5g in 100 mM PO_4 , pH 7.2, and pouring the slurry into a small disposable column (Econo-column, Bio-Rad). A 2.0 ml sample of a rabbit anti-serum, raised against T. rhodesiense fractions P_a or P_b (McLaughlin 1982) or a surface membrane fractions (see above) and previously dialyzed against 100 mM PO_4 buffer, was then slowly passed through the column (7.5 ml/hr.). The column was eluted with further PO_4 buffer until the U.V. monitor indicated the effluent to be free of protein. At this time the flagella pocket or surface membrane detergent extract was introduced onto the column. This extract was prepared by resuspending the final membrane pellets, prepared from fluorescamine labelled cells, in 1.5-2.0 ml 100 mM PO_4 , pH 7.2, containing 0.12% zwittergent 3-12 (Calbiochem). After centrifuging for 1.0h at 18,000 rpm ($30 \times 10^3 g$) the supernate was removed and the relative fluorescence measured. The sample was allowed to pass slowly into the column (7.5 ml/h) until protein breakthrough was recorded. At this time the effluent needle valve was turned off for 30 min. This permitted more complete binding of antigens in the detergent extract to the protein A bound IgG. Elution of unbound membrane compounds then proceeded using 100 mM PO_4 buffer containing 0.12% zwittergent 3-12 until the U.V. monitor registered a constant base line. The membrane antigen-antibody complexes were then displaced from the column using 100 mM glycine HCl, pH 3.0, containing 0.12% zwittergent 3-12. Fractions were collected throughout and each measured for relative fluorescence. These details are indicated in Fig. A.

ii. Lectin affinity chromatography. Either lentil lectin sepharose 4B or concanavalin A sepharose were packed into a 10 x 1 column. The fluorescamine labelled samples after dialyzing against three changes of 5 mM Tris 0.5 mM Ca^{2+} ; 0.5 mM Mn^{2+} , pH 7.2 were adjusted to 0.12% zwittergent 3-12.

The samples were centrifuged for 1.0h at 18,000 r.p.m. the supernate removed and the volume reduced to 1-2.0 ml by ultrafiltration (Amicon Um 10 filter). The concentrated membrane extract was applied to the column by upward displacement using a three way valve. After all of the sample was on the column a further 2-3.0 ml 5 mM Tris HCl, pH 7.2, containing 0.12% zwittergent 3-12 was passed through the column using a peristaltic pump adjusted to flow rate of 8.0 ml/h. To allow full binding of sugar residues to lectin the eluent flow was interrupted for 30 minutes. After this time further buffer was passed through the column until all unbound protein was removed. Approximately 15 ml 10 mM NaCl, 5 mM Tris-HCR, pH 7.2 containing 0.12% zwittergent 3-12 was then used to displace non-specifically bound material. Elution of glycoprotein was achieved either using 0.5 M α -methylmannoside followed by 10 mM acetate, 0.5 M NaCl, pH 4.0 or a linear gradient of α -methyl-mannoside (0.05-0.5M) in 100 mM NaCl, 5mM Tris, pH 7.2.

b. Gel Filtration

A 4 x 1 column of Sephadex G-100 was prepared in 100 mM formate buffer, pH 3.5 containing 0.15% zwittergent 3-12 and used in an attempt to dissociate antigens from immune complexes recovered from the Protein A-Sepharose column. Samples, adjusted to pH 3.5 with 0.1 M formic acid and containing 0.15% zwittergent 3-12 were then applied to the column. Elution commenced using the above formate buffer containing 0.15% zwittergent and 2.5 ml fractions collected and monitored as above.

c. Chromatofocussing

The technique of chromatofocussing is relatively recent being first described in detail by Sluytermans and Elgersama (1978) and Sluytermans and Wijdenes (1978). The method offers the resolution of conventional isoelectric focussing, but with the convenience of column chromatography. Thus a pH gradient is produced automatically during elution, without the need for

any externally applied electric field, and components are separated according to their iso-electric points.

For the separation of tryptic fragments of cell surface components and isolated flagella pocket membrane the following procedure was followed. A 40 x 1 column of Poly-buffer exchanger PBE 94 (Pharmacia) was prepared according to the manufacturer's instructions and washed with 10 column volumes of mM imidazole-HCl pH 7.4. The samples were dialyzed extensively using benzoylated dialysis tubing (m.W. cut off 3,000). Details of the release of cell surface components by limited trypsin peptidolysis and limited tryptic digestion of flagella pocket membrane are given below. If necessary the volume was reduced to 5-8 ml by ultrafiltration (Amicon UM-10 filter). The eluant was prepared by diluting Polybuffer 74 (Pharmacia) with water (1:8) and adjusting the pH to 4.25 with 0.1 M HCl. A total volume of 378 ml polybuffer was prepared and passed through the column after sample application. Fractions of 3.5 ml were collected using a FRAC 300 collector (Pharmacia) monitored at 280 mμ with a flow rate of 15 ml/h maintained using a PV3 peristaltic pump (Pharmacia). Each fraction was measured for relative fluorescence and every third fraction for pH. In some instances a gradient from pH 8.4 to pH 5.0 was used by equilibrating the column, and sample with 25 mM Tris-acetate pH 8.3 then eluting with a 3:1 mixture of PB 74 and PB 96 using a total of 30 ml polybuffer in 260 ml H₂O adjusted to pH 5.0.

3. Electrophoretic Analysis of Membrane Antigens

a. Polyacrylamide Gel Electrophoresis

Components isolated by either affinity chromatography or chromatofocussing were analysed with respect to purity and molecular weight using polyacrylamide gel electrophoresis (PAGE). Routinely this involved 7% gels 1:10 cross linked which contained 1% SDS. Gels were made up using 40 ml 0.1 M PO₄ buffer, pH 7.2, containing 2.8g acrylamide, 0.14g methylene

bisacrylamide, 2.0 ml 20% SDS and 100 λ TEMED. Polymerization was initiated by adding 250 λ ammonium persulphate (.16 g/ml) and a 16 x 17.5 gel, of 0.15 mm thickness cast between two glass plates. This gel slab was transferred to a vertical gel electrophoresis cell (Bethesda Biological Laboratories) containing 100 mM PO_4 /0.1% SDS, pH 7.2, in each electrode tank.

Each sample to be electrophoresed, including the standards used, was then adjusted to contain 1.0% SDS; 7.5 mM dithiothreitol; 5.0 mM EDTA; 20% sucrose and heated at 100°C for 3 minutes to promote complete protein monomer formation. A 30 λ aliquot of sample was then introduced into the sample slot and electrophoresis then proceeded for 3.0h at 25 mA constant current.

The separated components were visualized by means of the silver stain of Merril et al 1981. Alternative a modification of the mercury-Coommassie Brilliant Blue stain described by McLaughlin and Meerovitch 1976 and McLaughlin 1982 was used. This was prepared by dissolving .25g Coommassie Brilliant Blue G250 in 210 ml methanol + 40 ml glacial acetic acid and 250 ml sat. HgCl_2 . After soaking for 40 min. the gel was washed repeatedly with a destaining solution containing 250 ml ethanol, 650 ml water and 80 ml glacial acetic acid.

b. Immunoelectrophoresis. The methods used followed those described by McLaughlin 1982.

C. RESULTS

1. Isolation of Flagella Pocket and Surface Membrane Fractions.

Using the discontinuous sucrose gradient described in the Methods the results for marker enzyme and relative fluorescence enrichment shown in Table 1 were obtained. These reveal a comparative lack of contamination of both fraction with either lysosomes (proteinase) promitochondria (α -glycerophosphate dehydrogenase DCPIP linked) and glycosomes (NAD^+ linked α -glycerophosphate dehydrogenase).

2. Immuno-electrophoretic Analysis of Isolated Membranes

Fig. 1 shows the antigenic profiles obtained after CIEP of fractions recovered from the discontinuous gradient. The FPM fraction (Fig. 1F) exhibited at least three major precipitin peaks using anti-P_b - IgG w, x and y though peak w has been found to be variable in occurrence. As previously described there was no evidence of cross reaction between any of these components. The material removed from the 42/54% interface produced the precipitation profile shown in Fig. 1A. This same fraction was subjected to Percol gradient centrifugation; the upper surface membrane (SM) enriched fraction after CIEP against anti-P_a IgG gave the results shown in Fig. 1B. Noticeable was the loss of certain precipitin lines observed in the 42/54% interface material.

The material recovered from the bottom of the Percol gradient (glycosome enriched) and the 35/42% sucrose interface (mitochondria/lysosomes) gave the antigenic profiles shown in Figs. 1D and E. In agreement with previous results (McLaughlin 1982) both these fractions appear to exhibit limited reactivity.

The FPM fraction was further characterized using modified histochemical staining procedures (McLaughlin and Meerovitch 1975) to demonstrate enzymic activity present in immunoprecipitates. Fig. 2 shows the results obtained for a range of phosphatase substrates; the FPM fraction was found to be highly enriched in phosphatase activities. Unfortunately there was a loss of contrast during photographing the original plates; therefore, arrows have been used to highlight the position of the precipitin lines. Phosphatase activity toward all of the substrates used was detected with glucose-6-P₀, α -glycerophosphate and β -glycerophosphate. For the latter substrate (Fig. 2C) a second peak was discernable (2) which appears similar to that obtained using α -naphthol phosphate (Fig. 2D). This is in agreement with previous findings regarding differences in the distribution and properties of phosphatase

activity toward α and β -glycerophosphate respectively (see previous annual reports). The inclusion of an intermediate gel containing concanavalin A removed all of these phosphatase active immunoprecipitin lines. This is not surprising since most phosphatases studied to date are known to be glycoproteins.

3. Glycoprotein FPM Antigens. Prior results (McLaughlin 1982) demonstrated the glycoprotein nature of most of the FPM antigens. Fig. 3A shows that for FPM the results of CIEP using an intermediate gel containing Arachis hypogaea (peanut) lectin, are similar to those previously published using concanavalin A. A single antigen peak was found in the upper anti- P_b IgG containing gel, being the only component not reacting with PN lectin. Unlike concanavalin A, PN lectin did not result in a demonstrable precipitin zone. It can be concluded that apart from antigen N, the other FPM antigens contain galactose, mannose and possibly glucose in view of their reactivity with these two lectins.

A partially purified surface membrane fraction (42/54% gradient interface) was also analysed by CIEP using a PN lectin intermediate gel. A comparison of Fig. 3B with Fig. 1A shows reactivity between peak 2a and PN lectin whilst the antigenically cross reacting portion 2b appears not to react, possibly indicating that this is a non-glycosylated (at least with galactose) form of the antigen.

From experiments performed to date it would seem that for FPM antigens carbohydrate residues are not involved in their antigenicity. This conclusion is based on the lack of effect observed after incubation of a dialyzed FPM fraction with 2 mM NaIO_4 . The FPM fraction was extensively dialyzed against 10 mM PO_4 , pH 7.2, incubated for 30 minutes at 30°C with 2 mM NaIO_4 then analyzed by CIEP.

4) Membrane Association of Antigens.

A number of experiments have been undertaken and are still in progress, to investigate the nature of the association of antigens with the flagella pocket membrane. Increasing evidence has accrued since the initial studies of VSA for some involvement of hydrophobic interactions in its attachment to surface membrane. Current evidence indicates that there is an exposed variable N-terminal portion of VSA and a cross-reactive C-terminal portion to which carbohydrate residues are attached and which in situ carries a hydrophobic tail (Holder and Cross 1981).

The cross reactive between VSA obtained from different cloned isolates has been demonstrated to be a function of the attached carbohydrate sequences (Barbet, et al 1979). It has been postulated by Holder and Cross (1981) that the hydrophobic tail portion serves to anchor VSA in the lipid bi-layer of the surface membrane. Such an involvement of hydrophobic residues could explain the incomplete extraction of VSA noted in a number of reports (see review by Cross 1978) and the increased efficacy noted in the presence of detergent (i.e. Rovis, et al 1978). It should be noted that in surface labelling Trypanosomes, a recovered label may also be reacting with sites other than VSA, such as flagella pocket membrane.

The experiments reported below were designed firstly to remove more superficially exposed membrane antigens through limited trypsinolysis. Secondly has been an attempt to separate integral membrane protein antigens (i.e. those proteins associated with the hydrophobic core of the membrane) from hydrophilic more peripherally located proteins. Finally, at least so far, the role of membrane phospholipid in binding antigens has been assessed in terms of the relative importance of the phospholipid polar head group as compared to the fatty acyl side chain.

- a. Release of FPM antigens susceptible to trypsin cleavage. As a means

of removing exposed flagella pocket membrane (FPM) antigens a membrane suspension containing 6-12 mg protein of ml was prepared from Fluram labelled cells. To the membrane fraction, in 2.0 ml 250 mM sucrose, 5 mM calcium acetate, 20 mM Tris-HCl, pH 7.5 was added trypsin (Worthington TPCK) to a final concentration of 40 μ g/ml. After incubating at 30°C for 15 minutes. Lima bean trypsin inhibitor (Worthington) was added to a final concentration of 40 μ g/ml and the residual membrane removed by centrifugation for 1.0h at 18,000 r.p.m. using a Sorvall 33-34 rotor. Relative fluorescence was then measured using an aliquot of the supernatant and resuspended pellet. In the four experiments performed so far 30% of the relative fluorescence was recovered in the supernate, indicating susceptible cleavage points for certain labelled membrane proteins were relatively inaccessible to trypsin.

Analysis by CIEP (Fig. 4) of trypsin released material, revealed one antigenic component (a⁻) having a similar mobility to peak a (Fig. 1F) present in a Zwittergent/Triton x-100 extract of FPM. The co-identity of these components could be established by tandem CIEP. Several constituents much more anionic than any present in detergent extracts were also released. Presumably this reflects a high proportion of basic amino acid residues (i.e. arginine, lysine) in these cleaved peptides. Two of these anionic peptides, b¹ and b² showed complete cross reactivity whilst at least one further precipitin peak c was present which did not exhibit any cross reactivity.

Inspection of Fig. 4B reveals that in the presence of an intermediary gel containing concanavalin A, precipitin peak b is removed indicating the antigen to be a glycoprotein. The anionic peptides were not removed by concanavalin A; a glycopeptide was revealed that did not appear to be immunogenic (peak d) since there was no evidence of any corresponding precipitin peak in the antibody containing gel (Fig. 4A).

b. Release of Surface Antigens. Whole T. rhodesiense were also subjected to limited trypsinolysis: firstly to investigate the range of surface exposed antigenic groups released and then to compare these with the FPM peptides. Whilst extensive studies concerning the products of tryptic digestion of VSA have been published, there are few reports concerning trypsinization of whole cells and VSA in situ. It has been observed (Jackson, et al 1978) that after exposure to trypsin trypanosomes are no more susceptible to agglutination by concanavalin A. This would seem to be at variance with the previously referenced reports postulating the carbohydrate sequences of VSA to be inaccessible (Holder and Cross 1981).

For limited trypsinolysis Fluram labelled cells were suspended in 250 mM sucrose, 20 mM Tris-HCl, 2.5 mM calcium acetate, pH 7.5, containing 30 μ g trypsin per ml. After incubating at 30°C for 10 min. lima bean trypsin inhibitor was added to a final concentration of 30 μ g/ml. The trypanosomes were sedimented by centrifugation, 3000 rpm for 10 min., and the supernate containing the trypsin released surface components removed.

The relative fluorescence of the supernate was measured after concentration by ultrafiltration (Amicon UM 10 filter). From the absence of detectable fluorescence in the filtrate it was concluded that no loss of labelled components occurred during concentration. From 40-50% of the total relative fluorescence was recovered in the supernatant fraction. Subsequent fractionation of trypsinized cells revealed that 58-60% of this remaining fluorescence was associated with fraction P_b (FPM enriched). Of the remainder, 8-12% was found in fraction P_a (containing some of the surface membrane) and 12-18% in the "nuclear" fraction (also containing some surface membrane) and the rest in the final soluble fraction. These results pointed strongly to the fact that

under these experimental conditions trypsin was only removing surface membrane components and not exteriorly exposed flagella pocket membrane peptides.

The above conclusion was supported by the results of CIEP where the precipitin pattern shown in Fig.12 was obtained using anti-P_a but much less pronounced precipitin pattern discernable using anti-P_b IgG. Further analysis of the released surface peptides did, amongst other things confirm a lack of identity between individually purified surface peptides and FPM (see below).

1) Hydrophobic Interactions and Membrane Antigen Association. Effect of exposure to phospholipase activity. The importance for cell membrane structure of the interactions between membrane protein and phospholipid have been widely investigated. Whilst certain proteins are intimately associated with membrane lipid (integral membrane proteins) this association for others may be restricted to a few of the more hydrophobic amino acid residues. Proteins exhibiting limited interaction with membrane lipid may also have sequences exposed at the membrane surface. This, as was previously mentioned, has been postulated for the association of VSA with trypanosome surface membrane.

A useful approach to studying the role of phospholipids in a given cell membrane is the use of both phospholipases A and C as probes, (Roelefson and Zwaal, 1976) in particular for various specific membrane enzymes.

Phospholipase A effects the removal of one of the fatty acid side chains from the glycerol backbone of the phospholipid molecule and so would be expected to influence strictly hydrophobic bonding. Whilst hydrophobic interactions predominate, more polar types of bonding also appear to exert an influence, involving the charged phospholipid head groups and appropriately charged amino acid residues. Such bonding may in addition involve the participation of divalent metal ions such as Ca²⁺ or Mg²⁺. Phospholipase C cleaves the phosphorylated base from the phospholipids and would be expected to perturb

these more polar lipid-protein interactions.

In order to assess the degree to which phospholipase C exposure effected the release of FPM antigens, the procedure of McLaughlin and Muller 1981 was followed using trypsinized FPM from Fluram labelled cells. Bovine serum albumen however was omitted from the incubation mix and Sigma Type B. cereus phospholipase C (600 u/ml) was used. Utilizing FPM that had been subjected to limited trypsinolysis (see above) should have rendered membrane phospholipids more accessible to phospholipase action.

In contrast to approximately 30% of the total fluorescence label that was released after trypsin exposure only a further 4.5% was released after phospholipase C activity. This would indicate little interaction between the phosphorylated bases of FPM phospholipids and surface exposed proteins. However, analysis of the released proteins by CIEP (Fig. 5) revealed a variety of released components, most of which (Fig. 5B) were removed by an intermediary concanavalin A gel. This would appear circumstantial evidence at least for the involvement of interaction of more polar types between membrane phospholipid and FPM glycoprotein antigens.

ii. Separation of hydrophilic and hydrophobic flagella pocket membrane antigens.

To further assess the involvement of membrane lipid in binding FPM antigens an effort was made to isolate integral membrane proteins (more hydrophobic) from hydrophilic membrane protein. Numerous procedures for the differential extraction of membrane proteins according to their hydrophobic properties have been described (i.e. see review by Maddy and Dunn (1976)). Many of these methods require the use of organic solvents or detergents that often denature labile proteins. Aqueous solutions of the non-ionic detergent Triton X-114 undergo a temperature dependent phase separation and this was used by Bordier (1981) as a simple means of isolating hydrophobic from hydrophilic

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To further assess the involvement of membrane lipid in binding FPM antigens an effort was made to isolate integral membrane proteins (more hydrophobic) from hydrophilic membrane protein. Numerous procedures for the differential extraction of membrane proteins according to their hydrophobic properties have been described (i.e. see review by Maddy and Dunn (1976)). Many of these methods require the use of organic solvents or detergents that often denature labile proteins. Aqueous solutions of the non-ionic detergent Triton X-114 undergo a temperature dependent phase separation and this was used by Bordier (1981) as a simple means of isolating hydrophobic from hydrophilic

membrane proteins. In the present instance FPM (containing about 6 mg protein) was suspended in 10 ml 25 mM Tris HCl, pH 7.2, and to this was added 500 λ ice cold 10% Triton X114. Incubation at 30°C for 10 mins. caused the solution to emulsify resulting in the detergent forming minute droplets which could be sedimented from the bulk aqueous phase. In order to effect this separation the solution was centrifuged at 3000 r.p.m. for 10 minutes at room temperature using an HB4 swinging bucket rotor. This resulted in the formation of an upper aqueous phase containing hydrophilic membrane protein and a small lower phase enriched in detergent and containing integral membrane protein. The upper phase was reduced in volume by ultrafiltration to approximately 2-3 ml, before further analysis.

The antigenicity and lectin reactivity of the membrane antigens associated with each phase using CIEP is shown in Fig. 6. The most conspicuous difference between the antigenic profiles for the upper and lower phase fractions was the presence in the former (Fig. 6A) of two components a and b which totally cross reacted. These in turn partially cross-reacted with a third antigen designated component c. There is evidence from Fig. 6B that component c is also present in the upper phase. The inclusion of an intermediary concanavalin A - containing gel (Fig. 6C) removed antigens a and c but not the cross reacting antigen b. It is tentatively concluded that at least one of the FPM antigens is an integral (hydrophobic) membrane protein that is present in both a glycosylated (antigen a) and non-glycosylated form (b). These antigens partial cross react with a third more polar component (antigen c) which is also glycosylated. A poorly defined range of more hydrophilic antigens was demonstrated to be present in the upper phase and apart from component d these all appear to be glycoprotein (see Figs. 6B and D). Component d seems identical to antigen released after exposure to phospholipase C (Fig. 5B) which was also unreactive toward concanavalin A.

Unfortunately due to the very high background fluorescence exhibited by Triton it was not possible to measure the distribution of fluram labelled protein between the two phases. Clearly for this purpose a different labelling procedure such as radio-iodination would be more suitable.

5. Isolation of Flagella Pocket Membrane Antigens.

In order to further characterize flagella pocket membrane antigens, chromatographic procedures for their isolation are being evaluated. The first approach has been an attempt to dissociate intact, antigenic membranes constituent through the use of various detergents. Previous immunoelectrophoretic analysis, McLaughlin 1982, indicated the efficacy of the zwitterionic detergent, zwittergent 3-12, in combination with Triton X-100 in releasing membrane antigens, whilst having no obvious inhibitory effect upon antigen - antibody or lectin-glycoprotein binding.

The use of zwittergent has proved to have a number of other advantages: in contrast to Triton X-100 it exhibits little U.V. adsorption and so allows continuous monitoring of column effluent for protein. In order to use Triton it is necessary to use time consuming colorimetric procedures that are wasteful of sample material. Triton also has the added disadvantage of interfering with the measurement of relative fluorescence for those fractions containing labelled protein, a problem not encountered with zwittergent. Other detergents investigated, like Lubrol W or deoxycholate also exhibit none of the interference associated with Triton, but tend to come out of solution at low (cold room) temperatures. The only drawback so far in using zwittergent is the much greater cost and uncertainty so far as to its suitability for chromatofocussing. This may not be a problem since the use of zwittergent 3-12 in immuno-isoelectric focussing did not appear to influence the pH gradient (McLaughlin 1982).

a. Affinity Chromatography

The procedures investigated utilized the affinity of FPM protein for Protein A - Sepharose bound anti-P_b IgG and affinity toward concanavalin A Sepharose. The latter method appeared to be potentially useful in view of the glycoprotein nature of most FPM antigens, originally demonstrated by McLaughlin 1982 and further described in previous sections of this report. For both methods, work is in progress and the results to date will be described.

1) Lectin Affinity Chromatography.

The initial experiments, using a zwittergent extract of FPM, were not successful due to the incomplete removal of the sucrose used in the isolation of FPM. At least three changes of dialysis buffer 70·12% zwittergent in 100 mM PO₄, pH 7.2, were required, after which it was found necessary to reduce the sample volume to 2-3 ml. This allowed all the sample to be applied to the column before protein breakthrough at which time the flow of effluent was interrupted to promote more extensive binding of glycoprotein to the concanavalin A - Sepharose.

From results obtained so far 5-10% of the total protein applied is bound to the column and recovered after elution with 0.5M α -methylmannoside. For relative fluorescence 60% has been recovered after binding to the column--this figure when compared with the protein binding indicates an enrichment in labelled protein, i.e. exteriorly oriented FPM protein. Thus a significant proportion of the exteriorly situated FPM proteins are glycoprotein. Currently the use of a gradient from 0.05 - 0.5 μ M methylmannoside is being investigated as a means of trying to separate glycoproteins having different ligand affinities.

ii) Protein A-Sepharose Isolation of FPM antigens. The use of Protein A Sepharose bound anti P_b IgG as a means of isolating FPM antigens

has progressed somewhat further than lectin affinity chromatography. In the previous renewal proposal the intention was to raise a series of anti-sera to specific FPM antigens using individual immunoprecipitates removed from CIEP plates. The IgG isolated from each anti-sera was then to be coupled to CNBr activated Sepharose and used as an immunoabsorbent. However, in view of the greater convenience of Protein A Sepharose it was decided to first investigate the applicability of this adsorbant to isolating FPM antigens.

Extraction of an FPM fraction prepared from Fluram labelled trypanosomes with 0.12% zwittergent in 100mM PO_4 , pH 7.2, resulted in 75-89% of the relative fluorescence being recovered in the soluble fraction. On passing this zwittergent extract through a Protein A-Sepharose column containing bound anti- P_b IgG, 25-30% of the total relative fluorescence remains bound to the adsorbent. At this time it is not known whether the fact that 2/3 of the labelled protein is not bound accurately reflects the lack of immunogenicity of these exposed FPM proteins or is due to less than optimal antigen binding. It would seem worthwhile to investigate extending the period allowed for antigen binding from the present 0.5h. Other investigations using Protein A-Sepharose have employed time periods of up to 24 hrs. (Dissous et al 1981). An obvious test for the completeness of antigen binding would be to examine the unbound zwittergent extract using CIEP for antigen content.

After eluting the column with the 100 mM glycine HCl, pH 3.5, all of the bound Fluram labelled protein was eluted together with IgG. Gel filtration using Sephadex G-100 was used, as described in the methods, in an effort to dissociate antigens from IgG. However, all of the protein (as monitored at 280nm) and fluorescence (labelled antigen) eluted together in the void volume, no doubt due to a failure to dissociate antigen from IgG. This indicates a high binding affinity for this antigen - antibody complex and the

need for more drastic conditions for dissociation, such as the incorporation of organic solvents in the eluant buffer. For the present study the efficacy of both 10% dioxane (Andersson et al 1978) and 10% 2-chloroethanol (used by Zahler and Niggli (1977) as an effective solvent for membrane proteins) is being investigated. Whilst protein A-Sepharose, used in conjunction with SDS-PAGE, has been used previously by a number of investigators as an analytical tool for studying parasite surface antigens (Potocnjak et al 1980; Chang and Fong 1982) there appear to have been no attempts to dissociate the native antigens from IgG. The use of SDS-PAGE in the present investigation revealed only a limited number of individual peptides, apart from the prominent slow moving IgG band. The most conspicuous of these trypanosomal peptides (X, Fig.7) had a M.W. $\approx 45 \times 10^3$. This limited range of peptides compared to the number of precipitin lines after CIEP supports the probable incomplete removal of FPM antigens by the Protein A-Sepharose bound IgG. Crossed affini-electrophoresis (McLaughlin 1982) using a gel containing concanavalin A prepared using 0.15% zwittergent (figure not shown) produced a prominent precipitin line, that in view of its mobility was not IgG.

b. Chromatofocussing of Trypsin Cleaved Membrane Antigens

The membrane peptides cleaved after limited trypsinolysis of FPM prepared from Fluram labelled trypanosomes were fractionated using chromatofocussing as shown in Fig. 8. Two of the resulting peaks contained Fluram labelled peptides and eluted between pH 7.18 - 6.83 and pH 5.92 - 5.69. This coincided with the elution profile for antigenically active material (Fig. 9) as assessed by FRI using anti P_b IgG. A third component eluted between pH 6.42 and 6.1 but exhibited little fluorescence and was not antigenically active. The peak occurring at the start of the gradient, tubes 9-13, also contained labelled material and as the arrow on Fig. 9 indicates was weakly antigenic. The presence of antigenically active anionic membrane peptides

as indicated by CIEP of the unfractionated trypsin released peptides was previously discussed (see Fig. 4). A gradient using a higher initial pH would seem useful in this respect.

Concentration of the pooled antigenically active fractions, tubes 31 - 40 and 63 - 69, was affected using a UM10 Amicon filter after which the concentrated fractions were analysed using SDS-PAGE (Fig. 7). This revealed in both cases a single low molecular weight component having M.W. 13.5×10^3 (peak A2) and 12.0×10^3 (peak C2). Both concentrated pools were examined by CIEP giving similar profiles. Each exhibited a single slow moving precipitin band using anti FPM IgG (Fig. 12) but no reactivity was found using an anti SM IgG containing gel.

The range of trypsin released FPM antigens was compared with those obtained after limited trypsinolysis of whole trypanosomes. Fig. 10 shows the resulting elution profile after chromatofocussing the concentrated surface peptides (obtained as described above) using a column of PBE 94. The first peak (A) consisted of unbound material (i.e. having a pI higher than the column pH) which was resolved into several components by SDS-PAGE (Fig. 7). However, the use of FRI to monitor the antigenicity of the fractions Fig. 11A revealed an absence of reactivity toward anti- SM IgG. All of the remaining fractions were antigenic, particularly peaks D and E. In addition, these fractions, as well as those in peak C, reacted with a concanavalin A containing gel (Fig. 11B). Of much interest was the elution of Fluram labelled (surface exposed) peptides. Thus those fractions exhibiting demonstrable antigenicity (as judged by FRI) were relatively much more extensively labelled. Thus the ratio of labelled protein : total protein (relative fluorescence : adsorption at 280 nm) was for fractions A and B, 4.2 and 6.4 respectively. By comparison, for fractions C-E the ratios were 443, 210 and 330 indicating a high enrichment in surface exposed peptides.

Analysis of the pooled fractions A, C, D and E by SDS-PAGE (Fig. 7) disclosed each to have in common 2-3 peptides of M.W. 70×10^3 , 65×10^3 and 52×10^3 . Peak A contained in addition at least three other lower molecular weight minor components. Further analysis of the pooled fractions by CIEP using anti SM IgG (Fig. 12) has revealed for each 2-3 closely related components, which with the exception of precipitin peak b for pool C, show limited electrophoretic mobility. All of these components have been found to react with concanavalin A but to show little reactivity with anti-P_b-IgG (raised against crude FPM). This is shown in Fig. 10.

From the above evidence of extensive labelling, observed molecular weights and reactivity toward anti-SM IgG and concanavalin A it is concluded that these surface components are cleaved VSA. Whilst the principal reason for removing surface components from whole cells was to compare them with those obtained after mild trypsinolysis of FPM, certain other observations would seem in order. It is apparent that after limited exposure of whole cells to trypsin, as described for the present investigation, FPM components are not removed and the VSA is obtained largely intact. Possibly low levels of trypsin induce the release of VSA indirectly through influencing for instance membrane permeability. Bowles and Voorheis (1982) have described the release of VSA from T. brucei cells by inducing alterations in the Ca^{2+} flux across the surface membrane.

In contrast to T. brucei (Cross 1975), Olenick et al (1981) have revealed the VSA of T. rhodesiense to be somewhat more heterogeneous. Each variant possessed VSA of a single molecular weight but differences were observed between variants ($5.8 - 6.7 \times 10^3$) M.W. range. For individual variants VSA could be further resolved according to charge differences into 2-3 components after iso-electric focussing. These findings appear compatible with the range of molecular weight and pI values for VSA described in this investigation though, in the present instance, the use of a heterogeneous population of

T. rhodesiense limits any comparison. The reduced pI range reported above may be the result of the removal by trypsin of charged amino acid residues. In any event the observed differences in pI seem authentic properties of T. rhodesiense VSA since as reported by Olenick et al (loc. cit.) there is no evidence that they are the result of degradation during isolation. For the present study leupeptin (which inhibits proteolytic activity in T. rhodesiense see previous annual report) has been added to the concentrated surface released components with no effect on the results obtained. A final observation concerning the surface released components separated after chromatofocusing is the degree of cross reactivity that seems apparent from Fig. 11. It would be of interest to determine whether this cross-reactivity is related to that observed for VSA from different T. brucei clones (Barber & McGuire 1978) which has been ascribed to carbohydrate sequences believed attached near the N terminal end (Holder and Cross 1981). Incubation of fractions C-E with 2mMNaIO₄ (30°C for 30 minutes) does not affect their antigenicity, though as yet it is not known whether it eliminates cross reactivity.

6. Subcellular Distribution of Protective Antigens. In the last annual report the results of immunizing mice with T. rhodesiense isopycnic gradient fractions were given. This experiment has been repeated twice, once with the heterogeneous Wellcome CT strain of T. rhodesiense, and secondly using the CT clone D34 (WRAIR) isolate as challenge. In both cases protection was afforded to the immunized animals and was associated with various gradient fractions. The results obtained for the cloned strain are summarized in Table A of the Appendix.

For fraction P_a those particles recovered from the low density end of the gradient confer less protection than after isopycnic centrifugation of fraction P_b. For the latter fraction, gradient fractions 3-6 (containing flagella pocket membrane) had 13 survivors out of 20 animals; whereas, for

fraction P_a (which is not enriched in flagella pocket membrane) only 4 animals survived after 28 days.

For both fractions P_a and P_b those particles equilibrating at the high density part of the gradient (enriched in surface membrane) afforded protection. Thus for P_a gradient fractions 18-23 had 19 out of 25 survivors with 12 out of 23 survivors for P_b (2 mice died before challenge). Detracting from these results has been the scatter of positive results for fractions obtained at intermediate densities. Possibly sufficient tailing of FPM or SM particles into these intermediate fractions occurs to give rise to these anomolous results.

It was hoped to use as immunogens FPM and SM fractions isolated after using the discontinuous gradient described in this report. Unfortunately a 6 week hospitilization of the investigator's sole technical assistant has caused a temporary delay in this experiment. More meaningful results will be obtained in due course through the use of purified FPM antigens isolated using the methods previously detailed.

D) CONCLUSIONS

The information obtained previously (McLaughlin 1982) established the flagella pocket and surface membrane to be the predominant antigenic sites in bloodstream T. rhodesiense. From the results of this present investigation there is ample evidence that the principal FPM antigens are distinct from surface membrane associated antigens including VSA. Additional evidence has also been obtained demonstrating the ability of FPM antigens, as well as SM antigens, to protect mice against a challenge infection.

One area of interest is the nature of the attachment of intact antigens, or those sequences that exhibit antigenicity, to the flagella pocket membrane. Two of the FPM antigens would seem to associate with the core of the membrane having distinctly hydrophobic properties. One of these was present as both a

glycosylated and non-glycosylated protein, the former possibly being the form in which the nascent polypeptide chains are transported from the endoplasmic reticulum/Golgi apparatus for insertion into the membrane. It is unlikely that these integral membrane antigens would be of importance in conferring protection against infection.

There was a greater range of hydrophilic, therefore more peripherally localized antigens, though these have not been adequately resolved. Most of these antigens appear to be glycoprotein, apart from one component that was also extractable after phospholipase C exposure. Treatment with phospholipase C also revealed the apparent importance of interaction of a more polar type between membrane phospholipid and FPM antigens. If the carbohydrate groups are buried within the membrane, as they appear to be for VSA (Holder and Cross 1981) then interaction between charged groups on these glycosyl sequences and the polar head groups of phospholipid could be important. Preliminary evidence suggests that these carbohydrate sequences are not necessary for the expression of antigenicity.

Attempts to purify FPM antigens have yielded a single polypeptide, as deduced from SDS-PAGE of M.W. $\approx 45 \times 10^3$, lower than reported values for VSA. This same antigen does exhibit reactivity toward concanavalin A. Limited trypsinolysis of intact cells has released VSA largely intact, though a range of M.W. and PI values were exhibited. There was no evidence of cleavage into the large N terminal and smaller C terminal fragments as originally reported by Johnson and Cross 1979 for purified VSA. No FPM antigens were cleaved, indicating the incubation time was not sufficient for trypsin access.

Using isolated FPM at least two closely related peptides (M.W. $\approx 12,500$) with different iso-electric points were released. These proved to be antigenic using antibodies raised against a crude FPM fraction, but not with anti surface-membrane antibodies. These low molecular weight antigenic peptides

were not released from whole cells and would seem to be fragments of antigens limited to the flagella pocket membrane.

A recent paper by Mancini et al 1982 describes the isolation of surface membrane from T. brucei, and has revealed, in agreement with our findings a range of glycosylated polypeptides apart from VSA. In addition some of these components were believed to occupy an integral (i.e. not exposed) localization. Unfortunately the method used by these investigators to rupture the trypanosomes (sonication) clearly removes microtubules from the surface membrane thus causing them to sediment at a lower density than for previous investigations (see McLaughlin 1982). In addition 3'-nucleotidase activity was found to be wholly soluble whereas the enzyme is clearly membrane bound in T. rhodesiense broken with glass beads (McLaughlin 1982). The conclusion to be drawn from this is that the conditions for cell breakage used by Mancini et al (loc. cit.) were too drastic. For this reason they fail to distinguish between surface membrane and flagella pocket membrane. As the results obtained in the present study show, antigenically at least these two sites are quite different.

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Enzyme	Membrane Fraction	
	SM	FPM
Phosphatase		
-glycerophosphate	10	15
-glycerophosphate	4	25
Arylamidase	3	28
Mg - ATPase	12	2
-glycero PO_4 dehydro NAD	0	0
-glycero PO_4 dehydro DCPIP	0	0.2
Relative Fluorescence	145	131

Table 1. Enrichment in specific activity relative to the original cell homogenate for certain marker enzymes and Fluram labelling. Enzyme assays and the determination of specific activity were as previously described (McLaughlin 1982).

TABLE A Immunization of mice using fractions recovered after isopycnic centrifugation of *T. rhodesiense*, (Wellcome CT strain). Fractions P_a and P_b were subjected to isopycnic centrifugation as previously described (McLaughlin 1982). Each gradient fraction was prepared for injection and administered as described in the previous annual report. For each fraction, groups of 5 mice were used with two groups of 5 mice as controls. These latter groups were given a 1:3 mixture of Freund's complete adjuvant and buffered sucrose. Mice were challenged with 2000 trypomastigotes of the Wellcome CT strain clone. All of the control group mice were dead within 4 - 5 days.

Fraction Number	Mice Surviving After 28 days	
	P _a Gradient	P _b Gradient
1	1	0
2	0	0
3	1	3
4	2	4
5	0	4
6	1	2
7	2	1
8	4	4
9	2	2
10	1	4
11	3	4
12	3	3
13	2	4
14	3	2
15	1	0
16	2	1
17	1	4
18	3	4
19	3	3
20	3	3
21	4	3
22	3	1
23	3	0
Control Group	0	0

Fig A. Attachment and alution of FPM proteins from a Protein A - Sepharose column containing immobilized anti P_b - IgG.

The FPM proteins were obtained by extracting a washed FPM fraction with 0.12% zwittergent. 1 = zwittergent extract of FPM fraction applied to column. S = eluant flow stopped for 0.5 all antigen to react with immobilized anti P_b - IgG. 2 = elution with 100 mM glycine - HCl, pH 3.0, commenced.

Fig. 1. Crossed immunoelectrophoresis of T.rhodesiense subcellular fractions.

The fractions were obtained using the discontinuous sucrose gradient and Percol gradient described in the Methods. All fractions and gels were prepared in 0.12% zwittergent 3-12 and 0.4% Triton X-100. (A) Material removed from 42/54 interface after discontinuous gradient centrifugation of fraction pa. (B) The upper less dense fraction (surface membrane enriched) obtained after Percol density gradient centrifugation of fraction pa 42/54. (C) as for F using anti Pa - IgG. (D) as for (B) except the dense (glycosome) fraction was used after Percol density gradient centrifugation. (E) Material removed from the 35/42 interface (lysosome/mitochondria) after discontinuous gradient centrifugation of fraction Pa. (F) flagella pocket membrane (FPM) fraction removed from 23/35 interface after gradient centrifugation.

For A,B,C,D,E anti Pa IgG was used, for F anti P_b - IgG.

Fig. 2. Demonstration of phosphatase activities associated with immuno - precipitates obtained after CIEP of flagella pocket membrane.

In all cases anti P_b IgG was used in the gels with all other conditions as described in Fig. 1. The demonstration of enzymic activity follwed methods described by McLaughlin and Meerovitch (1975).

(A) Glucose - 6 - phosphate (B) α - glycerophosphate (C) β - glycerophosphate (D) α - naphthol phosphate.

Fig. 3. Reactivity of flagella pocket and surface membrane antigens with peanut lectin.

CIEP was performed with an intermediate gel containing 40 μ g/cm² PN lectin with (A) having an upper gel containing anti P_b IgG and (B) an upper gel containing anti Pa - IgG. (A) washed FM pellet as antigen (B) fraction Pb 42/54 (crude surface membrane).

Fig. 4. Antigenicity of FPM peptides released after limited trypsinolysis.

(A) CIEP of FPM trypsin cleaved peptides against anti- P_b IgG
(B) as for A with the lower gel containing 80 μ g/cm² concanavalin A.

Fig. 5. Released of antigenic components from trypsinized FPM membrane after exposure to phospholipase C.

(A) CIEP of released components against anti P_b IgG. (B) As for A with a lower gel containing 80 μ g/cm² concanavalin A.

Fig. 6. Comparison of hydrophobic and hydrophilic membrane protein antigens after Triton X-114 extraction of FPM.

(A) lower phase components (hydrophobic membrane antigens) demonstrated

using CIEP with anti Pb IgG. (B) Upper phase (hydrophilic) membrane protein demonstrated using CIEP as for (A). C and D as for A and B respectively with an intermediary gel containing 80 μ g concanavalin /cm².

Fig. 7. Analysis of all surface and FPM antigen fractions by SDS-PAGE. A,C - trypsin released FPM peptide antigens : pooled antigenic fractions (see Figs. 8 and 9) after chromatofocussing. B = pooled fractions recovered from protein A - sepharose immobilized anti- Pb IgG column, D,E,F,G = fractions A,C,D and E eluted from PBE 94 column after chromatofocussing trypsin released cell surface peptides.

Standards, trypsin (15,000 M.W.); myosin (18,400 M.W.); aldolase (40,000 M.W.); hexokinase (51,000 M.W.); bovine serum albumen (66,000 M.W.).

Fig. 8. Chromatofocussing elution profile of peptides released after limited trypsinolysis of FPM.

A 40 x 1 column of PBE 94 was equilibrated with 25ml Tris-acetate pH 8.4 and the sample applied after dialysis against the same buffers. For elution polybuffers PB 74 and PBab were mixed according to the methods and a flow rate of 17ml/hour maintained.

x x relative fluorescence of content of approximately every other tube collected.

o o pH of every other fraction.

— Absorbance at 280 nm.

Fig. 9. Fused rocket immunoelectrophoresis (FRI) of fractions recovered after chromatofocussing of trypsin cleaved FPM peptides (see Fig. 8).

Antigen wells received 12 μ l of each fraction. The first antigen well received an aliquot from fraction 10 (protein breakthrough) and succeeding fractions were used for the remaining antigen wells (to fraction 80). The samples were electrophoresed into an anti-Pb containing IgG.

Fig. 10. Elution profile for trypsin released cell surface peptides after chromatofocussing on PBE 94.

Surface peptides cleaved after limited trypsinolysis of whole cells were concentrated by ultrafiltration and dialysed against 25mM imidazole - HCl pH 7.4. This dialyzed solution was then applied to a 40 x 1 cm column of PBE 94. Symbols used are as in Fig. 8.

Fig. 11. Fused rocket immunoelectrophoresis of fractions recovered after chromatofocussing trypsin released cell surface peptides (see Fig. 10).

(A) Fractions electrophoresed into gel containing anti SM-IgG

(B) as for (A) but with intermediate concanavalin A containing gel.

Fig. 12. CIEP of pooled fractions recovered after chromatofocussing trypsin released cell surface peptides and FPM peptides.

A = Pooled fractions A B = Pooled fractions B (both obtained after chromatofocussing FPM peptides) C = Pooled fractions C after chromatofocussing cell surface peptides D = as for C but pooled fractions D; E = as for D but intermediate concanavalin A gel.

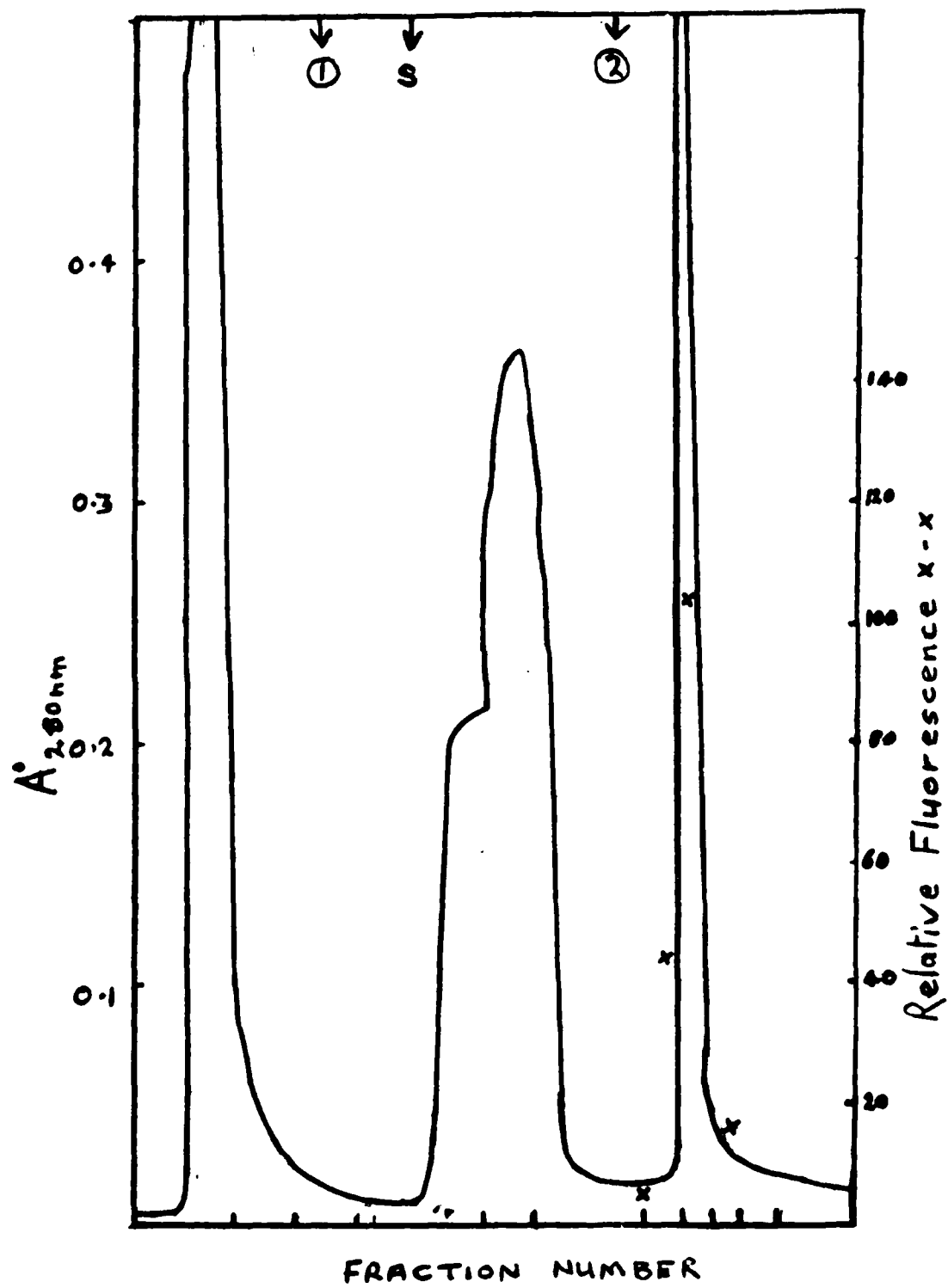


Fig A

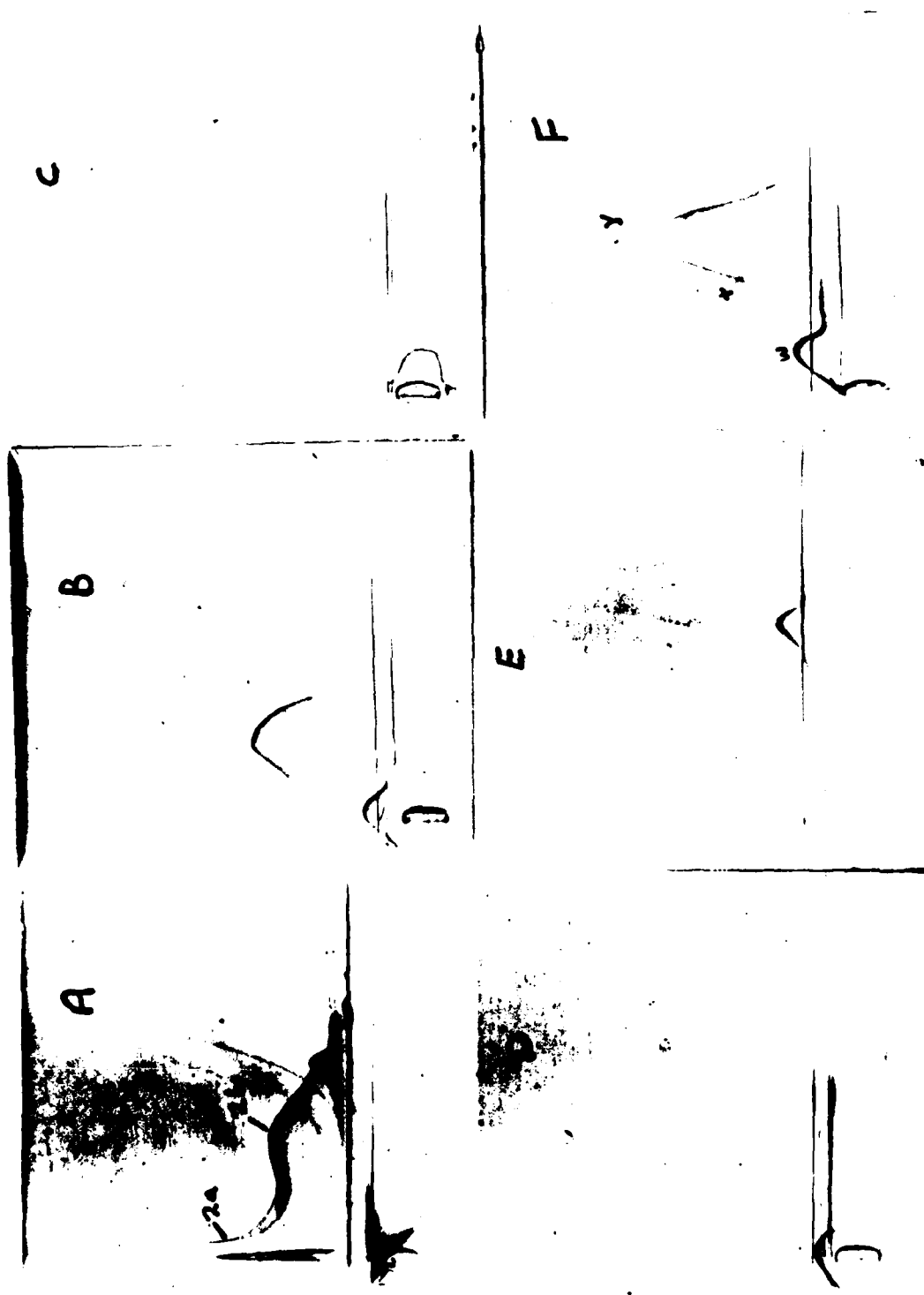
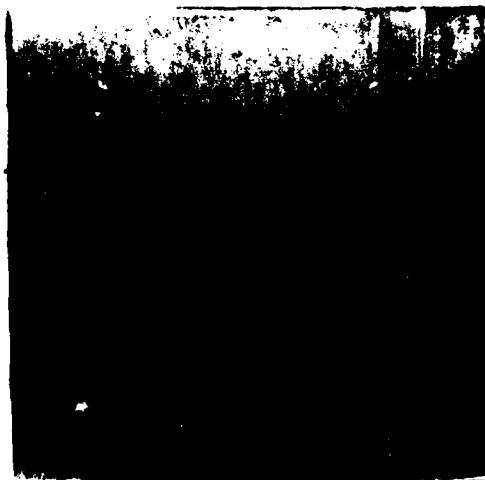


Fig 1

Fig 2

B

↖ ↗



R

↖ ↗

C

↖ ↗



Fig 3

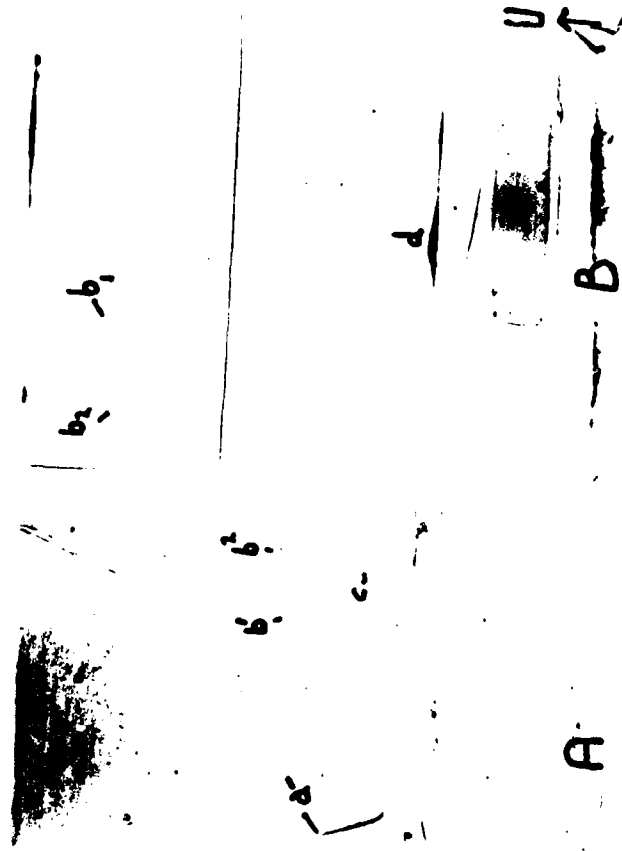


Fig 4

Fig 5

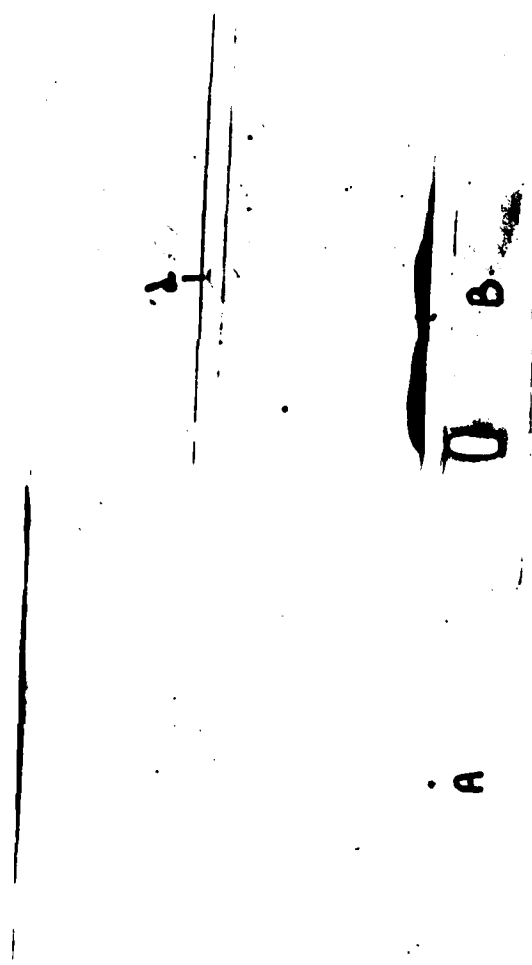
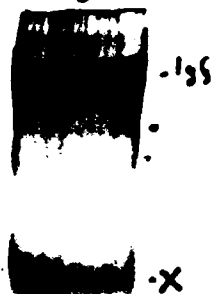




Fig 6

A B C D



D E F G

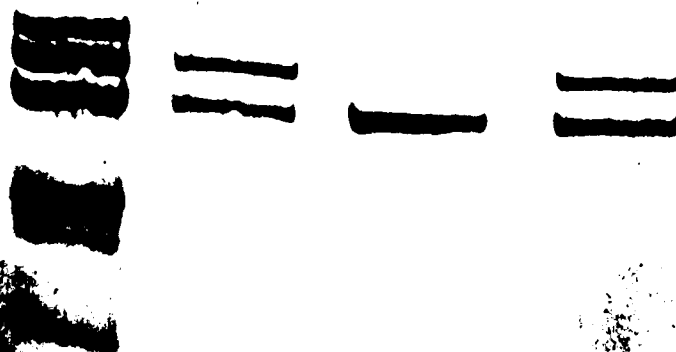


Fig 7

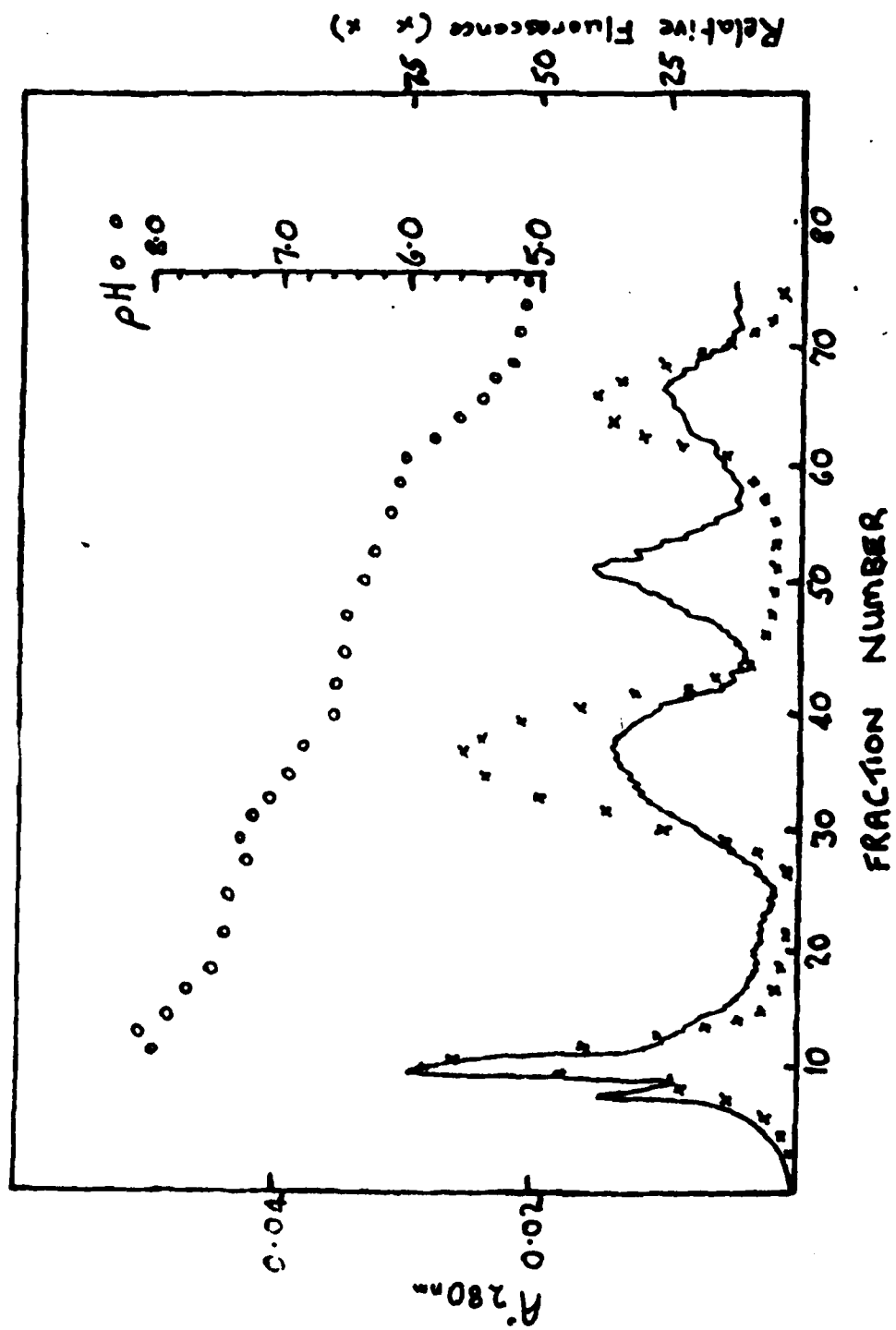


Fig. 8



10 20 30 40 50 60 70 80

Fig 9

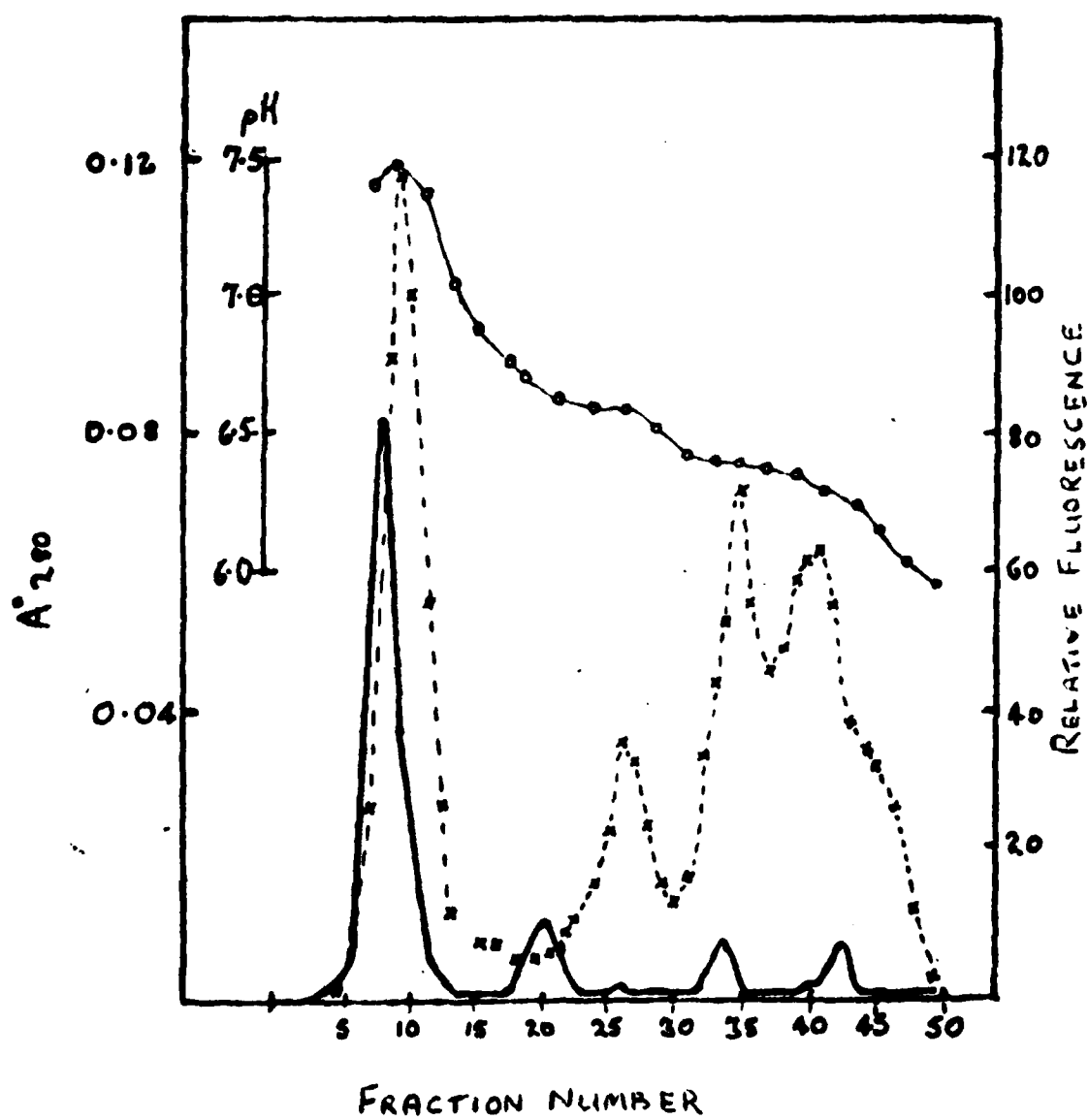
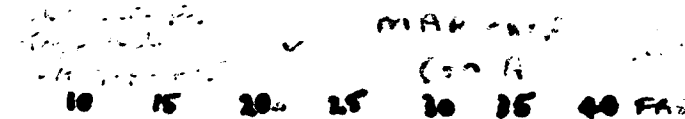


Fig 10



A



B

Sm IgG

Con A



Fig 11

Figure 11

Figure 12

A

B

D

E

ATE
LMED
-8