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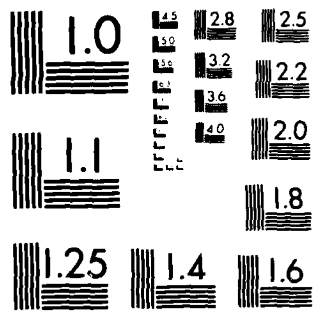

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DISTRIBUTION AND CHARACTERIZATION OF ANTIGENS FOUND IN *SUBCELLULAR FRACTIONS OF AFRICAN TRYPANOSOMES*

Annual Report

John McLaughlin

August 1981

Supported By

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FORWARD

Experiments involving laboratory animals have been in accordance with all relevant guidelines regarding humane treatment.

SUMMARY

This report describes the experiments to determine the subcellular localization of antigens in the African trypanosome Trypanosoma rhodesiense. Most of the antigenic components are associated with the surface membrane and flagellar pocket membrane. One prominent lysosomal antigen is present. At least 5 of the flagellar pocket antigens are glycoprotein and two of these display identical electrophoretic mobilities with surface membrane glycoproteins.

Preliminary experiments show the surface membrane and lysosomal fractions to be the most protective against challenge infection with T. rhodesiense. The lysosomal and flagellar pocket membrane fractions were also protective. Neither the promitochondrial or glycosomal fraction appeared particularly antigenic or to have protective potential.

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A. INTRODUCTION

This report details work performed during the period August 20, 1980 through August 21, 1981 being the last 6 months of the second year of this project and the first 6 months of the current (second) renewal period (March 1, 1981-February 28, 1982).

The major effort was expended in a study of the distribution and partial characterization of the antigens associated with particle fractions recovered from cell homogenates of Trypanosoma rhodesiense. Using fused rocket immunoelectrophoresis of fractions obtained after isopycnic centrifugation, it was possible to localize the subcellular distribution of antigens by comparing the immunoprecipitin profiles with those for previously established marker enzymes and a covalent surface label. Using crossed immunoelectrophoresis, affini-immunoelectrophoresis and immuno-isoelectric focussing it has been possible to further characterize pooled fraction from those parts of the gradient displaying the most diverse array of antigens.

B. METHODS

1) Maintainance and collection of parasites

The procedures followed were as described in previous annual reports, all infected animals being provided by the Rane Laboratory, University of Miami.

2) Enzyme Assays

Methods used for most of the enzyme activities measured in this report have been detail previously (Reports 1 and 2). The following assays, utilized in this report were not then described:

a) Neutral proteinase (elastase like proteinase)

To 15mM n-t-Boc-alanine-2-nitrophenol in 75mMPO, pH 6.5 was added enzyme (fraction) to a final volume of 1.0ml.. The appearance of the free nitrophenate ion was followed spectrophotometrically at 37°C by measuring absorbancy at 410nm. Specific activities were calculated using a calibra - tion curve.

b) Membrane associated leucine-aminopeptidase

The overall procedure for this assay was described in the previous annual report. Further investigation has revealed the membrane associated enzyme has no divalent metal requirement, unlike the "typical" leucine aminopeptidase present in the cell sap of T. rhodesiense. The protocol now followed uses 100 λ 75mM HEPES, pH 7.8, 250 λ 100mM leucinamide, and 40-80 λ enzyme in a final volume of 500 λ. Released ammonia was then measured as described in the second annual report.

c) 3'-Nucleotidase

The assay mix contained 10mM 3'-AMP 30mM Tris-HCl, pH 8.5, containing 2mM $CoCl_2$, 0.1% Triton X-100 and enzyme in a final volume of 1.0ml. After a 20 minute incubation at 30°C the reaction was stopped by adding 250 λ 6% sulfosalicylic acid then after centrifuging at 3000rpm for 10 minutes the supernate removed and released orthophosphate determined using the method of McLaughlin and Meerovitch 1975.

3) Antigenic analysis of sub-cellular fractions

The second annual report described in part the immunoelectrophoretic procedures employed. However certain modifications have been made so the methods now in use are presented below.

a) Immuno-electrophoretic Procedures

i) Fused Rocket Immunoelectrophoresis. The technique of Svendsen 1973 was adapted for present use as follows. A 1% solution of agarose (Bio-Rad) was prepared in 50mM Tris-133mM glycine, pH 8.55, to which one of a range of detergents was added. The actual detergents and their concentrations are given for individual experiments as described in the results section.

Gels were cast using 10ml molten agarose solution on a 70 x 100mm sheet of hydrophilic support film (F.M.C.) and a row of 25 sample holes cut, 20mm from the short edge, using a 2.0mm gel punch and template. A 70 x 75mm area of gel immediately above the sample holes was removed and replaced with 6.5ml molten agarose, cooled to 60°C, containing 0.35-0.55ml of the appropriate lgG fraction. For fused rocket affinielectrophoresis the molten agarose contained concanavalin A at 100_{μ} g/cm^2.

For antigen samples 100 λ aliquots of each fraction obtained after isopycnic gradient centrifugation was removed and one of a range of detergents added, the type and concentrations being stated for individual experiments. Details of procedures for isopycnic gradient centrifugation of T. rhodesiense have been given in previous annual reports and a related publication (McLaughlin 1981). The distribution of selected marker enzymes and a covalent surface label is presented in the results section. The detergent treated fractions were applied in sequence to each antigen well (15-20λ) using a positive displacement micropipettor (5ma) and allowed to diffuse for 40 min. Electrophoresis was initiated, using the same Tris-glycine buffer as above in the electrode tanks, with a contant voltage of 1.5V/cm (actual output 90V) for 18.0hs. The gels were then washed in repeated changes of 0.5M NaCl over a 48h period, washed in distilled water for 2-3h and stained using the Coommassie Brilliant Blue R-25-/$HgCl_2$ procedure described by McLaughlin and Meerovitch (1975).

Where immunoprecipitates were to be stained for enzymic activity *electrophoresis was performed* at 4°C. Subsequent washing and staining are described in the results section for individual enzymes.

ii) Crossed Immunoelectrophoresis. Gradient fractions exhibiting the most antigenic diversity as revealed by FRI were further analyzed using crossed or tandem immuno-electrophoresis (McLaughlin and Meerovitch, 1975; Kroll, 1973). Detergents used for antigen extractions and first and second dimension electorphoresis are given for individual experiments. For first dimension electrophoresis a series of 7.0 x 1.5mm sample slots were formed in a 1% agarose layer cast on a 70 x 100mm glass plate. The use of glass plates, rather than the plastic support film facillitated subsequent removed of gel strips for second dimension electrophoresis. After applying a 25λ pooled sample, electrophoresis was performed with bromophenol blue stained serum albumin as a marker, for 1.5-2.0h using a potential difference of 7.0V/cm. Both gel and electrode buffers were as detailed above. For tandem crossed immunoelectrophoresis an additional row of sample slots was formed 10mm above the first and contained the second antigen sample to be compared for cross reactivity.

After first dimension electrophoresis strips of agarose containing the electrophoresed samples were removed to a sheet of gel support film 1.5cm from the cathodic end. The area above the sample strip was covered with molten buffered agarose containing antibody or lectin, prepared as above. For some experiments split gels were used with an intermediate concanavalin. A containing gel and an antibody containing gel above. The area below the sample strip was covered with buffered agarose and electrophoresis commenced using the same buffer as above with a constant voltage of 1.5V/cm for 18h. Washing and staining of gels was performed as for crossed immunoelectrophoresis.

iii) Immuno-isoelectric focussing. Gels for iso-electric focussing were prepared using agarose specially prepared for iso-electric focussing (Agarose IEF, Pharmacia) by modifying the manufacturers recommended procedure. Into 12-0ml deionized water were dissolved 0.15g agarose IEF and

1.5g sorbitol, at which time 0.75ml 2% Zwittergent 3-12 and 1.5ml 2% Triton X-100 were added. Hjelmeland et al. (1979) found mixtures of Zwitterionic and non-ionic detergents to improve iso-electric focussing of membrane proteins. The usefulness of Zwitter-ionic detergents is discussed in the results section. After cooling the gel solution to $70^{o}C$ 0.9ml ampholyte solution (Pharmalyte, Pharmacia) was added, pH range as indicated for each experiment. The molten agarose was then poured over a 110 x 100mm sheet of gel support film. A series of five sample slots (7.0 x 1.5mm) were formed across the middle of the gel which was then allowed to set at $4^{o}C$ for 2-3 hours. The pooled samples to be analyzed, corresponding to those used for crossed immuno electrophoresis were solubilized by adding Triton x-100 and Zwittergent 3-12 to a final concentration of 0.25% and 0.15% respectively. After applying a 25λ sample to each slot a constant voltage of 15V/cm (actual output 160V) was applied for 24 hours, with 0.05M H_2SO_4 in the anode tank and 0.5M NaOH in the cathode tank.

The support film containing the focussed samples were cut into strips which were placed gel side down across a 70 x 100mm sheet of clean support film. The samples were then electrophoresed into an antibody or lectin containing gel as described for crossed immunoelectrophoresis. Since agarose IEF gels proved to be rather friable it was not possible to remove gel sample strips intact from glass plates. For this reason the gels were cast on support film and transfered still adhering to the film for electrophoresis.

Antibody production and the isolation of lgG fraction was performed as described in the second annual report except that it was found that 3 antigen injections rather than 5 were sufficient prior to subsequent booster immunizations.

C. RESULTS

1) Further investigations of fluorescamine labelling of *T. rhodesiense*. In the second annual report it was concluded that no exclusive surface membrane enzyme(s) was present that could be conveniently assayed, a view still valid as will be discussed below in section C-2. For that reason the use of a surface membrane covalent labelling procedure employing the fluorogenic reagent fluorescamine was investigated. The procedure employed and results regarding the distribution of the label after gradient centrifugation were also discribed.

Since that time it has been found that labelling is as effective using a fluorescamine solution containing 5mg/ml acetone as the original 10mg/ml. The present procedure is described as a footnote to Fig. 1. The distribution after differential centrifugation of relative fluorescence following the labelling of intact as opposed to disrupted cells is shown in Fig. 1. In the former case, Fig. 1a, cells were maintained in iso-osmotic sucrose, labelled, disrupted with glass beads, and fractionated as previously described (see previous annual reports). In the latter instance the cells were first disrupted then labelled and subsequently fractionated (Fig. 1b). As can be seen from Fig. 1b, fluorescence is distributed almost equally, based on relative specific activity, between the various fractions, with 50-60% of the total fluorescence being found in the final soluble fraction (cell sap). This differs from intact cells (Fig. 1a) where fluorescence of the soluble fraction is greatly reduced whilst that for the particulate fractions, especially the nuclear pellet and fraction P_b are much increased. This indicates that for intact cells the surface membrane serves to restrict access of fluorescamine to cytoplasmic components (i.e. cell sap). Such results complement findings previously reported that it is surface and flagella pocket membrane that is principally being labelled.

Using the lower concentration of fluorescamine and ensuring that only freshly isolated trypanosomes are used, the amount of fluorescence recovered in the final soluble fraction has been reduced from 20% to 12-17%. This is considerably more than the 5% upper limited suggested as acceptable by Hubbard and Cohn 1976 for other cell surface labels. However this higher recovery almost certainly reflects certain characteristics unique to trypanosomes. It is probable that labelling of the soluble contents of the flagellar pocket occurs but a more important factor is the almost certain detachment of labelled surface glycoprotein coat (VSA). The ease of removal of this coat material was revealed in the original description of attempts to surface label *Trypanosoma brucei* (Cross, 1975).

2) *Final conclusions regarding marker enzymes.* Certain conclusions regarding the subcellular localization of the various *T. rhodesiense* marker enzymes studied were presented in both of the previous annual reports. In addition some of these results, specifically those concerning certain glycosomal enzymes, have also been published (McLaughlin, 1981).

Some additional enzyme activities have also been investigated for their potential interest.

a) *3'-Nucleotidase.* Although there is no evidence for any specific 5-nucleotidase, an active Co^{2+} stimulated 3'-AMP is present (specific activity = $70 \pm 12 mU^{-1} mg\ protein^{-1}$). The enzyme is optimally active at pH 8.8 in the presence of 2mM Co^{2+} and has a K_m=0.8mM. Goltlieb and Dwyer (1981) found a highly active 3'-nucleotidase in the trypanosomatid, *Leishmania donovani* which was localized exclusively in the surface membrane. From distribution plots obtained after gradient centrifugation, Fig. 2, it is evident that the *T. rhodesiense* enzyme is not a surface membrane enzyme. Part of the activity is associated with a particle population having an equilibrium density of 1.13 with a broad shoulder equilibrating around ρ =1.17. The lower density particles occur in that part of the gradient where flagella pocket membrane would be expoited. However the equilibrium density for 3'-nucleotidase is slightly higher than that for established flagella pocket marker enzymes (i.e. acid phosphatase, arylamidase).

b) Neutral proteinase (n-t Boc-alanine-2-nitrophenol). This artificial low molecular weight substrate has been used widely as a substrate to demonstrate elastase activity (Barrett, 1977) and was of interest in view of the extensive breakdown of connective tissue seen in African trypanosomiasis (Goodwin et al., 1973). If such a proteolytic enzyme were found in T. rhodesiense it would be of interest to investigate possible immunoinhibition. Whole cell homogenates do hydrolyze this substrate (110nmoles p-nitrophenol per hr per mg protein), with activity being distributed principally in fraction P_a as shown in Fig. 2b. The activity associated with this fraction was found to be optimally active at pH 6.0-6.5 with a K_m=6.25mM. From the results of inhibitor studies activity was unaffected by thiol blocking agents (chloromercuribenzoate), serine protease inhibitors such as phenylmethane sulfonyl fluoride. In addition trypsin inhibitor and leupeptin, which inhibits the T. rhodesiense thiol proteinase, were without effect. The tosylated chloromethyl ketone of lycine (TLCK) partially inhibited enzyme activity (52%) suggesting more than one enzyme was responsible for hydrolysis of the substrate. Activity against the unblocked substrate, alanine-2-naphthylamide, unlike n-t-Boc-ala-pnitrophenol was identical to that splitting the other amino acid naphthylamides i.e. fraction P_b was more enriched than fraction P_b and the enzyme was highly susceptible to inhibition by mercuriberzoate and TLCK.

A more specific alanine tripeptide substrate for elastase (Succ-(Ala)$_3$-p-nitroanilide) was not hydrolyzed by T. rhodesiense. Only slight breakdown of elastin congo red is evident thus indicating this proteinase not to be synomomous with true elastase.

Leucine aminopeptidase. This enzyme was of interest since it has a surface membrane localization in a number of cells. These were indications of part of this activity being associated with the surface membrane in T. rhodesiense (second annual report). Since then it has been found that there is a leucine aminopeptidase activity present which requires no divalent metal (in contrast to the cell sap enzyme) and which shows similar distribution profiles to the various arylamidases (amino acid naphthylamidase). Indeed there is clear evidence from substrate competition plots that the arylamidase and aminopeptidase activities occurring in faction P_b are due to the same enzyme.

Table 1 shows the range of enzyme activities that have been detected in T. rhodesiense and their probable subcellular localization. These results are based upon previous investigations with T. rhodesiense and published work on other bloodstream African trypanosomes.

3) Antigenic Analysis

The T. rhodesiense gradient fractions used for antigenic analysis were characterized with regard to the distribution of surface label and a range of marker enzyme activities. For the most part the localization of these markers with specific subcellular sites has been established in previous annual reports. Fig. 4 displays the enzymatic and surface labelling profiles of the fractions used for immunoelectrophoresis. It should be noted that in these experiments the high speed pellet, that was subject to isopyenic gradient centrifugation, was derived from a post-nuclear supernatant fractions obtained by centrifuging the initial homogenate at 3500rpm (SS 34 rotor) rather than 2500rpm for 4 minutes, (see second annual report). This modification was necessary because of the intense smearing and poor resolution of precipitin lines associated with the high density particle antigens. For this reason the proportion of surface membrane present was less and this is apparent from the reduced levels of marker enzymes such as β-glycerophosphate and leucine aminopeptidase present in the high density region of the gradient.

a) Fused Rocket Immunoelectrophoresis. The distribution of antigens extracted in the presence of two non-ionic detergents (Triton X-100 and Lubrol W) are shown in Fig. 7 using gels containing both anti P-A and anti P-B IgG. The principal immunoprecipitates were formed between density increments 1.10-1.15 (flagella pocket membrane) and 1.20-1.22 (surface membrane). Also present in all cases was a broad precipitin peak (ly), sometimes as in Figs. 7B, C and E with double peaks, formed between demities 1.130-1.20. This corresponds with the sedimentation profile of the lysosomal thiol proteinase. Also apparent in Triton extracted samples, but only using anti P-B IgG, were two peaks formed at the very bottom of gradient (Fig. 7B, C). These correspond to the sedimentation of the various glycosomal marker enzymes. At the very top of the gradient (first 3 fractions) were 2-3 small peaks presumably formed by cell sap components still present in the high speed pellet prior to centrifugation. The other principal organelle, the promitochondrion, from marker enzyme evidence equilibrated in that part of the gradient (p 1.14) showing little evidence of antigenicity. Possibly the precipitin line (l) present in the two Lubrol extracted samples (Fig. 8 E and F) is of mitochondrial origin.

As was expected gels containing anti P-A IgG were more reactive toward surface membrane antigens (Figs. 7A, D) whilst anti P-B IgG containing gels were more reactive toward flagella pocket membrane. From Figs. 7E and F Lubrol did not appear to extract the range of antigens from flagella pocket membrane seen after exposure to Triton. However one very prominent antigen was extracted (fp) with some evidence of partially identity with the lysosomal antigen (ly) from Fig. 7F. From the profiles obtained it was possible to discern at least 8 antigens components present in the surface membrane fractions and 4 in the flagellar pocket membrane. The use of crossed immunoelectrophoresis proved more useful in resolving the number of individual antigens.

The use of the zwitterionic detergents (zwittergent series) have been claimed to produce more complete extraction of integral membrane proteins than non-ionic detergents without the denaturing effects of solvents such as sodium dodecyl sulphate (Gonenne and Ernst, 1978). Fig. 8 A-I shows a series of immunoprecipitation profile using gradient fractions after exposure to Zwittergent 3-12 both alone and in the presence of a non-ionic detergent. The use of Zwittergent alone was not entirely successful. Whilst certain antigens appeared to be extracted as before (flagellar pocket antigen fp) much of the precipitation occurred before the samples had migrated into the antibody containing gel. This probably was due to increased cathodic migration of IgG due to binding of Zwittergent. With 0.1% Zwittergent, Fig. 8C, which is below the critical micellar concentration (0-15%) antigen samples migrated into the antibody gel but with clear evidence of "ghosting" (see arrows).

Figures 8D-I show the influences of non-ionic detergents on the pattern obtained with Zwittergent. From Fig. 8E, F, where 0.5% Triton X-100 was employed in addition to 0.1% Zwittergent, the ghosting effect is eliminated and the extraction of both flagellar pocket and especially surface membrane antigen appear extensive. However there is still some precipitation of samples before migrating into the antibody gel. The same problem is encountered using Zwittergent in the presence of Lubrol W (Fig. 8H,I) plus extensive smearing of precipitin lives. In summary whilst Zwittergent in the presence of Triton X-100 appeared to effect excellent solubilization of membrane antigens it was not possible to totally overcome the problem of increased cathodic migration of IgG.

The possible usefulness of sonication is shown in Fig. 8J. The sonicated samples (2.0 min exposure per sample 300W, Braun sonifier) were electrophoresed into an anti-P-A IgG containing gel in the presence of 0.5% Triton X-100. The result were not very satisfactory, though antigen 1 may represent more complete extraction of the mitochondrial antigen (Fig. 7).

In order to identify gradient fractions containing lectin binding components (presumably glycoproteins) gradient fraction were extracted with 0.1% Zwittergent (Fig. 9A) or 0.5% Triton X-100 and electrophoresis into a concanavalin A containing gel. Marked reactivity is associated with the surface membrane fraction (sm) and to a lesser extent flagellar pocket membrane (FP). In Fig. 9B a split gel was used the upper gel (1) containing anti P-B IgG and the lower gel (2) concanavalin A. Noticeable is the obvious reduction in the range of antigenic components associated with both FP and SM, especially the former. It also appeared that Zwittergent was far superior in extracting lectin binding proteins than Triton X-100.

b) Crossed Immunoelectrophoresis. By means of crossed immunoelectrophoresis it was possible to further resolve the flagellar pocket and surface membrane antigens. From Fig. 10A it is apparent that with Triton X-100, fraction pooled form densities 1.195-1.22 (surface membrane) up to 10 antigenic components were extracted.

The inclusion of the proteinase inhibitor leupeptin (which totally inhibits the thiol proteinase of T. rhodesiense) during cell disruption has not influenced the subsequent pattern of precipitin lines. In common with studies of VSA it could be argued that some specific proteinase unaffected by more common inhibitors (as for instance cathepsin D)) is present.

Tandem crossed immunoelectrophoresis permitted a direct comparison of the antigens found in flagellar pocket and surface membranes. From Fig. 10B there is no evidence of any extensive cross reaction between antigens from these two sites. Possibly the apparent merging of precipitin lines of antigens b and x may be indicative of some cross reactivity.

Figures 11A, B show results obtained using crossed immuno-affinielectrophoresis with an intermediate gel containing concanavalin A. The apparent efficacy of Zwittergent 3-12 in extracting lectin binding components was used for the first dimension electrophoresis. At least 4 concanavalin A reactive proteins were distinguished in the surface membrane with component M being the most perminent.. As many as 5 reactive proteins were present in the flagellar pocket membrane extract, two of which (M^- and N^-) exhibited identical mobilities to those detected in the surface membrane.

Whilst Con A absorbed out most of the antigenically active material from the flagellar pocket membrane extract (Fig. 11B), a diverse array of antigens still remained in the surface membrane preparation (Fig. 10A). It must be remembered that some of the precipitin peaks almost certainly represent antigens not associated with either of these subcellular sites, since undoubtedly low levels of other cell components are present (i.e. flagella fragments in the denser part of the gradient). In reference to the number of surface membrane antigens noted in this investigation, Vooheis et al. (1979) reported at least 10 polypeptides that were separated after SDS-acrylamide gel electrophoresis of a T. brucei surface membrane preparation. None of these however were considered to be glycoprotein based upon negative Schiff staining, the author concluding that all of the surface glycoprotein coat was cost during membrane isolation. There is evidence from the study of Rovis et al. (1980) that up to 40% of the total VSA in T. congolense requires dsetergent treatment for extraction. The surface membrane glycoproteins detected in the present study may not be derived from surface coat glycoprotein. There is no evidence at present to suggest that they are the result of degradation of VSA. Clearly there is a need to establish the reactivity of this range of glycoproteins against antibodies to purified VSA.

Other studies suggest trypanosomatid surface membrane to possess a range of glycoproteins. In a study of the glycosylation of T. brucei surface membrane glycoproteins Brett and Voorheis (1980) found up to four protein acceptors for various sugars supplied. An extensive range of surface membrane glycoproteins has been demonstrated by Dwyer 1981 in Leishmania donovani, 24 of the 40 total membrane peptides containing carbohydrate moieties.

c) Immuno-isoelectric Focussing. The results obtained by this procedure have been somewhat disappointing so far as the separation of surface membrane antigens in concerned. Between the pH limits pH 5.0-8.5 (Fig. 12C,D) produced a range of prominent antigens having pI=7.2—at least 4 antigens could be discerned. At pH=5.6-6.6 a broad precipitin band formed showing two peaks, other minor components could be seen in Fig. 12C. In both Figs. 12C and D was a faint precipitin peak having an identical pI (5.9) to the major component found in flagellar pocket membrane (Fig. 12A). The most obvious finding was the difference in the iso-electric points of the two principal antigens associated with flagellar pocket and surface membrane. The flagellar pocket membrane showed evidence of a second major antigen with a pI=6.5 and a broad shoulder to pH 7.2. With the pH interval 6.5-9.0 Fig. 12B most of the flagella pocket antigens, as expected from Fig. 12A are crowded between pH 6.1-6.7.

d) Enzymatic activity of immunoprecipitates. By using appropriate substrates and modifying established histochemical staining procedures it is possible to demonstrate visually any enzymatic activity associated with immunoprecipitates. This phase of our investigation, in conjunction with studies of the immunoinhibition of surface membrane, flagellar pocket and lysosomal enzymes is still in progress.

Failure to demonstrate enzyme activity in immunoprecipitin lines could be due to immune inhibition of a given enzyme. In general the larger the substrate molecule the more susceptible is an enzyme to immune inhibition.

i) Acid phosphatase. Using β-glycreophosphate as substrate the histochemical procedure of Wachstein & Meisel, 1957 as described in the legend to Figure 13A. A single prominent peak was stained coincidental with those density fractions (flagellar pocket membrane) where most of the acid phosphatase was assayed biochemically. It was not possible to directly correlate the peak with the overall pattern after protein staining. Sicne this phosphatase is clearly a potent immunogen it should be possible to use crossed immunoelectrophoresis to advantage to correlate this enzyme with the overall range of flagellar pocket antigens present.

ii) 3'-nucleotidase. Fig. 13B demonstrates the presence of 3'-nucleotidase activity however due to insufficient washing of the gel prior to ammonium sulphide exposure the pattern produced in too diffuse. Precipitation is associated with those fractions somewhat denser than the putative flagella pocket membrane, as was found after assaying the fractions directly (section C2). No precipitation was associated with those fractions where activity distribution plots (Fig. 4) demonstrated a prominent shoulder of activity. A very diffuse area of reaction product was found in that part of the gradient immediately above where surface membrane equilibrated.

iii) "Leucine aminopeptidase". The procedure followed was as described by Uriel (1971) the substrate used (leucyl-4-methoxy-2-naphthylamide) however is known to be non specific for leucine-amino peptidase and is also hydrolysed by a range of amino acid naphthylamidases (arylamidase) according to Barrett and Poole (1969).

It was found that a single flagellar pocket immunoprecipitate was stained for activity, the precipitin line showing only slight migration into the antibody gel. Unfortunately the azo dye produced by the coupling procedure faded before photography, preventing inclusion of a figure of the stained gel.

The results obtained to date demonstrate that the above methods, and other histochemical staining procedures, in the presence of appropriate inhibitors should permit the identificaiton of enzymatically active antigens. These results can be correlated with those studies of immunoinhibition of specific enzymes which is in progress (section C5).

4) Attempts to Clone Antigenic Variants of T. rhodesiense

This aspect of the proposal has seen the least progress, and for a very basic reason. As will be stated in more detail below, it has not proved possible to infect rabbits with the CT Wellcome strain of *T. rhodesiense* maintained at the Rane Laboratory. The procedures followed were initially those described by Cross, 1975, referenced in the previous proposal, and were undertaken by Dr. Arba Ager, of the Rane Laboratory and consultant for the project.

In all instances New Zealand white rabbits, 4-6 weeks old, were used and infected:

(a) intraperitoneal using up to 5.0ml freshly drawn mouse blood containing 10^7 trypanosomes/1ml.

(b) by injection of 1.5ml whole infected mouse blood directly into the marginal ear vein.

Blood samples were withdrawn on a daily basis for up to 3 weeks but no viable organisms were noticed except for 5-6 per field up to the second to third day. In addition we have attempted to immunosuppress rabbits using 6-mercapto purine (Schwartz, 1976) but this was without effect. Simple gel diffusion analysis of sera withdrawn from rabbits does show two very weak precipitin lines reacting with the anti P_a and anti-P_b sera.

The Wellcome CT strain was received at the Rane Laboratory in 1972 from WRAIR and has been maintained continuously since that time by serial blood passage in ICR/HA Swiss mice. Unfortunately the original stock received was never frozen to allow for any comparative studies. It seems quite certain that the Rane Laboratory line of this strain possesses no infectivity toward rabbits. It is interesting in this regard that we have found the minimum 100% lethal dose in Swiss ICR mice to be 1000 trypanosomes for this same parasite. This compares with infections using _T. brucei_ of as low as 50 trypomastigotes (Cross, 1975). In addition the Wellcome CT strain maintained here, as was reported previously (McLaughlin, 1981) is totally devoid of any oligomycin sensitive ATPase activity, indicative of a loss of infectivity to the tse-tse fly vector (Opperdoes, _et al_, 1976).

We have received, through the courtesy of Mr. K. Esser (WRAIR) a cloned isolate of the Wellcome CT strain (Wellcome CT-cp3-BT4) which we intend to compare with the Rane Laboratory line. In addition we received a series of antigenic variants (WRAT-at 1p9; 3p5; 4p6 and 50) which will be used for subsequent investigations of stable antigens.

5) Immunoinhibition of Membrane Associated Enzymes

For the experiments in this section enzymes were assayed using procedures already described, with certain pH modifications to avoid dissociation of any enzyme-antibody complexes. To date three enzyme activities have been investigated: acid phosphatase; thiol proteinase and leucine aminopeptidase. Fractions enriched in flagellar pocket membrane, lysosomes and surface membrane respectively were used as a source of enzyme. A 45-150 λ aliquot was added to increasing amounts of either anti P_A or P_B lgG (2.5mg/ml) to a final volume of 500 λ containing 100mM NaCl, 40mM acetate, pH 6.8, and 0.2% Triton X-100. The samples were left at room temperature for 15 minutes then placed on ice for 1.0h. The appropriate substrate, and buffer was added to yield a final concentration equivalent to that in the normal assay. The pH of the final assay mixes were acid phosphatase, pH 6.5; thiol proteinase pH 6.5, aminopeptidase pH 7.5.

The degree of inhibition produced is shown in Fig. 15 from which it is apparent that the proteinase is far more susceptible than either of the other activities. A maximum of 30% acid phosphatase, 55% aminopeptidase and 80% of the proteinase was susceptible to inhibition. The first two enzymes especially acid phosphatase are markedly antigenic (see Fig. 13) however despite this the phosphatase is not very susceptible to inhibition.

6) Immunoprophylactic Potential of P_A and P_B Derived Gradient Fractions

A series of experiments has been initiated to investigate the degree of protection against T. rhodesiense induced by subcellular fractions recovered after isopycnic gradient centrifugation.

a) Fractions used for immunoprophylaxis

Both the distribution plots and antigenic profiles indicated overlap of fractions. For that reason further resolution between fractions was affected by gradient centrifugation of the large granule (P_A) fraction and small granule fraction (P_A) rather a high speed pellet. Fig. 14 shows the distribution profiles of various peptidase activities and demonstrates the increased resolution (see Fig. 4). Identifiable were distinct activities associated with flagellar pocket (low density fractions): lysosomes (mid-density range) and surface membrane (high density fractions). In the latter case activity toward leucinamide and to a lesser extent carboxypeptidase activity toward cbz-glutamyl-tyrosine, was most noticable. The distinct surface membrane leucine aminopeptidase was discussed previously (Section C2). The distribution of promitochondrial and glycosomal enzymes were not further resolved.

b) <u>Immunization of mice and subsequent challenge with T. rhodesiense</u>

The gradient fractions obtained were mixed were Freunds complete adjuvant (Hoechst-Calbiochem) using 1.5ml fraction plus 0.75ml adjuvant (final concentration 33%). For each fraction five mice each received 0.4ml mixed antigen injected subcutaneously into the shoulder area. Antigen was administered on days 0, 5, 14, 20, 25 as was adjuvant mixed with 0.5M sucrose (in the same proportions) to a series of controls. Thus for the 24 gradient fractions recovered each from fractions P_A and P_B five replicates plus two controls were established making a total of 336 mice in all. Mice were challenged 12 days after the final antigen injection using 1000 trypanosomes per mouse administered in 0.2ml saline diluted infected mouse blood. This number of parasites was established as the minimum number required to cause a 100% mortality rate.

At the time of writing this report it is now 10 days since the mice were challenged and all control animals died after 4-5 days. All groups of mice that were immunized have survivors. For the P_A fraction random survivors are seen from fractions 1-10, with a 75% survival rate for mice immunized with fractions 11-14 (lysosomal population). The high density gradient fractions from fraction P_A (16-22), and to a lesser extent fraction P_B (15-18) containing surface membrane have a 100% survival rate.

The other protective fractions are those from the top of the P_B gradient containing flagellar pocket membrane (fractions 3-9) where 55% of the mice are surviving.

The protection induced by the surface membrane fractions is not unexpected and may well be due to variant specific antigen still membrane associated. Further experiments are required to verify the protection induced by the lysosomal and flagella pocket membrane. The subcellular components plus the surface membrane are the most antigenically active particulate cell fractions. It had been expected that more rapid deaths (i.e. after 3 days) might have been produced by some of these membrane fractions. Clayton et al., 1979 reported an immunosuppressive effect induced by a "membrane" fraction from T. brucei. However the nature of the fraction used by these authors is unclear. In addition Sacks et al. (1980) report that the immuno suppressive effect varies according to the virulence of the strain of trypanosome.

It should be possible to give further details of this experiment in the next quarterly report. A further experiment is planned using more concentrated antigen and attempting to reduce the amount of adjuvant.

GENERAL DISCUSSION

The results presented in this report are the first to systematically analyze the subcellular distribution of antigenic components in one of the African trypanosomes. Recent studies have been reported for Trypanosoma cruzi (Gonzalez Cappa et al, 1980) but were mainly concerned with establishing the antigenicity of flagella fractions. It is quite evident now that the early study of Brown and Williamson (1964) was ineffective due to a failure to adequately characterize the organelles and membrane systems present in the fractions prepared. In addition the fractionation scheme itself was crude as were the methods used to demonstrate antigenicity. The notion has since persisted (i.e. Bawden, 1975) that soluble (in fact surface glycoprotein) components are the only antigenically important cell constituents. More recently Rovis and Baekkeskov (1980) fractionated T. brucei and analysed the resulting subcellular fractions for their reactivity toward antibodies to purified VSA. They found as expected reactivity limited to the "surface membrane" fractions. There is some doubt, as stated in previous annual reports concerning the authenticity of the marker enzymes used by these authors. In addition the inherent overall antigenicity of the subcellular fractions was not investigated, in contrast with the present report.

The results of this study to date have demonstrated a range of antigenic components associated with subcellular sites other than the surface membrane, though this appears to exhibit the most diversified range of antigens. Thus at least 6-7 antigenic components are associated with the flagella pocket of which at least 5 appear to be glycoprotein. The lysosome fraction also exhibits one prominent antigenic component. Cross reactivity between these antigens has been noted, though only to a limited degree. Further work on purified antigens is required as suggested in the renewal proposal. At least two of the surface and flagellar pocket membrane glycoproteins were electrophoretically identical. Iso-electric focussing however has so far indicated the antigens to have quite dissimilar iso-electric points, though resolution has not been optimal.

Results so far, show all of the fractions capable of confering some degree of protection, though whether such protection would prove is variant specific is not known. Certainly the flagellar pocket antigens are of much interest since this is a site in contact, if somewhat limited, with the external environment. The flagella pocket is of importance for nutrient uptake (Vickerman & Preston, 1976) in African trypanosomes and could possibly be a potential site for antibody inhibition of such processes. None of the flagellar pocket enzymes investigated so far has proved to be very susceptible to immune inhibition. The flagellar pocket might also be a useful site for drug action/uptake. Possibly the incorporation of antibodies to flagellar pocket antigens into liposomes containing drugs would target drugs to this site where uptake would presumably be more efficient.

An important issue concerning the surface membrane antigens is the degree to which the glycoproteins detected in this study are related to the surface glycoprotein coat. Are they degradation products (due to surface membrane peptidase, such as the aminopeptidase described previously) precurssors of surface glycoprotein or quite unrelated being authentic membrane components rather than a surface coat?

ACKNOWLEDGEMENTS

It is a pleasure to thank Dr. Arba Ager and his staff at the Rane Laboratory, University of Miami for our supply of infected blood. Invaluable technical support was provided by Ms. Lydia Lopez and Mr. Howard Perlman.

FIGURE LEGENDS

Fig. 1 Distribution of relative fluorescence after fluorescamine labelling of (a) disrupted cells (b) whole cells.

For labelling four successive 50 λ aliquots of fluorescamine (5mg/ml anhydrous acetone) were added to washed intact trypanosomes or disrupted trypanosomes in 20ml 250mM sucrose; 2mM EDTA; 1mM KCl; 10mM HEPES, pH 8.5, with constant mixing. After making the volume up to 80-100ml with buffered sucrose (pH 7.5) the intact cells were centrifuged (2,500rpm, 10 mins, SS-34 rotor) and twice washed with buffered sucrose. After disrupting these labelled cells the homogenates were fractionated as previously described.

Fig. 2 pH activity curve for 3'-nucleotidase activity using acetic acid, sodium acetate-tris buffers (75mM).

Fig. 3 Distribution of 3'-nucleotidase activity, using 10mM 3'-AMP as substrate after differential centrifugation (see methods). Relative specific activity plot was calculated as described in previous annual reports. Fractions from left to right show nuclear pellet large granule fraction (Pa) small granule fraction (P_b) and soluble fraction.

Fig. 4 Isopycnic centrifugation of a T. rhodesiense high speed pellet (see comments in section C-2,3). Centrifugation conditions and presentation of results were described in the second annual report.

Recoveries range from 78% (protein) to 108% (relative fluorescence). Fractions obtained from identical gradients and high speed pellets used for antigenic analysis.

Fig. 5 pH activity curve for peptidase activity toward n-t-Boc-alanine-2-nitrophenol. Procedure for assay as described in methods; buffers 60mM Po_4.

Fig. 6 Distribution of peptidase activity toward n-t-Boc-alanine-2-nitrophenol after differential centrifugation. Details as described for Fig. 3.

Fig. 7 Fused rocket immunoelectrophoresis of T. rhodesiense fractions obtained after isopycnic gradient centrifugation (see Fig. 4) using non-ionic detergents to extract antigens. A,B,C,D all used 0.5% Triton X-100 to extract samples and in agarose gels. E and F used 0.4% Lubrol W. A;D;E antibody containing gel used anti-P_A lgG, B,C,F antibody containing gel used P_B lgG.

Fig. 8 Fused rocket immunoelectrophoresis of fractions obtained after isopycnic gradient centrifugation using Zwittergent 3-12 alone or in the presence of non-ionic detergents. C,F,I,J samples precipitated in gels containing anti P_A lgG. The following detergents were used: (A), fractions and gel contained 0.25% Zwittergent 3-12 (B) fractions contained 0.25% Zwittergent, gel 0.1% Zwittergent (C) fractions and gel contained 0.1% Zwittergent (D) fractions contained 0.25% Zwittergent, gel 0.5% Triton X-100 (E,F) fractions and gel contained 0.5% Triton X-100/0.1% Zwittergent (H,I) fractions and gel contained 0.4% Lubrol W/0.1% Zwittergent (J) fractions sonicated, see methods, then electrophoresed into gel containing 0.5% Triton X-100. Arrow shows position of first sample and successive bars, 1.117 and 1.23 density increments.

Fig. 9 Fused rocket affini-Z of gradient fractions A, samples extracted with 0.15% Zwittergent 3-12 and electrophoresed into gel containing Zwittergent and 80 μ/cm^2 concanavalin A. B samples extracted with and electrophoresed into gel containing 0.5% Triton X-100 Upper gel (1) contains anti P_B lgG, lower gel (2) contains concanavalin A as for (A).

Fig. 10 Crossed immunoelectorphoresis of pooled gradient fractions exhibiting most diversified range of antigens. (A) pooled surface membrane fractions in the presence of 0.5% Triton X-100, gel contained same detergent and anti P_B lgG (B) tandem crossed immunoelectrophoresis, first sample slot contained pooled surface membrane fractions, second sample slot contained pooled flagella pocket membrane, both extracted with 0.5% Triton X-100. Gel contained same detergent and anti P_B lgG (C) crossed immunoelectrophoresis of pooled flagella pocket fractions, detergent extraction and antibodies used as in A and B (D) pooled flagella pocket fractions using 0.15% Zwittergent as detergent in first dimension electrophoresis and 0.1% Zwittergent 0.4% Triton X-100 for second dimension electrophoresis with anti P_b lgG (E) fraction P_B, recovered after differential centrifugation (see first annual report) in the presence of 0.5% Triton X-100. Other conditions as for A-C.

Fig. 11 Crossed immuno-affinielectrophoresis of pooled surface membrane (A) and flagellar pocket membrane (B) fractions. Electrophoresis conditions as for Fig. 10D. Upper gel (A) contained anti P_A lgG, upper gel (B) contained anti P_B lgG. Both lower gels contained concanavalin A (80μ g/cm^2).

Fig. 12 Immuno-isoelectric focussing of pooled surface membrane (C,D) and flagellar pocket membrane (A,B). Conditions as given in methods, electrophoresis into gels containing 0.5% Triton X-100 10.1% Zwittergent 3-12 with anti P_B lgG in A and B, anti-P_a lgG in C and D. Intervals at base of plates indicates 0.5 pH unit increment.

Fig. 13 Enzyme activities associated with immunoprecipitin lines formed after fused rocket immunoelectrophoresis (FRI) of gradient fractions in the presence of 0.5% Triton X-100 10.1% Zwittergent. For both A and B anti P_b lgG was used. (A) Demonstration of acid phosphatase activity. The gel after FRI was allowed to soak for 3-4 hrs in two changes of 0.15M NaCl, 100mM acetate, pH 6.5, to remove non-specifically bound enzyme activity. The gel was then incubated at 37°C in a solution containing 10ml 12.5% β-glycerophosphate; 5.0ml 0.5M acetate, pH 5.0; 12ml 0.2% lead nitrate and 23ml deionized water. After 3 hrs the gel was washed in running tap water for 2.0 hrs, rinsed for 2-3 minutes. Immunoprecipitin lines displaying acid phosphatase activity showed brown deposits of lead sulphide. (B)

3'-nucleotidase activity demonstrated using the same method as for (A) with an incubation solution containing 20ml 3'-AMP solution (125mg/100ml) 20ml 0.2M Tris, pH 7.8, 0.5ml 100mM $CoCl_2$; 1.0ml 2% lead nitrate, 3.5ml H_2O.

Fig. 14 Isopycnic density gradient centrifugation of the large granule (P_a) and small granule (P_b) fractions obtained from a _T. rhodesiense_ post nuclear supernate.

The two fractions (P_a, P_b) were obtained as described in previous annual reports from a post nuclear supernate fraction after centrifuging the whole cell homogenate at 3,000rpm for 4 mins. Gradients were prepared as previously using 24% and 53% sucrose solutions.

Plots show distribution of various peptidases using a range of synthetic low molecular weight substrates.

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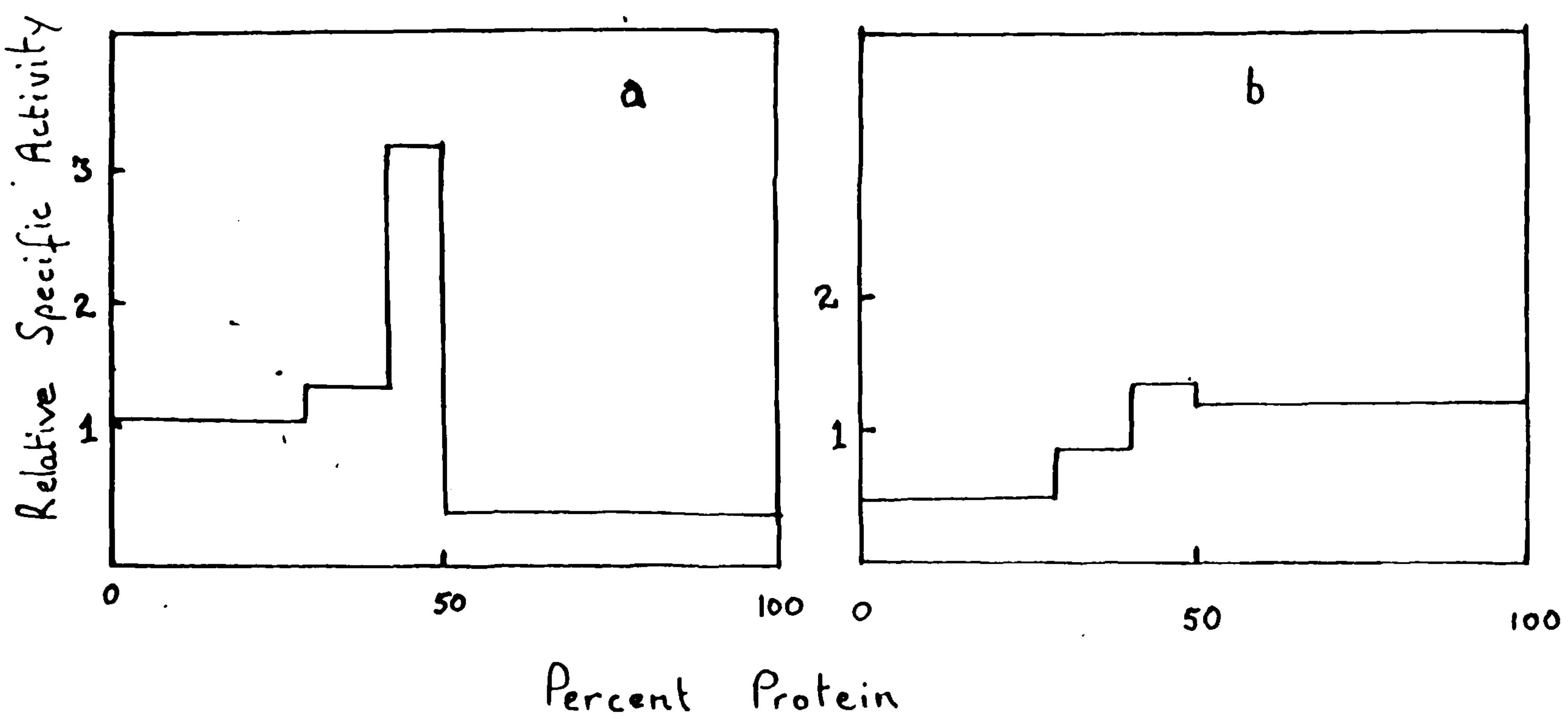
Flagellar Pocket	tartrate sensitive phosphatase arylamidases (major) "leucine aminopeptidase" carboxypeptidase 3'- nucleotidase (possible)* neutral proteinase (possible)*
Promitochondrion	Mg-ATPase/GTPase (oligomycin insensitive) α-glycerophosphate dehydrogenase (DCPIP linked)
Lysosome [a]	Thiol proteinase Carboxypeptidase (major) Neutral proteinase (partial) Arylamidase (partial) 3'-nucleotidase (minor)
Surface membrane	tartrate insensitive phosphatase Mg-ATPase (vanadate sensitive) leucine aminopeptidase (no Me^{2+})

Table 1. Association of selected enzymes with subcellular sites in *T. rhodesiense*.

* These both sediment to slightly denser positions than other flagellar pocket enzymes.

a Contains enzymes not all of which contain typically acid lysosomal pH optima.

Fig. 1



Relative Specific Activity
a
b
3
2
1
2
1
0
50
100
0
50
100
Percent Protein

Fig 2

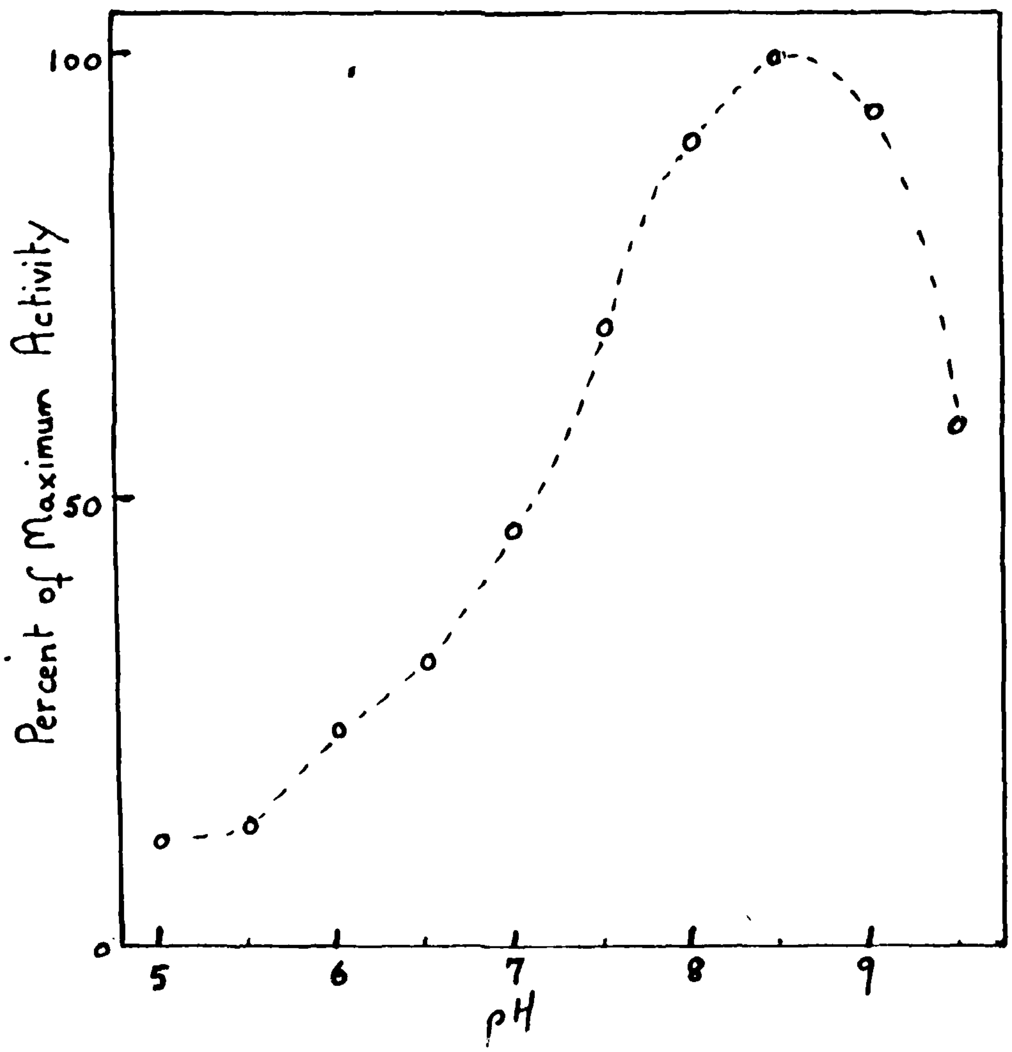
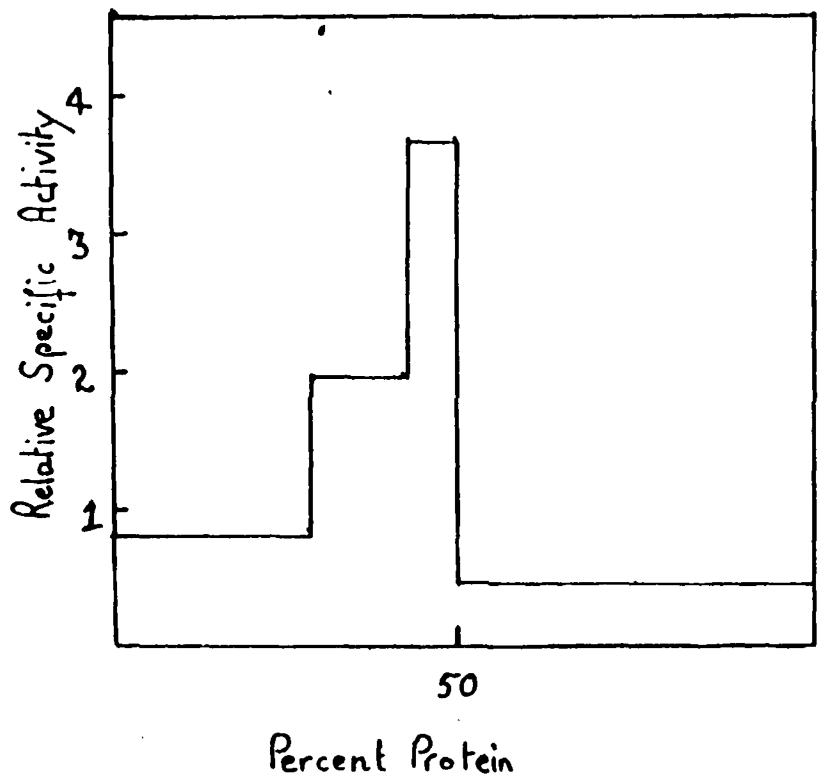


Fig 3



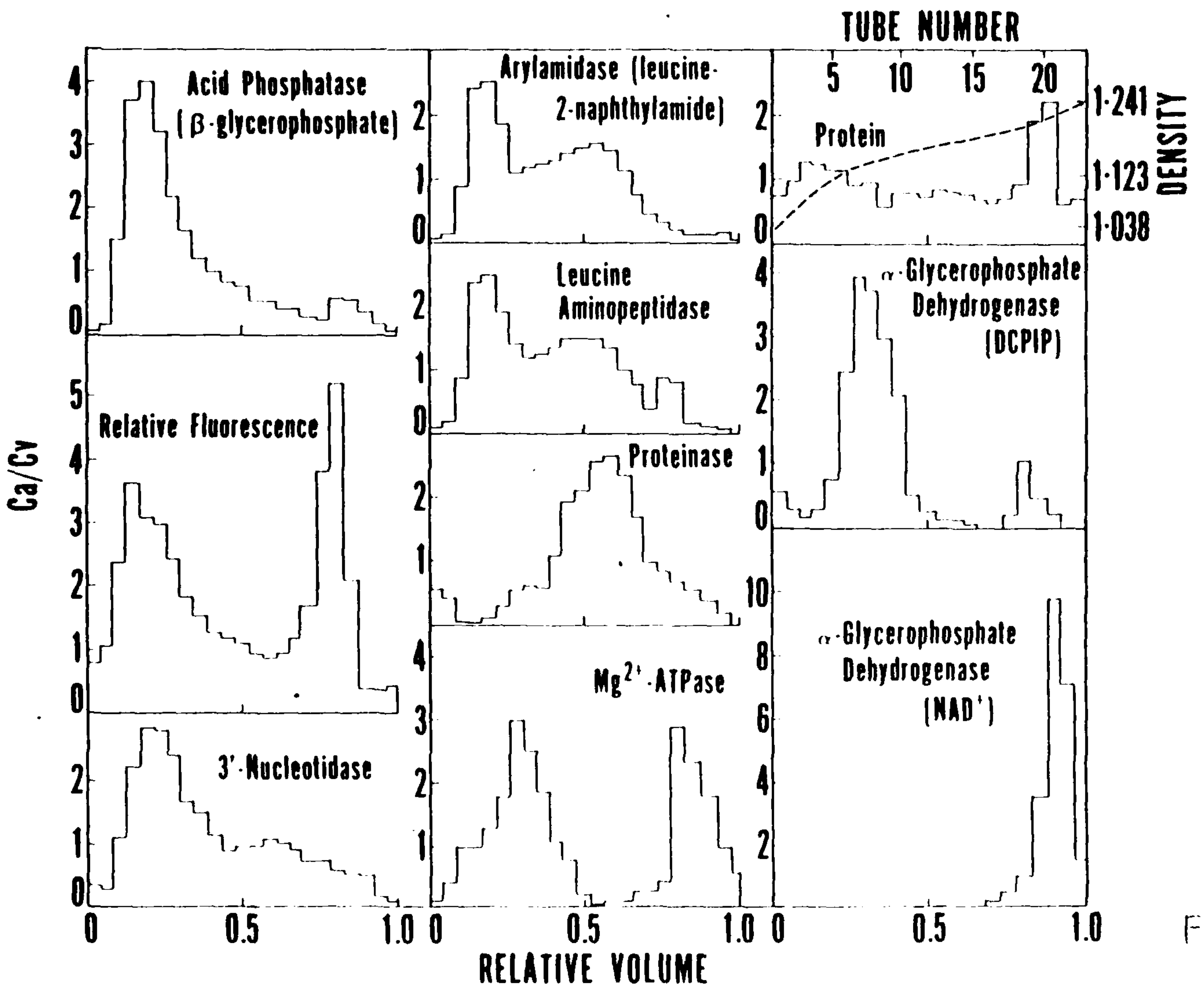

TUBE NUMBER
5
10
15
20
Ca/Cv
Acid Phosphatase
(β-glycerophosphate)
Relative Fluorescence
3'-Nucleotidase
Arylamidase (leucine-
2-naphthylamide)
Leucine
Aminopeptidase
Proteinase
Mg2+-ATPase
Protein
α-Glycerophosphate
Dehydrogenase
(DCPIP)
α-Glycerophosphate
Dehydrogenase
(NAD+)
1·241
1·123
1·038
DENSITY
0
0.5
1.0
RELATIVE VOLUME

Fig. 4

Fig 5

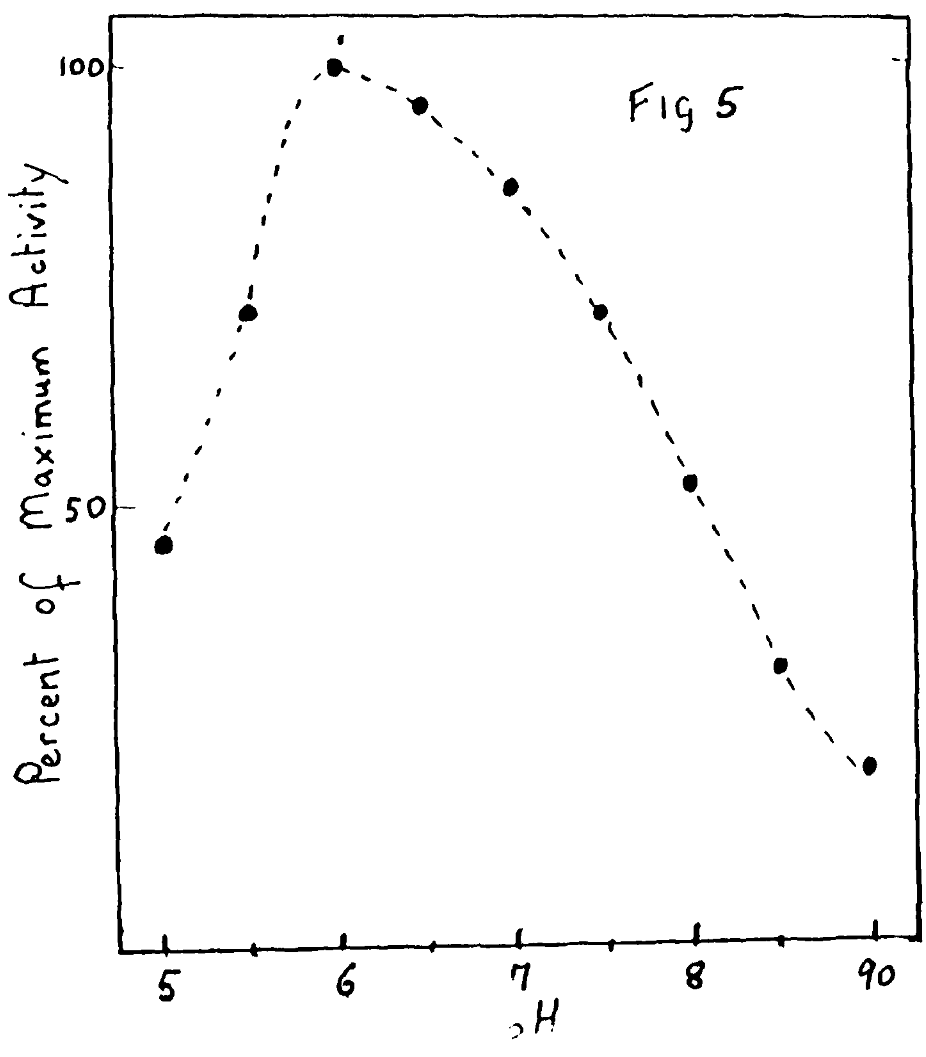


Fig 6

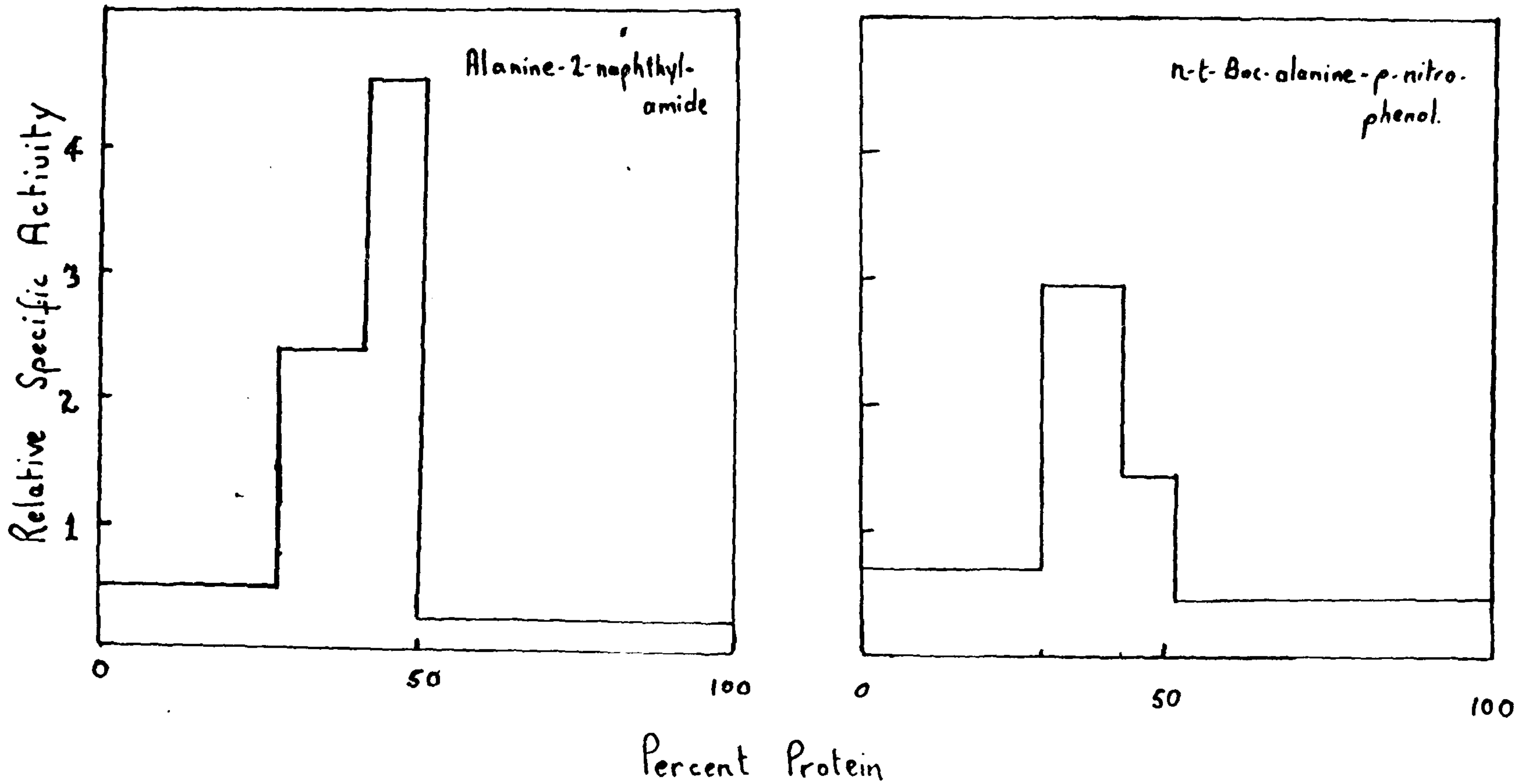

Alanine-2-naphthyl-amide
n-t-Boc-alanine-p-nitro-phenol.
Relative Specific Activity
1
2
3
4
0
50
100
0
50
100
Percent Protein

Fig 7

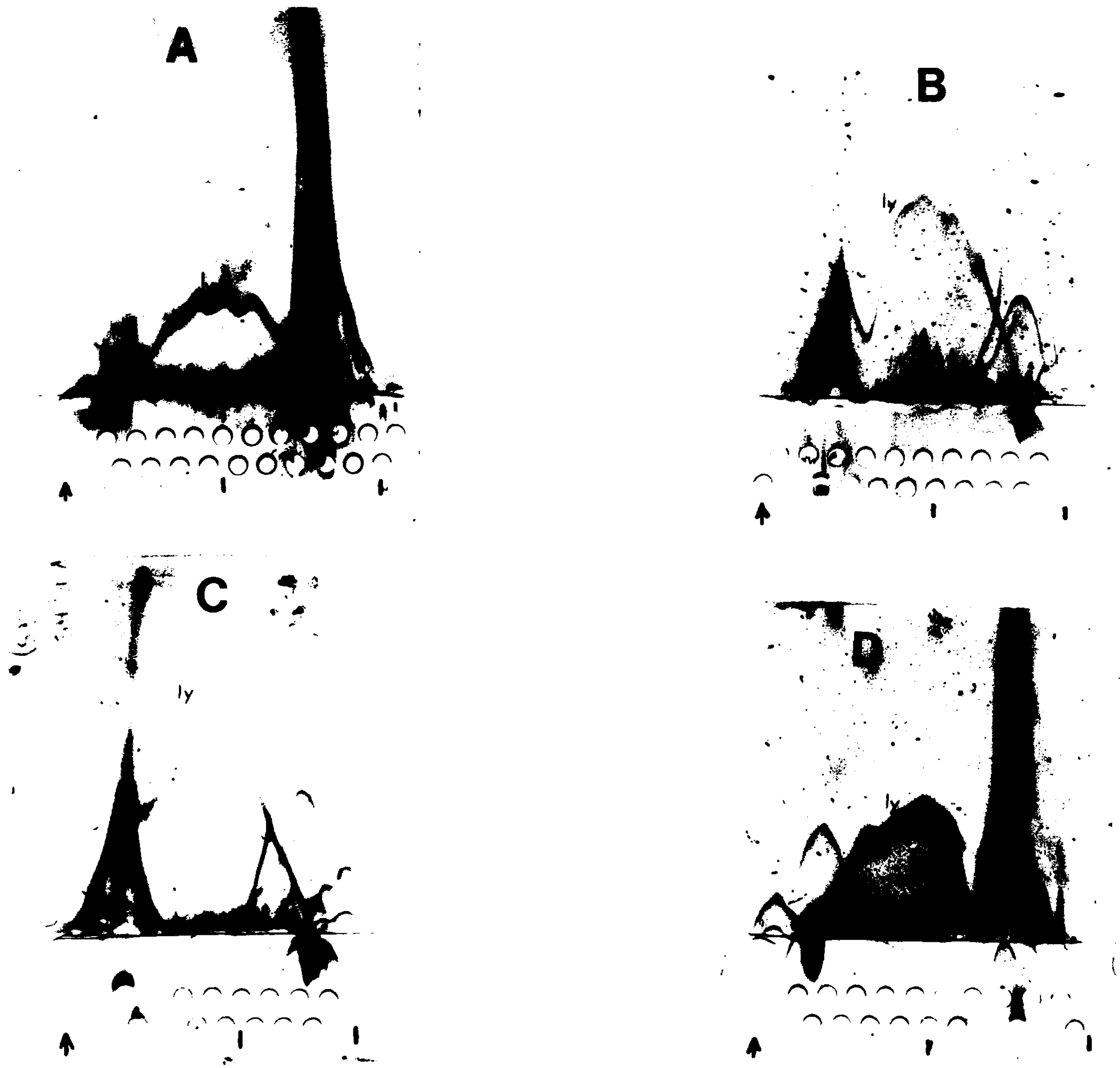
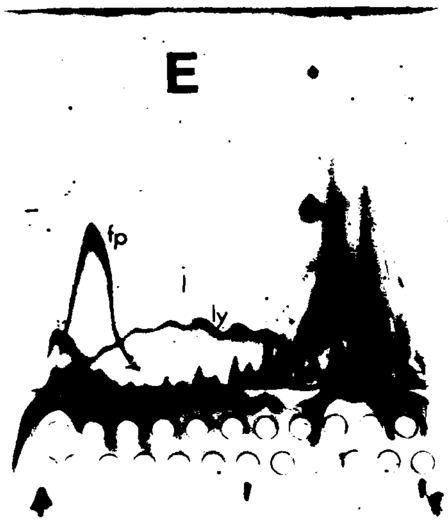


Fig 7a



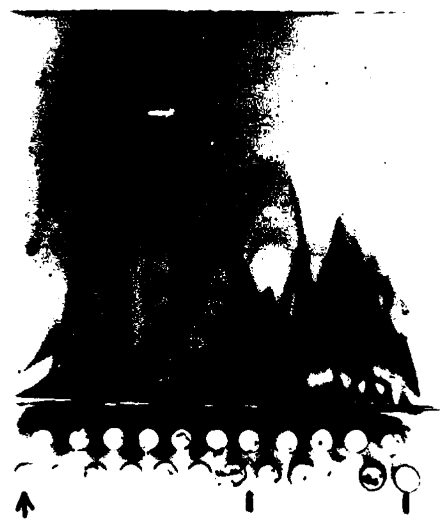


Fig 8

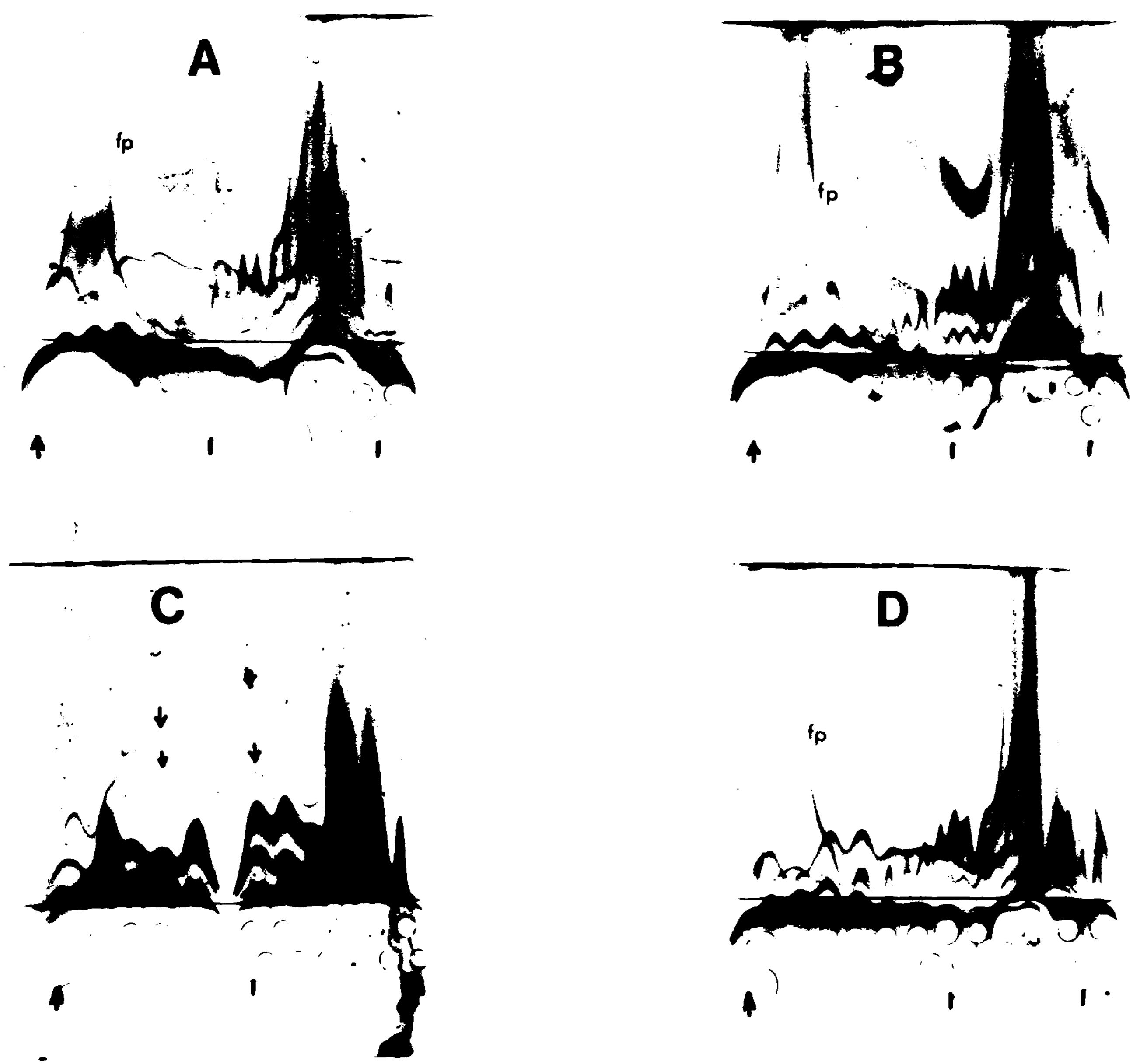


Fig 8a

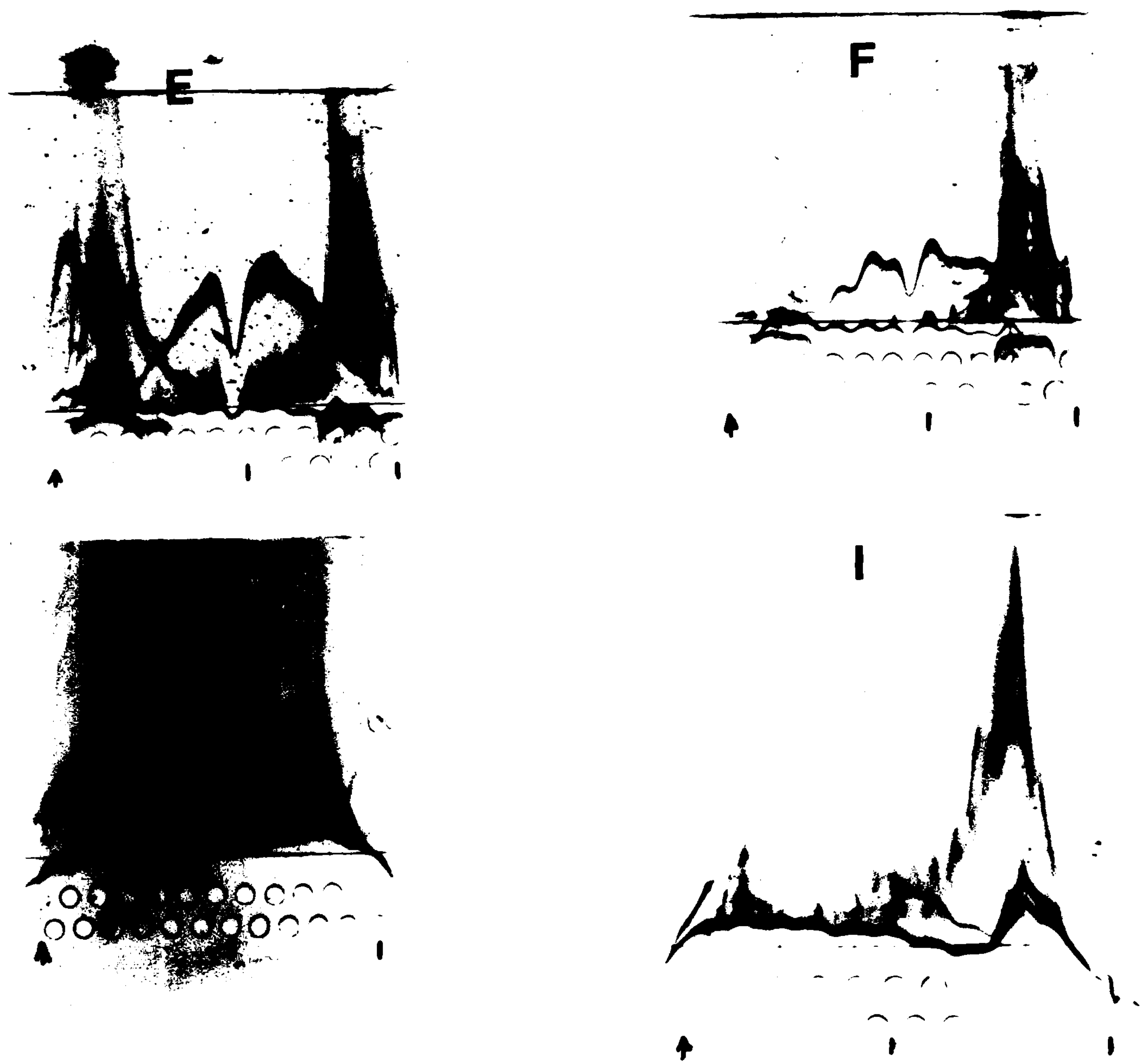


Fig 86

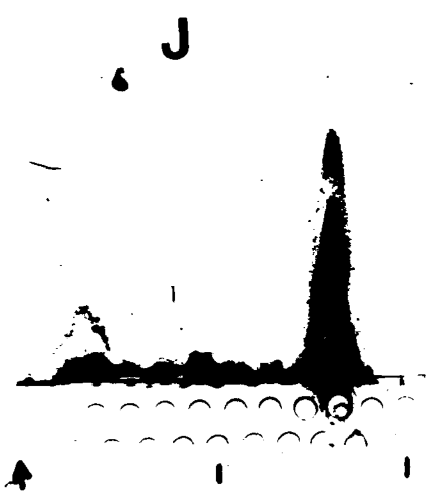


Fig. 9

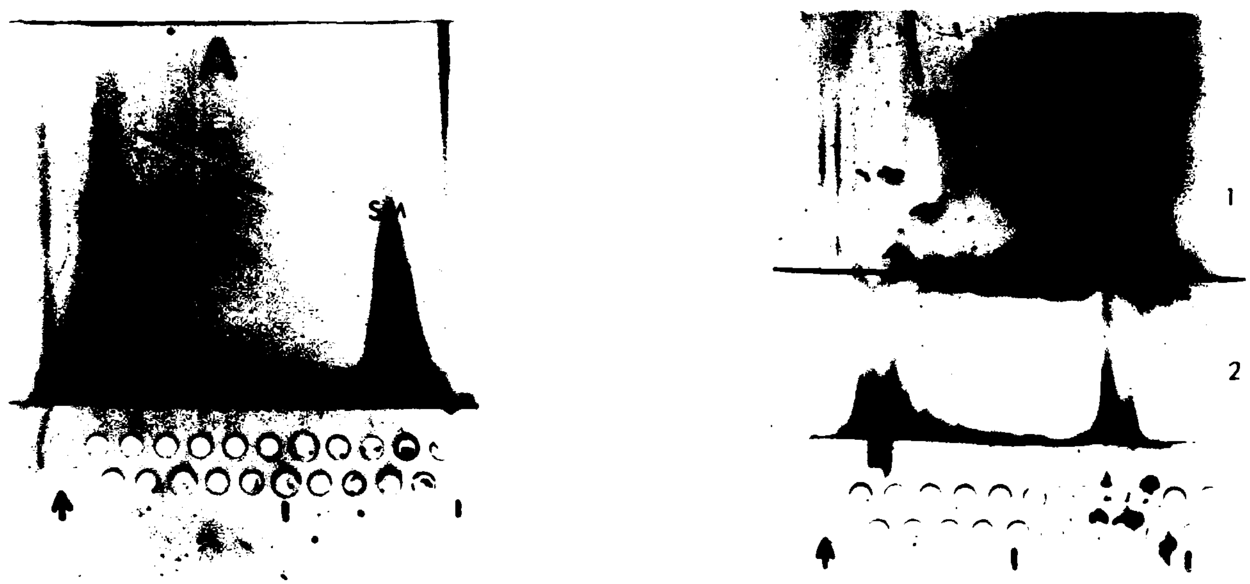


Fig 10

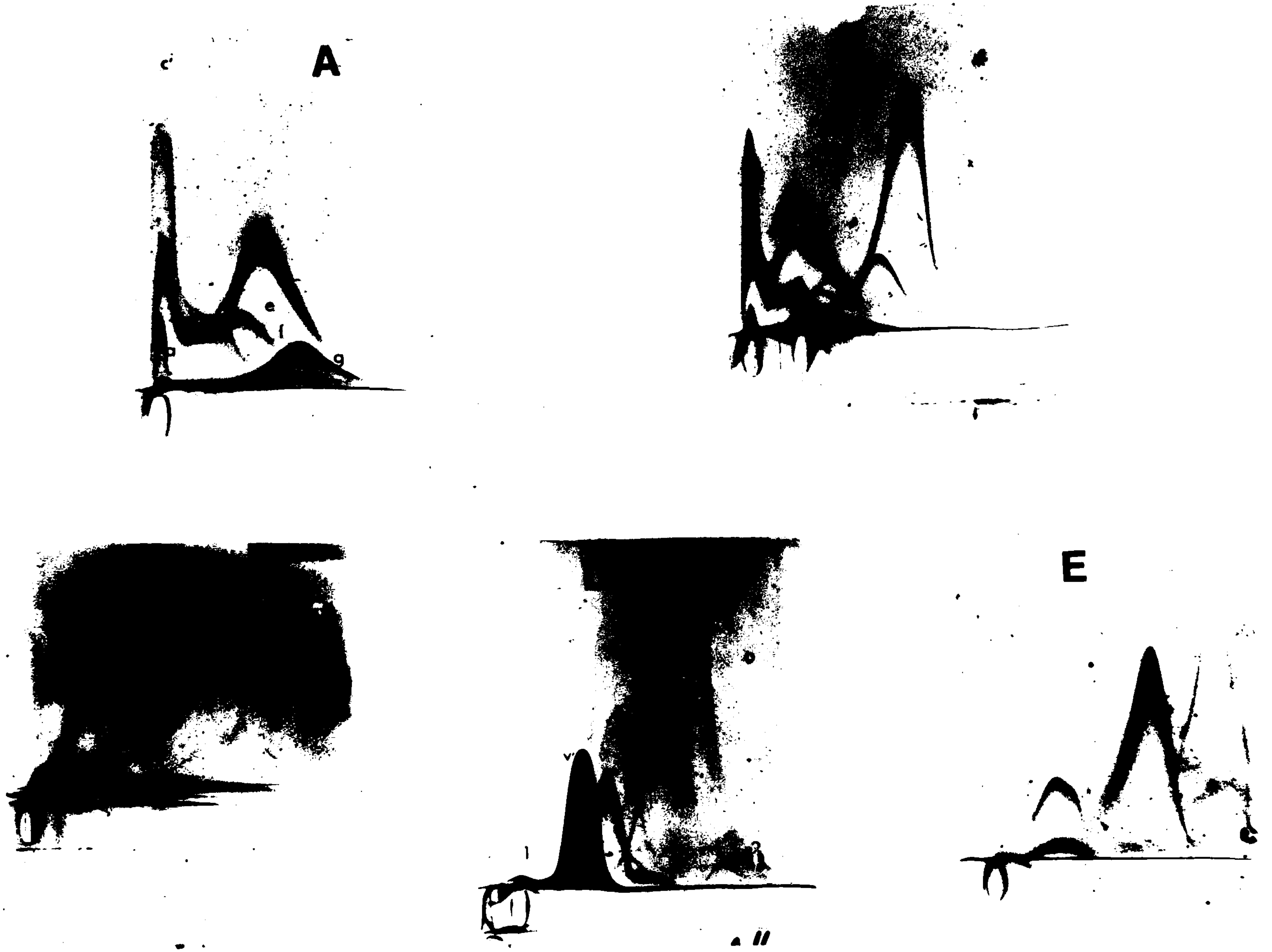


Fig 11

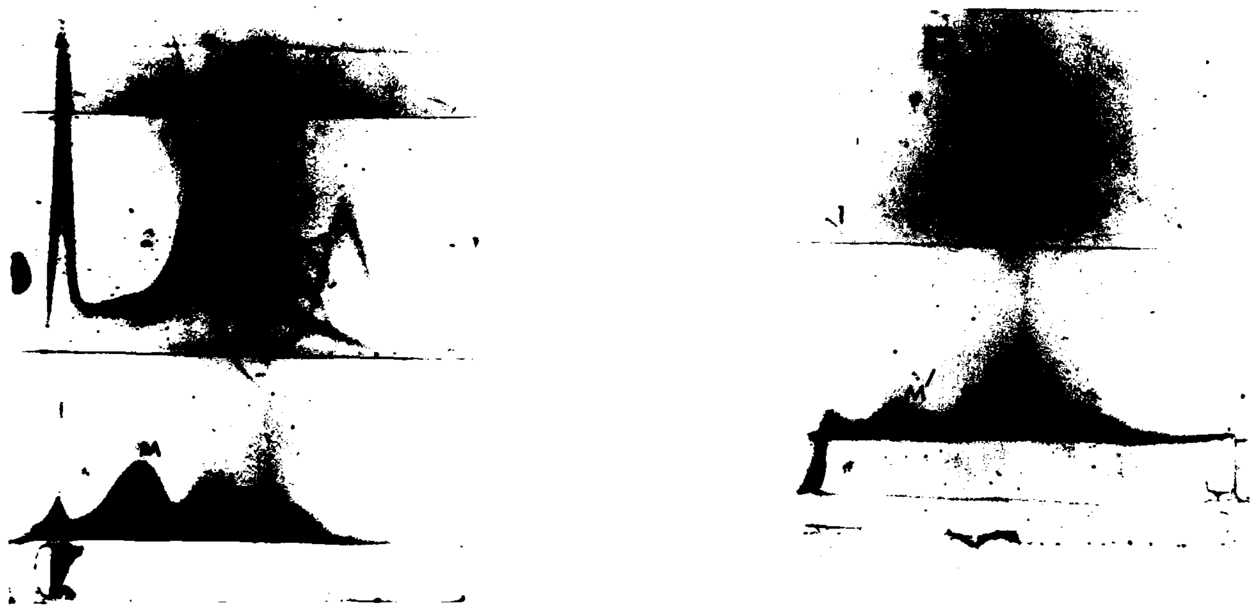


Fig 12

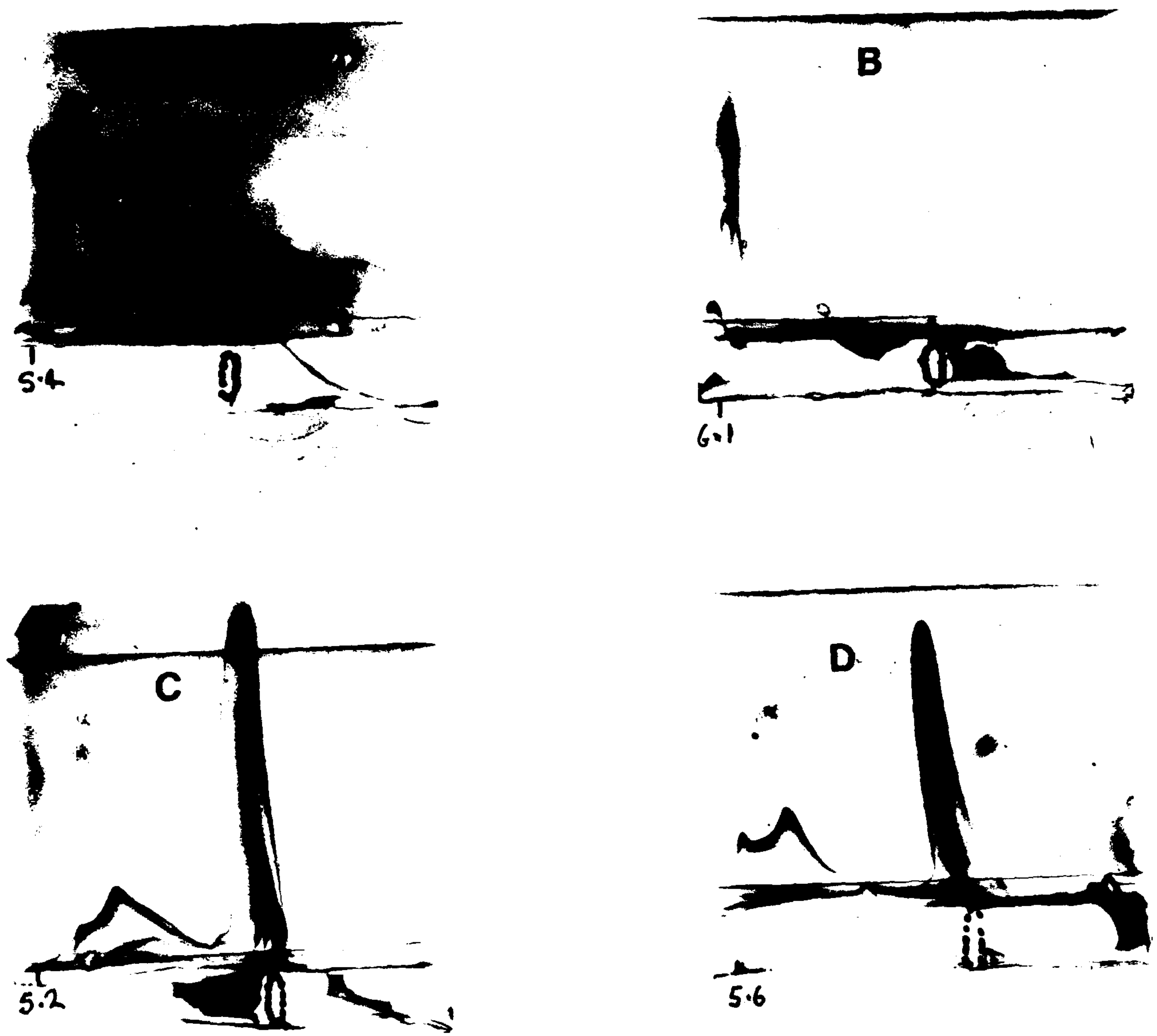
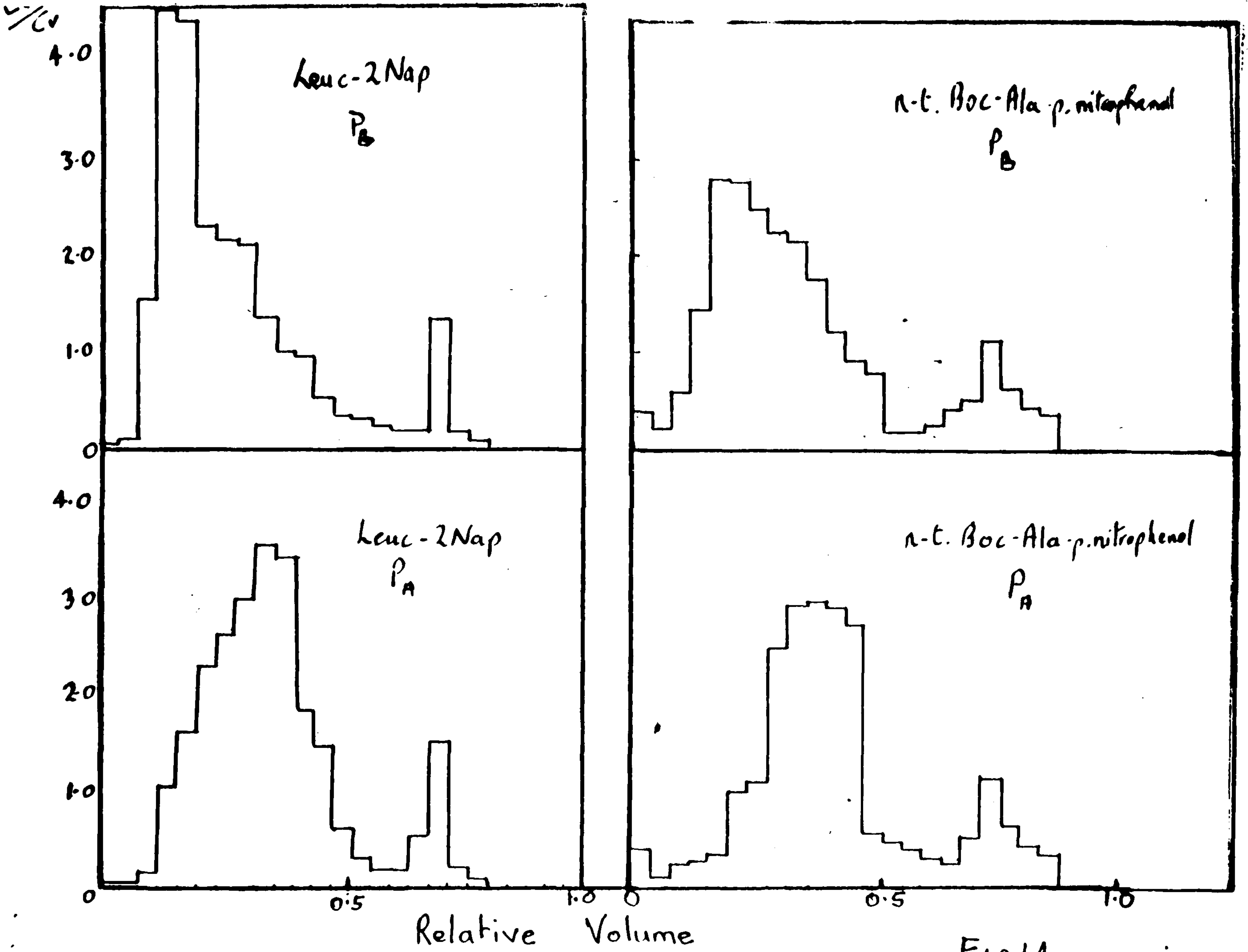
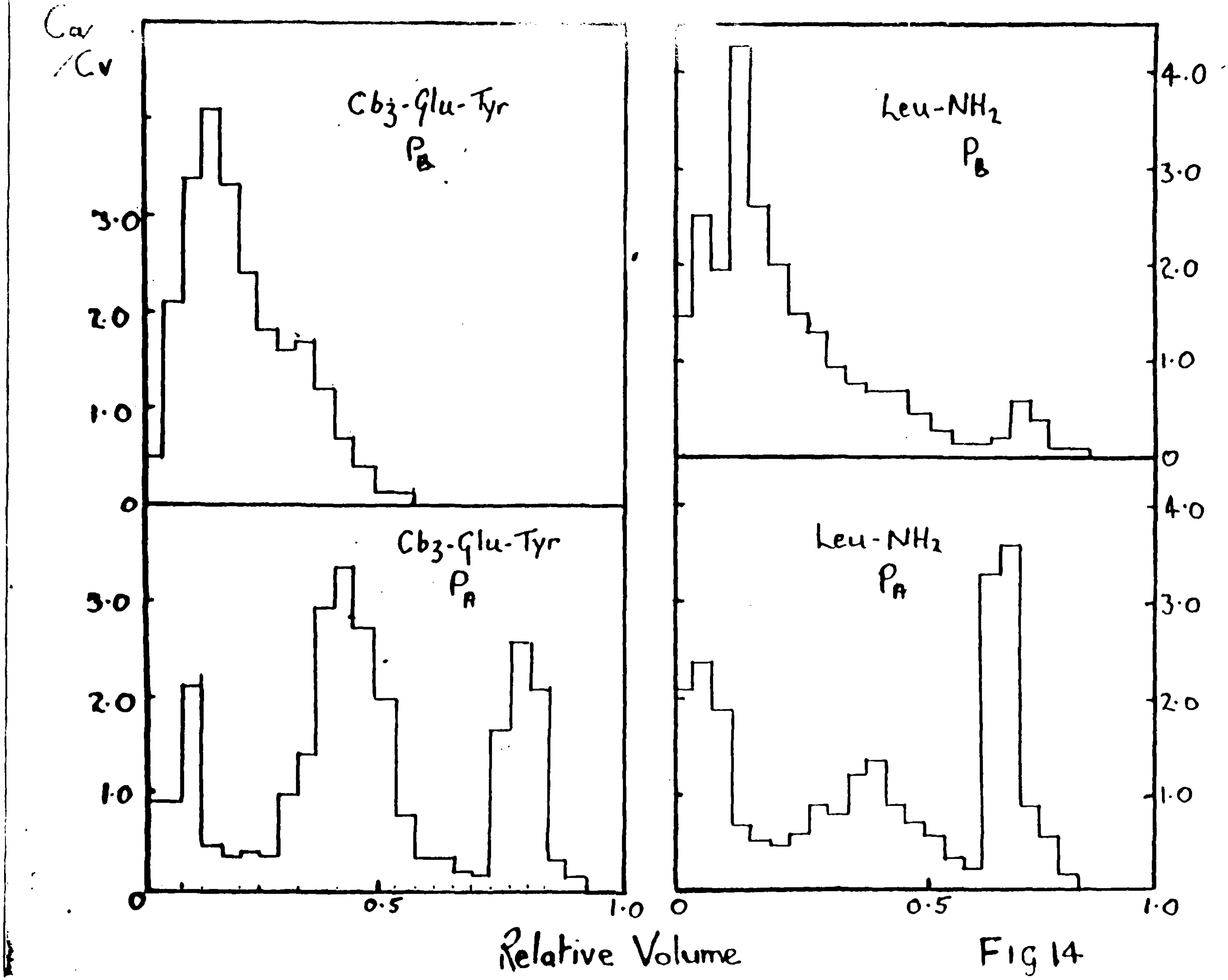


Fig. 13



v/Cv
4.0
3.0
2.0
1.0
0
Leuc-2Nap
P_B
Leuc-2Nap
P_A
n-t. Boc-Ala-p. nitrophenol
P_B
n-t. Boc-Ala-p. nitrophenol
P_A
0.5
1.0
Relative Volume

Fig 14



Ca/Cv
Cb3-Glu-Tyr
P_B
Cb3-Glu-Tyr
P_A
Leu-NH2
P_B
Leu-NH2
P_A
0
1.0
2.0
3.0
4.0
0.5
Relative Volume

FIG 14

MED

-8