FIBRIN CLOT DISSOLUTION IN AN ACID ENVIRONMENT

by

Jacqueline Anne Law

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DECLARATION

I hereby declare that this thesis is a record of the work carried out by me; that the thesis is of my own composition and that no part of it has previously been presented for a higher degree.

The research was carried out in the Department of Biochemistry of the University of St Andrews and in the Department of Biochemistry of the University of Edinburgh, under the supervision of Dr. G.D. Kemp of the University of St Andrews.

ACADEMIC RECORD

I began a course of study in Haematology and Blood Transfusion at the David Dale College, Glasgow in September 1968 and gained Associate Membership of the Institute of Medical Laboratory Technology (now Sciences), by examination in June 1970. In July 1976 I matriculated as a research student in the Department of Biochemistry of the University of St Andrews.

CERTIFICATE

I hereby certify that Jacqueline Anne Law has spent seven terms engaged in research work under my direction and that she has fulfilled the conditions of the Resolution of the University Court, 1974 no.2, and that she is qualified to submit the accompanying thesis for the degree of Master of Science.

ACKNOWLEDGEMENTS

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ABSTRACT

A fraction has been isolated from out of date male plasma containing an acid protease which is capable of dissolving stable fibrin clots suspended in 1% monochloracetic acid. By polyacrylamide disc gel electrophoresis, in the presence of sodium dodecyl sulphate, this fraction is found to contain two constituents. The major one has an estimated molecular weight of 64,000. This major constituent has been isolated using slab gel electrophoresis and contains the enzyme activity of the fraction. From using immunoelectrophoresis techniques, it seems that this major constituent is associated in some way with a degraded form of albumin. Attempts to remove this albumin contaminant by various affinity chromatography techniques are described.

Several techniques for assaying acid proteinases are evaluated as a technique for the assay of this activity, including fibrin blue assay, acid denatured haemoglobin assay, radial diffusion in agarose gel containing skimmed milk assay and an assay using the synthetic substrate N-acetyl-L-phenylalanyl-L-diiodotyrosine.

Pepsin has been shown to be present in plasma, but there are factors against this isolated activity being pepsin, including its inability to be bound by aluminium hydroxide gel and its reduced sensitivity with the acid denatured haemoglobin assay.

The exact nature of the activity remains to be elucidated when further purification has been achieved. However, since pepstatin, a powerful inhibitor of carboxy proteinases, inhibits the activity of this fraction against acid denatured haemoglobin it might be assumed that the activity isolated belongs within the group of enzymes known as carboxy proteinases.

1. Introduction

Haemostasis reaches a climax with the formation of a stable fibrin clot from liquid plasma. This is achieved by means of a series of enzyme reactions, where proenzymes in the plasma are activated in a cascade reaction (Esnouf, 1977). This cascade terminates with the action of thrombin on fibrinogen to form fibrin, which is then acted upon by a plasma transamidase enzyme called fibrin stabilising factor, or factor XIIIa (F XIIIa). This in turn has been activated by thrombin in the presence of Ca^{++}.

Fibrinogen is a high molecular weight protein (MW 340,000), consisting of three pairs of polypeptide subunits α(A), β(B) and γ chains. These are held together by numerous disulphide bridges. Thrombin removes a peptide A from the N terminus of the α(A) chain of fibrinogen (fibrinopeptide A), which it has been suggested by Blomback *et al* (1972) leads to a conformational change which exposes the β(B) chain to thrombin action, with the release of a further peptide, fibrinopeptide B. This leaves the soluble fibrin monomer, which, as the final stage of blood coagulation, is rendered insoluble to the action of dilute acid and alkali and to the action of proteolytic enzymes present in the plasma.

Activated F XIII is the enzyme responsible for this; it catalyses the formation of $\varepsilon(\gamma$-glutamyl)-lysine isopeptide bonds rapidly between γ chains to form $\gamma\gamma$ dimers and more slowly between α chains to give an α polymer (McKee *et al* 1970). This now stable cross linked fibrin is hereinafter referred to as a stable fibrin clot.

Stable fibrin clot formation can be determined by the ability of the clot to withstand dissolution after thirty minutes incubation at $37^{o}C$ in either 1% monochloracetic acid (MCA) or 5M urea. A comparat-

ive study concerning the respective sensitivities of different F XIII assays showed that the critical activity for clot stabilisation is somewhat lower in the urea test than in the assay using acid (Tyler 1966). However, this statement has recently been disputed by Ikemori *et al* (1975), who found that most fibrin clots formed from plasma with normal F XIII level, which were insoluble in urea, dissolve when exposed to MCA at 37°C. This has also been observed by Shanberge *et al* (1973), who concluded that an acid proteinase, probably pepsin, markedly influences clot dissolution in acid.

Ragaz *et al* (1976) reported an unusual bleeding disorder in a female patient referred to them in their department in Berne, Switzerland. It clinically resembled F XIII deficiency. The patient had a history of easy bruising and after several operations had shown a tendency to significant post-operative haemorrhage and markedly delayed wound healing, giving distended abdominal scars. For a more recent operation, epsilonaminocaproic acid was administered. This is a known inhibitor of plasminogen, one of the enzymes involved in fibrinolysis.

At the time of this Swiss patient's first examination and thereafter, she was on oral contraceptive as treatment for menorrhagia and had no symptoms suggesting disorder of the gastrointestinal tract.

No haematological abnormalities were found, including F XIII measurements made by the method of Lorand *et al* (1969). In the thromboelastogram, the maximal amplitude was insignificantly decreased. However, her plasma clots were completely dissolved in 1% MCA within three hours, but remained insoluble over forty eight hours in 5M urea. Since the patient's clots were dissolving in 1% MCA,

a defect of F XIII fibrin polymerization had to be excluded. This was done and if anything, the patient's fibrin polymerization appeared to be more efficient than normal.

It was discovered that the patient's plasma markedly enhanced dissolution of stable fibrin clots from normal plasma or purified normal fibrinogen at low pH. Such dissolution was more rapid at 37°C than at room temperature and could be delayed by cooling. This was indicative of a plasma factor, probably a proteinase, in the patient's plasma.

Urea denatured haemoglobin was used as a substrate (Anson 1938). After sixteen hours incubation the patient's plasma exhibited more than twice the enzyme activity present in normal plasma; her father showed 194% of activity, but her mother and sisters showed no significantly different activity from that of normal plasma. An attempt at a pH optimum was made using the patient's plasma, giving a pH optimum of between pH2.5 and 3.5.

The enzyme was inactivated by heating at 60°C for ten minutes, as was purified pepsinogen. Neutralisation following acid treatment inactivated the enzyme as with purified pepsinogen, but in contrast to purified red cell protease (Reichelt *et al* 1974).

Ragaz *et al* (1976) concluded that the acid clot solubility test is not a specific indicator of F XIII deficiency and that this patient's plasma exhibited an increase in pepsin-like activity.

Shanberge *et al* (1972) found that clots formed from normal young adult females are more stable than those from males to acid dissolution but those formed from females on oral contraceptive pills are significantly more stable than those of males or females not on oral

contraceptives. They concluded that this increased stability of fibrin clots to acid dissolution was not related to the increased fibrinogen level or to the level of F XIII in such individuals.

In 1973, Shanberge *et al* purified an enzyme which they thought could be responsible for the dissolution of stable fibrin clots. It was found to have a molecular weight of 44,000 and a pH optimum of 2.0.

Ikemori *et al* (1975) also isolated an enzyme from plasma. It was found to have a molecular weight of around 40,000 and a pH optimum of 2.0. They also tested the purified enzyme by immunodiffusion with specific pepsinogen antiserum. This gave reaction lines.

Patients with pernicious anaemia are known to have achylia and plasma was taken from them and added to normal plasma clots in 1% MCA. These clots did not dissolve. Similar findings were made with plasma from women taking oral contraceptives. Ikemori *et al* (1975) then added their purified enzyme and the clots dissolved. They concluded that the increased stability of fibrin clots seen in women taking oral contraceptives was in fact due to decreased levels of their purified enzyme.

The above work of Ikemori *et al* (1975) makes the female patient of Ragaz *et al* (1975) seem even more unusual, in that she was taking oral contraceptives and yet exhibited increased clot dissolution.

As early as 1952 Mirsky *et al* reported that plasma from healthy subjects contains at least three systems which display proteolytic activity:-

i) one active at pH3.5-4.0 is independent of the presence of the stomach and is inactivated at pH1.5

ii) one active at pH1.5-3.0 is independent of the presence of the stomach and is not affected by alkalination.

iii) one dependent upon the presence of a functioning gastric mucosa, which is inactivated by alkalination.

They concluded that the major proportion of activity of plasma at pH1.5 is due to the system which behaves like pepsin and its presence is attributed to the autocatalytic conversion of pepsinogen.

Hoar and Browning (1956) confirmed the findings of Mirsky *et al* (1952) and reported that plasma pepsinogen levels in patients' with peptic ulcer disease are significantly higher than the normal value. Conversely, they are significantly lower in patients with pernicious anaemia.

Ruenwongsa *et al* (1975), in their work with an acid protease in human seminal plasma, isolated an enzyme which they originally thought was pepsin. The enzyme could hydrolyze acid denatured haemoglobin at a rate comparable with that of hog pepsin, but not synthetic substrates such as N-acetyl-L-phenylalanyl-L-diiodotyrosine. Further irreversible inhibitors of pepsin, such as ρ-bromophenoxyl bromide and 1,2-epoxy-3-(ρ-nitrophenoxy) propane did not inhibit this enzyme to the same extent. This, they suggest, could be a difference between the specificity of the enzyme from seminal plasma and pepsin and possibly even a difference in the active site of the enzyme.

Laing (1975) confirmed the findings of Ragaz *et al* (not published until 1976), finding more acid proteinase activity in the Swiss patient's plasma than in normal plasma.

Taylor (1976) after further work on the patient's plasma,

found that activity after 55% saturation with ammonium sulphate was split between the precipitate and the supernatent; and that activity was not bound to aluminium hydroxide gel. She also found a difference in activity of pepsin and the enzyme found in the Swiss patient's plasma on different substrates. She concluded that the activity which she had found was probably a different one from that of Ikemori *et al* (1975), who found activity only in the 55% precipitate. It had also bound to aluminium hydroxide gel.

Laing (1975) and Taylor (1976) followed the activity of the plasma enzyme, using an adaption of the technique of Nelson *et al* (1961). This involves dying a powdered fibrin with indigo carmine and measuring spectrophotometrically the dye released by the activity in the plasma. However, Laing (1975) discovered that the presence of even small amounts of sodium chloride could cause an inactive solution to appear active, by releasing more dye than the solution itself. Taylor (1976) tested the fibrin blue against sodium chloride solutions of 0.15M, 0.3M, 0.4M, and 0.5M. Dye was released by these various concentrations of sodium chloride, but the dye release was not proportional to the sodium chloride concentrations. To overcome this she dialyzed her samples against neutral buffer before assaying them for activity.

The optical density readings which were taken by both Laing (1975) and Taylor (1976) were made after two hours incubation. It would be much more satisfactory to measure the activity of the enzyme by its initial velocity.

Problems of keeping the assay mixture in suspension and of standardizing the amounts of substrate for each assay had arisen.

The first aim of the present project was to look at the fibrin assay and to examine the possibilitiies of finding another suitable assay.

Since the activity sought is of a fibrinolytic nature, plasminogen free plasma was prepared and used as the starting material for the project.

Working concurrently with, but in opposition to, the coagulation system is the fibrinolytic system. This consists of a series of proenzymes, which are triggered by the action of the first activated clotting factor, activated Hageman Factor (F XIIa). This activates an activator in plasma, which causes the cleavage of plasminogen to form plasmin. Plasmin is an active fibrinolytic enzyme in the plasma.

In view of the difficulty of determining whether female blood donors were taking oral contraceptives and in the light of the work of Shanberge *et al* (1972) it was decided to use male donor plasma for the purification procedure outlined in this project.

The aim of the following project was to isolate the enzyme responsible for the dissolution of fibrin clots in an acid environment.

2. Preliminary studies on the fibrin blue assay

2.1 preparation of unstained fibrin

Fibrin was prepared using the method of Taylor (1976), from recalcified bovine plasma. This gave a suspension of fibrin which had a tremendous variation in particle size and it was felt that for the assay to have any chance of being reproducible, the particle size would have to be standardized.

At this stage it was decided to dry the fibrin overnight in an incubator at 37°C and to attempt to grind the fibrin in an automatic mortar and pestle, then finally to grade the powder. This proved to be tedious and the results were less than satisfactory, since the dried fibrin seemed particularly resistant to powdering.

Dried desiccated blood fibrin (Difco Laboratories, Detroit) was obtained and this was easily graded by passing through Endecott mesh sieves of grade 100 and 200. The material caught between 100 and 200 mesh was used.

2.2 choice of staining solution and method of staining

Since the indigo carmine dye used by Nelson seemed unsatisfactory for the reasons already given, it was decided to test the stability of a coomassie blue dye/fibrin complex. Coomassie blue is not particularly soluble in distilled water and a minimum of methanol was therefore used to make it dissolve.

staining solution

0.5g coomassie blue R250

10ml methanol

90ml 0.018M HCl

The solution was filtered before use.

Nelson *et al* (1961) had dyed the fibrin by heating at 80°C, but this seemed unnecessary with the fibrin/coomassie blue mixture as dye uptake appeared to be unaltered by temperature. It was decided to dye the fibrin in a 37°C waterbath. To ensure maximum contact of the fibrin particles with the staining solution, the mixture of fibrin and dye for staining was placed on an underwater magnetic stirrer.

Nelson *et al* (1961) used 0.018M HCl to wash the dyed fibrin and this solution was used, but containing 20mg of chloramphenicol/ 1000ml of 0.018M HCl, which was added as a bacteriostat.

The fibrin blue was centrifuged in a bench centrifuge, the supernatant removed and the pellet of fibrin blue washed with 0.018M HCl until the supernatant was clear. This took six to eight washings.

Fibrin blue was being used at a concentration of 450mg fibrin/ 200ml of 0.018M HCl. 90ml of staining solution was used to dye the fibrin.

2.3 stability of fibrin blue

The fibrin blue was tested with 0.5M NaCl solution and 7% gelatin. 2ml of each of these solutions were allowed to mix with 20ml of fibrin blue solution, for two hours at 37°C and the optical density of their supernatants read at 610nm, using supernatant from washed fibrin as a blank. No difference was seen between the sample and the blank. It was therefore assumed that the fibrin dyed with coomassie blue was stable with at least 0.5M NaCl and that the dye could not be eluted from the fibrin by the influence of gelatin, a protein.

2.4 removal of fibrin particles before reading optical densities

If an attempt to assay the activity of plasma was to be made using initial velocities, a method of removing aliquots of reaction mixture at different times throughout the reaction had to be devised.

Centrifugation as a method of removing the fibrin blue particles might seem satisfactory, but so long as the fibrin blue particles remain in contact with the enzyme mixture the reaction proceeds. So for reproducible time course studies, an immediate cessation of the reaction is required.

Several methods of stopping the reaction were tried, including cooling by plunging into liquid nitrogen, denaturing of the proteins by the addition of concentrated alkali, precipitation of proteins with 5% trichloracetic acid and boiling on its own. This drastic treatment of the fibrin blue reaction mixture caused the dye to be strongly eluted from the fibrin. Even if this had proved satisfactory, fibrin particles would still have had to be removed.

13mm Swinnex disc filter holders (Millipore U.K. Ltd.) fitted on to the end of a 5ml disposable syringe, with Whatman no.541 filter paper cut to size as a filter pad, were then tried. These were found to be ideal and had the advantage that the filter holders could be easily cleaned with distilled water, then dried with acetone for re-use.

2.5 spectrophotometric investigation

2.5.1 maximum absorbance

A 1/2 dilution of plasma in 0.05M phosphate buffer at pH7.0 was made and added to an aliquot of fibrin blue solution. This was allowed to react for two hours to give colour elution from the fibrin blue. Once cleared of fibrin particles, the supernatant was diluted 1/10 with distilled water. Using clear supernatant from the washed fibrin blue as a blank, readings were made on a spectrophotometer over various wavelengths, to see which gave the maximum absorbance. 610nm was found to be the most suitable. It was therefore decided that optical density readings would be performed at 610nm, using the clear supernatant of washed fibrin blue as a blank, hereinafter simply called the blank.

2.5.2 linearity of dye solution

The Lambert and Beer Law states that the extinction is proportional to the concentration of the absorbing substances and to the thickness of the layer.

A sample with colour elution was prepared as described above.

Aliquots of the supernatant cleared of fibrin particles were diluted to 12.5%, 25%, 50% and 75% with distilled water and, using a blank, their extinctions were read at 610nm.

A graph of optical density readings versus the dilutions of eluted dye was plotted. This gave a straight line with optical density readings between 0.05 and 1.1. It was decided that samples having an optical density reading greater than 0.8 should be diluted with distilled water so that they might fall within the linear portion of the graph.

2.6 initial velocity studies

The fibrin blue assays carried out by Nelson *et al* (1961), Laing (1975) and Taylor (1976), were carried out using a single reading to estimate the enzyme activity after a fairly long incubation. Laing and Taylor both experienced problems with keeping the fibrin in suspension during the incubation period.

In order that the conditions for each assay may be reproduced as far as possible, it is essential when measuring enzyme activity to do this at the initial stages of the reaction, where the influence of products being formed or substrate being exhausted will be at a minimum.

The problem of mixing the fibrin blue during the reaction was overcome by placing the reaction vessel over an underwater magnetic stirrer. It was important to attempt to achieve a standard mixing rate. The flow rate of the water gushing from the exit of the magnetic stirrer was measured and the same magnetic flea used each time. The flow rate of the water was 100ml/sec and the flea was 2.5cm long.

Fibrin blue solution was kept in an even suspension by gently mixing on a magnetic stirrer. 50ml were removed by means of a 10ml blowout pipette. This was allowed to equilibrate to 37^{o}C by placing in a waterbath and allowing to mix for five minutes. 5ml of plasma diluted 1/4 with 0.05M phosphate buffer pH7.0 were added and a stopwatch immediately started.

Aliquots of approximately 1ml were removed by means of the Swinnex disc filter holder described above, at thirty second intervals. These were diluted as necessary and read at 610nm against a blank. A graph was plotted of optical density readings versus time and a

tangent drawn through the origin to the curve obtained. The rate of the reaction was calculated and the activity was expressed in units of activity, where a unit of activity is defined as an increase in absorbance of 0.01 per minute.

The results of several tests using the same batch of fibrin blue were satisfactorily reproducible, although variations were seen between batches.

It was decided that freeze dried bovine serum should be used as a standard. Results obtained with each batch of assays would in future be based on a standard activity of 6.25 units of activity/ml sample. The strength of the bovine serum sample was 30mg/ml.

Taking aliquots from the reaction vessel at thirty second intervals was found to be unnecessary and thirty seconds, two minutes, five minutes, ten minutes, fifteen minutes and twenty minutes proved sufficient. This allowed the volume of fibrin blue solution used for each assay to be reduced to 20ml and, more importantly, to reduce that of the enzyme sample to 2ml.

2.7 effect of substrate concentration

When substrate concentration is plotted against initial velocity, a section of rectangular hyperbole is obtained.

To test this statement, a series of different concentrations of fibrin blue was made. The 100% solution was taken as 450mg fibrin/200ml of 0.018M HCl and from this, solutions of 150%, 80%, 70%, 60%, 50%, 25%, and 12.5% were made.

Assays were performed with all the above concentrations of substrate, using 20ml of fibrin blue solution and 2ml of plasma diluted

with 0.05M phosphate buffer pH7.0. All assays were performed in duplicate. A graph of activity versus substrate concentrations was plotted (figure 1).

The graph does show the shape of a rectangular hyperbole, with the reaction beginning to fall off at the higher substrate concentrations. The low activity seen in the 150% concentration may be due to experimental error, since at this concentration of fibrin particles it was difficult to remove all the particles with the first filtration.

It was decided to use the method of Lineweaver and Burke (1934) to plot the reciprocal of the activity versus the reciprocal of the substrate concentration. This gives a straight line which cuts the base line axis at -1/Km.

Experimental data of activity and substrate concentrations from the above experiment were used to plot the graph (figure 2). From this the Km is seen to be a substrate concentration of 133.3% (3.0mg/ml).

2.8 effect of enzyme concentration

The velocity of the enzyme reaction should be proportional to the enzyme concentration.

As stated earlier, difficulty occurred in the filtration procedure when fibrin particles became concentrated. For the experiment which follows, it was decided to make the concentration of fibrin blue 1.7mg/ml.

To test the statement given above, a range of dilutions of plasma was made, 6.25%, 12.5%, 25%, 35%, 50%, 70%, 90%, in

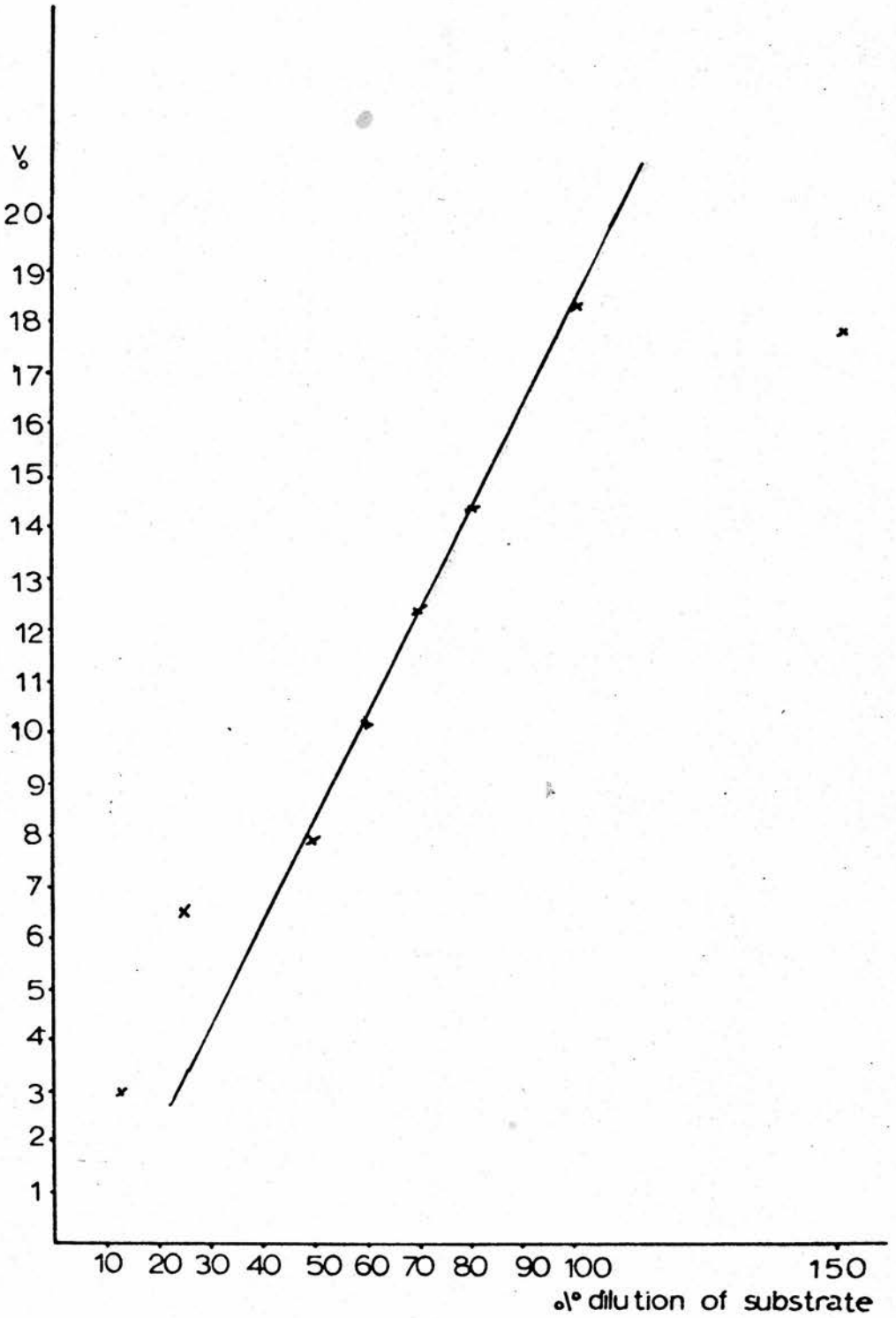

figure 1 the effect of substrate concentration. A graph of initial velocities versus percentage dilutions of dyed fibrin (2.25mg/ml equals 100%)

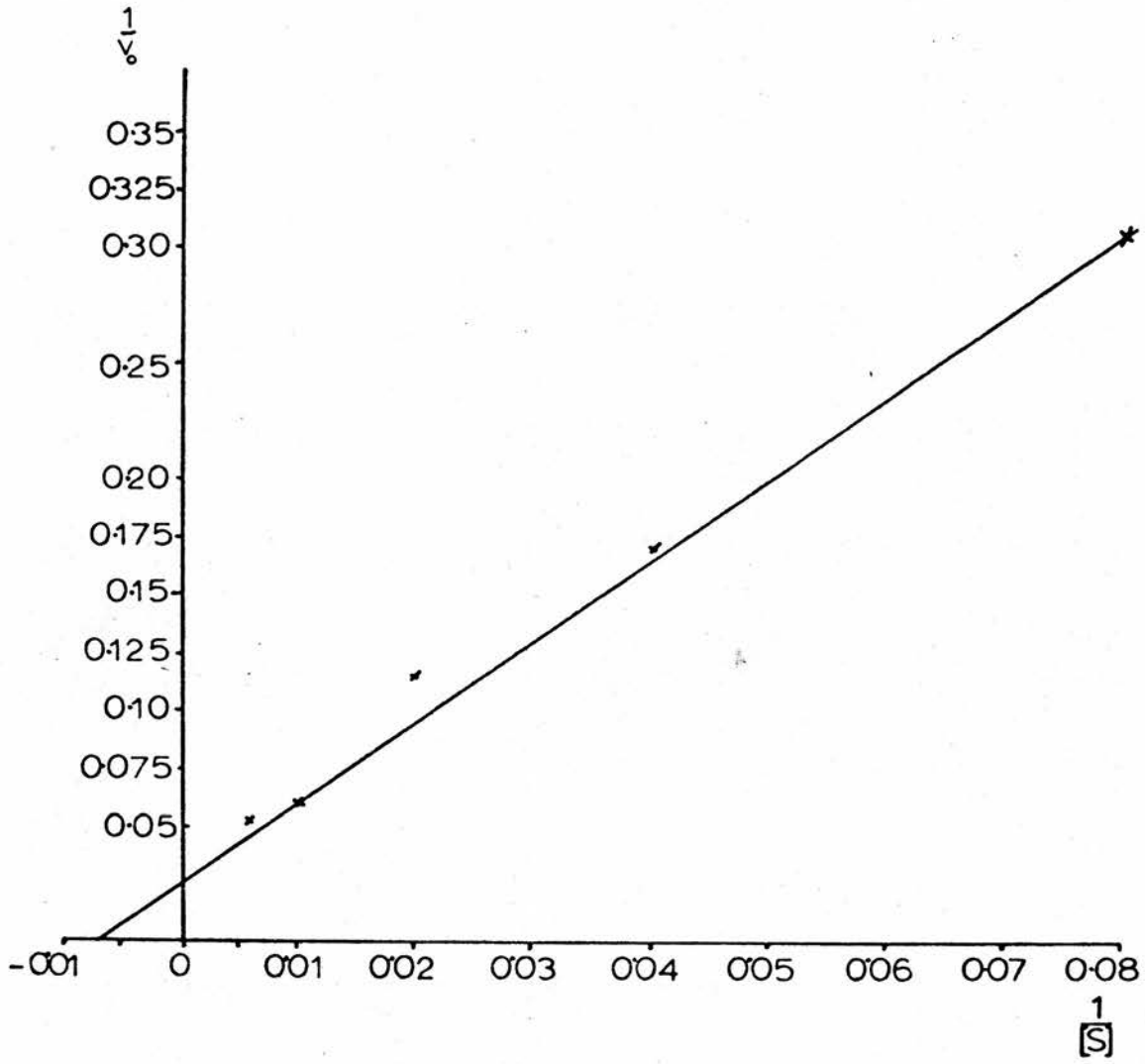

figure 2 The estimation of the Km of a 1/4 dilution of plasma using the method of Lineweaver and Burke (1934). KM equals a substrate concentration of 133.3%

0.05M phosphate buffer pH7.0. Assays with fibrin blue were performed in duplicate on each of these dilutions. A graph was plotted of activity versus plasma concentration (figure 3).

The line obtained is essentially linear, with a tendency to stray from linearity at its extremities. This non-linear rate change is a commonly observed phenomenon in proteinase reactions and it has been described by Dixon and Webb (1966).

This experiment was repeated using various concentrations of pepsin (human Sigma), from 2.5µg/ml - 100µg/ml. This gave a similar line to figure 3, again showing deviations from linearity at its extremities.

2.9 conclusion

The concentration of the fibrin blue used for these experiments has been below the Km, but as can be seen from figure 3, a linear relationship can be seen, provided the concentration of the enzyme is not too high.

The substrate is insoluble and as the reaction proceeds, presumably the hydrolysis of the substrate by the enzyme will make more substrate available. It was decided to proceed with the enzyme purification using the procedure outlined in the following methods section as the assay. Care was taken to ensure that the substrate concentration was kept constant and that each batch of fibrin blue was standardized as already described.

Bearing in mind that these experiments were carried out with plasma, which contains a wide variety of enzymes capable of influencing activity and of proteins which could be preferentially hydro-

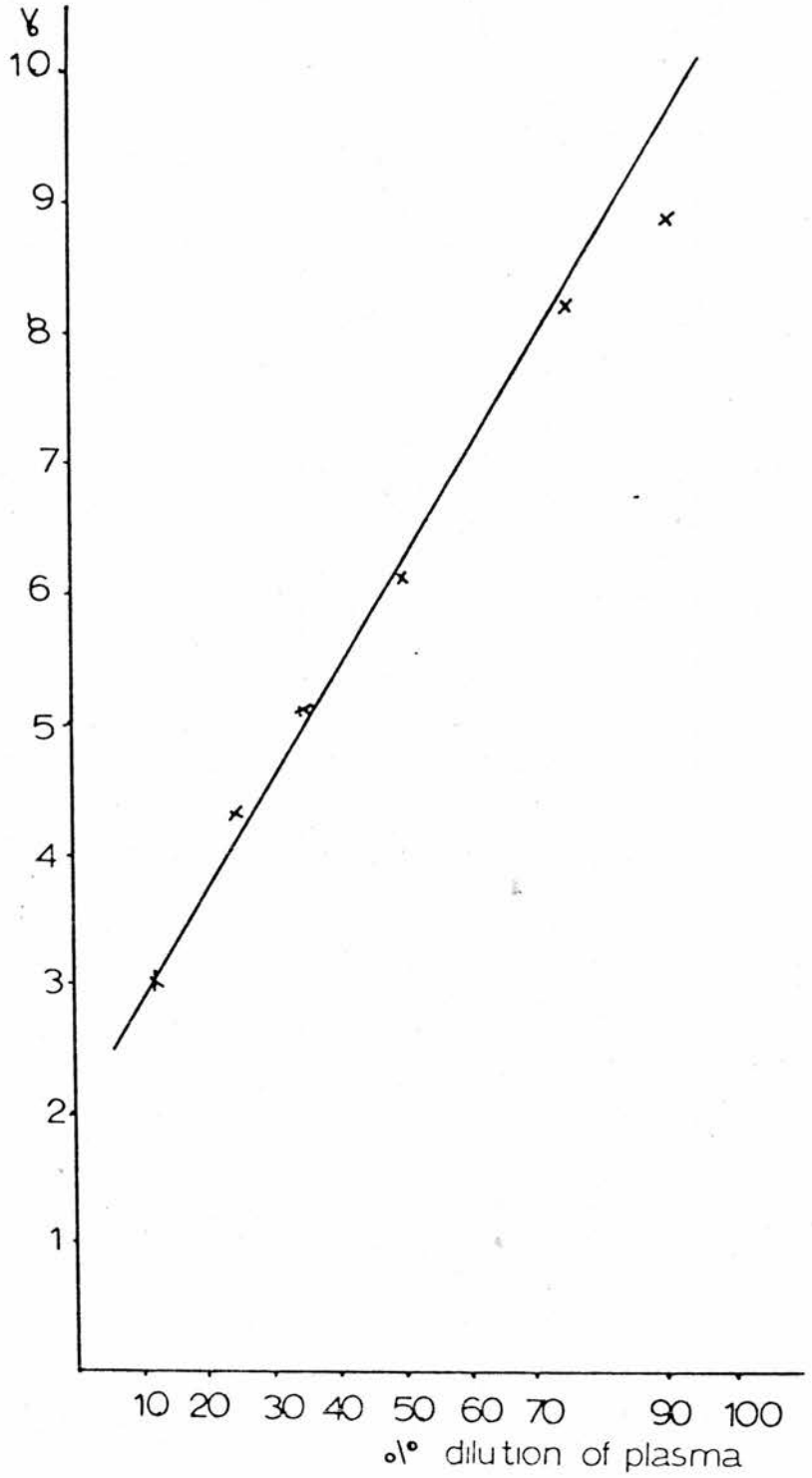

figure 3 The effect of enzyme concentration. A graph of initial velocities versus percentage dilutions of plasma

lysed by the acid proteinase in preference to the fibrin blue, it would be advantageous to perform more intense kinetic studies once the enzyme has been purified.

3 Methods: The Assays

3.1 Fibrin blue assay

3.1.1 preparation of dyed fibrin

450mg of desiccated fibrin

90ml of coomassie staining solution

2 litres of 0.018M HCl washing solution

The fibrin was mixed with the staining solution and placed over an underwater magnetic stirrer for thirty minutes in a water-bath at 37°C. The fibrin blue was then washed several times with 0.018M HCl, until the supernatant was clear, then made up to 266.6ml with 0.018M HCl.

3.1.2 the assay

20ml of the above fibrin blue solution was taken and allowed to equilibrate to 37°C for five minutes in a waterbath. The fibrin blue was kept in suspension by placing the beaker over an underwater magnetic stirrer. 2ml of the sample to be tested was pipetted into the reaction beaker and a stopwatch immediately started. Aliquots were removed at thirty seconds, two, five, ten, fifteen and twenty minutes. The optical density of these aliquots was then read on a spectrophotometer at 610nm against a blank. The initial velocity of the sample was then calculated by plotting optical density versus time and drawing a tangent to the curve of these points.

3.1.3 screening of fractions from column chromatography

Each fraction collected during the purification of the plasma was screened for the presence of activity as follows.

1ml of fibrin blue was pipetted into a series of test tubes, one for each fraction collected. 0.1ml of fraction to be tested

was pipetted in and these tubes were shaken as vigorously as possible in a waterbath at 37°C for one hour. They were then removed from the bath and allowed to sit on the bench at room temperature for approximately thirty minutes, to allow the fibrin particles to settle. The optical density of each fraction was then read in a spectrophotometer at 610nm, by carefully removing the supernatant with a pasteur pipette and placing it directly into a 1ml plastic cuvette. The optical density readings thus obtained were directly plotted on the elution pattern.

3.2 Clot dissolving assay

3.2.1 preparation of clots

Small clots were prepared, 0.1ml M/20 $CaCl_2$ to 0.3ml of plasma were incubated at 37°C for sixteen hours to ensure stable clot formation. After this time had elapsed, one of the clots was tested for stable clot formation by placing it in 5M urea at 37°C for thirty minutes. As the clot failed to dissolve, stable clot formation was seen to have taken place. These small clots were then washed against 1% NaCl solution for three days at 4°C, changing the NaCl solution twice daily. The clots were transferred to distilled water for two hours before use.

3.2.2 the assay

2.6ml of 1% monochloracetic acid (MCA) was added to 0.4ml of test sample and a small clot added. This mixture was placed in a waterbath at 37°C and shaken vigorously. From time to time the clot mixtures were removed from the waterbath and the presence or absence of a clot observed.

Each sample was set up in duplicate and a set of controls put

up each time. The control was a mixture of a small clot and 2.6ml 1% MCA. Only if the clot in the control was still visible were the times of disappearance of the test clots recorded.

3.3 Acid denatured haemoglobin assay

This method is based on that of Tang (1970).

3.3.1 preparation of haemoglobin (Hb)

A 10% solution of Hb (Armour Pharmaceutical bovine Hb enzyme substrate powder) was made in distilled water. This was dialyzed against distilled water for twenty four hours at 4°C, changing the distilled water three times. Once dialyzed, the Hb solution was centrifuged at 2500 x g for thirty minutes to remove any sediment, the supernatant removed for use and 2.5mg of thiomersal added as a bacteriostat.

3.3.2 preparation of acid denatured Hb substrate

This should be prepared freshly on the day of assay.

3.5ml of 0.3M HCl were added to 10ml of 10% Hb solution and the volume adjusted to 50ml with distilled water. The pH of the substrate was adjusted to 2.5 using concentrated HCl. This should be equilibrated at 37°C just prior to performing the assay.

3.3.3 the assay

0.25ml of enzyme solution with 0.25ml of 0.1M sodium citrate buffer pH2.5 was pre-incubated at 37°C. 2.5ml of the warmed acid Hb solution were then added. The mixture was then incubated at 37°C for sixteen hours.

5ml of 5% trichloracetic acid were then added to stop the reaction, by precipitating the proteins. This was allowed to stand

for ten minutes and then filtered through Whatman no.3 filter paper.

The filtrate was read on a spectrophotometer at 280nm. All assays were set up in duplicate. A blank was also set up with each sample. This was treated in the same way as above, but the trichloracetic acid was added before the sample was incubated.

The activity is expressed as the average of the change in optical density between the sample and the blank.

3.4 N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT) assay

The method is that of Tang (1970).

3.4.1 preparation of substrate

2mM APDT* in 5mM NaOH was prepared by weighing out 124.4mg of APDT and dissolving it in 5ml of 0.1M NaOH. Once dissolved, the solution was made up to 100ml with distilled water.

3.4.2 preparation of ninhydrin

750ml of methyl cellosolve had removed from it any peroxides which may have formed during storage, by bubbling oxygen free nitrogen through it for thirty minutes.

To test for the presence of peroxides, equal volumes of 5% potassium iodide and methyl cellosolve were mixed and allowed to stand for two minutes. If the colour of solution should change from straw coloured to deep yellow, peroxides are present.

250ml of 4M sodium acetate buffer pH5.5 were prepared and oxygen free nitrogen bubbled through for thirty minutes.

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* The APDT was generously provided by Dr. A.P. Ryle of the Department of Biochemistry of the University of Edinburgh

20g of ninhydrin and 3g of hydrindantin were mixed with the methyl cellosolve and buffer solutions. The mixture was decanted into an analyzer bottle and oxygen free nitrogen pushed through the system to remove air, for about fifteen minutes.

3.4.3 the assay

0.5ml of the sample to be tested, 0.25ml of 0.208M HCl and 0.25ml of APDT substrate were taken and incubated at 37°C for one hour. All tests were performed in duplicate.

1ml ninhydrin was added to each and they were put in a boiling waterbath for fifteen minutes, then cooled in cold water. 5ml of 60% ethanol was added and shaken for thirty seconds.

Blanks were set up by adding ninhydrin before APDT. The samples were then read on a spectrophotometer at 570nm against the blanks.

3.5 Radial enzyme diffusion into skimmed milk containing agarose gel

The method is based on that of Lowenstein *et al* (1976).

3.5.1 materials

1g of agarose was dissolved in 100ml of distilled water by boiling and allowed to cool to 55°C, then kept at that temperature.

1g of skim milk powder was reconstituted by gradually adding 30ml of 0.1M sodium acetate buffer pH5.5.

3.5.2 preparation of plates

10cm x 10cm x 0.1cm glass plate was acid washed and steeped in methanol to ensure that it was grease free. The plate was then covered in a thin layer of agarose, by wiping with a gel moistened paper tissue. Once this was dry the plate was placed on a level

board.

13.5ml of 1% agarose at 55°C were mixed with 1.5ml of reconstituted milk powder. This mixture was poured over the glass plate and allowed to set.

The gels were then incubated in 100ml of appropriate buffer. To reach pH2.8, 0.1M sodium acetate/formate was used. 0.015M sodium azide was added. pH2.1 0.2M HCl/KCl buffer was used.

The gels were removed after two hours at 37°C in the buffers. 2.5mm wells were then punched in the gel plates and 25µl of sample to be tested was placed in each well.

The plates were then incubated for twenty hours at 37°C. They were then allowed to dry out slowly at room temperature. The area of digestion could easily be seen as a clear ring surrounding the wells, but once the plates had dried out, this area was not so well defined. It was therefore decided to stain the gels before drying.

3.5.3 staining solution

1.5g napthalene black

250ml methanol

250ml distilled water

50ml glacial acetic acid

3.5.4 destaining solution

250ml methanol

250ml distilled water

50ml glacial acetic acid

The gels were stained in staining solution for two minutes, then placed in the destaining solution for at least forty eight

hours, changing this solution several times, until the digested areas were clear of dye. The plates were then dried as described above.

3.5.5 measurement of activity

The activity of the samples applied was measured as the area of the clear ring of digestion seen around the well. This could be done by placing the plate on a piece of graph paper on a light box and counting the squares.

4 Methods: Analytical and preparative

4.1 SDS disc gel electrophoresis

The method is a modification of that described by Weber and Osborn (1969).

4.1.1 acrylamide stock

38.7g acrylamide

2.66g N',N' methylebisacrylamide (bisacrylamide)

made up to 100ml with distilled water and stored in a dark bottle at 4°C.

4.1.2 buffer

0.5g sodium dodecyl sulphate (SDS)

0.38ml N,N,N',N', tetramethylethylendiamine (TEMD)

120g urea

30ml 0.1M disodium hydrogen phosphate

20ml 0.1M sodium dihydrogen phosphate

made up to 250ml with distilled water and stored at 4°C.

4.1.3 catalyst

0.14g ammonium persulphate in 100ml of distilled water, made up fresh for use.

4.1.4 chamber buffer

0.01M phosphate buffer

0.1% SDS

this should have a pH of 7.1.

4.1.5 tracking dye

0.025g bromophenol blue in 50ml of distilled water.

4.1.6 5% gel solution

2.5ml acrylamide stock

10ml of buffer

7.5ml of distilled water

0.03g ammonium persulphate

This was poured into gel tubes 5mm x 75mm with chamber buffer carefully layered on top, so that a straight running edge would be obtained. Gels could be stored overnight at room temperature in this state.

Samples for disc gel electrophoresis were dialyzed overnight at 4°C against chamber buffer. They were then applied to the top of the gel, mixed with one drop from a Pasteur pipette of both tracking dye and glycerol. The amount of sample varied but was approximately 10µl of a 10mg/ml solution.

Chamber buffer was used in the electrode tanks and with the anode at the top, the gels were run with a current of 2mA/gel at a voltage of about 60V. The run was allowed to proceed until the tracking dye had reached the bottom of the gel, which took approximately two hours.

After measuring the length of the gel and the distance which the tracking dye had run, the gels were removed from their tubes and stained for 30-45 minutes. They were then destained overnight with several changes of destaining solution.

4.1.7 staining solution

1.25g coomassie brilliant blue R250

227ml methanol

227ml distilled water

46ml glacial acetic acid

4.1.8 destaining solution

675ml distilled water

250ml methanol

75ml glacial acetic acid

The position of the protein bands on the gels was recorded using, in St Andrews, a Vitatron TLD 100 densitometer with 0.25 x 2.5cm slit width, 570nm filter and a chart speed of 60mm/min, and in Edinburgh a Gifford gel scanner with the wavelength at 580nm a scan rate of one and a chart speed of 30mm/min.

Measuring from the top of the peaks to the start of the gel, the corresponding molecular weights were calculated, using the method of Weber and Osborn (1969).

4.1.9 standards

albumin human fraction V

albumin egg

myoglobin whale skeletal muscle type 11

all were supplied by Sigma and stored desiccated and frozen. A stock solution of each, 5mg/ml in distilled water, was made up and kept frozen. A solution for use was made up as follows:-

0.1ml of stock

0.9ml of chamber buffer

10µl of 1% mercaptoethanol

this was incubated at 37°C for three hours and stored frozen for use.

10µl of each standard was mixed with the tracking dye and glycerol and run along with the samples.

4.2 SDS Slab gel electrophoresis

20ml of 5% gel solution were poured into 7.3cm x 8.3cm glass plates. The samples were made up as before and run at 15mA for approximately two and a half hours, until the tracking dye had reached the bottom of the gel. The gels were then removed from the plates and a small portion down one side cut off. The remaining gel was stored at $4^{o}C$ in a Petrie dish. The smaller portion was stained for twenty minutes, then quickly destained by placing it in destaining solution containing some DEAE-cellulose and gently agitating it. By following this procedure the protein bands were visible within thirty minutes.

The smaller portion was then aligned with its parent gel and the individual protein bands cut out. These were broken up with the aid of a mortar and pestle and eluted, with several changes of chamber buffer. The eluted material was then separated from the gel pieces by filtering through Whatman no.1 filter paper, then dialysed for three days with two changes per day against 0.1M phosphate buffer pH7.1 at $4^{o}C$.

At the end of this time a small aliquot was taken and saturated potassium chloride added as described by Van Heyningen (1973). The K^{+} would form an insoluble precipitate with any remaining SDS; since no precipitate was seen, it was assumed that all the SDS had been removed. Each sample was then freeze dried to concentrate the protein. Immunoelecrophoresis, disc gel electrophoresis and spec-

ific activities were then performed on each sample.

4.3 Immunoelectrophoresis

4.3.1 chamber buffer

2% sodium barbitone brought to pH8.2 with concentrated HCl.

4.3.2 I.D.agar

1.5% I.D.agar (Oxoid) was dissolved in 1% sodium barbitone buffer pH8.2. 0.05M sodium azide was added as a bacteriostat.

Glass microscope slides were cleaned with dichromate sulphuric acid glass cleaner, then stored in alcohol for use. Three slides were flooded with 12ml of molten agar. Troughs and wells were cut in the agar surface. Samples were placed in the wells, with tracking dye in one of them. The samples were run at a constant voltage of 160V until the tracking dye had run to the end, taking approximately three hours. Specific antiserum was then put in the troughs and allowed to diffuse in a damp chamber for twenty four hours at room temperature. Slides were then washed for forty eight hours in 1% NaCl solution, then for two to three hours in distilled water. They were then dried overnight in an oven at $37^{o}C$.

Once dried, the slides were stained in solution 4.1.7 for two to three minutes, then left to destain overnight in solution 4.1.8.

The antisera used were:-

anti albumin (rabbit)

anti human serum (rabbit)

anti α_2 macroglobulin (rabbit)

all supplied by Behring and kept at $4^{o}C$.

4.4 Davies disc gel electrophoresis

The method is that described by Davies (1964).

4.4.1 gel solutions

Solution A	Solution B
48ml 1M HCl	48ml 1M HCl
36.6g Tris	5.98g Tris
0.25ml TEMED	0.5ml TEMED
distilled water to 100ml	distilled water to 100ml
pH8.9	pH6.7

Solution C	Solution D
28g acrylamide	10g acrylamide
0.74g bisacrylamide	2.5g bisacrylamide
distilled water to 100ml	distilled water to 100ml

Solution E	Solution F
4mg riboflavin	40g sucrose
distilled water to 100ml	distilled water to 100ml

Solution G

0.14g ammonium persulphate

distilled water to 100ml

4.4.2 tank buffer

6g Tris

28.8g glycine

distilled water to one litre pH8.3 dilute ten times for use.

4.4.3 7% separation gels

1 part of solution A

2 parts of solution C

1 part of distilled water

mixed before pouring with 4 parts of solution G

This solution was poured into gel tubes as for disc gel electrophoresis, except that they were finally layered with distilled water until set. A layer of stacking gel was then poured on top of the separation gel.

4.4.4 3% stacking gel

1 part of solution B

2 parts of solution D

1 part of solution E

4 parts of solution F

This was left to polymerize using a long wave ultraviolet light for twenty minutes.

Samples were applied as for disc gel electrophoresis and run at 1mA/gel until a tight band had formed in the stacking gel, then at 2mA/gel until tracking dye reached the end of the gel.

Gels were then stained and destained as in disc gel electrophoresis and the positions of the bands of protein noted as before.

4.5 Davies slab gel electrophoresis

Glass plates 15cm x 14cm were used. A plug was formed at the bottom of the plate with 20% acrylamide solution.

4.5.1 20% acrylamide solution

8.35ml of solution C

2.32ml of solution A

1.07ml of distilled water

0.75ml of solution G

10µl TEMED

Once this had set, 30ml of separating gel solution was poured down the plate on top of the plug. A layer of secondary butanol was then carefully layered on top to give a smooth running edge. Before use, the secondary butanol was washed off with solution B and 10ml of stacking gel were then poured in on top of the separating gel and polymerized as before. Samples were applied as before and run in a Raven slab gel electrophoresis tank at a constant voltage of 150V.

Slab gels were then treated as in section 4.2, except that they were eluted with 0.1M phosphate buffer pH7.1, then directly freeze dried. Immunoelectrophoresis and activities were again performed on each sample.

4.6 Protein estimation, micro biuret techniques

The method is that of Itzhaki and Gill (1964).

reagent(i)

0.21% copper sulphate in 30% sodium hydroxide

reagent(ii)

30% sodium hydroxide

Reagents can be stored at room temperature for at least six months.

The protein was estimated as follows.

A_1 2ml distilled water + 1ml reagent(i)

A_2 2ml protein + 1ml reagent(i)

B_1 2ml distilled water + 1ml reagent(ii)

B_2 2ml protein + 1ml reagent(ii)

All mixtures were mixed vigorously on a vortex mixer and allowed to stand for five minutes so that colour development could reach its maximum. Optical density of the mixtures was read at 310nm as follows:-

A_2 against A_1 to give Δ A

B_2 against B_1 to give Δ B

The difference between Δ A and Δ B is the optical density of the solution.

The protein content of the solution is read off a standard curve made using bovine albumin fraction V (Sigma).

4.7 Purification

Out of date male plasma was used for this purification. It was generously supplied, first by the East of Scotland Blood Transfusion Service (Dundee) and, later, by the South of Scotland Blood Transfusion Service (Edinburgh).

4.7.1 removal of plasminogen

The following method is based on that of Deutsch and Mertz (1970).

Lysine sepharose 4B was swollen and washed with 0.05M phosphate buffer pH7.5. A column 15cm x 1.8cm was packed and equilibrated with phosphate buffer. Ten column volumes of plasma were passed through

the column at a flow rate of 10-15ml/hr. The column was then washed with phosphate buffer until the absorbance of the effluent fell below 0.05 at 280nm. The effluent was plasminogen free plasma. Plasminogen was then eluted from the column, which was then re-equilibrated.

4.7.2 ammonium sulphate precipitation

The first batch of plasma was brought to 70% saturation with ammonium sulphate and allowed to mix for eighteen hours at $4^{o}C$, before being centrifuged at 2500 x g at a temperature of $4^{o}C$ for fifteen minutes. The precipitated material was then reconstituted in 0.85% sodium chloride and dialyzed for three days against the sodium chloride which was changed twice a day. The non-diffusible material was used for the next step.

The second batch of plasma was first brought to 50% saturation and then to 70% saturation with ammonium sulphate; each fraction was then treated as above.

4.7.3 aluminium hydroxide gel fractionation

This method was based on the method of Ikemori *et al* (1975).

gel preparation. 50ml of 0.2M aluminium sulphate were heated to $63^{o}C$. 5ml of 58% ammonium hydroxide were added to 45ml of distilled water previously heated to $67^{o}C$. The ammonium hydroxide solution was poured into the ammonium sulphate in a steady stream. Temperature was maintained at $62-63^{o}C$. while the mixture was stirred for ten minutes. The gel was then washed with distilled water until no free sulphate could be detected by testing with barium nitrate.

fractionation. One tenth volume of aluminium hydroxide gel was added to the non-diffusible material from above. It was stirred

vigorously with a magnetic stirrer at room temperature for one hour. The mixture was then centrifuged at 2500 x g for thirty minutes at 18°C and the supernatant was collected and used for the following purification. The gel was then washed six times with 100ml of 0.85% sodium chloride. The first supernatant from these washes was added to the original supernatant. The gel was then extracted twice with 125ml of 0.5M phosphate buffer pH7.0 and the eluted material collected.

4.8 ion exchange chromatography

4.8.1 batch tests

The activity in plasma was found to stick to both DEAE-cellulose and CM-cellulose at pH's 8, 7, 6, 4, and 3 when batch tests were carried out by Laing (1975).

4.8.2 DEAE-cellulose 52 (Whatman)

The DEAE-cellulose resin was precycled, degassed and the fines removed.

A column 8cm x 2.5cm was then packed with the resin and allowed to equilibrate by pumping starting buffer (0.05M Tris/HCl pH7.6) through the settled resin column. The flow rate was adjusted to 30ml/hr. A sample of plasma was dialyzed for sixteen hours at 4°C against the starting buffer. Once the column had equilibrated, the plasma was applied to the column and fractions collected every twenty minutes.

Starting buffer was pumped through until no more protein was seen in the effluent, then buffer pH5.6 was applied until again no protein was coming off. Then a 1ml NaCl solution was applied to

clear the column of protein.

4.8.3 CM-cellulose 52 (Whatman)

This resin was precycled and the fines removed.

A column 8cm x 2.5cm was packed and equilibrated as described above, using a 0.05M sodium acetate buffer pH4.0. A sample of plasma was dialyzed against acetate buffer. Fractions were collected as above until the effluent contained no protein. 0.05M sodium acetate buffer pH5.4 was then applied, followed by 1M NaCl to clear the column of protein.

4.8.4 Sulphopropyl Sephadex (SP-Sephadex)

The SP-Sephadex was supplied by Pharmacia.Ltd. of Stockholm. This resin does not require precycling, but should be allowed to swell at room temperature for one to two days in the buffer which is to be used for the experiment. The buffer should be changed several times.

The method used with SP-Sephadex is a modification of the method of Hagenmaier and Foster (1971).

A column 20cm x 1.8cm was poured using the swollen SP-Sephadex in a starting buffer which was 0.02M sodium acetate buffer pH4.3 0.18M NaCl. The sample to be applied was dialyzed for sixteen hours at 4^{o}C against this buffer.

Once the column had equilibrated, the sample was applied and starting buffer pumped through the column at the rate of 30ml/hr. Fractions were collected every twenty minutes. When the effluent was free of protein 0.02M sodium acetate buffer 0.27M NaCl was pumped through and fractions collected until no further protein was seen in the effluent.

4.8.5 Sulphoethyl Sephadex (SE-Sephadex)

The SE-Sephadex was supplied by Pharmacia Ltd. of Stockholm. This resin was used in exactly the same way as described above, only a Pasteur pipette column 8cm x 0.5cm was made.

4.9 Gel filtration

4.9.1 Sephadex G100

This was supplied by Pharmacia Ltd. of Stockholm. It was swollen for three days at room temperature in an excess of running buffer, 0.01M phosphate buffer pH7.1. It was then degassed in a Buchner flask for a few minutes before being packed into a column 45cm x 2.5cm. Great care was taken in packing the column and the operating pressure used during the experiment was kept to about 150mm.

To test the efficiency of the packing, Dextran blue (0.2%) was applied and phosphate buffer pumped through the column at a rate of 20ml/hr. The void volume of the column was also measured as the Dextran blue passed out of the column. Samples to be applied to this column were freeze dried and made up to a minimum volume with distilled water. Fractions were collected every five minutes.

4.9.2 Sephadex G200

This was supplied by Pharmacia Ltd. of Stockholm. It was treated in a similar manner to that described above, except that once the column had been packed, the buffer was allowed to feed through the column by gravity. Samples to be applied to the column were freeze dried and made up to a minimum volume with distilled water. Fractions were collected every twenty minutes.

4.10 Affinity chromatography

4.10.1 Blue sepharose CL-6B

This method is a modification of that of Pharmacia Fine Chemicals (1976). The sepharose was supplied by Pharmacia Ltd. of Stockholm as a freeze dried powder and the required amount was swollen and washed in distilled water on a sintered glass filter for about fifteen minutes. 1g of freeze dried powder required about 200ml of distilled water for washing.

As only a small amount of this powder was available, only a batch test was possible and a small column 3cm x 1.8cm was packed and equilibrated with starting 0.05M Tris/HCl buffer at pH7.0,which contained 0.1M KCl. The sample to be applied was dialyzed against the starting buffer for sixteen hours at $4^{o}C$. The sample was applied and starting buffer pumped through at a flow rate of 24ml/hr until the effluent was clear of protein.

0.05M Tris/HCl buffer at pH7.0 containing 1.5M KCl was then applied until no further protein was eluted. Fractions were collected every five minutes.

4.10.2 preparation of activated sepharose

The following method is based on that of Chibber *et al*(1974).

materials. 40g of sepharose 4B (Pharmacia) in 100ml of distilled water. A 5% cyanogen bromide solution in distilled water was prepared. The powder was weighed in the cold and dissolved in an ice bath. All these procedures must be carried out in a fume cupboard. *N.B.* CNBr only keeps for about one year.

One litre of 0.1M sodium bicarbonate was made up in cold distilled water and its pH corrected to 8.4.

0.5M NaOH

ice cubes made from distilled water

procedure. This was carried out in a fume cupboard. Using ice cubes to maintain a temperature of 18-20°C, the CNBr was added to the suspended agarose while stirring the mixture. During this operation a pH meter was used to monitor the pH level which had to be kept at pH11 using 0.5M NaOH. The reaction was allowed to proceed for ten minutes. It was then stopped by washing in a Buchner filter with the ice cold $NaHCO_3$.

This activated sepharose was now ready to have the following attached:-

4.10.3 anti-albumin

1ml of anti-albumin 4.1.9 was added to 5g of the activated sepharose

4.10.4 albumin (Human Sigma)

5ml of 4mg/ml albumin in 0.2M sodium bicarbonate pH9.0 were added to 5g of the activated sepharose.

Both sepharose conjugate mixtures were left to gently mix for twenty four hours on a roller mixer at 4°C.

2.5mg of lysine were then added to each mixture to block any spare CNBr groups. This was left as before for twenty four hours at 4°C.

The conjugates were then washed in a Buchner filter with one litre of 1M NaCl and 2 litres of distilled water. They were stored at 4°C with 0.02% sodium azide as a bacteriostat.

4ml of protein sample were added to each conjugate and they were left as before for sixteen hours at 4°C.

Conjugates were then centrifuged in a bench centrifuge and the supernatant removed and kept as material unbound. Material was eluted by washing with 1M NaCl.

Both sets of unbound and eluted material were dialyzed for sixteen hours against two changes of 0.01M phosphate buffer pH7.1, then freeze dried.

4.10.5 dextran blue sepharose

Activated sepharose was used from the above. Dextran blue was attached using the method of Travis and Pannell (1973).

0.5g of dextran blue in 50ml of 0.1M sodium bicarbonate pH9.5 was added to 10g of activated sepharose and the whole mixed as above

The sepharose-blue dextran conjugate was then washed in a Buchner filter with 2 litres of 0.1M sodium bicarbonate pH9.5, 2 litres of 6M urea, 2 litres of distilled water and 2 litres of 0.05M Tris/HCl 0.5M NaCl pH8.0. The sepharose was stored in this at $4^{o}C$ prior to use.

A Pasteur pipette column 8cm x 0.5cm of the blue dextran sepharose was made and equilibrated with the Tris/HCl buffer above. The sample was applied and the Tris/HCl buffer washed through until the eluent was clear of protein. 6M urea was then washed through to clear the column of protein. The material washed through with the Tris/HCl buffer (unbound material) and material eluted with the 6M urea (bound material) were dialyzed against 0.01M phosphate buffer pH7.0 for sixteen hours at $4^{o}C$, changing the buffer once. Both fractions were then freeze dried.

4.11 pH activity of the enzyme

The source of enzyme activity used was the material from the first batch of plasma which had not been bound by aluminium hydroxide gel.

A batch of fibrin blue solution was made. 40ml aliquots of the solution were washed six times in the appropriate buffer solution. The buffers used were pH1.2-2.2 0.2M HCl/0.2M KCl and pH2.3-3.9 0.1M potassium hydrogen phthalate 0.1M HCl. The pH of the final wash was recorded as the pH of that particular assay. The fibrin blue assay was performed on each sample.

A pH activity curve was then constructed, plotting all the obtained activity values versus the pH of the final wash.

4.12 Pepstatin inhibition of the enzyme

Pepstatin* was supplied as 1mg/ml solution in ethanol.

Pepsin solutions (hog sigma) at concentrations of 10µg/ml, 100µg/ml and 500µg/ml were prepared. 50µl of pepstatin was then added to 5ml of each pepsin solution and the mixture incubated for forty five minutes at 37°C. Activity of the pepstatin/pepsin mixture was then assayed using the acid denatured haemoglobin method and fibrin blue method. Activity of the original pepsin solution was also assayed with the above methods. Since total inhibition of the pepsin solutions was observed, the method of inhibition obviously worked. It was therefore decided to try plasma in place of the pepsin

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* I am indebted to Dr. A.J. Barrett of the Strangeways Laboratory, Cambridge, who generously gave the pepstatin for this experiment.

solution. Further tests were also carried out using material which was not bound to aluminium hydroxide gel and a fraction from one of the CM-cellulose columns.

The above three solutions were mixed with pepstatin at the same concentrations as the pepsin solutions above and incubated at 37^{o}C for forty five minutes. Activity of both the starting material and the pepstatin mixture was worked as above, but the clot dissolving assay was also included.

Throughout the whole of this project, plasma or fractions used were kept at 4^{o}C, or frozen whenever storage was necessary.

5 Results

5.0 First batch of plasma

A brief summary of these results was given in Law and Kemp (1977). A diagrammatic representation of the following purification is given in figure 4.

5.1 Removal of plasminogen

The plasma from two out of date donations of blood was pooled, giving approximately 450ml of plasma. 600ml of plasminogen free plasma were obtained by passing the plasma through lysine sepharose. The specific activities of this starting material are given in table I.

5.2 Ammonium sulphate precipitation I

The precipitate obtained after 70% saturation was dialyzed and 440ml of material were then obtained as shown in table I.

5.3 Aluminium hydroxide gel I

Two fractions were obtained (see table I):-

5.3.1 material which was not bound by the $Al(OH)_3$ gel

5.3.2 material eluted by phosphate buffer from the $Al(OH)_3$ gel.

This showed a low activity with the fibrin blue assay, but as the gel had not been washed free of all protein before elution, this was taken to be residual activity. It was apparent from this result that the 0.85% sodium chloride washes should be given until no activity is seen and only then should phosphate buffer elution of the $Al(OH)_3$ gel be performed.

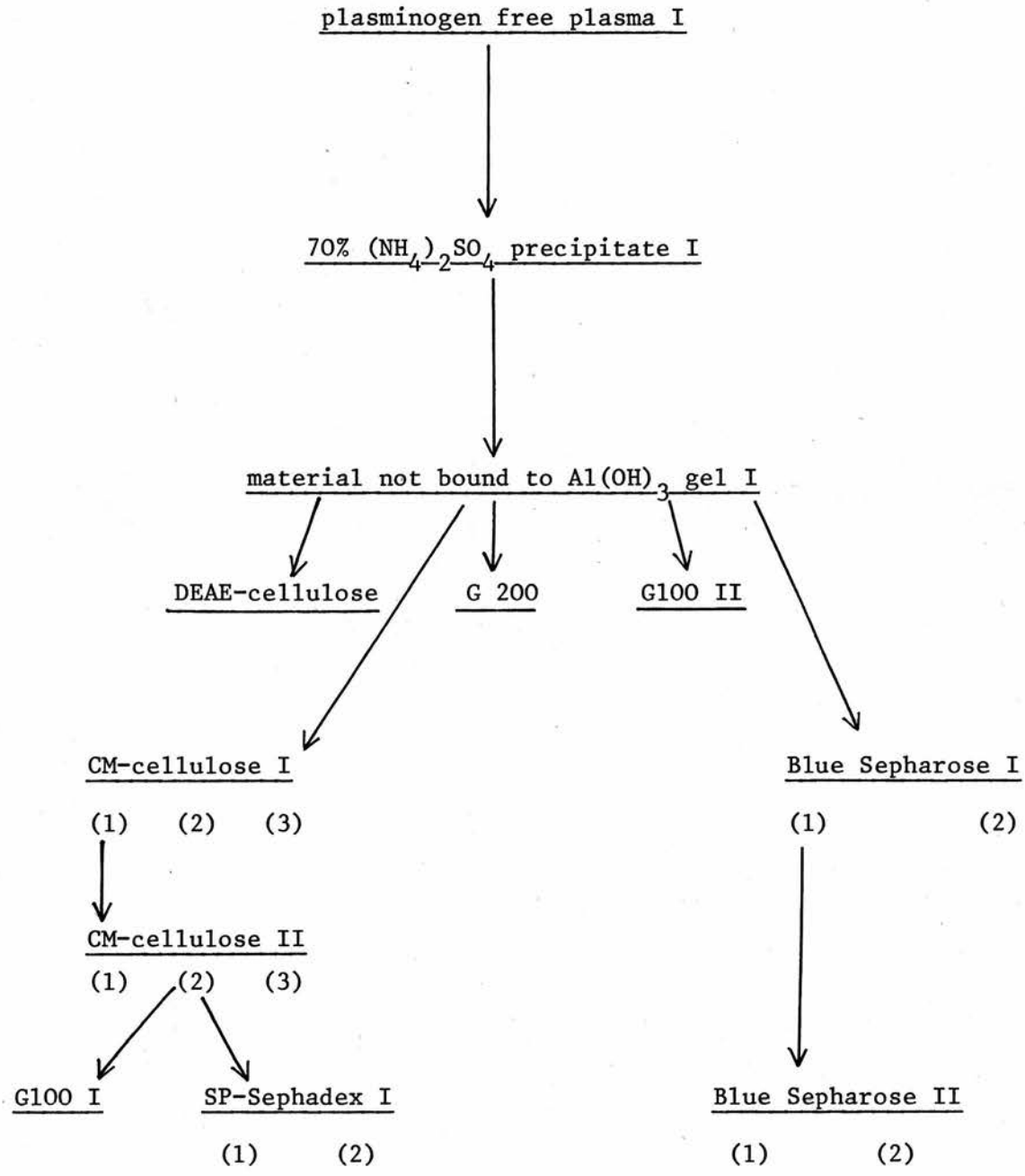


figure 4 purification of the first batch of plasma

Procedure	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg protein x 10^{-1})	Purification	Yield %
plasminogen free plasma I	600	7.1	4260	16.75	4.23	1.0	100
70% $(NH_4)_2SO_4$ precipitate I	440	7.4	3234	20.5	3.5	0.8	75
material not bound to $Al(OH)_3$ gel I	796	9.4	7482	14.0	6.7	1.6	176
DEAE-cellulose	100	4.8	484	12.0	4.0	1.2	90
CM-cellulose I pool 1	190	3.9	741	3.5	11.0	2.6	137
CM-cellulose I pool 2	114	3.0	342	1.3	23.1	5.3	64
CM-cellulose I pool 3	94	1.1	103	1.15	9.7	2.2	20
CM-cellulose II pool 1	108	2.0	216	0.45	44.4	10.5	153
CM-cellulose II pool 2	92	1.1	101	0.3	36.6	8.7	72
SP-Sephadex I pool 1	75	1.8	135	0.11	164	54.6	31
SP-Sephadex I pool 2	44	1.9	82	0.05	380	32	78

Table I Purification of the first batch of plasma

5.4 Ion exchange chromatography

It was decided to try ion exchange chromatography for the next stage of the purification.

5.4.1 DEAE-cellulose

100ml of fraction 5.3.1 were applied to 30g of DEAE-cellulose. All the material applied was bound to the column (figure 5, overleaf). One peak of activity was eluted (table I).

SDS disc gel electrophoresis (figure 6, overleaf) and immuno-electrophoresis (figure 7) were performed on the pool.

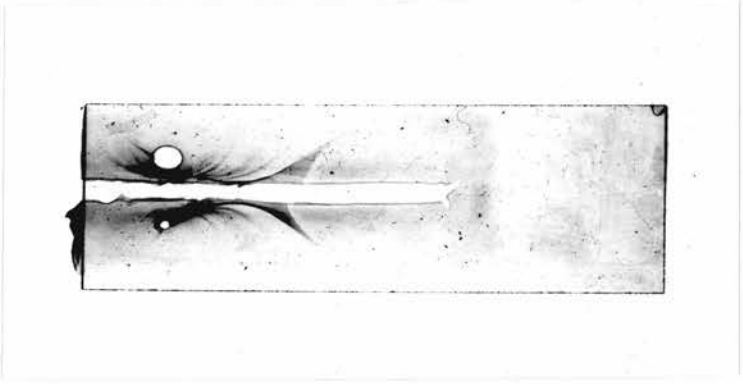


figure 7 immunoelectrophoresis of two aliquots of the pool from DEAE-cellulose. Anti-human antiserum in the trough.

Since the above procedure had given no separation, it was decided to try CM-cellulose.

5.4.2 CM-cellulose I

100ml of fraction 5.3.1 were applied to 30g of CM-cellulose (figure 8). Some material passed through, but it had a very low specific activity and was therefore disregarded.

The material eluted between pH4.6 and pH5.3 showed the greatest activity and was pooled to form pool 1. Two other pools were created,

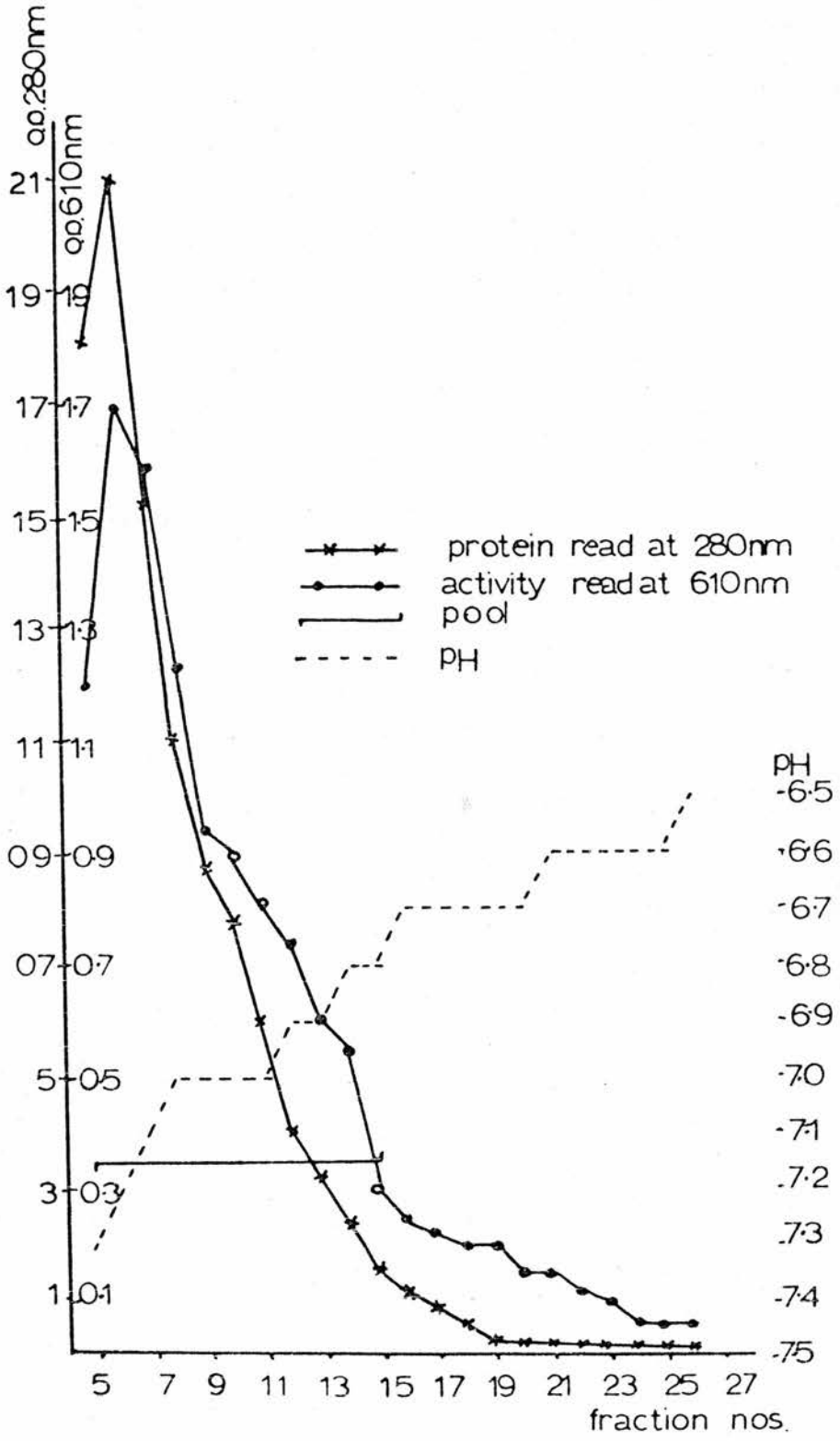


figure 5 elution pattern from DEAE-cellulose column. The sample was applied in the equilibrating buffer, 0.05M Tris/HCl pH7.6. Pool eluted with buffer pH5.6

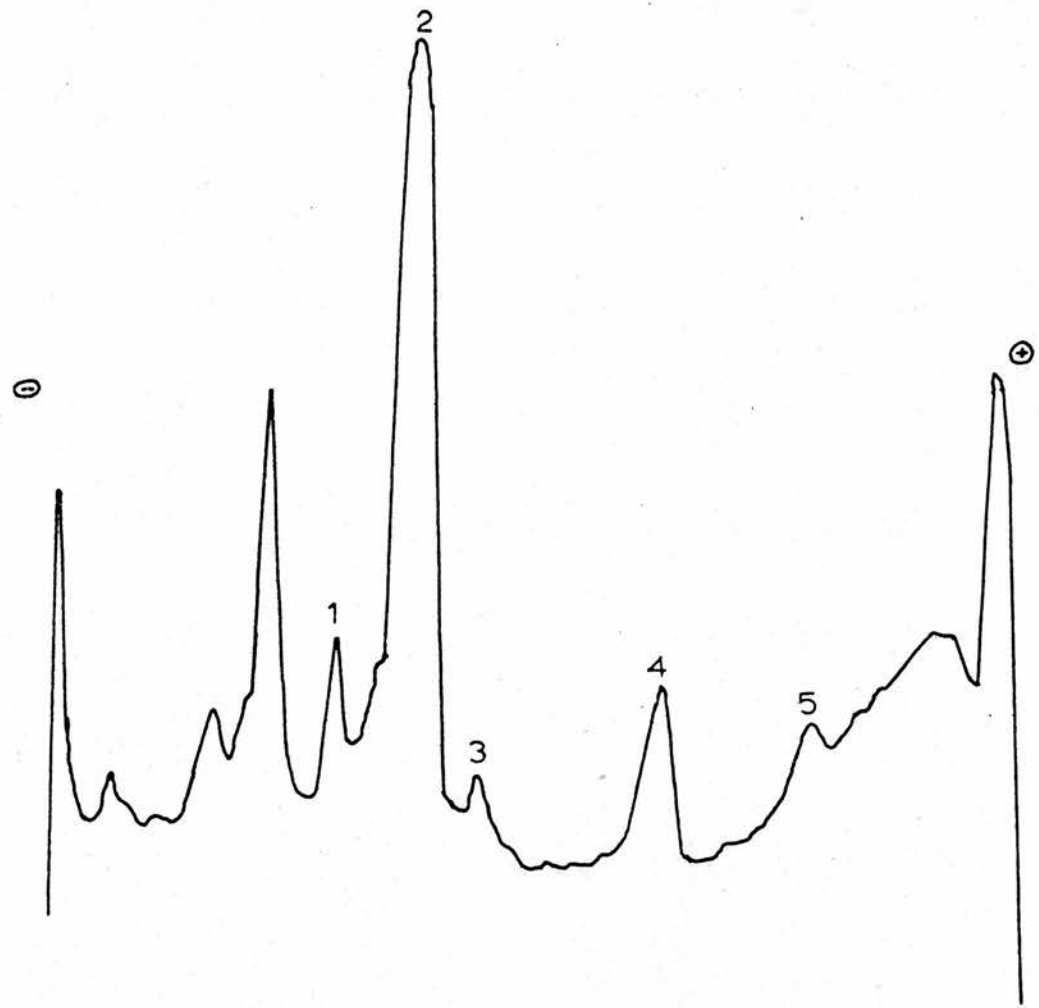


figure 6 densiometric scan of an aliquot of the pool from DEAE-cellulose. Estimated molecular weights are:- peak 1, 68,000; peak 2, 54,000; peak 3, 44,000; peak 4, 24,000 and peak 5, 15,500. The unlabelled peaks are greater than 70,000

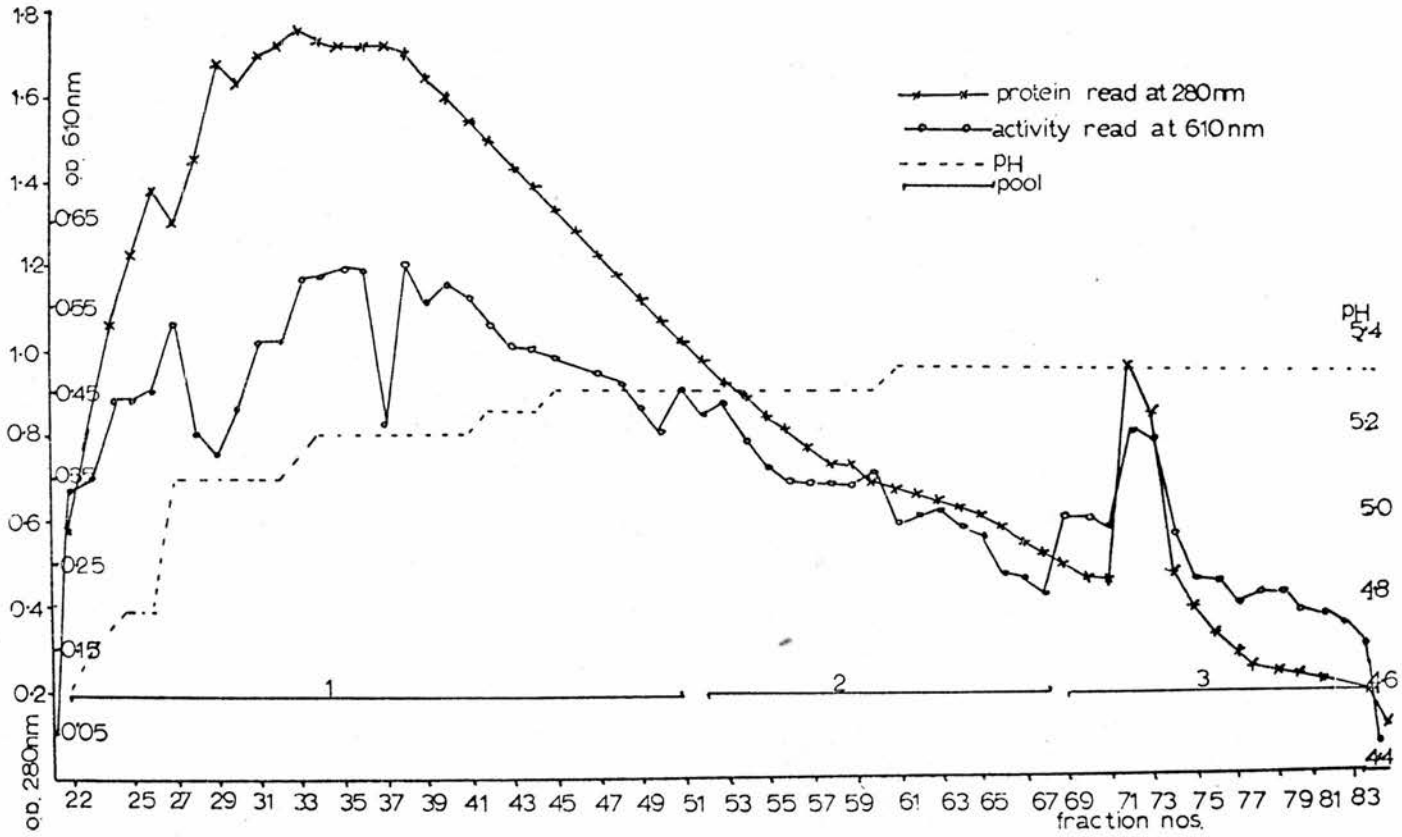


figure 8 elution pattern from CM-cellulose column I. Sample applied in equilibrating buffer, 0.05M acetate buffer, pH4.0. Elution buffer applied at tube 1 was same buffer, pH5.4

tubes 51-68 (pool 2) and tubes 69-83 (pool 3). These are shown in table I.

SDS disc gel electrophoresis on the above pools showed pool 1 to contain the least variety of protein bands (figure 9).

5.4.3 CM-cellulose II

It was decided to reapply some of pool 1 CM-cellulose I to a further CM-cellulose column.

50ml were applied in the same manner as above and the elution pattern shown in figure 10 was obtained. The material eluting between pH4.0 and pH5.0 was pooled (pool 1), while tubes 39-50 formed pool 2 and tubes 51-62 formed pool 3. Pools 1 and 2 had very similar protein bands on disc gel electrophoresis (figure 11), but the specific activity of pool 1 was greater, as can be seen from table I. Pool 3 showed very little activity or protein and was therefore disregarded.

Immunoelectrophoresis of pool 1 against antihuman antiserum and anti-albumin antiserum showed precipitation lines for albumin only (figure 12) and anti-α_2 macroglobulin antiserum showed no precipitation lines.

It would appear that at this stage the major contaminant was probably a degraded form of albumin. Wilson and Foster (1971), while conducting conformational studies on bovine plasma albumin noted the presence of a proteolytic enzyme, active against the F form of albumin at approximately pH3.8, and also against acid expanded bovine plasma albumin. Hagenmaier and Foster (1971) achieved a partial purification of this enzyme using SE-Sephadex G50. At this stage, SE-Sephadex was unavailable and so SP-Sephadex was used.

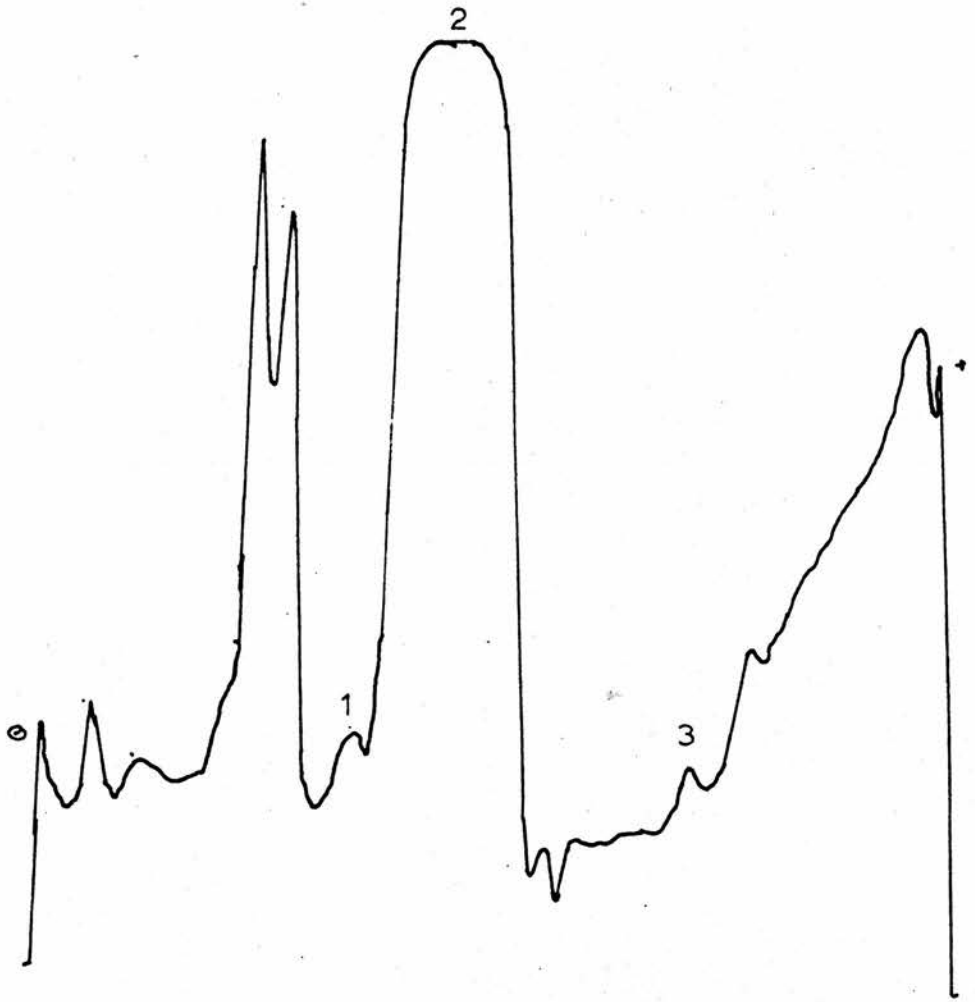


figure 9 densiometric scan of an aliquot from pool 1, CM-cellulose I. Estimated molecular weights are:- peak 1, 64,000; peak 2, 47,000 and peak 3, 23,000. The unlabelled peaks are greater than 70,000

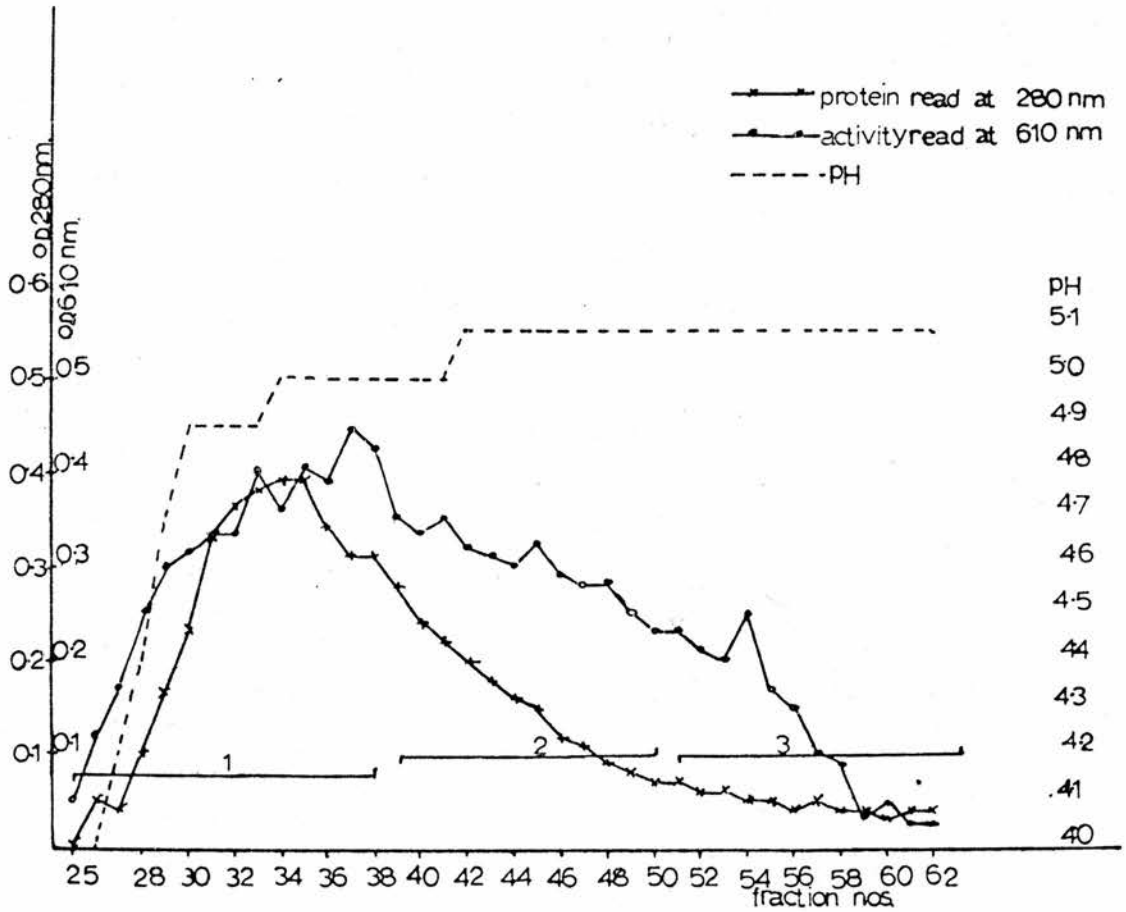


figure 10 elution pattern from CM-cellulose column II. Sample applied in equilibrating buffer, 0.05M acetate buffer pH4.0. Pools eluted with same buffer pH5.4

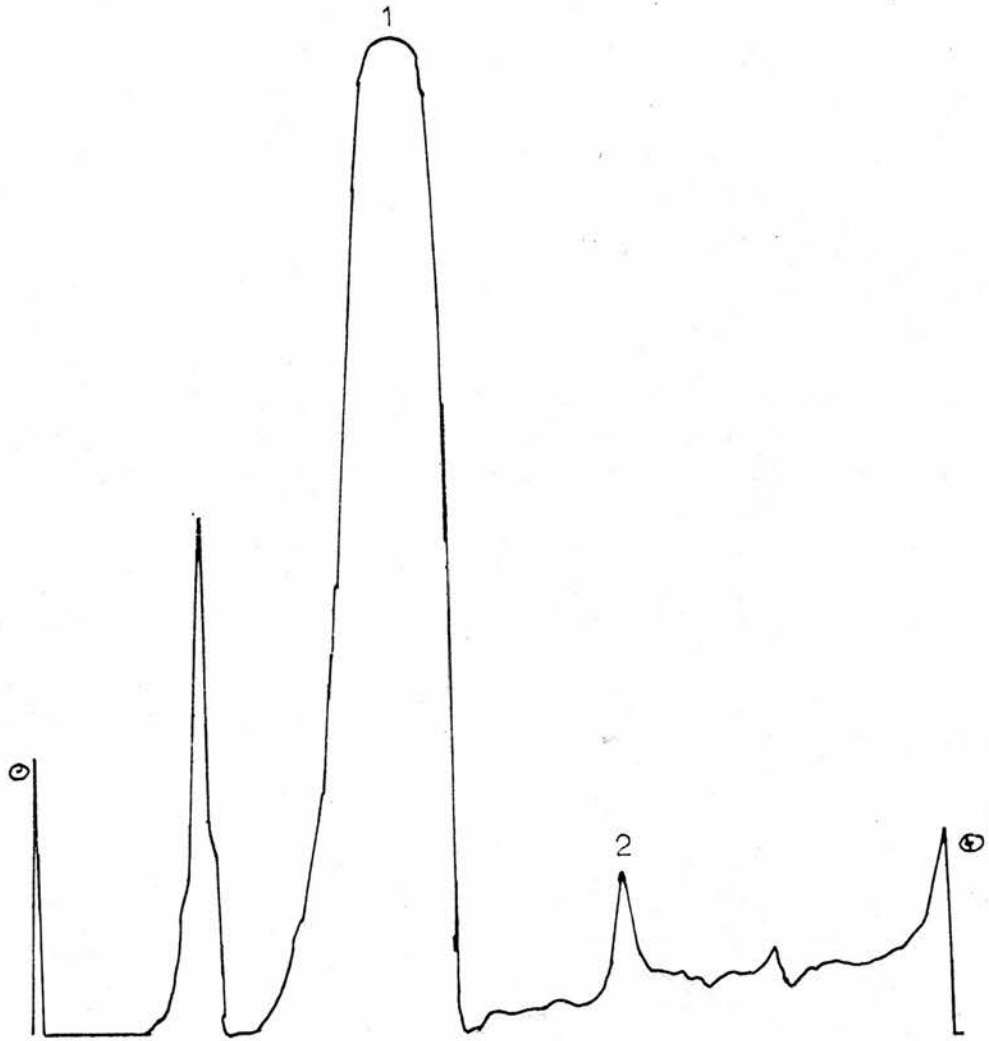
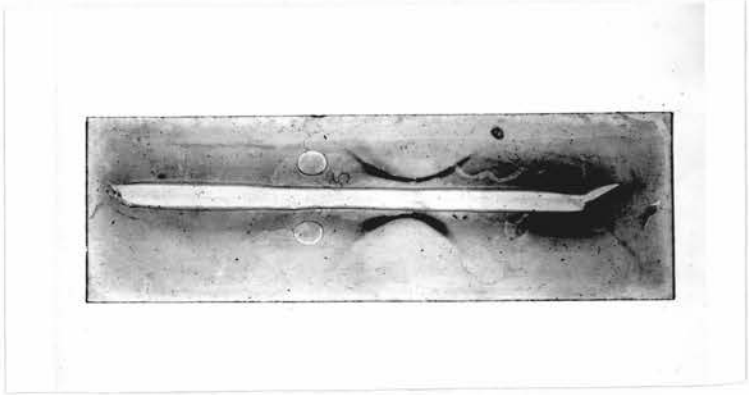
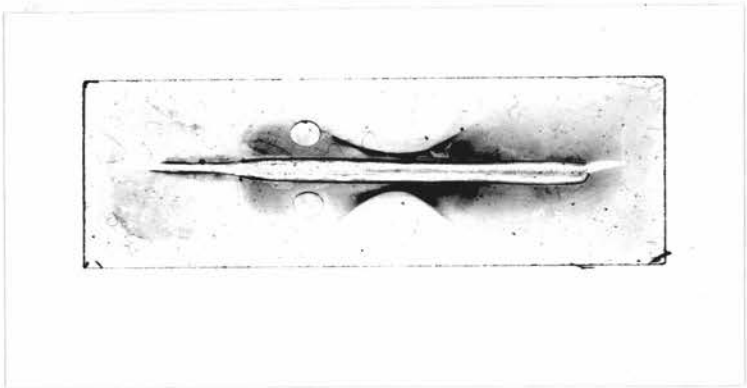


figure 11 densiometric scan of an aliquot from pool I CM-cellulose II. Estimated molecular weights are:- peak 1, 57,000 and pool 2, 28,000. The unlabelled peaks are greater than 70,000



a)



b)

figure 12 immunoelectrophoresis of aliquots of pool 1 from CM-cellulose II.

a) anti-human antiserum in the trough

b) anti-albumin antiserum in the trough

5.4.4 SP-Sephadex I

50ml of material from pool I CM-cellulose II were applied to SP-Sephadex.

The enzyme activity was absorbed to the column and then eluted with the acetate buffer containing 0.27M NaCl. This gave two peaks of activity(figure 13), which were taken and separately pooled. The second pool had a significantly higher specific activity as shown in

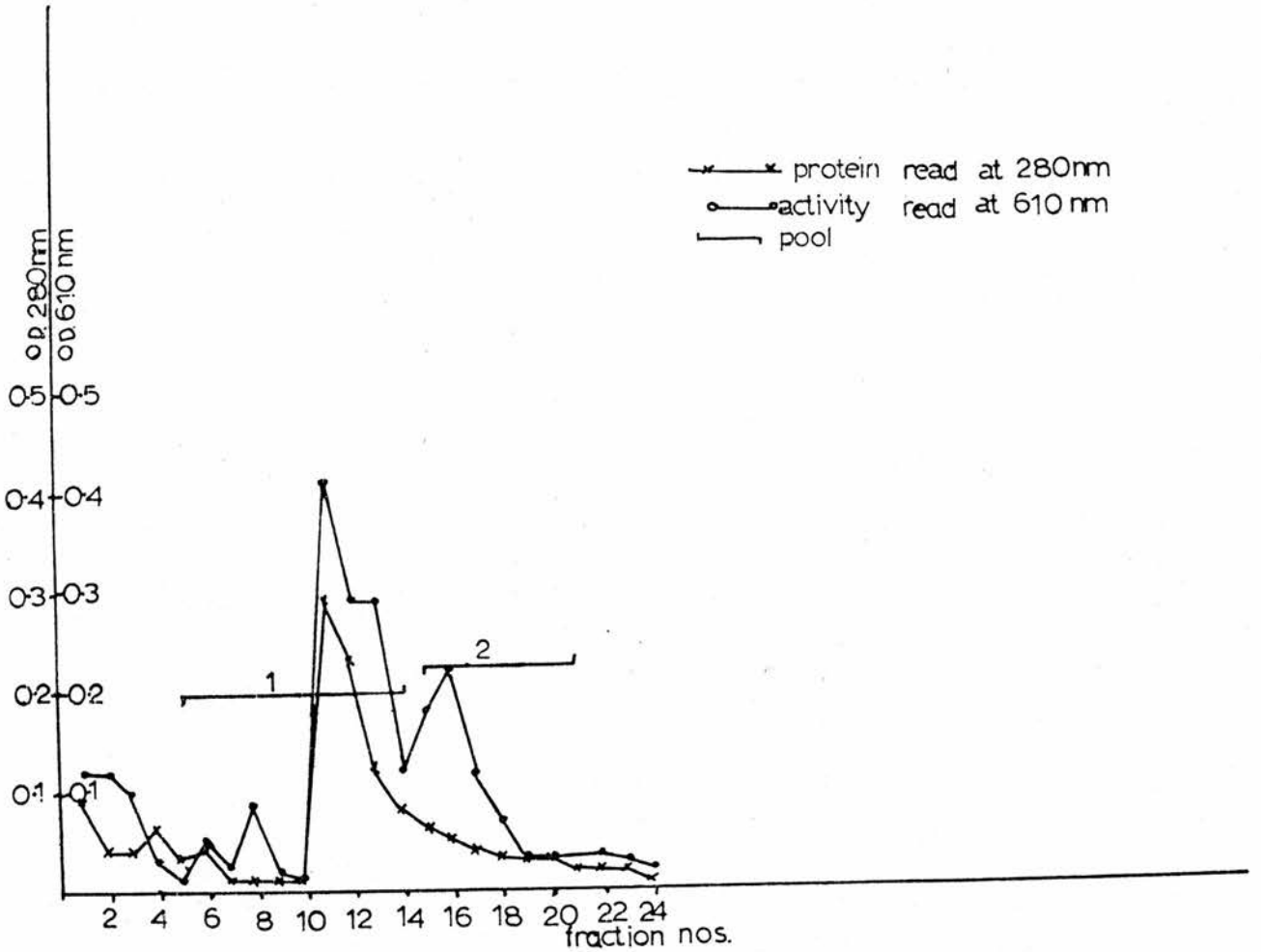
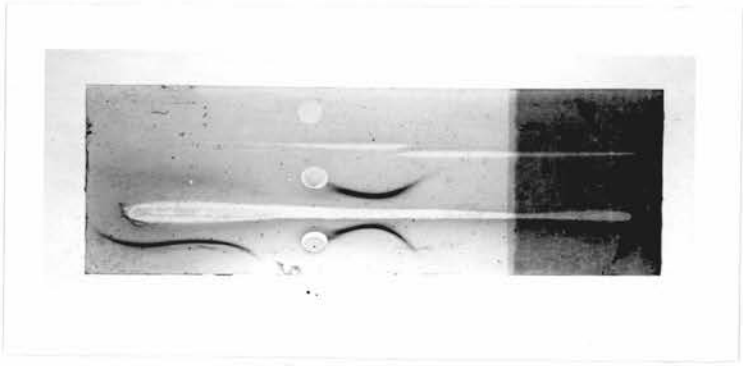


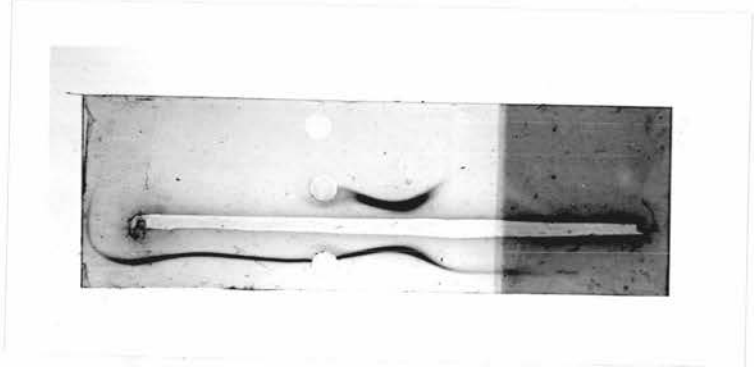
figure 13 elution pattern from SP-Sephadex column I. Sample applied in equilibrating buffer, 0.02M acetate buffer pH4.3, 0.18M NaCl. Pools eluted with same buffer, containing 0.27M NaCl, applied after tube 5

table I. An aliquot of each pool was subjected to SDS disc gel electrophoresis and the second pool showed only two major constituents as can be seen in figure 14 (overpage).

Immunoelectrophoresis against anti-human antiserum and anti-albumin antiserum showed only albumin precipitation lines (figure 15). Anti-α_2 macroglobulin antiserum showed no precipitation.



a)



b)

figure 15 Immunoelectrophoresis of aliquots of pools 1 and 2 from SP-Sephadex I

a) anti-human antiserum in the trough

b) anti-albumin antiserum in the trough

The above purification procedure gave a fraction which had a high

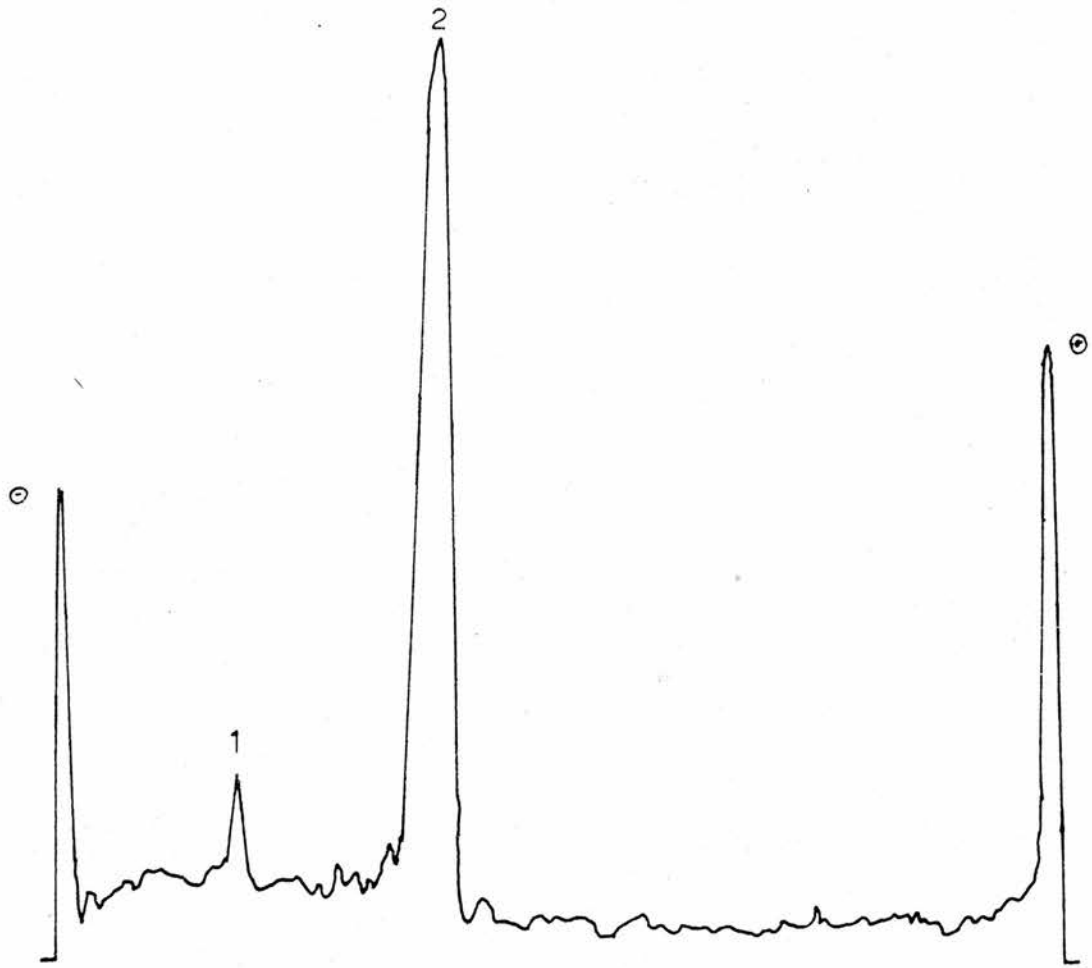


figure 14 densiometric scan of an aliquot from pool 2 SP-Sephadex I. Estimated molecular weights are:- peak 1, 84,000; peak 2, 52,000

specific activity and contained only two constituents, both of which should be separable by gel filtration.

5.5 Gel filtration

5.5.1 Sephadex G100 I

50ml of pool 1 CM-cellulose II were freeze dried then redissolved in 4ml of distilled water. Once this had been applied to the column, buffer was pumped through. After the void volume, three peaks of protein were seen (figure 16), but only one showed activity (table II).

SDS disc gel electrophoresis and immunoelectrophoresis again showed a similar pattern to the SP-Sephadex above.

The above results still indicate the presence of an albumin contaminant. It was decided to go back and use two further aliquots of the material which had not been bound to aluminium hydroxide gel and subject them to Sephadex G100 and G200 gel filtration.

5.5.2 Sephadex G100 II

34ml of fraction 5.3.1 material from $Al(OH)_3$ gel were freeze dried then redissolved in 4ml of distilled water. This was applied to the Sephadex column and buffer was pumped through. After the void volume, two large peaks of protein were seen and several small ones (figure 17). Activity was seen only with the two large peaks. The two peaks were divided into three pools (table II).

SDS disc gel electrophoresis of an aliquot of each pool showed the presence of a wide variety of protein bands (figures 18, 19, 20). Immunoelectrophoresis of an aliquot of each pool against anti-human antiserum showed several precipitation lines (figure 21), while

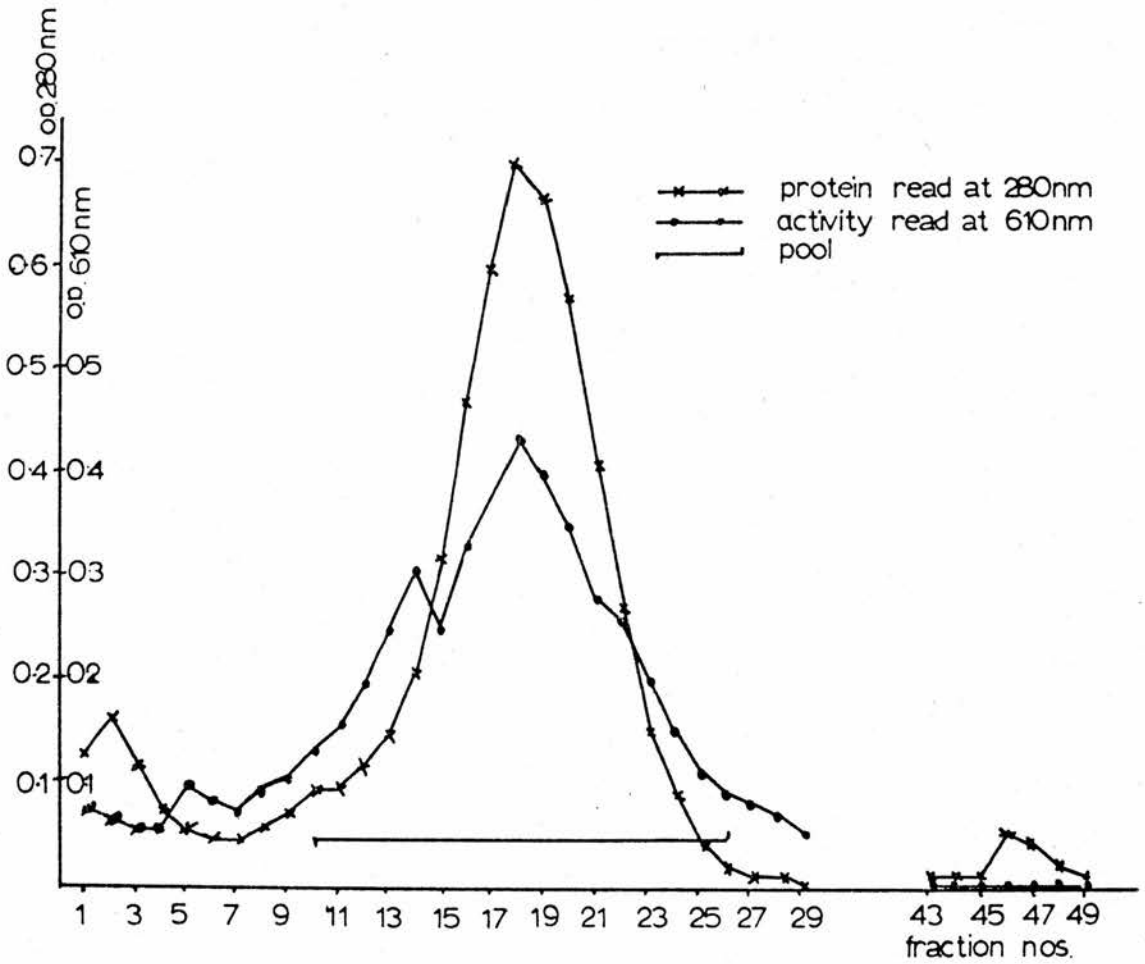


figure 16 elution pattern from Sephadex G100 column I. Sample applied in 0.01M phosphate buffer pH7.1

Procedure	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg protein x 10^{-1})	Purification	Yield %
Sephadex G100 I pool 1	35	3.0	105	3.2	9.4	2.2	42
Sephadex G100 II pool 1	28	4.3	120.4	3.0	11.5	2.7	66
Sephadex G100 II pool 2	16	6.5	104	3.7	14.2	3.4	57
Sephadex G100 II pool 3	32	5.6	179.2	3.0	15.1	3.7	98
Sephadex G200 pool 1	12	1.6	19.2	1.5	1.2	0.3	7
Sephadex G200 pool 2	40	3.0	120	3.3	9.9	2.3	45

Table II Gel filtration of the first batch of plasma

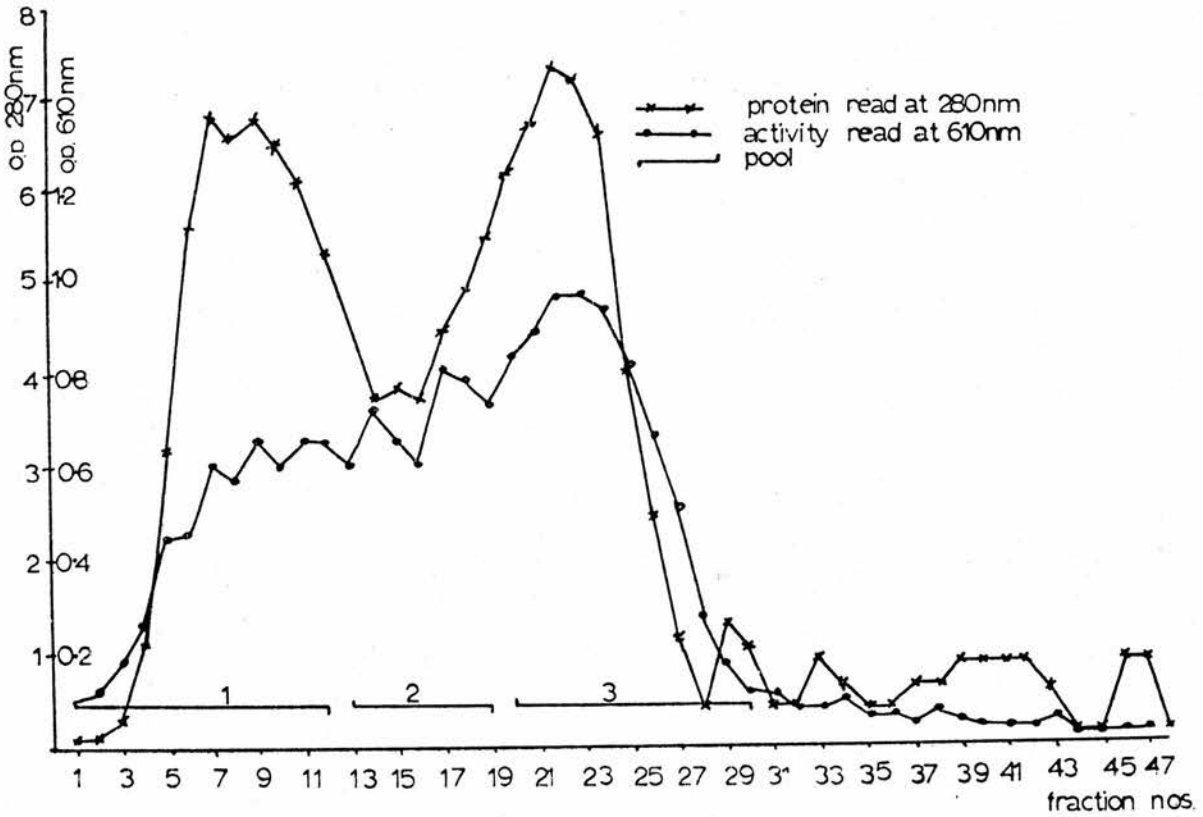


figure 17 elution pattern from Sephadex G100 column II. Sample applied in 0.01M phosphate buffer pH7.1

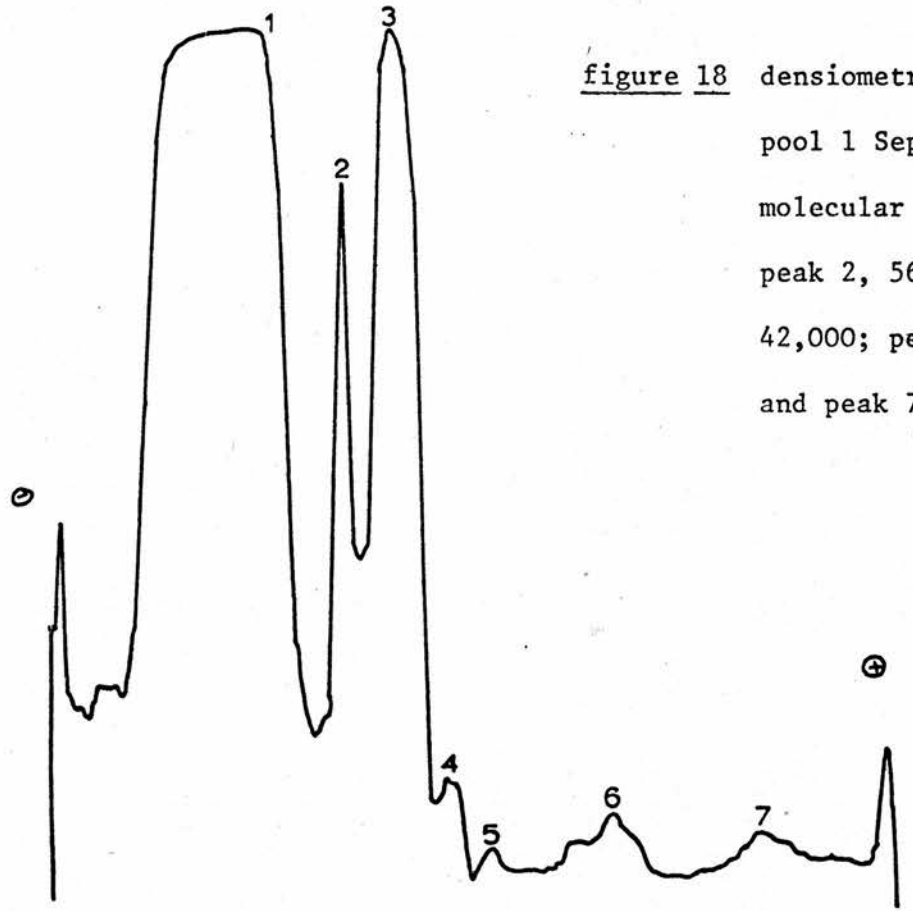


figure 18 densiometric scan from an aliquot of pool 1 Sephadex G100 II. Estimated molecular weights are:- peak 1, 68,000; peak 2, 56,000; peak 3, 48,000; peak 4, 42,000; peak 5, 37,000; peak 6, 26,000 and peak 7, 14,000

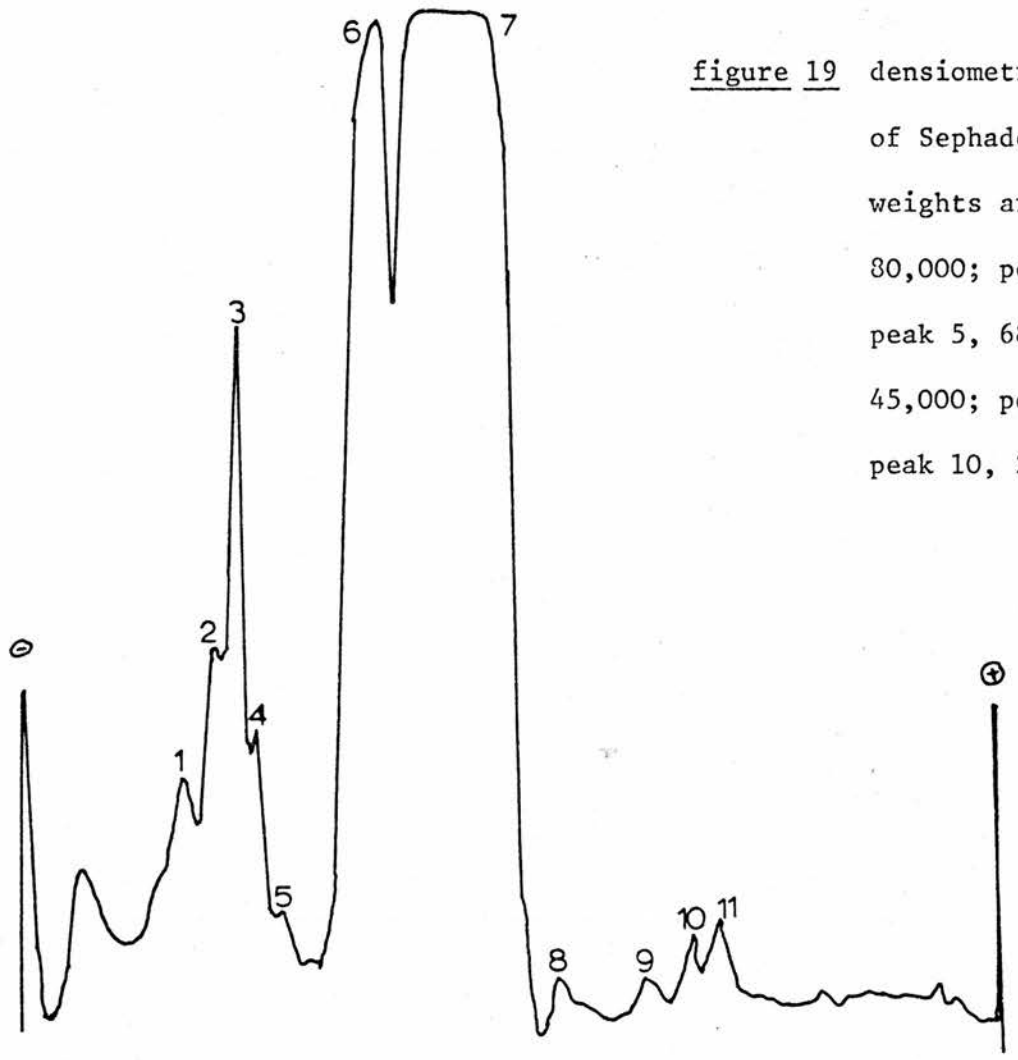


figure 19 densiometric scan of an aliquot from pool 2 of Sephadex G100 II. Estimated molecular weights are:- peak 1, 85,000; peak 2, 80,000; peak 3, 77,000; peak 4, 73,000; peak 5, 68,000; peak 6, 54,000; peak 7, 45,000; peak 8, 35,000; peak 9, 28,000; peak 10, 25,000 and peak 11, 23,000

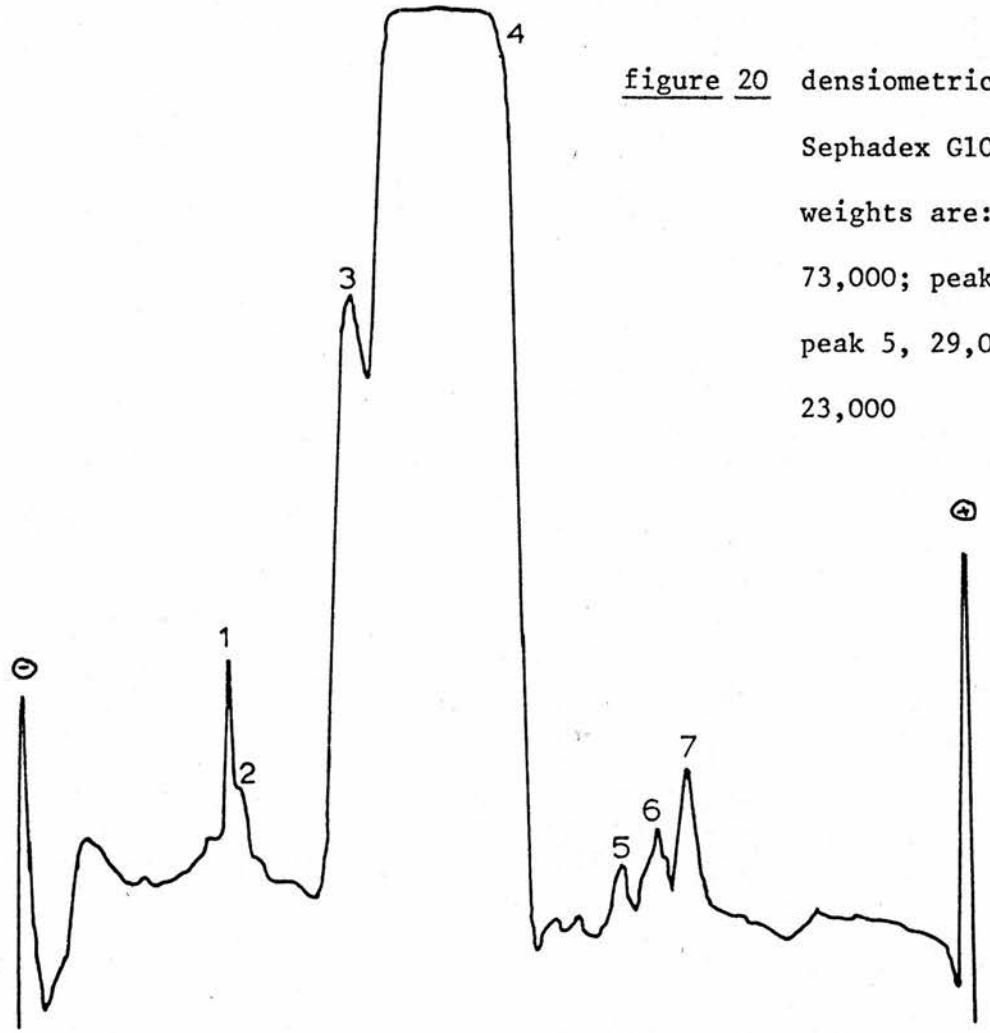


figure 20 densiometric scan of an aliquot from pool 3 Sephadex G100 II. Estimated molecular weights are:- peak 1, 75,000; peak 2, 73,000; peak 3, 56,000; peak 4,45,000; peak 5, 29,000; peak 6, 26,000 and peak 7, 23,000

anti-albumin antiserum showed a precipitation line. Anti-α_2 macroglobulin antiserum showed no precipitate.

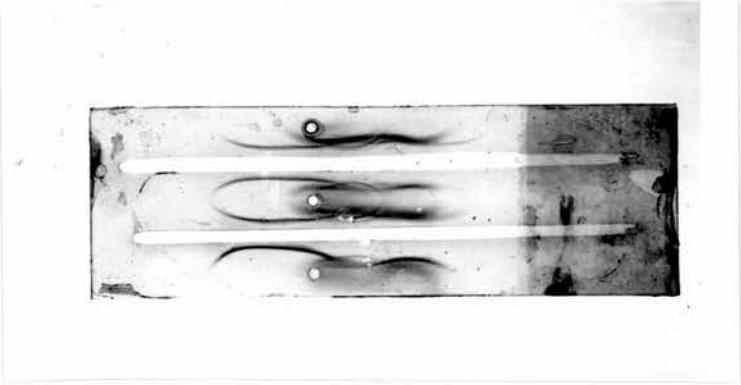


figure 21 immunolectrophoresis of an aliquot of pools 1, 2 and 3 from Sephadex G100 II. Anti-human antiserum in the troughs.

5.5.3 Sephadex G200

50ml of fraction 5.3.1 were freeze dried then redissolved in 5ml of distilled water. The material was carefully layered on to the top of the Sephadex and buffer allowed to feed through the column by gravity. After the void volume, three major peaks of protein were seen (figure 22). This was made into two pools (table II).

SDS disc gel electrophoresis on aliquots of the pools showed that pool 1 (figure 23) contained equal quantities of high and low molecular weight protein bands, whereas in comparison, pool 2 (figure 24) had more high molecular weight material than low.

Immunoelectrophoresis of aliquots of pool 1 against anti-human antiserum showed fewer precipitation lines than did pool 2 (figure 25). Anti-albumin antiserum showed precipitation lines with both pools. Anti-α_2 macroglobulin antiserum showed no precipitation lines.

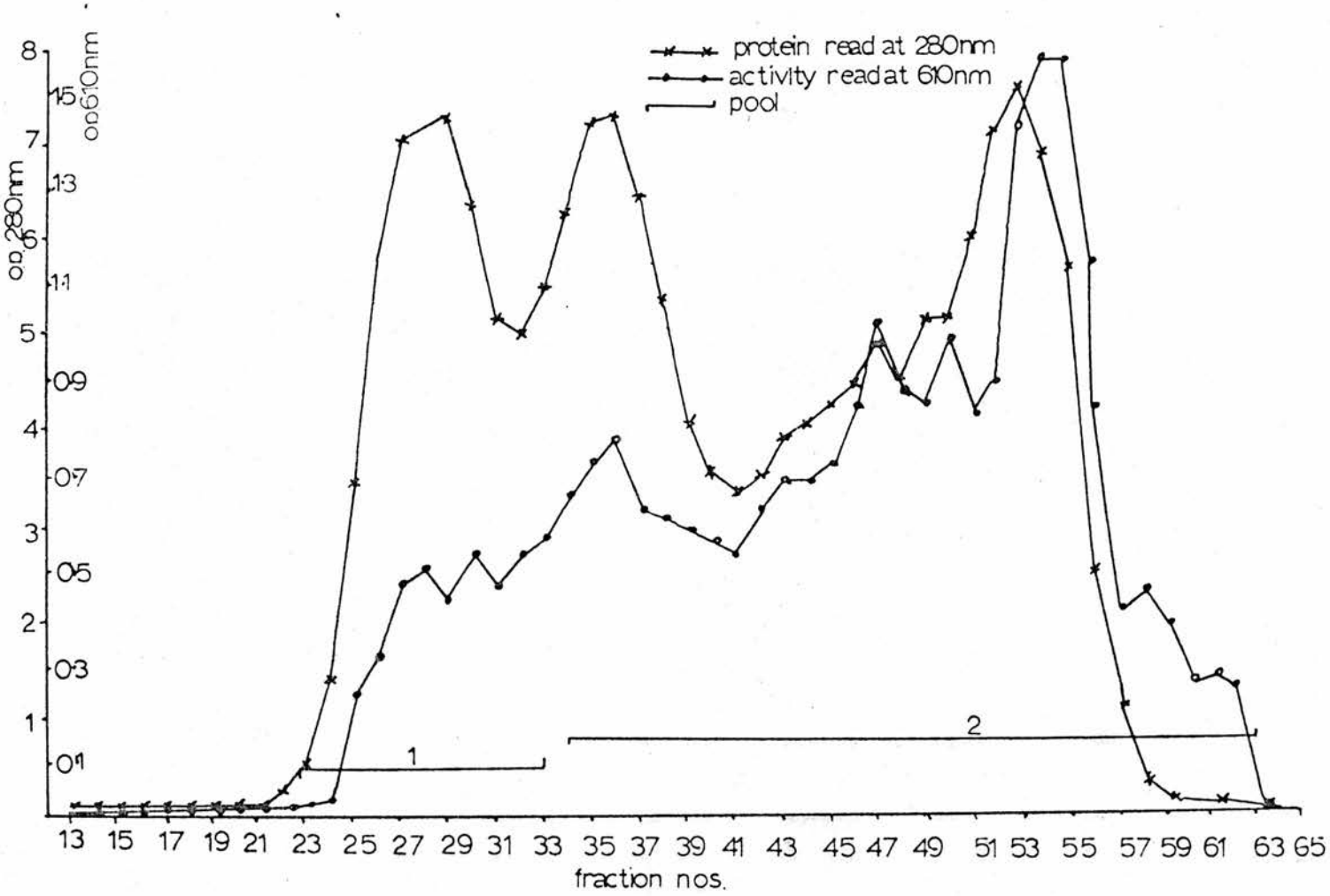
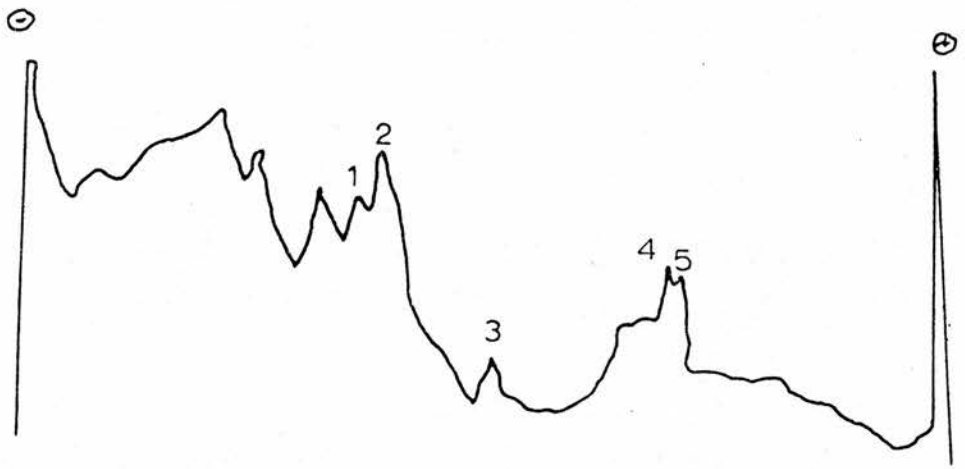


figure 22 elution pattern from Sephadex G200 column. Sample was applied in 0.01M phosphate buffer pH7.6

figure 23 densiometric scan of an aliquot from pool 1 Sephadex G200. Estimated molecular weights are peak 1, 75,000; peak 2, 69,000; peak 3, 51,000; peak 4, 32,000 and peak 5, 31,000. Unlabelled peaks are greater than 80,000



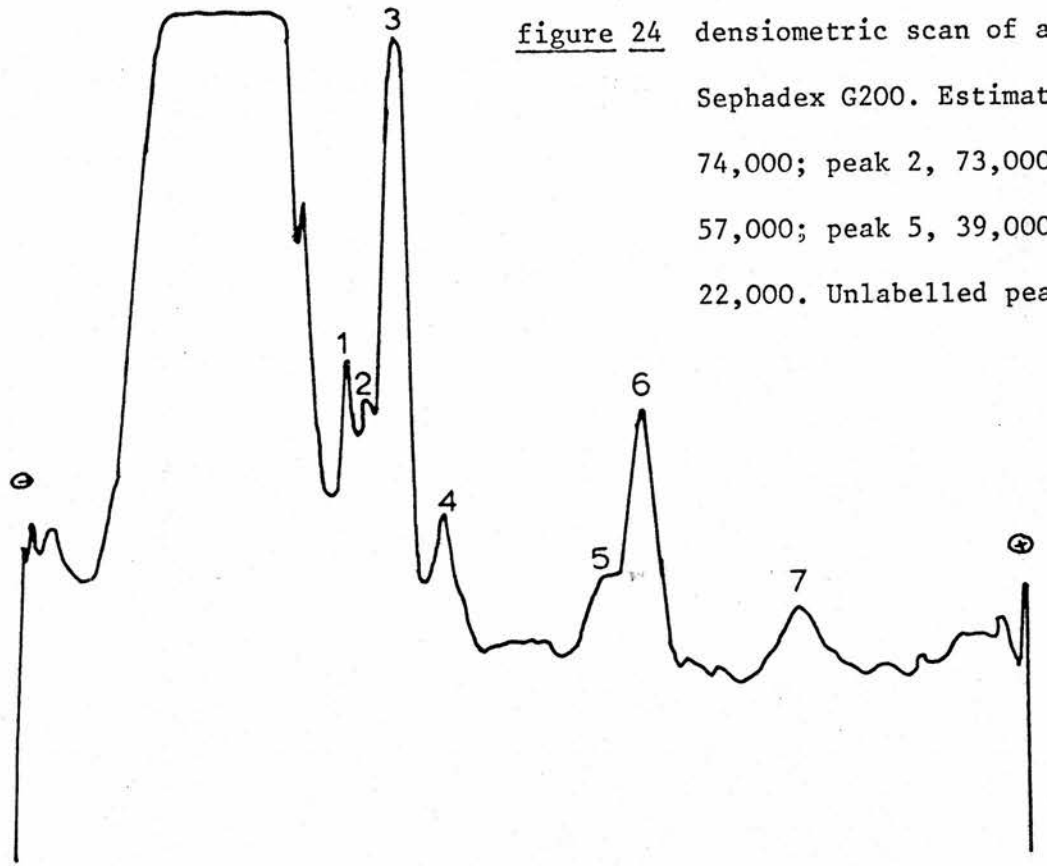
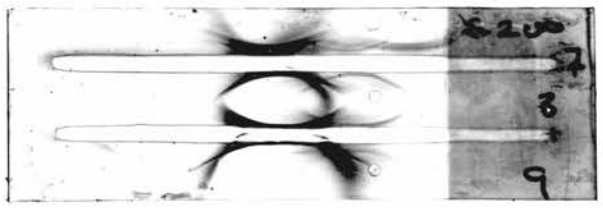


figure 24 densiometric scan of an aliquot from pool 2 Sephadex G200. Estimated molecular weights are peak 1, 74,000; peak 2, 73,000; peak 3, 64,000; peak 4, 57,000; peak 5, 39,000; peak 6, 33,000 and peak 7, 22,000. Unlabelled peaks are greater than 80,000



pool 1

pool 2

figure 25 immunoelectrophoresis of an aliquot of pools 1 and 2 from Sephadex G200. Anti-human antiserum in the troughs.

Since all the above results show the persistent presence of an albumin contaminant associated with the greatest activity against fibrin blue, it was decided to try to remove the albumin by affinity chromatography. Serum or plasma albumin has a unique ability to bind dyes very tightly.

5.6 Affinity chromatography

Blue Sepharose CL-6B is commercially produced by covalently attaching Cibacon blue F3G-A to cross linked agarose gel Sepharose CL-6B.

5.6.1 Blue Sepharose I

3ml of fraction 5.3.1 of $Al(OH)_3$ gel fractionation were applied to the Blue Sepharose and buffer pumped through. Material was immediately seen to come off the column, making it obvious that it had not been bound (figure 26). The buffer containing 1.5M KCl was then pumped through the column. This caused more protein to be eluted, which was disregarded.

The material collected was made into two pools (table III).

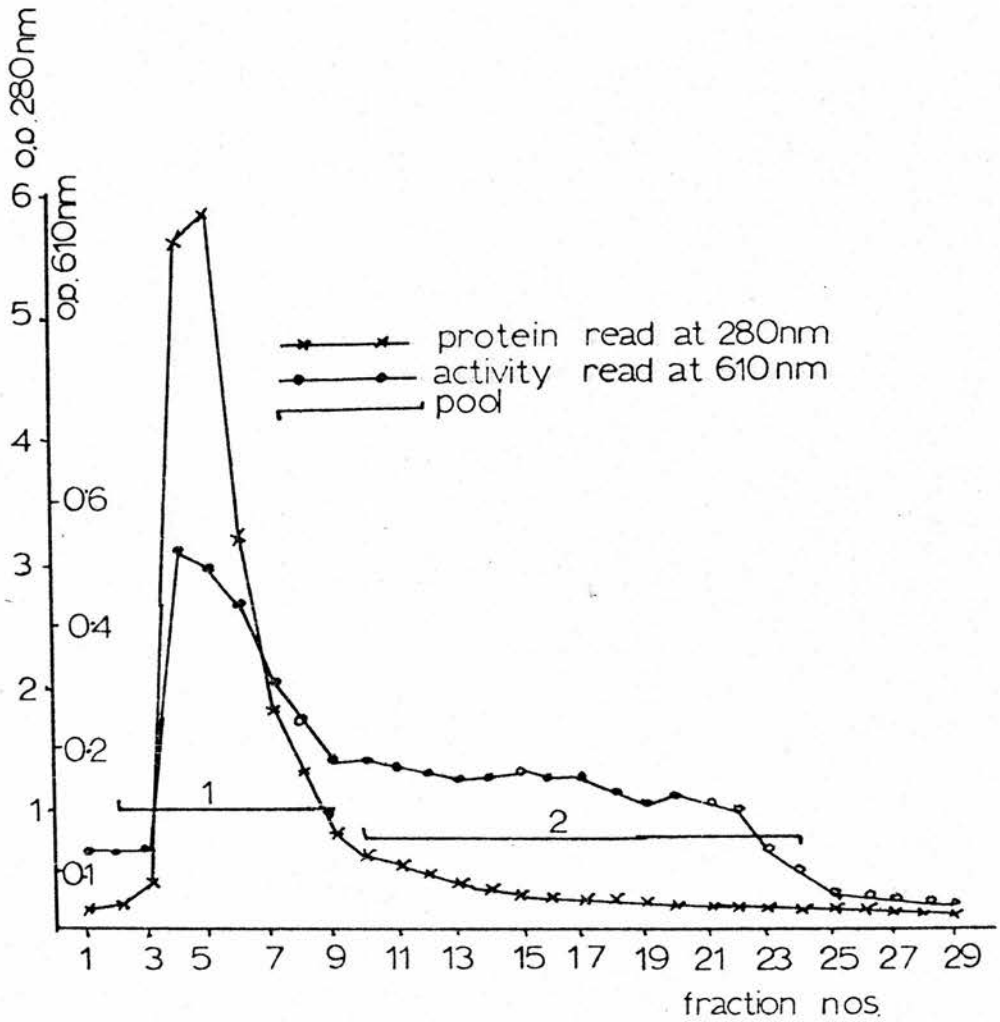


figure 26 elution pattern from Blue Sepharose column I.
Sample was applied in equilibrating buffer,
0.05M Tris/HCl pH7.0, 0.1M KCl

Procedure	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg protein x 10^{-1})	Purification	Yield %
Blue Sepharose I pool 1	10	1.1	11.0	2.5	4.5	1.0	68
Blue Sepharose I pool 2	16	0.6	9.6	0.53	1.1	0.3	60
Blue Sepharose II pool 1	5.5	0.6	3.3	0.39	14.4	3.4	30
Blue Sepharose II pool 2	9.0	0.24	2.16	0.5	4.6	1.1	20
Blue Sepharose II pool 3	9.5	0.2	1.9	0.43	4.7	1.0	17

Table III Affinity chromatography of the first batch of plasma

SDS disc gel electrophoresis was performed on an aliquot of each pool. This showed a wide variety of protein bands. Immunoelectrophoresis against anti-human antiserum showed a mixture of precipitation bands, while anti-albumin antiserum showed a precipitation line. Anti-α_2 macroglobulin antiserum showed no precipitation lines.

5.6.2 Blue Sepharose II

It was decided to reapply 6.8ml of the material from pool 1 above. As before, buffer was pumped through and the unbound material passed out of the column. This was made into two pools (figure 27). 1.5M KCl was then pumped through. This caused more material to be eluted, which was in turn used to form another pool, pool 3 (table III).

SDS disc gel electrophoresis was performed on an aliquot of each of the pools as shown in figures 28, 29 and 30 (overleaf). Immunoelectrophoresis was carried out on an aliquot of each pool against anti-human antiserum (figure 31) and against anti-albumin antiserum, which showed precipitation lines. Anti-α_2 macroglobulin antiserum showed no precipitation lines.

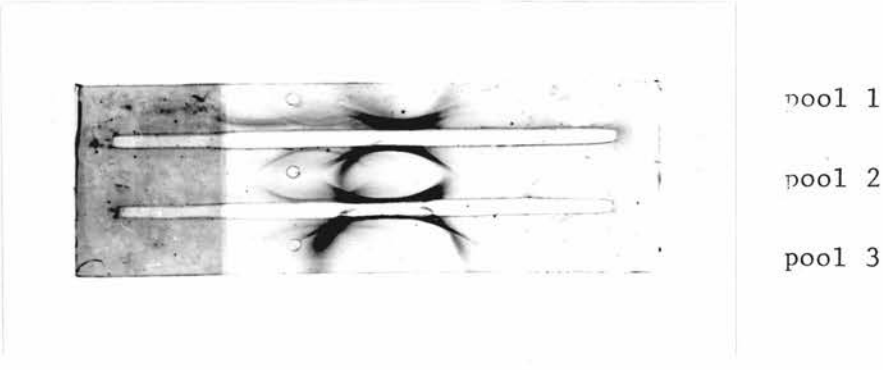


figure 31 immunoelectrophoresis of an aliquot of pools 1, 2 and 3 from Blue Sepharose II. Anti-human antiserum in the trough.

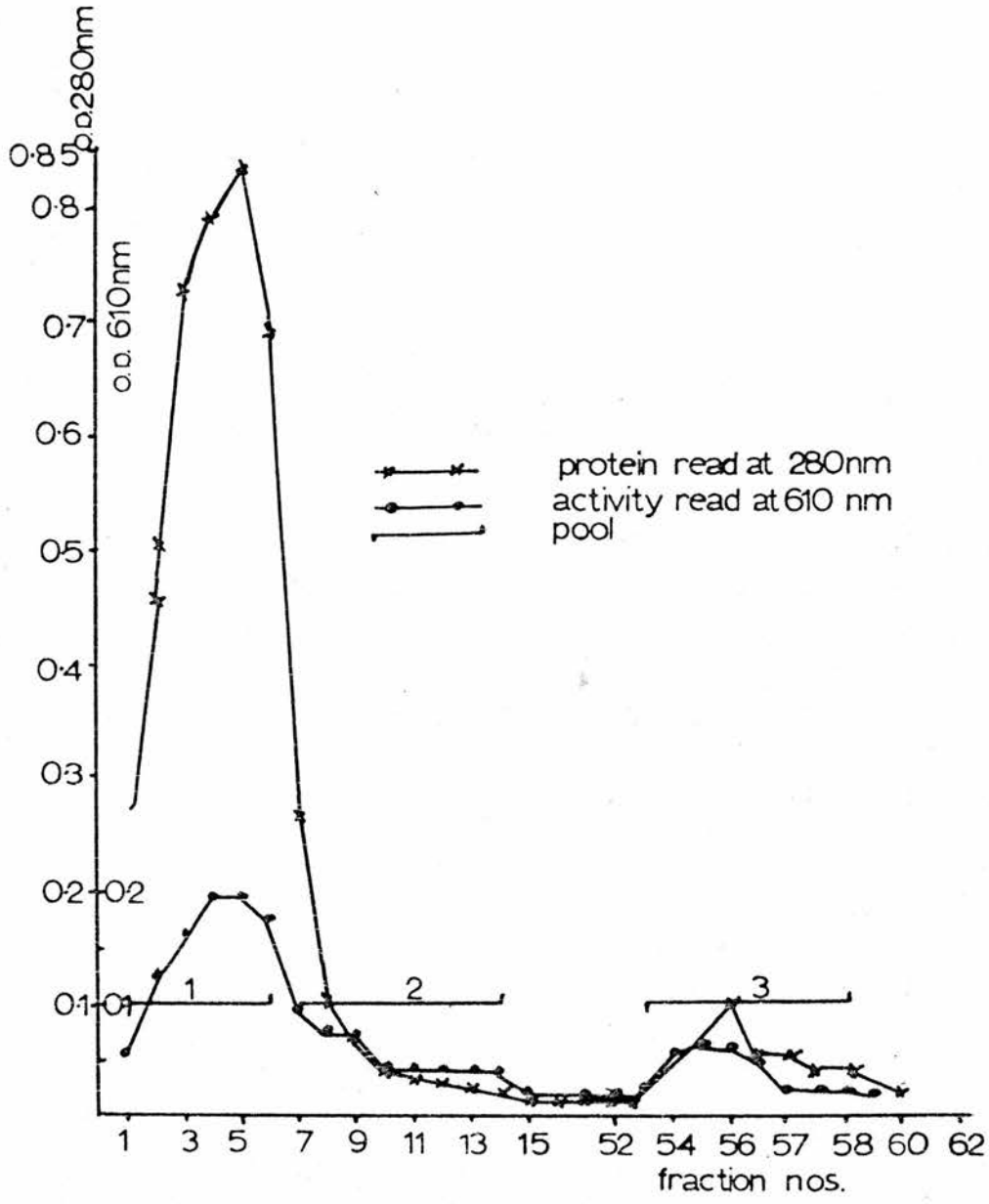


figure 27 elution pattern from Blue Sepharose column II. Sample applied in equilibrating buffer 0.05M Tris/HCl pH7.0, 0.1M KCl. Pool 3 eluted with some buffer containing 1.5M KCl, applied after tube 14

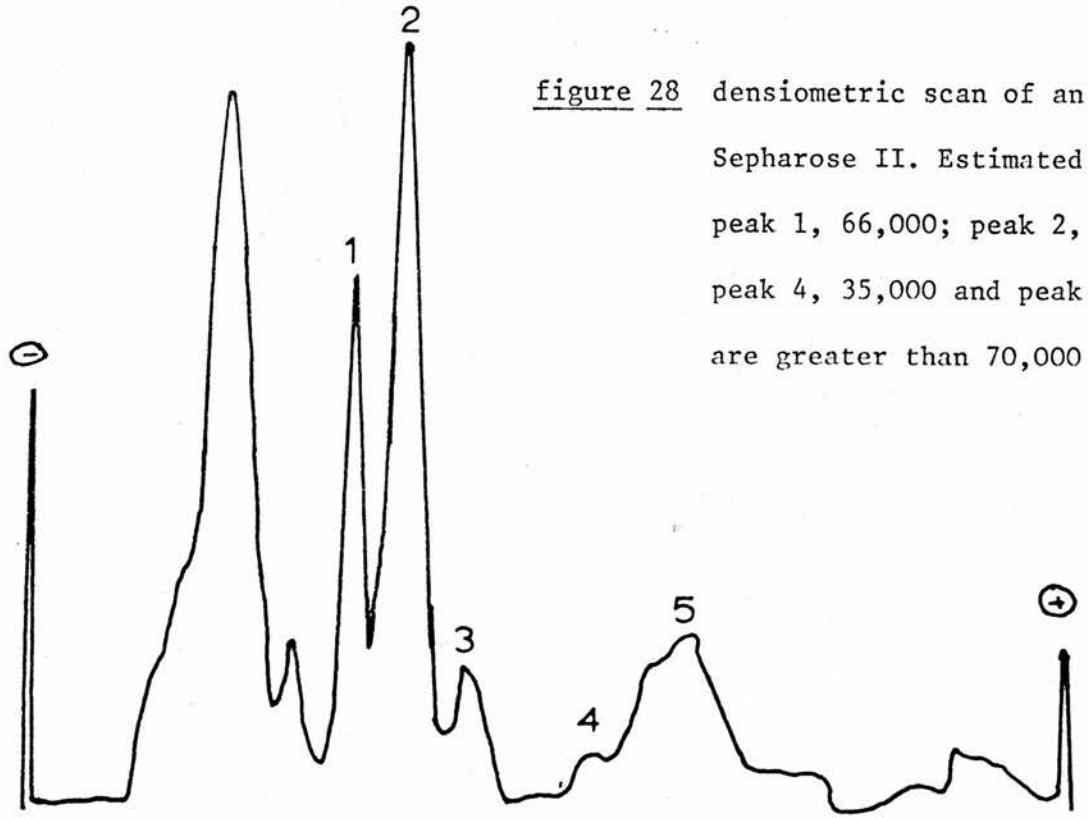


figure 28 densiometric scan of an aliquot from pool 1 Blue Sepharose II. Estimated molecular weights are:- peak 1, 66,000; peak 2, 59,000; peak 3, 50,000; peak 4, 35,000 and peak 5, 26,000. Unlabelled peaks are greater than 70,000

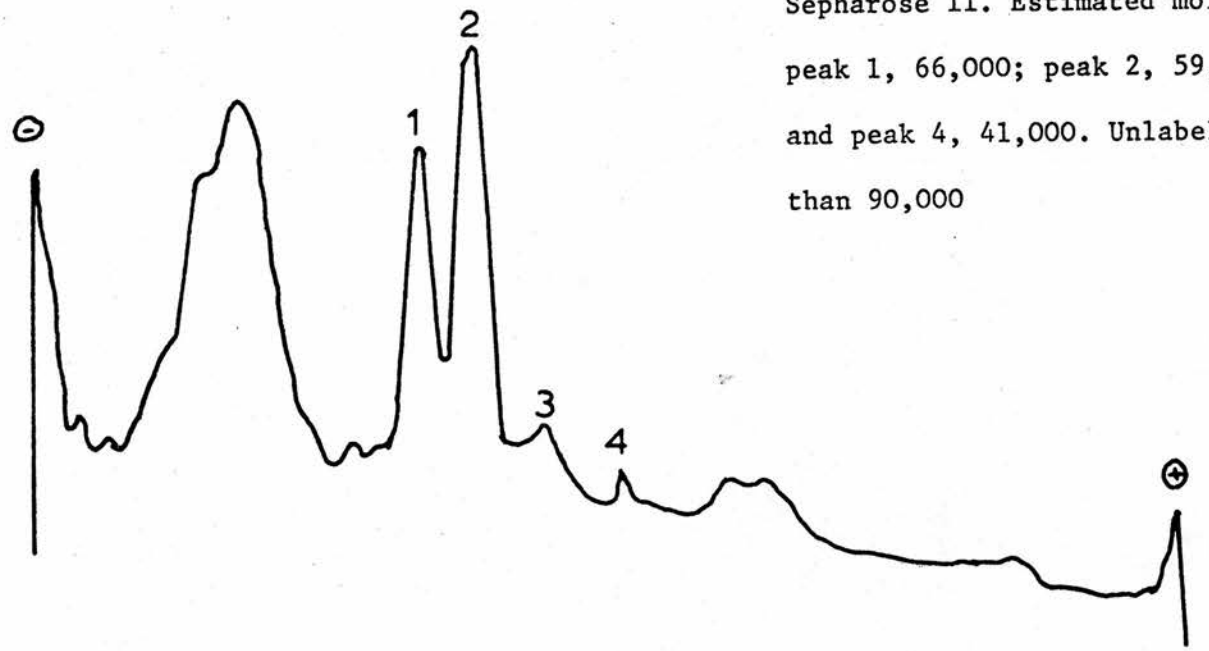


figure 29 densiometric scan of an aliquot from pool 2 Blue Sepharose II. Estimated molecular weights are:- peak 1, 66,000; peak 2, 59,000; peak 3, 50,000 and peak 4, 41,000. Unlabelled peaks are greater than 90,000

figure 30 densiometric scan of an aliquot from pool 3 Blue Sepharose II. The estimated molecular weight of peak 1 is 66,000

6 Second batch of plasma first purification

A diagrammatic representation of this purification is given in figure 32. It was decided for this purification to make a cut in the ammonium sulphate precipitation at 50%. Laing(1975) had found a reasonable level of activity in this fraction and, since human albumin is contained in the 64% precipitate, it should be free of albumin. A 70% precipitate was then made of the 50% supernatant, which gives the fraction with the greatest activity, but containing albumin. Since the cation exchange resin had given the best purification, it was decided to continue using CM-cellulose.

6.1 plasminogen free plasma was prepared as previously described. This gave 750ml of plasminogen free plasma as starting material. Half was stored frozen and the other half used in the steps described below (table IV).

6.2 ammonium sulphate precipitation II

365ml of the plasminogen free plasma was taken to 50% saturation with ammonium sulphate. Once the protein precipitate had been removed by centrifugation, the supernatant then left was taken to 70% saturation. The precipitate thus formed was dialyzed and 180ml of material was then available as shown in table IV.

6.3 aluminium hydroxide gel II

Two fractions were obtained (table IV);-

6.3.1 material which was not bound by $Al(OH)_3$ gel

6.3.2 material eluted by phosphate buffer. Bearing in mind what had been discovered previously, the $Al(OH)_3$ gel was washed six

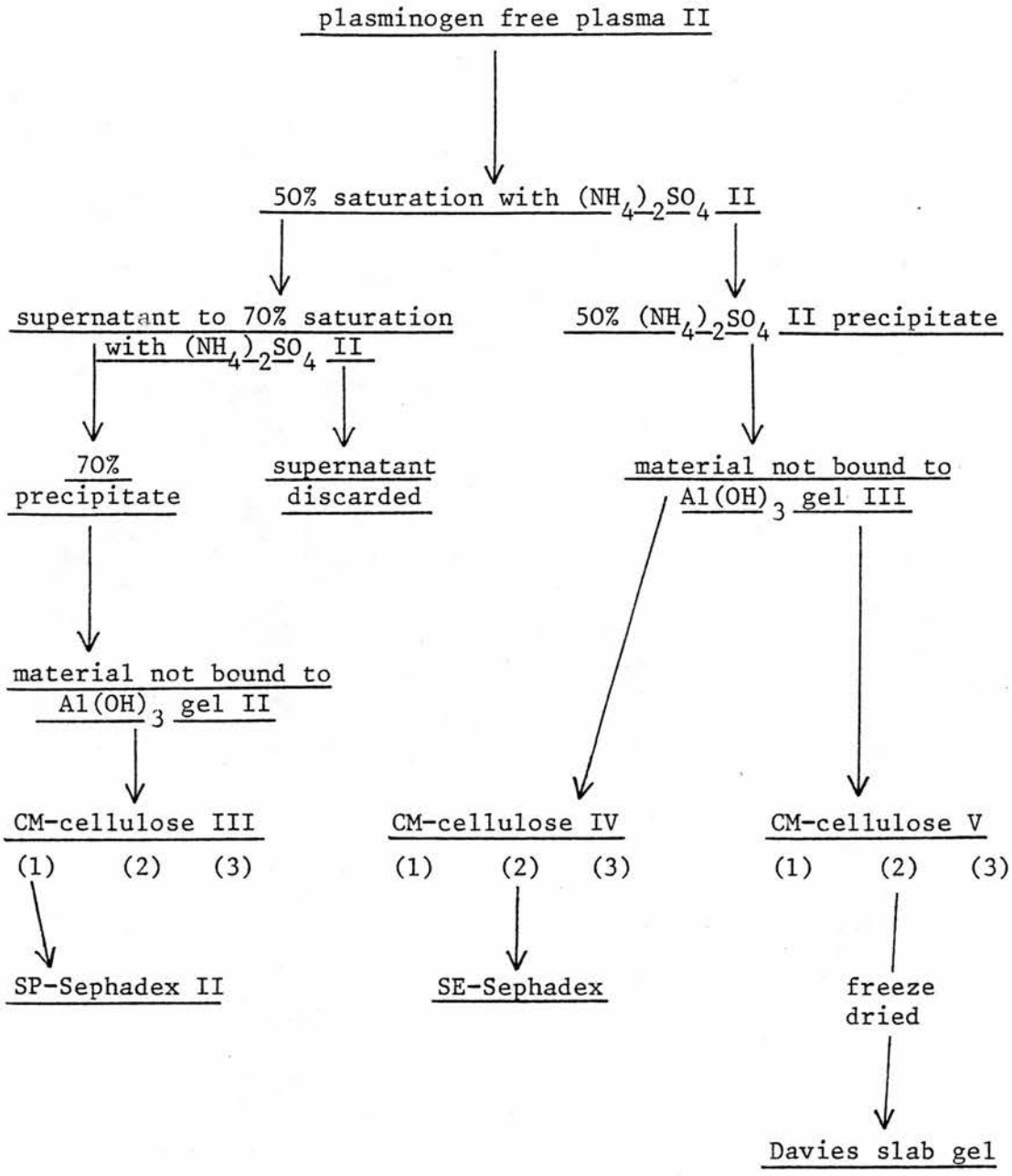


figure 32 first and second purifications of the second batch of plasma

Procedure	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg protein x 10^{-1})	Purification	Yield %
plasminogen free plasma II	365	7.7	2811	18.5	4.2	1.0	100
70% $(NH_4)_2SO_4$ precipitate II	180	8.6	1548	23.0	3.7	1.0	55
material not bound to $Al(OH)_3$ gel II	292	6.4	869	12.0	5.3	1.3	66
CM-cellulose III pool 1	90	2.8	252	1.3	22.4	5.4	37
CM-cellulose III pool 2	27	6.8	184	21.3	3.2	0.8	27
SP-Sephadex II pool 1	57	1.0	57.0	0.15	66.6	16.1	61
SP-Sephadex II pool 2	33	1.1	36.3	0.34	32.4	7.7	24

Table IV First purification of the second batch of plasma

times with 0.85% sodium chloride until no more protein or activity was seen in the supernatant. The gel was then eluted with the phosphate buffer. This eluted a small amount of protein which showed no activity.

6.4 CM-cellulose III

70ml of fraction 6.3.1 from the $Al(OH)_3$ gel fractionation II were applied to approximately 35g of CM-cellulose. All the material was bound and once the eluting buffer had been pumped through and a pH of 4.5 reached, material with activity was eluted (figure 33). One pool was made of this material (table IV). To clear the column of protein, 1M NaCl was pumped through. This gave a peak of protein and activity which was pooled to form pool 2.

SDS disc gel electrophoresis was performed on an aliquot of pool 1 (figure 34). Immunoelectrophoresis was performed against anti-human antiserum and anti-albumin antiserum, giving a precipitation line. Anti-α_2 macroglobulin antiserum showed no precipitation lines.

6.5 SP-Sephadex II

20ml of pool 1 from CM-cellulose III was applied. Material which was unbound passed through the column and was pooled (pool 1). The eluting buffer containing 0.27M NaCl was pumped through and more material was collected as pool 2 (table IV; figure 35).

SDS disc gel electrophoresis was performed on an aliquot of each of these pools (figures 36, 37). Immunoelectrophoresis was performed against anti-human antiserum and anti-albumin antiserum, giving albumin precipitation lines only (figure 38).

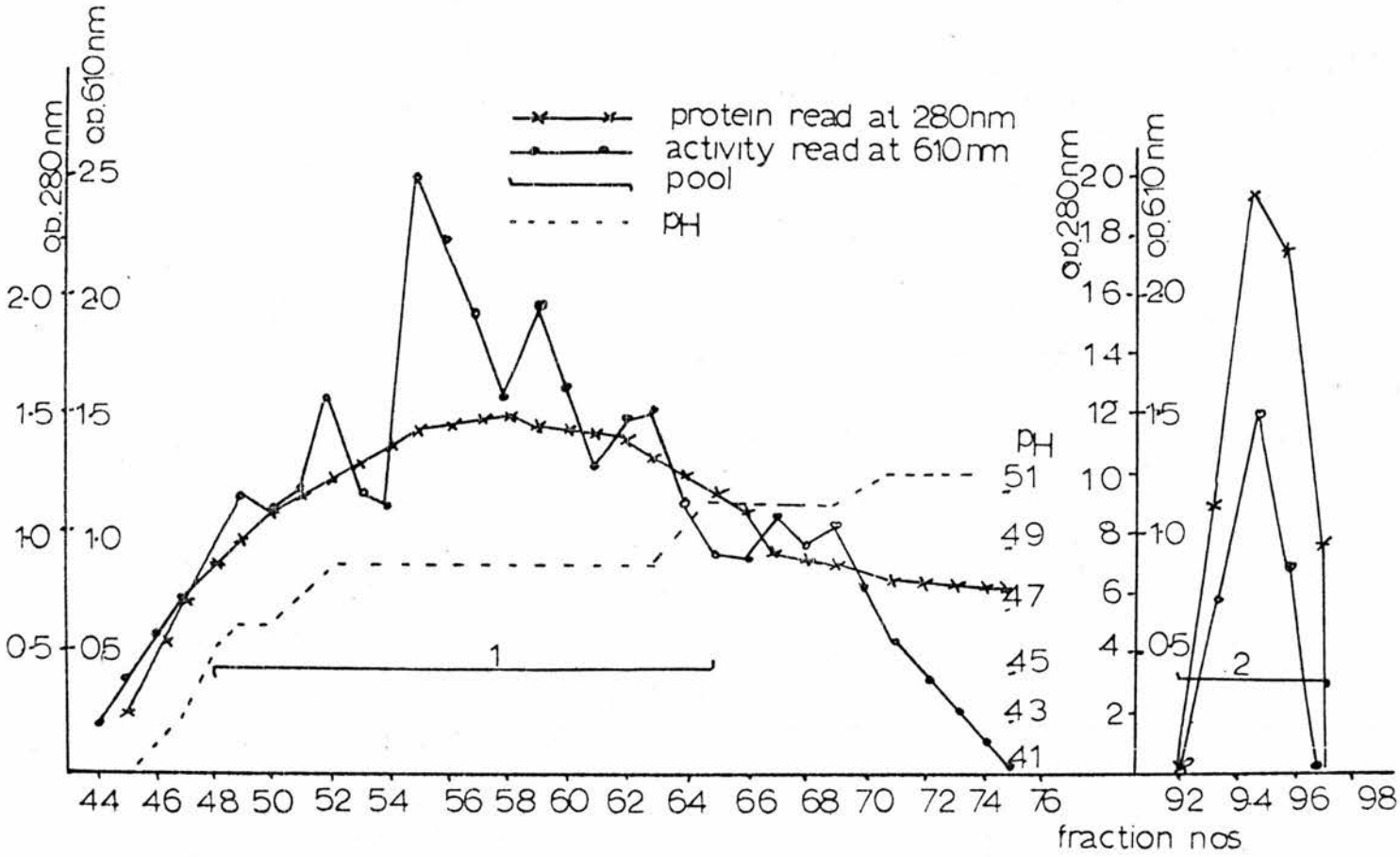


figure 33 elution pattern from CM-cellulose III. Sample applied in equilibrating buffer, 0.05M acetate buffer pH4.0. Pool 1 eluted with same buffer pH5.4; pool 3 with same buffer containing 1M NaCl. This was applied after tube 76

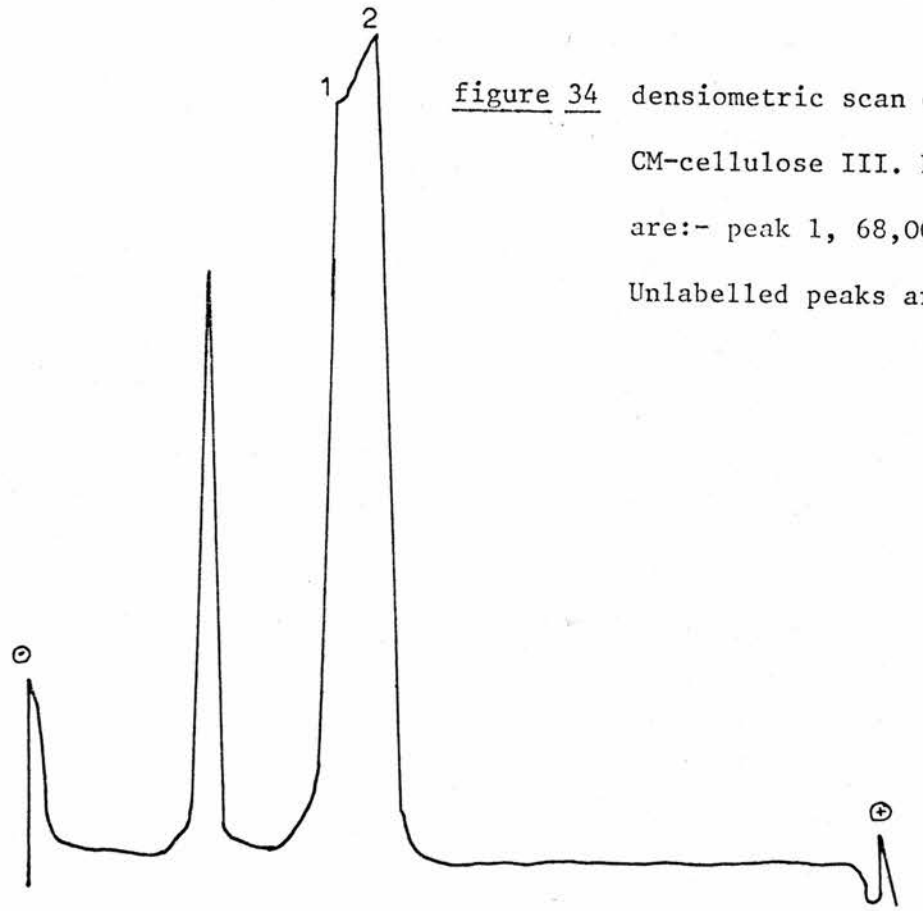


figure 34 densiometric scan of an aliquot from pool 1 CM-cellulose III. Estimated molecular weights are:- peak 1, 68,000; peak 2, 56,000. Unlabelled peaks are greater than 90,000

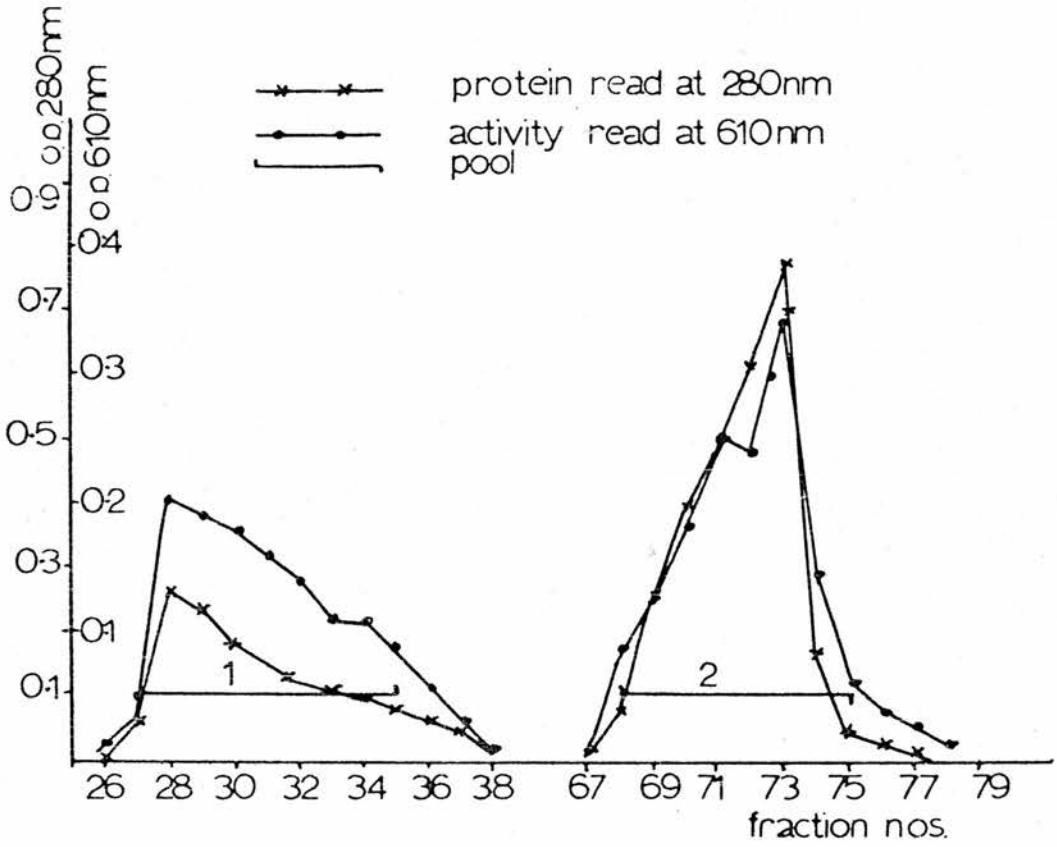


figure 35 elution pattern from SP-Sephadex column II. Samples applied in equilibrating buffer 0.02M acetate buffer pH4.3, 0.18M NaCl. Pool 2 eluted by same buffer containing 0.27M NaCl, applied after tube 36

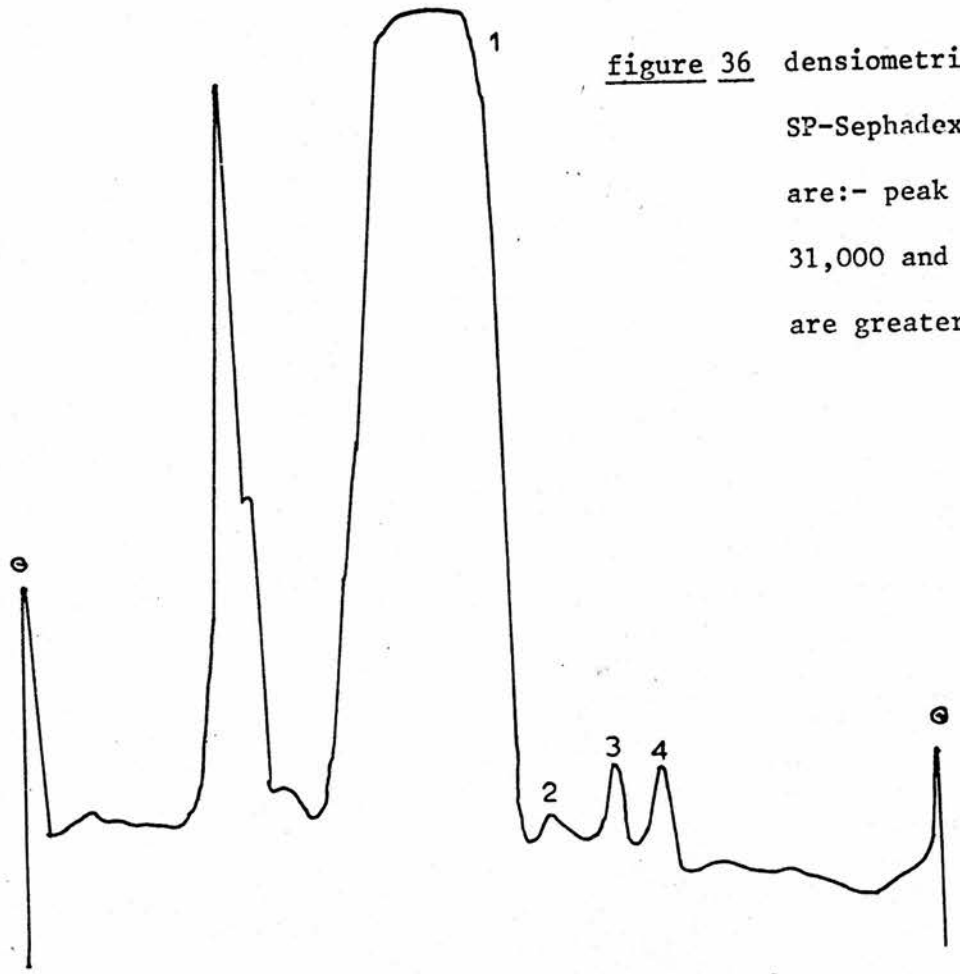


figure 36 densiometric scan of an aliquot from pool 1 SP-Sephadex II. Estimated molecular weights are:- peak 1, 51,000; peak 2, 36,000; peak 3, 31,000 and peak 4, 26,000. Unlabelled peaks are greater than 80,000

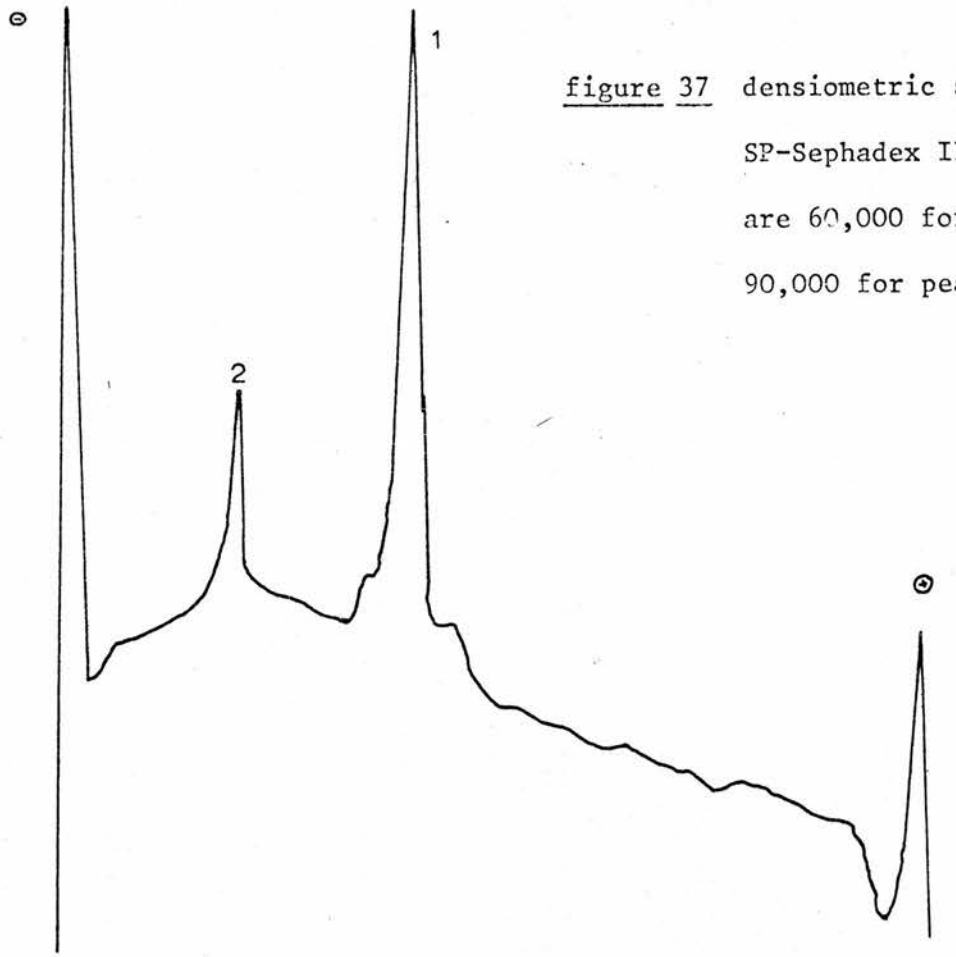
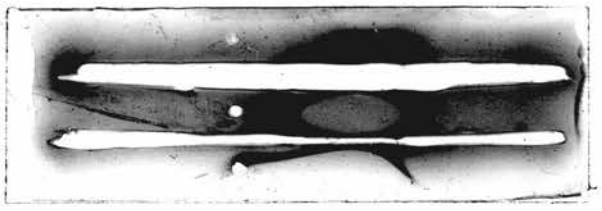


figure 37 densiometric scan of an aliquot from pool 2 SP-Sephadex II. Estimated molecular weights are 60,000 for peak 1 and greater than 90,000 for peak 2

Anti-α_2 macroglobulin gave no precipitation lines.



pool 1

pool 2

figure 38 immunoelectrophoresis of aliquots from pools 1 and 2 of SP-Sephadex II. Anti-human antiserum in the trough.

From the above results it is obvious that the cut in the ammonium sulphate precipitation at 50% removes activity, which is to the detriment of the specific activities, which are much lower than in the previous purification procedure.

For the following purification it was decided to use the 50% precipitate.

7. Second batch of plasma second purification

A diagrammatic representation of this purification is given in figure 32. For this purification, the 50% ammonium sulphate precipitate formed above was used. After it had been reconstituted and dialyzed, 130ml were available for the next step.

7.1 aluminium hydroxide gel III

Two fractions were obtained:-

7.1.1 material which was not bound by $Al(OH)_3$ gel

7.1.2 material which was eluted by phosphate buffer (see table V)

7.2 CM-cellulose IV

55ml of fraction 7.1.1 from $Al(OH)_3$ gel III were applied to approximately 35g of CM-cellulose. Starting buffer was pumped through the column and unbound material passed out of the column (figure 39). This was pooled to form pool 1. The material eluting between pH4.4 and 5.4 was pooled as pool 2. 0.5M NaCl in the eluting buffer was then applied to the column, giving another peak of protein and activity which formed pool 3 (Table V). 1M NaCl was then pumped through to clear the column. This gave a small peak of protein which was disregarded, as it gave very little activity.

SDS disc gel electrophoresis was performed on an aliquot of pools 2 and 3 (figures 40 and 41).

Immunoelectrophoresis was performed on an aliquot of pools 2 and 3 against anti-human antiserum (figure 42). Anti-albumin anti-serum gave precipitation lines in each pool, while anti-α_2 macroglobulin antiserum gave no precipitation lines.

Procedure	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg protein x 10^{-1})	Purification	Yield %
plasminogen free plasma II	365	7.7	2811	18.5	4.2	1.0	100
50% $(NH_4)_2SO_4$ precipitate II	130	7.2	936	26.5	2.7	1.5	33
material not bound to $Al(OH)_3$ III	225	4.7	1058	11.5	4.1	1.0	38
material eluted from $Al(OH)_3$ gel	300	-	-	0.3	-	-	-
CM-cellulose IV pool 1	27	2.1	57	1.1	19.1	4.5	8
CM-cellulose IV pool 2	90	2.4	216	0.6	40.0	9.6	31
CM-cellulose IV pool 3	39	7.8	304	2.75	28.4	6.7	182
CM-cellulose V pool 1	41	3.6	148	2.4	15.0	3.5	20
CM-cellulose V pool 2	125	2.1	263	0.2	105.0	25.2	35
CM-cellulose V pool 3	150	1.1	165	1.3	8.5	2.0	22

Table V Second purification of the second batch of plasma

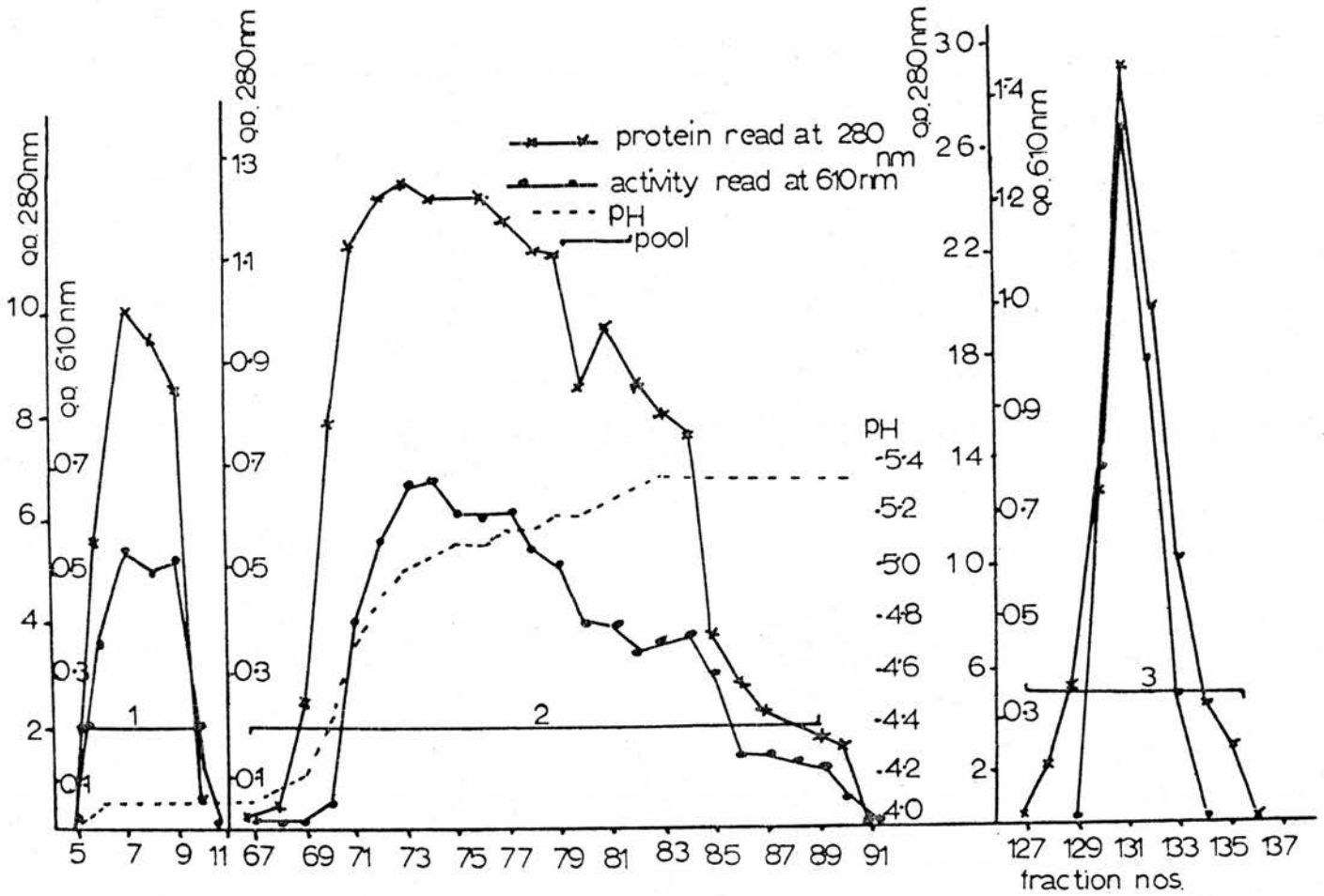


figure 39 elution pattern from CM-cellulose column IV. Sample applied in equilibrating buffer, 0.05M acetate buffer pH4.0. Pool 2 eluted with same buffer pH5.4 applied after tube 10 and pool 3 same buffer containing 0.5M NaCl applied after tube 92

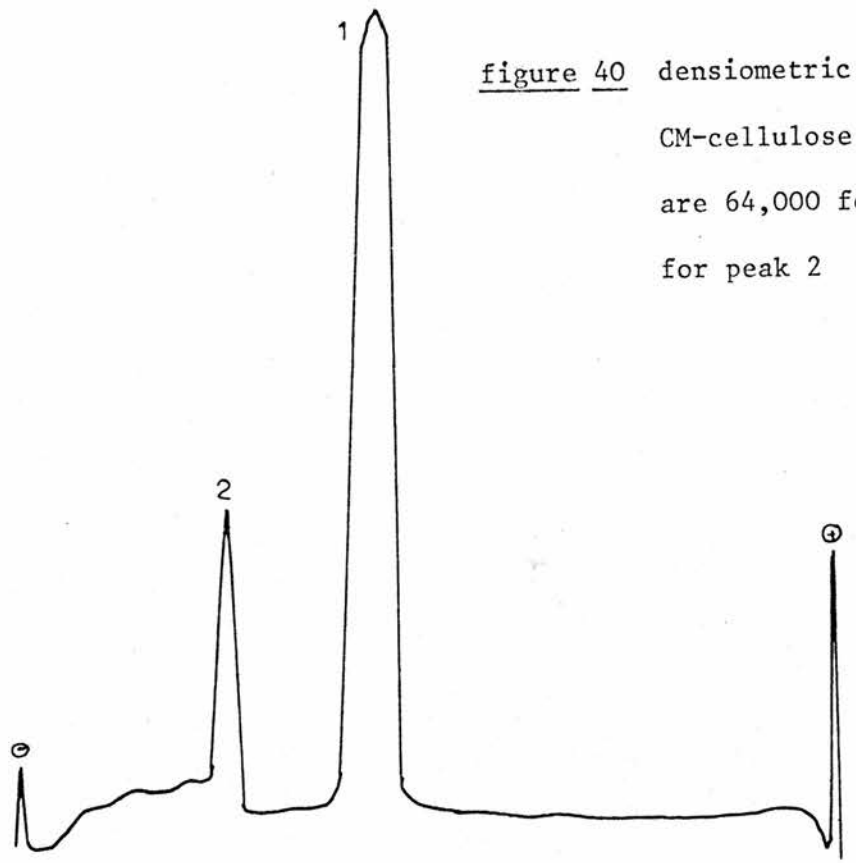


figure 40 densiometric scan of an aliquot from pocl 2 CM-cellulose IV. Estimated molecular weights are 64,000 for peak 1 and greater than 90,000 for peak 2

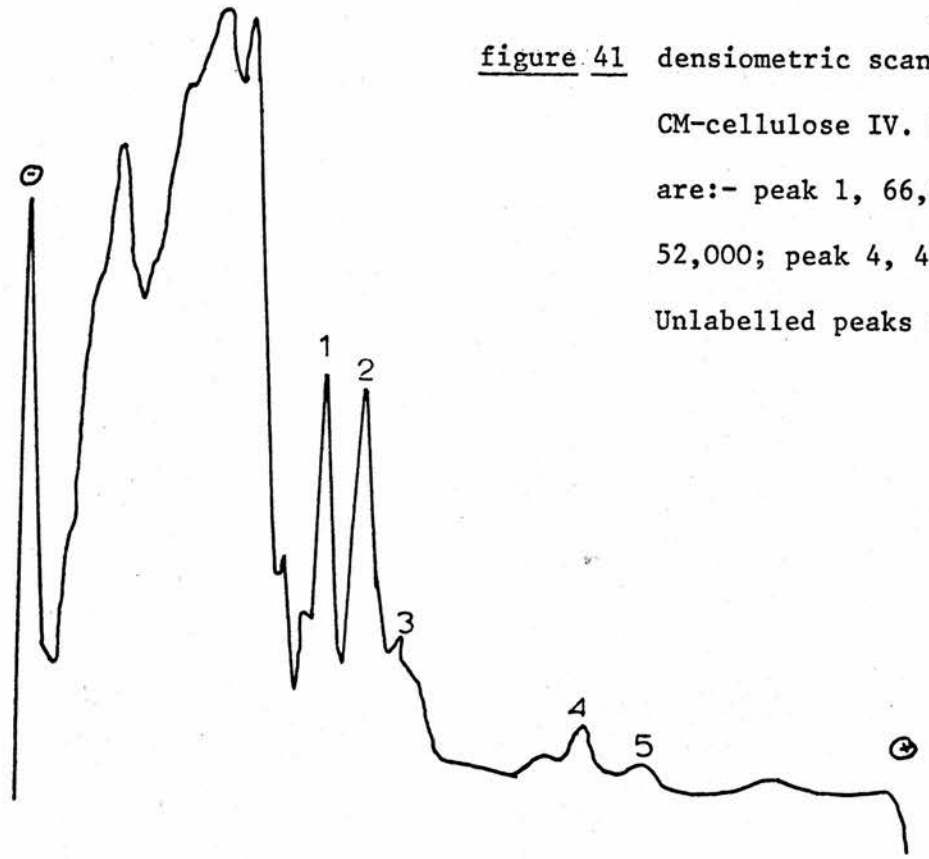


figure 41 densiometric scan of an aliquot from pool 3 CM-cellulose IV. Estimated molecular weights are:- peak 1, 66,000; peak 2, 60,000; peak 3, 52,000; peak 4, 49000 and peak 5, 23,000. Unlabelled peaks are greater than 70,000

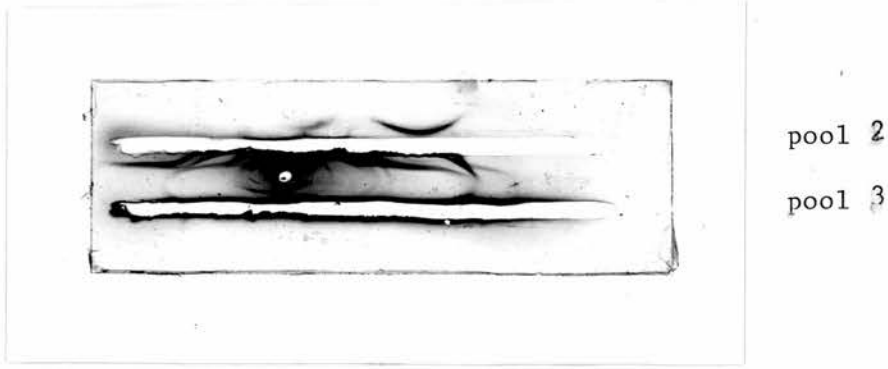


figure 42 immunoelectrophoresis of an aliquot of pools 2 and 3 from CM-cellulose IV. Anti-human antiserum in the trough.

7.3 CM-cellulose V

60ml of fraction 7.1.1 from $Al(OH)_3$ gel were applied as above. The elution pattern for this fractionation was similar to that of figure 39. Three pools were formed (table V).

SDS disc gel electrophoresis on aliquots of pools 2 and 3 gave similar pictures to those of figures 40 and 41. Similarly, the immunoelectrophoresis precipitation lines were as in figure 42.

7.4 SE-Sephadex

A small amount of SE-Sephadex became available and so it was decided to perform a trial run.

5ml of CMcellulose IV pool 2 were applied and a peak of protein eluted with the starting buffer, pool 1. When the eluting buffer was pumped through the column, another peak of protein was seen, pool 2. So little activity or protein was seen in both pools, that the calculation of activities *etc.* proved impractical. SDS disc gel electrophoresis of both pools gave similar results to those of figures 40 and 41 and immunoelectrophoresis gave similar results to figure 42.

8 Second batch of plasma third purification

A diagrammatic representation of this purification is given in figure 43.

8.1 185ml of plasminogen free plasma II were used as starting material (table VI).

8.2 ammonium sulphate precipitation III

The precipitate formed after 50% saturation was dialyzed and 92ml of material were then available, as shown in table VI.

8.3 aluminium hydroxide gel IV

The fractionation was carried out and two fractions were obtained:-

8.3.1 material which was not bound by $Al(OH)_3$ gel

8.3.2 material which was bound and then eluted by phosphate buffer (table VI).

8.4 CM-cellulose VI

35g of CM-cellulose were taken and 70ml of fraction 8.3.1 from $Al(OH)_3$ gel were applied. Starting buffer was pumped through the column and a small peak of protein was eluted. It showed little or no activity and was disregarded. The material eluting between pH4.6 and 5.2 was pooled as pool 2 (figure 44). 0.5M NaCl was then pumped through the column. SDS disc gel electrophoresis gave similar pictures to that of figures 40 and 41. Immunoelectrophoresis also showed the same precipitation lines as in figure 42.

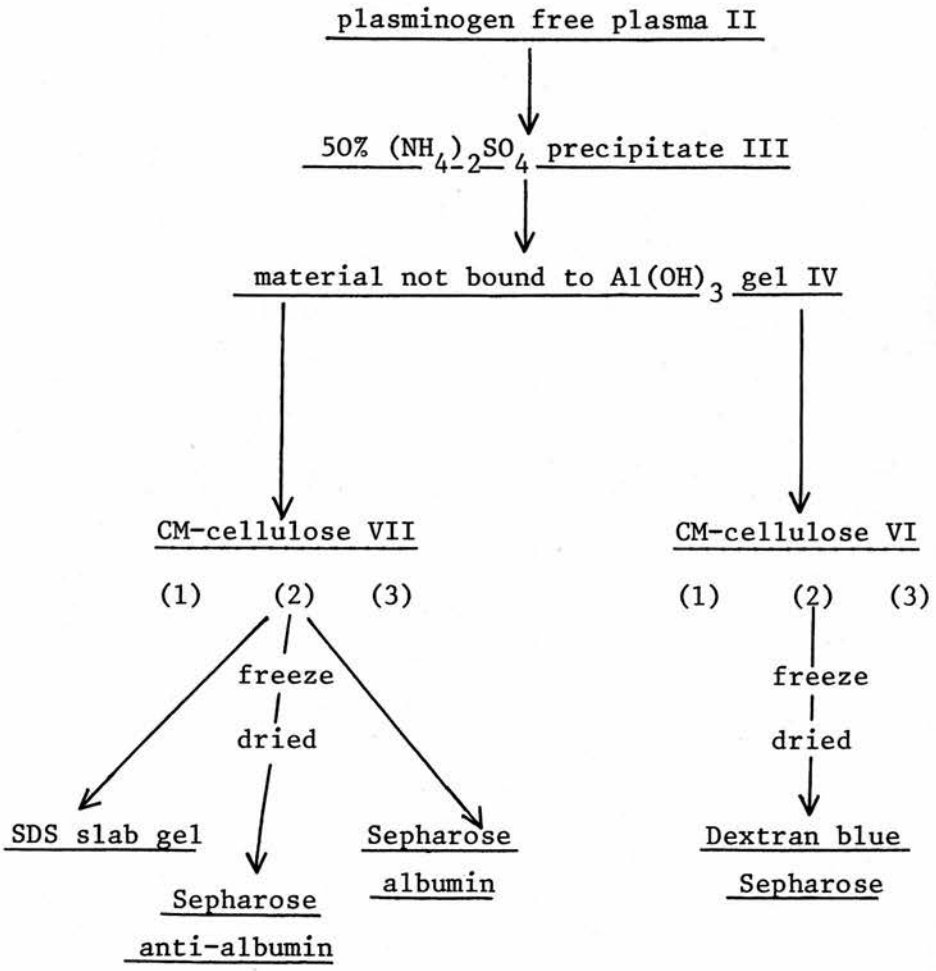


figure 43 third purification of the second batch of plasma

Procedure	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg protein x 10^{-1})	Purification	Yield %
plasminogen free plasma II	185	7.7	1425	18.5	4.2	1.0	100
50% $(NH_4)_2SO_4$ precipitate III	92	4.8	442	15.0	3.2	0.8	31
material not bound to $Al(OH)_3$ IV	160	3.6	576	6.2	5.8	1.4	40
material eluted from $Al(OH)_3$ gel	340	-	-	0.5	-	-	-
CM-cellulose VI pool 1	185	2.1	389	0.35	60.0	14.0	62
CM-cellulose VI pool 2	45	3.2	144	2.6	12.3	2.9	23
CM-cellulose VII pool 1	225	2.3	518	0.37	62.2	15.0	65
CM-cellulose VII pool 2	83	2.9	241	1.7	17.1	4.1	29

Table VI Third purification of the second batch of plasma

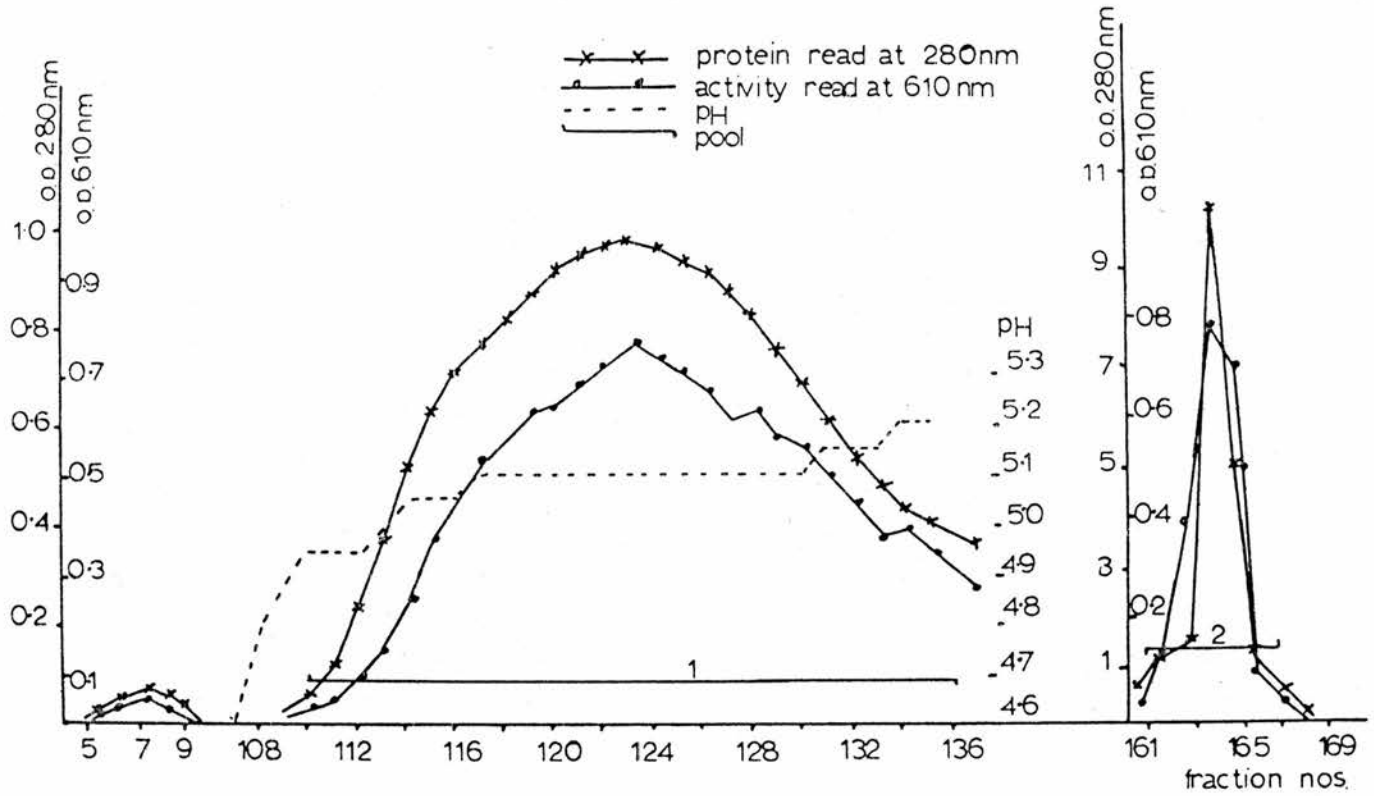


figure 44 elution pattern from CM-cellulose column VI. Sample applied in equilibrating buffer, 0.05M acetate buffer pH4.0. Pool 1 eluted by same buffer pH5.4 applied after tube 9 and pool 2 by same buffer containing 0.5M NaCl, applied after tube 136

8.5 CM-cellulose VII

90ml of fraction 8.3.1 from $Al(OH)_3$ gel IV were applied as above. The elution pattern for this fractionation was similar to that of figure 44.

Two pools were formed (table IV).

SDS disc gel electrophoresis was performed on aliquots of each pool (figures 45 and 46). The molecular weight of the main component of pool 2 of this fraction appears to have a reduced molecular weight compared with that of the other similarly prepared pools. A possible explanation will be given in the discussion section. Immunoelectrophoresis showed precipitation lines as in figure 42.

The fractions collected throughout the preceding purifications have all shown activity against fibrin blue, but it was not certain whether this activity would still dissolve native fibrin clots. To make the activity approximate to that of plasma, fractions were concentrated by freeze drying (table VII).

Table VII

	activity (units/ml)	protein (mg/ml)	specific activity (units/mg protein x 10^{-1})
90ml of CM V	2.1	0.2	105
concentrated to 45ml	3.0	0.4	75
150ml of CM VI	2.1	0.35	60
concentrated to 50ml	6.3	1.05	60
200ml of CM VII	2.3	0.37	62.1
concentrated to 50ml	9.1	1.14	79.8

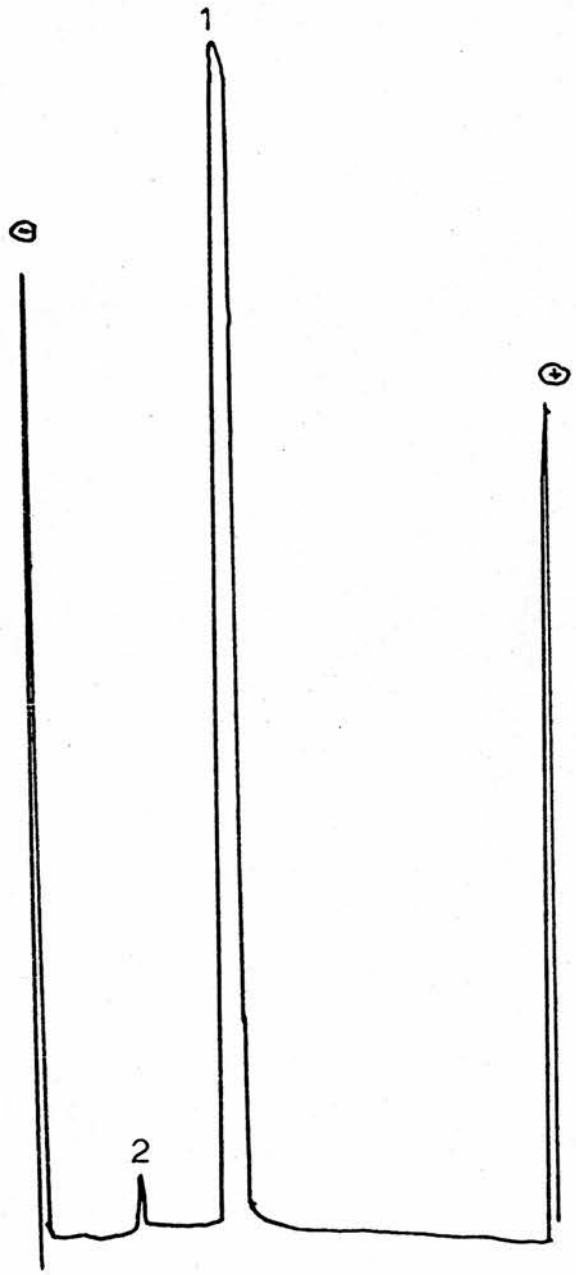


figure 45 densiometric scan of an aliquot from pool 1 CM-cellulose VI. Estimated molecular weights are 54,000 for peak 1 and greater than 90,000 for peak 2

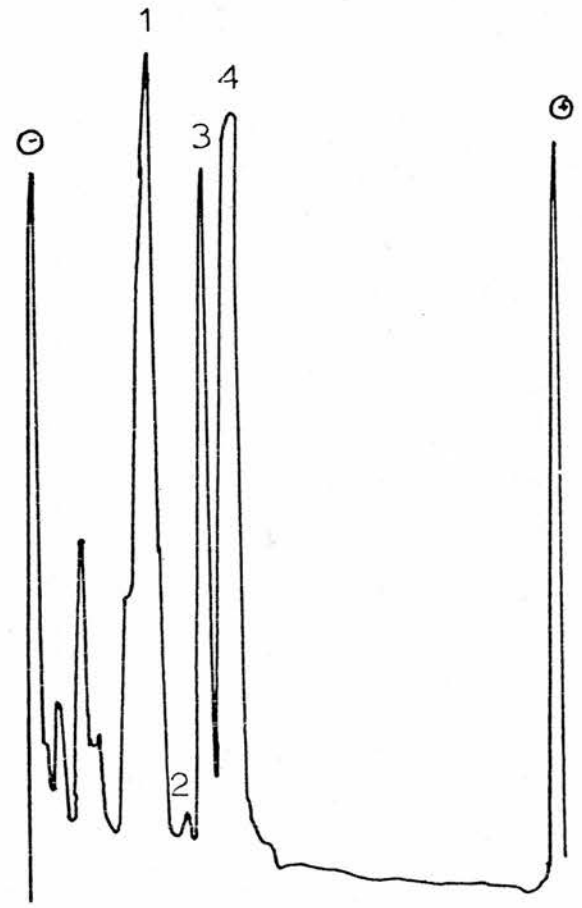


figure 46 densiometric scan of an aliquot from pool 2 CM-cellulose VI. Estimated molecular weights are:- peak 1, 54,000; peak 2, 62,000; peak 3, 66,000 and peak 4 76,000. Unlabelled peaks are greater than 90,000

The clot dissolving assay was performed using the concentrated samples. The samples were set up in duplicate.

Table VIII

	hours incubation at 37°C			
fractions	16	24	30	39
Plasma	X***	----------		
CM V	0*	0	0	0
CM VI	0	&**	&	X
CM VII	0	&	X****	-
control	0	0	0	0

* 0 indicates the presence of a clot

** & indicates a partially dissolved clot

*** X indicates the absence of a clot

**** One clot dissolved completely. The other showed small granular particles

The results shown above would indicate that the activity isolated with the fibrin blue assay, after concentration, has the ability to dissolve stabilized native fibrin.

It was decided to determine the activity of the concentrated second pool of CM-cellulose VII against acid denatured haemoglobin. Samples were originally set up at pH3.1. This was the pH recommended by Tang (1970). No reaction was seen. The pH was then lowered to 2.5 and the samples retested. The incubation period for each sample was sixteen hours at 37°C. The samples listed below were set up in

duplicate.

The difference in optical density readings between the blanks and the samples shows the presence of more peptide material in the sample than in the blank. This peptide material has been digested from the acid denatured haemoglobin by the action of acid proteinase in the sample.

Table IX

sample	sample reading	blank	Hb digestion
plasma	0.735†	0.355	Δ 0.38
conc. CM VII pool 2	0.49	0.27	Δ 0.21

† results are given as optical density readings at 280nm

The results given in table IX indicate activity in the fraction against acid denatured haemoglobin.

9 Further purification procedures

9.1 SDS slab gel electrophoresis

A repeat disc gel electrophoresis run on the concentrated CM-cellulose still showed a pattern of protein bands similar to figure 45.

Using a slab gel technique it was decided to prepare samples of the protein bands individually to see whether either had a greater specific activity and was free of albumin.

The sample of each band was prepared as previously described, using material from concentrated CM VII, pool 2. Three fractions were collected from each gel:-

i) 54,000 band

ii) high molecular weight band (high MW band)

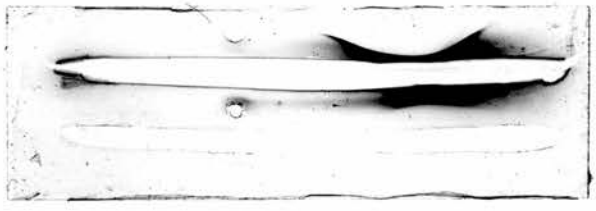
iii) residual gel (the slab gel left once the above bands had been removed).

SDS disc gel electrophoresis of the individual bands of material showed that they had been successfully eluted from the slab gels.

Table X

sample	activity (units/ml)	protein (mg/ml)	specific activity (units/mg protein x 10^{-1})
54000 band	3.3	0.66	50.0
high MW band	0.7	0.1	14.3
residual gel	-	-	-
conc. CM VII pool 2	9.1	1.14	98.8

The results listed in table X show once again that the highest specific activity is seen in the sample containing the most protein. Aliquots of these samples were subjected to immunoelectrophoresis against anti-human antiserum and what seemed like the greatest concentration of albumin was seen in the 54000 band, as can be seen in figure 47. Immunoelectrophoresis against anti-albumin antiserum showed precipitation lines. Anti-α_2 macroglobulin antiserum showed no lines.



54,000 band

high MW band

residual gel

figure 47 immunoelectrophoresis on aliquots of the material eluted from SDS slab gels. Anti-human antiserum in the trough

The acid denatured haemoglobin assay was also performed on the samples listed below.

Table XI

sample	sample reading	blank	Hb digestion
conc CM VII pool 2	0.49†	0.27	Δ 0.22
54000 band	0.41	0.30	Δ 0.11
high MW band	0.38	0.30	Δ 0.08
residual gel	0.40	0.40	Δ 0.00

† results are given as optical density readings at 280nm

The results given in the above table show the greatest activity in the 54,000 band, although it should be noted that the amount of activity in the high MW band is more than would have been expected from the fibrin blue assay results.

9.2 Davies gels

SDS disc gels separate proteins according to charge and size; Davies gels separate proteins purely by size. It was therefore decided to subject an aliquot of concentrated CM-cellulose V, pool 2 to Davies gel separation. Protein separation was obtained as shown in figure 48. Two distinct bands and one small band were noted and so it was decided to see whether they could be separated individually, using a Davies slab gel.

On staining a small piece off the side of the slab gels, the small band could not be seen. However, it may become evident in the residual slab gel material collected after the two bands have been cut away.

Three fractions were collected:-

i) band 3

ii) band 2

iii) residual gel

An aliquot of each of the above fractions was once again subjected to Davies disc gel electrophoresis. Bands, as expected, were seen from band 3 and band 2 fractions, but no bands were seen in the residual gel. Band 1 must therefore be so weak as to be undetectable.

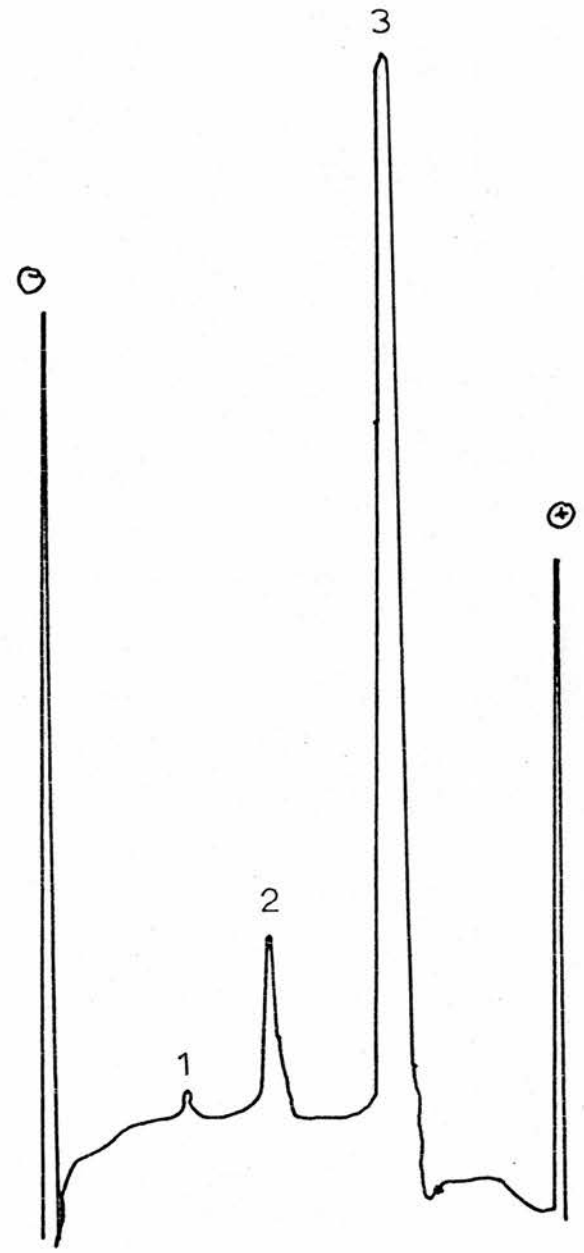


figure 48 densiometric scan of an aliquot from pool 2 CM-cellulose V, subjected to Davies gel separation

Table XII

sample	activity (units/ml)	protein (mg/ml)	specific activity (units/mg protein x 10^{-1}
conc. CM V pool 2	3.0	0.4	75.0
band 3	3.4	0.38	89.5
band 2	0.5	0.08	62.5
residual material	-	-	-

The results in this table again show that the highest specific activity is seen with the highest protein concentration. This is also the sample which contains the greatest quantity of albumin when immunoelectrophoresis is carried out against anti-human antiserum (figure 49). Immunoelectrophoresis against anti-albumin antiserum showed a precipitation line, but anti-α_2 macroglobulin antiserum showed none.

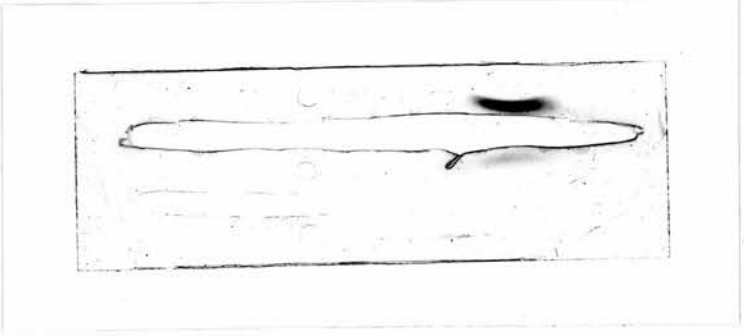


figure 49 Immunoelectrophoresis of aliquots from the material eluted from Davies slab gel. Anti-human antiserum in the trough.

The acid denatured haemoglobin assay was also performed on the samples, giving the results listed below.

Table XIII

sample	sample reading	blank	Hb digestion
conc. CM V pool 2	0.45†	0.30	Δ 0.15
band 3	0.38	0.29	Δ 0.09
band 2	0.23	0.21	Δ 0.02
residual gel	-	-	-

† Results are given as optical density readings at 280nm

This table again shows the greatest activity in band 3, which contains the majority of the material applied to the slab gel.

9.3 Affinity chromatography

From the results obtained so far, it would appear that the greatest enzyme activity can be associated in some way with albumin, since both slab gel techniques were unable to give a sample free of albumin but with activity. It was therefore decided to try affinity chromatography using several sepharose complexes. In an effort to be economical with materials, only pilot fractionations were performed, to assess the suitability of the techniques tried.

9.3.1 sepharose anti-albumin conjugate

This fractionation was performed using 4ml of concentrated CM-cellulose VII, pool 2. Two fractions were obtained:-

i) material not bound by sepharose anti-albumin conjugate

ii) material eluted from sepharose conjugate by 1M NaCl

Aliquots of both fractions were subjected to SDS disc gel electrophoresis (figures 50 and 51) and immunoelectrophoresis against

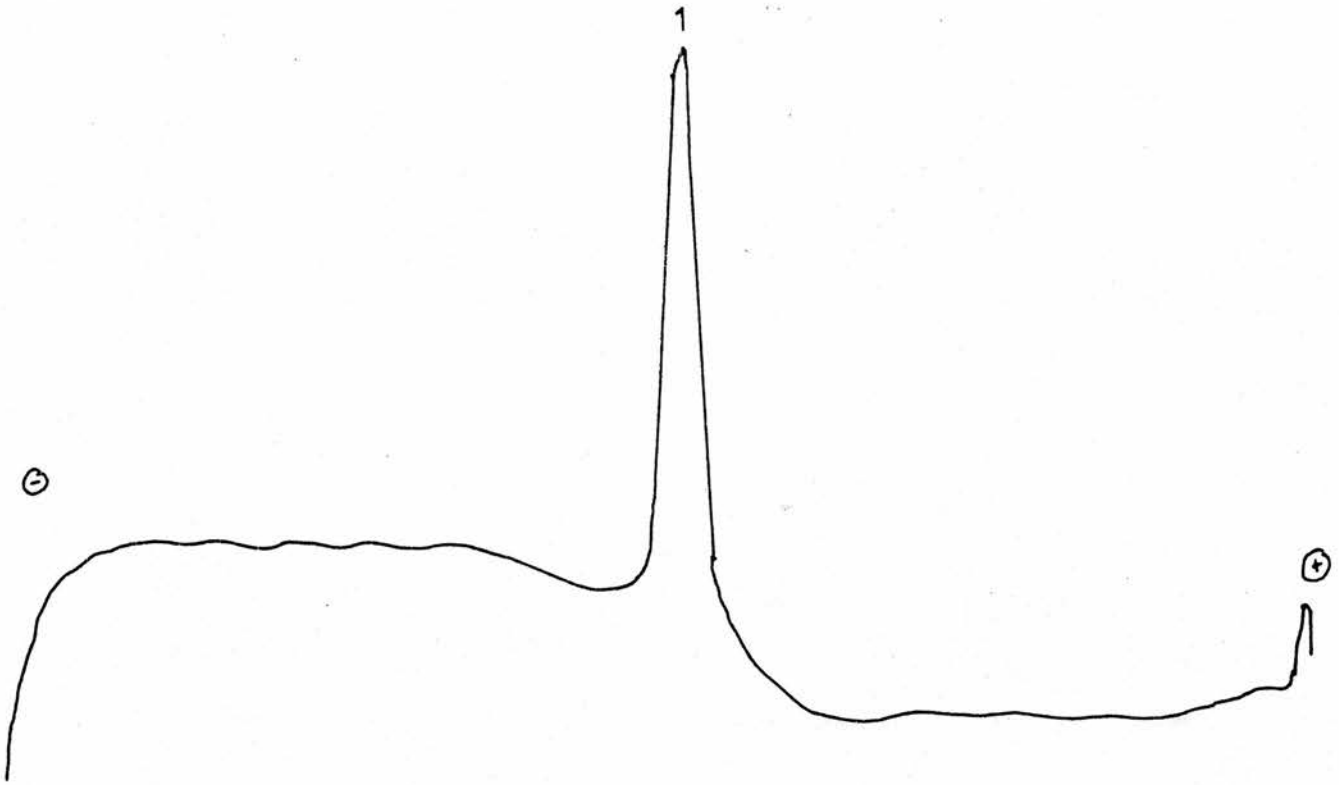


figure 50 densiometric scan of an aliquot from the material which was not bound by the sepharose anti-albumin conjugate. The estimated molecular weight of peak 1 is 50,000

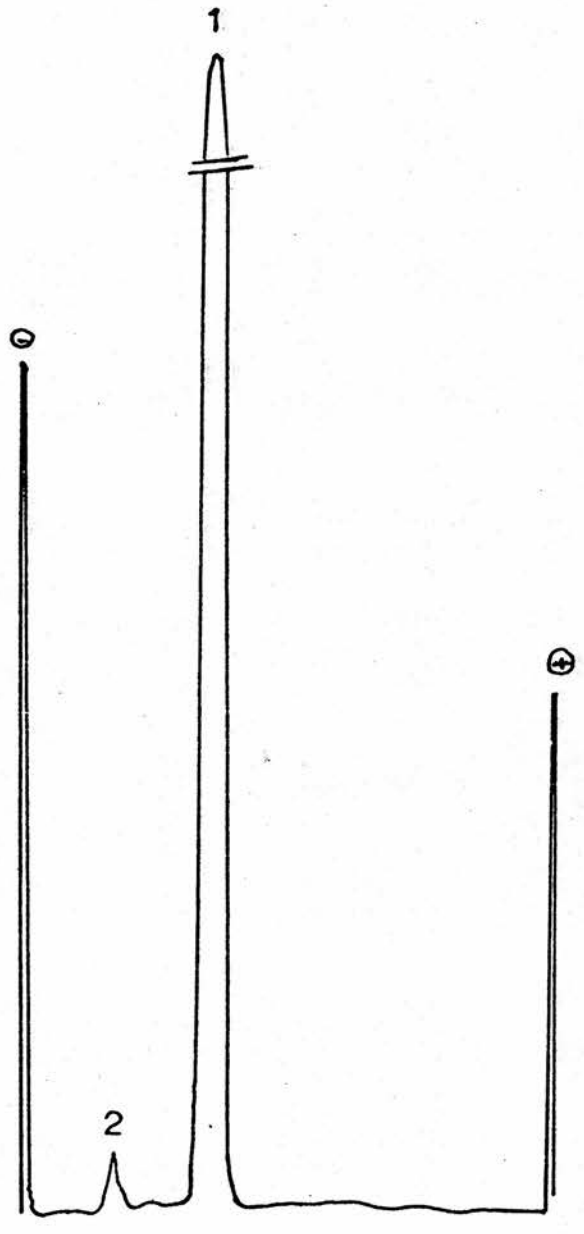
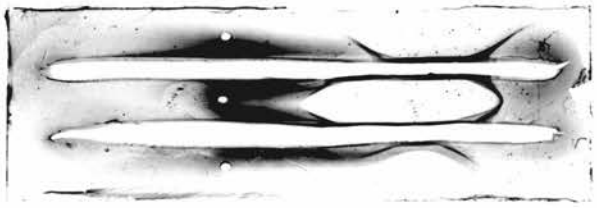


figure 51 densiometric scan of an aliquot of the material eluted from the sepharose anti-albumin conjugate. The estimated molecular weight of peak 1 is 54,000 and of peak 2, greater than 90,000

anti-human antiserum (figure 52); anti-albumin antiserum showed a precipitation line, but anti-α_2 macroglobulin antiserum showed none.



unbound material

eluted material

figure 52 immunoelectrophoresis of aliquots from the fractions collected from the sepharose anti-albumin conjugate. Anti-human antiserum in the trough

Table XIV

sample	activity (units/ml)	protein (mg/ml)	specific activity (units/mg protein x 10^{-1}
conc. CM VII pool 2	9.1	1.14	79.0
unbound material	3.2	0.79	40.5
eluted material	1.4	0.18	77.7

9.3.2 sepharose albumin conjugate

4ml of concentrated CM-cellulose VII, pool 2 were again used. This gave two fractions:-

i) material not bound by sepharose albumin conjugate

ii) material eluted from sepharose conjugate by 1M NaCl

Aliquots of both fractions were subjected to SDS disc gel electrophoresis (figures 53 and 54). Immunoelectrophoresis showed similar precipitation lines to figure 52.

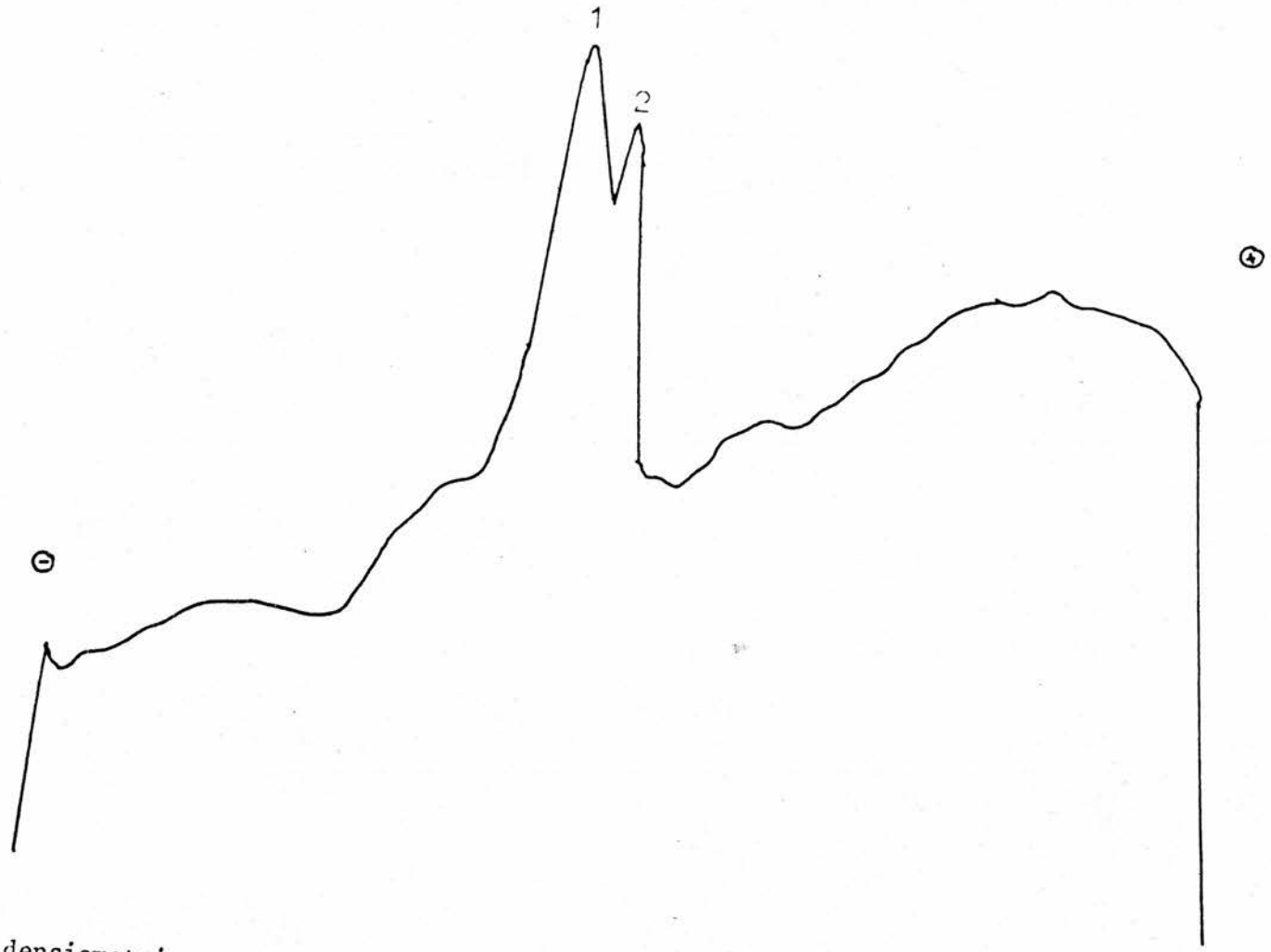


figure 53 densiometric scan of an aliquot from material not bound by the sepharose albumin conjugate.

Estimated molecular weights are 55,000 for peak 1 and 50,000

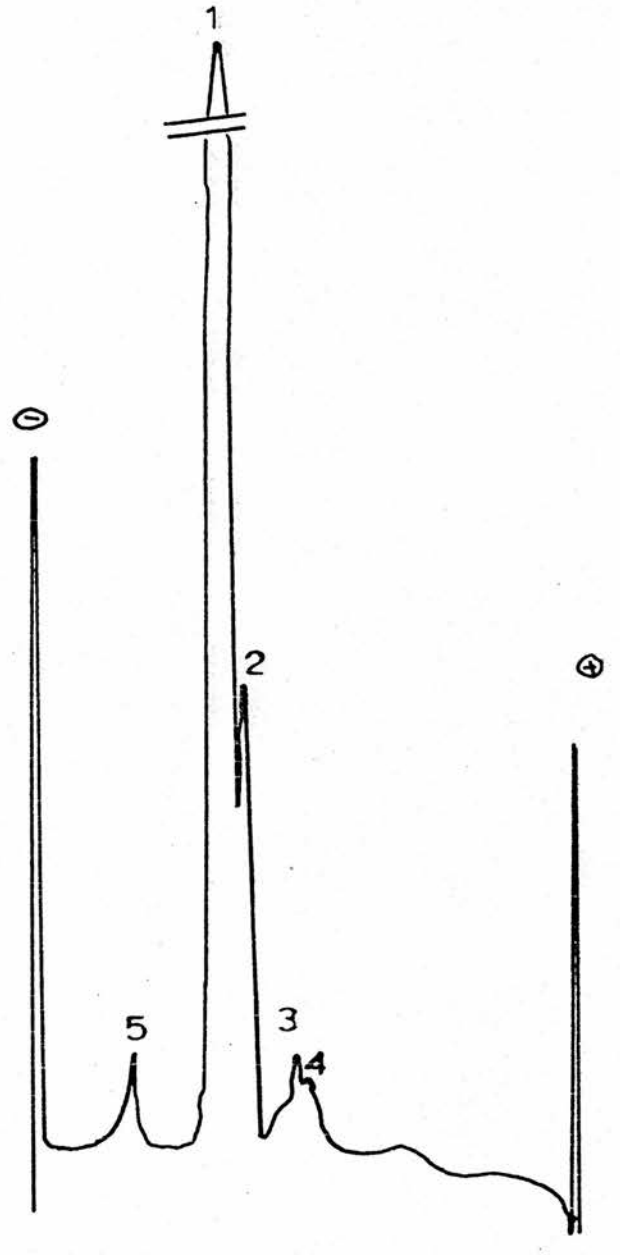


figure 54 densiometric scan of an aliquot from the material eluted from the sepharose albumin conjugate. Estimated molecular weights are:- peak 1, 54,000; peak 2, 49,000; peak 3, 39,000; peak 4, 36,000; peak 5, greater than 90,000

Table XV

sample	activity (units/ml)	protein (mg/ml)	specific activity (units/mg protein x 10^{-1}
conc. CM VII pool 2	9.1	1.14	79.8
unbound material	2.6	1.22	21.8
eluted material	0.9	0.20	45.0

9.3.3 sepharose dextran blue conjugate

This was performed following the method of Travis *et al* (1973). 4ml of concentrated CM-cellulose VI, pool 2 were used and two fractions were obtained:-

i) material not bound by dextran blue conjugate

ii) material eluted by 6M urea

Aliquots of both fractions were subjected to SDS disc gel electrophoresis (figures 55 and 56). Immunoelectrophoresis showed similar precipitation lines to figure 52.

Table XVI

samples	activity (units/ml)	protein (mg/ml)	specific activity (units/mg protein x 10^{-1}
conc. CM VI pool 2	6.3	1.05	60.0
unbound material	2.2	0.7	81.4
eluted material	0.5	0.17	29.0

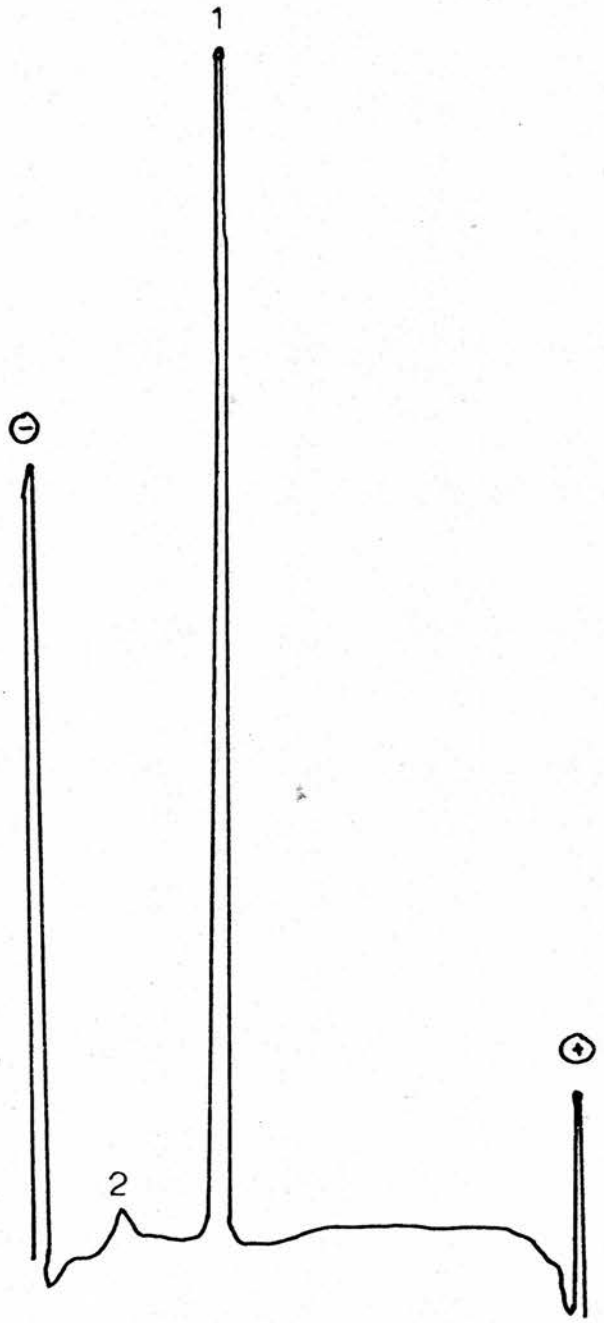


figure 55 densiometric scan of an aliquot from the material not bound by the sepharose dextran blue conjugate. Estimated molecular weights are 64,000 for peak 1 and greater than 90,000 for peak 2

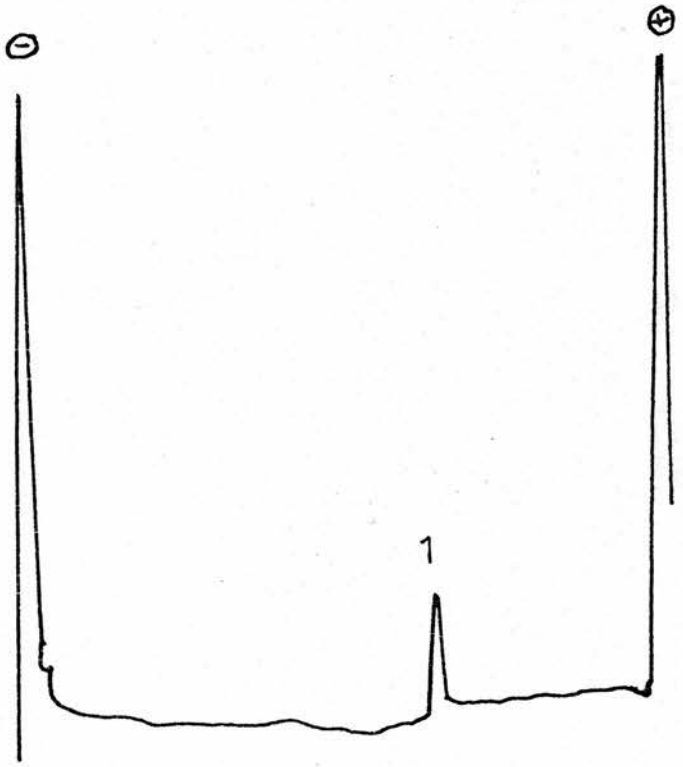


figure 56 densiometric scan of an aliquot from the material eluted from the sepharose dextran blue conjugate. Estimated molecular weight of peak 1 is 64,000.

10 pH activity profile of the enzyme

This was performed and a pH profile of the enzyme obtained as shown in figure 57. This gave an optimum pH for activity of 2.4. A second peak at 1.6 was also observed.

11 Pepstatin inhibition of the enzyme

The first experiment using pepsin at different concentrations showed total inhibition, when the reaction mixture was tested (pepsin + pepstatin) against fibrin blue and acid denatured haemoglobin for activity.

Plasma was treated in a similar way to the pepsin solutions. This reaction mixture showed total inhibition when tested against acid denatured haemoglobin and, in the clot dissolving assay, undissolved fibrin clots were still visible after thirty six hours. However, activity was still present in the fibrin blue assay to the same extent as in the uninhibited plasma. This seemed to cast serious doubts on the specificity of the fibrin blue assay, as will be discussed later.

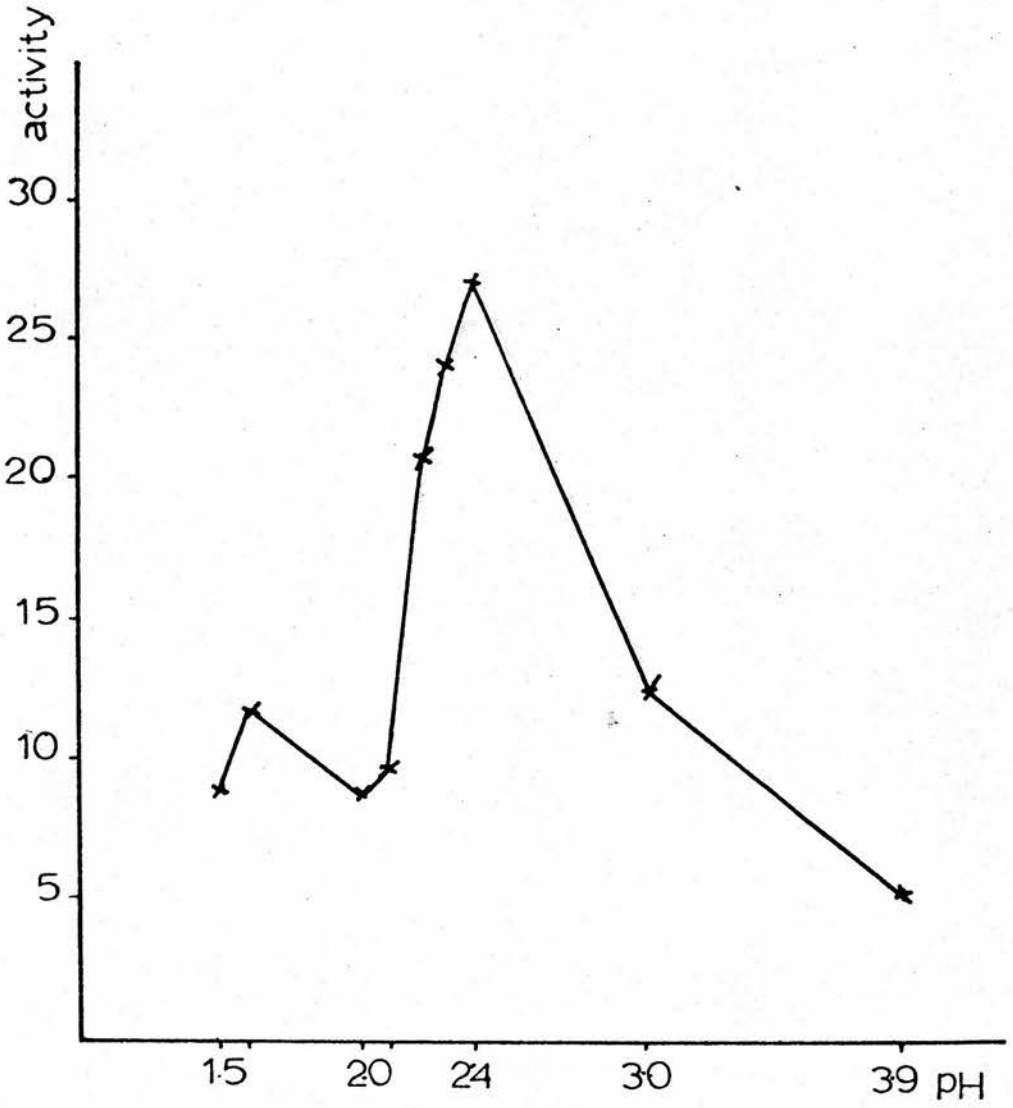


figure 57 a graph of the pH activity profile of the material not bound by aluminium hydroxide gel

12 Discussion

12.1 Purification

A 70% ammonium sulphate precipitate was made from the first batch of plasminogen free plasma, since, during batch tests, this fraction was found to have the highest activity with fibrin blue and the clot dissolving assay had shown it to give the fastest dissolution of clots.

The activity with fibrin blue was not bound to the aluminium hydroxide gel, although, as explained earlier, a little carry over of activity was seen in the phosphate eluted material. The fact that the activity was not bound to the gel would suggest that the activity sought here was different to to that of Ikemori *et al* (1975), as their activity was eluted from the gel.

Figure 5 shows the elution pattern from DEAE-cellulose. From the large number of bands seen (figure 6) and precipitation lines (figure 7), fibrin blue activity would appear to have the same charge as the majority of the proteins applied to this column and so they have all been eluted together. Since this anion exchange resin had given no separation, it was decided to try a cation exchange resin, CM-cellulose.

CM-cellulose I was made into three pools, the first of which showed the least number of protein bands and the greatest activity. It was decided to reapply 50ml of this pool to another column, CM-cellulose II. The effluent from this was formed into two pools. The first showed a tenfold purification and from figure 11 it can be seen that it only contains three protein bands, a major band at 57,000 and minor bands at 28,000 and in the region of 100,000. This

pool in figure 12a gave a sharp precipitate line, yet shows no constituent of the molecular weight of albumin (MW 68,000).

SP-Sephadex I fractionation was made into two pools. The material which was more positively charged and was not absorbed by the resin went to make the first pool. The second pool was eluted by the increased ionic strength of the buffer. Hagenmaier *et al* (1971) had separated their proteinase activity in pool 1, the albumin being eluted in pool 2. However with the above fractionation a thirty two fold increase in activity was seen in the second pool, indicating that the activity still seemed to be associated with a possible albumin fragment.

Figure 14 shows that pool 1 of the SP-Sephadex I fractionation contained only two constituents, the major one having a molecular weight of 52,000 and the minor 84,000. These should be separable on Sephadex G100.

Pool 1 of CM-cellulose II showed similar protein bands to those of SP-Sephadex pool 1 and as there was more of this pool available, it was decided to subject an aliquot to gel filtration (Sephadex G100 I). Three peaks of protein were detected, the second one having the only measurable activity, but still containing the albumin contaminant.

Hagenmaier *et al* (1971) found that at pH's above 5.0 the proteinase which they had isolated seemed to have no activity against albumin. Both CM-cellulose and SP-Sephadex fractionations were performed below this pH. If a fraction which had not been at a pH lower than 5 was taken, the activity may still be free of albumin and easier to separate.

Some of the material which had not bound to aluminium hydroxide

gel was taken and applied to Sephadex G100 II and Sephadex G200. Protein separation was seen, but these methods failed to separate albumin from activity.

It was then decided to try affinity chromatography as a means of removing the albumin contaminant. Only a small amount of Blue Sepharose CL-6B was available and so it was decided to try a pilot scheme before possibly ordering more.

The material collected from the first column had not been bound by the Blue Sepharose and still contained the albumin contaminant. It may have been that the column had been overloaded and so it was decided to reapply some of this material to a second column to see whether this would remove more of the albumin. Again material passed through unbound and this formed pools 1 and 2, while a third pool was formed from the material which was eluted.

All pools showed traces of albumin, but pool 3 obviously contained free albumin and showed very little activity. The greatest activity was still seen with the greatest protein concentration, pool 1, which also gave precipitation lines (figure 31) and so contained albumin.

The lack of success in removing albumin by this technique may be due to the fact that the configuration of this albumin contaminant has been changed by the proteolytic activity present in the plasma and so its ability to bind to dye to any extent has been lost.

During the purification procedure followed, the percentage recovery has been in excess of one hundred at several stages. This could be due to the removal of some inhibitor, or, since the protease sought is of unknown specificity, merely due to the removal

of other protein (and therefore potential substrate) as the purification progresses.

For the first purification of the second batch of plasma it was decided to take the plasma to 50% saturation with ammonium sulphate and then to take the remaining supernatant to 70% saturation with ammonium sulphate. CM-cellulose and SP-Sephadex resins were used for the purification. Disc gel electrophoresis and immunoelectrophoresis pictures were similar to the previous purification. However, specific activities were lower and no further purification was seen. The splitting of the ammonium sulphate precipitation appeared to remove activity to the detriment of the specific activity, while not removing the contaminating albumin complex.

Human albumin is claimed to be contained in the 64% precipitate of ammonium sulphate fractionation of plasma (Steinbuch, 1972). It was therefore decided for the next purification (second purification of the second batch of plasma) to take the plasminogen free plasma to 50% saturation with ammonium sulphate and to use the material from this precipitation to see whether a fraction with activity but free of the albumin contaminant could be obtained.

Two pools were again obtained from the aluminium hydroxide gel fractionation in spite of the precautions taken to wash the gel clear of the protein before eluting with the phosphate buffer. The first pool (unbound material) contained the activity and was used for the rest of the purification. The second pool contained very little protein and no activity against fibrin blue. Although no activity against fibrin blue was seen, it would be reasonable to conclude that the protein found was a trace of the proteolytic

activity isolated by Ikemori *et al* (1975).

Aliquots of the material which had not been bound to the aluminium hydroxide gel were applied to CM-cellulose columns IV and V. More resin was used than in the previous purification and this achieved a better separation.

With both CM-cellulose IV and V, protein passed through the column unbound and was pooled as pool 1; pool 2 was obtained as the pH was raised by the applied eluting buffer.

Figure 40 shows that there were only two protein constituents to this pool. The major one had a molecular weight of 64,000 which, as can be seen in figure 42 again showed only albumin precipitation lines. The third pool in both fractionations was eluted with an increase in ionic strength and contained a wide variety of proteins.

The pilot fractionation of CM-cellulose IV with the available amount of SE-Sephadex was unsuccessful as a means of removing albumin from activity.

A third purification was made, using the same plasminogen free plasma but making a different 50% ammonium sulphate precipitation. The aluminium hydroxide gel fractionation was made into two pools as above. CM-cellulose fractionations VI and VII gave the same results as above, except that less material passed through unbound, and what did was discarded.

It was noticed that the major constituent of CM-cellulose VII pool 2 had a significantly lower molecular weight, more within the range of the constituents of pool 2 of SP-Sephadex I. This could have been caused by the protein constituent of the main band being digested by the proteolytic activity which had been separated with it.

This sample had been left at $4^{o}C$ over a period of a week before being subjected to SDS disc gel electrophoresis. The other CM-cellulose fractions (IV, V and VI) had been subjected to SDS disc gel electrophoresis and frozen immediately they had been collected. So the molecular weights seen in the pools from these columns are probably a truer representation of the facts. In future, care would have to be taken to ensure that samples were frozen immediately they had been obtained.

The molecular weights of the main constituent of the second pools from CM-cellulose fractionations IV, V and VI are very close to that of albumin, and, since the immunoelectrophoresis showed a distinct precipitation line in the place of albumin, it would be appropriate to find whether this is in fact albumin. A way of confirming this was to run a sample of albumin beside an aliquot of CM-cellulose VI pool 2 on a split SDS disc gel. Figure 58 shows such a gel and it can be seen that the material on the right of the gel, which is the CM-cellulose aliquot, has run slightly in front of the albumin band (on the left), indicating that the molecular weight of the main constituent must be slightly less than that of albumin. This tends to confirm the result found in section 8.4, demonstrated in figure 40, which gave an estimated molecular weight of 64,000.

An aliquot of the above CM-cellulose pool, when concentrated, was rerun on SDS disc gel electrophoresis. No extra bands were seen. This was the hoped for result, as concentration of this pool could have caused other bands of protein to appear.

SDS slab gel preparation of the concentrated CM-cellulose VII

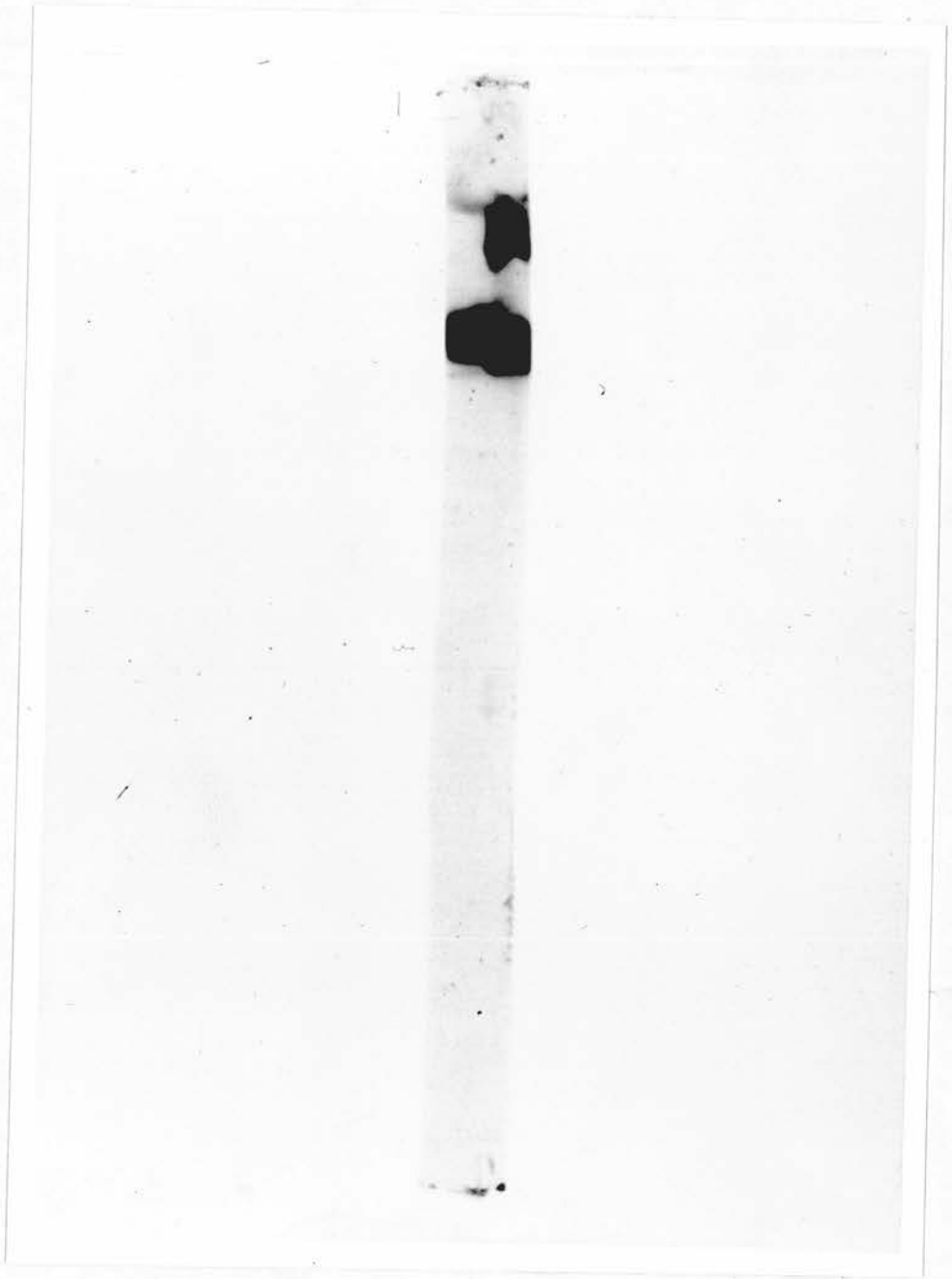


figure 58 SDS disc gel which has been split. Material applied on the right is an aliquot from CM-cellulose VI pool 2. On the left is an aliquot of a 10mg/ml solution of human albumin

pool 2 enabled fractions of the individual bands to be prepared. Activity with both fibrin blue and acid denatured haemoglobin assays was concentrated i.e. 54,000 band. Negligible activity was seen in the high MW band. Immunoelectrophoresis again showed the presence of the albumin contaminant. An attempt was made to make this comparative by applying five times more material from the high MW band in the agar well than from the 54,000 band. The albumin precipitate line appeared stronger in the 54,000 band than in the high MW band.

Davies slab gels were performed on concentrated material from CM-cellulose V pool 2. The activity was concentrated in band 3, with activity in band 2 being negligible. Again, five times more material was applied to the agar well of the second band and the albumin precipitation line appeared stronger in band 3.

It would appear from the results of both types of slab gels that the major activity is confined to one band, namely the 54,000 molecular weight band.

Affinity chromatography was again tried; the sepharose-ligands were prepared in the laboratory. Sepharose anti-albumin conjugate was used in the hope that this would bind the albumin contaminant and thus release the activity to pass freely out of the column. The albumin contaminant could then be eluted from the column.

However, activity stuck to the material attached to the sepharose, but due to uncertainty of specificity of the anti-albumin, non-specific binding may have taken place. The majority of the activity and protein was unbound by the sepharose. This may have been due to overloading, or to the loss in ability of the digested albumin to react with the anti-albumin attached to the sepharose.

Sepharose albumin conjugate was used in the hope that this would bind the activity and thus remove it from the albumin contaminant. Once again some material with activity passed through the column, but this may have been due to overloading.

Activity stuck to the sepharose material which when eluted contained small molecular weight fragments. These could be products of digestion of albumin. The albumin actually stuck to the sepharose may have been digested and a way of substantiating this would be to attach radioactively labelled albumin to the sepharose and to examine the radioactive counts in the eluent.

Sepharose dextran blue conjugate was also used to take advantage of albumin's ability to bind this dye and thus leave the activity free. Again the majority of the material was unbound. This may have been due to the digested albumin having lost its strong affinity for the dye.

The above affinity chromatography techniques were unsuccessful in giving fractions of activity free of the albumin contaminant. In the future it might be advantageous to attempt to remove the albumin from the plasma as the first step in the purification. Travis *et al* (1973) removed 96% of the total albumin component of plasma by adsorption to sepharose blue dextran.

12.2 Pepstatin inhibition

Pepstatin is a low molecular weight potent inhibitor of acid proteinases. Discovered in culture filtrates of various species of *Actinomyces* (Umezawa *et al* 1970). It inhibits nearly all acid proteinases including pepsin, renin, cathepsin D, chymotrypsin,

gastricsin, Proteases B from *Aspergillus niger* and several other proteinases of microbiological origin. It does not inhibit neutral and alkaline proteinases.

Pepstatin is a hexapeptide containing two residues of an unusual amino acid, 4-amino-3 hydroxy-6 methylheptanoic acid (for convenience called statine by Tang (1976)). These two statyl residues are thought to be the main structural component responsible for the pepstatin inhibition of pepsin, by reacting with the two aspartyl residues present at the active site of the pepsin (Marcinszyn *et al* 1976).

Pepstatin inhibited the ability of the enzyme isolated in this project to digest acid denatured haemoglobin, but not to elute dye from fibrin blue. This is not too surprising a result, as pepstatin shows the ability to inhibit such a wide range of acid proteinases.

12.3 The assays

12.3.1 fibrin blue

The fact that pepstatin inhibited the ability of the enzyme purified in this project to digest denatured haemoglobin but not its activity against fibrin blue could point to the presence of two separate enzyme systems; one inhibited by pepstatin, which reacts with the acid denatured haemoglobin and another not inhibited, which reacts with fibrin blue.

This, however, would seem unlikely as it had been noticed that the dye eluted from fibrin blue by different concentrations of pepsin appeared to give a differently coloured eluted material in proportion to activity. This was substantiated by the fact that this eluted

solution gave maximum spectrophotometric absorbance at 570nm against the 610nm found with the solution eluted by the activity in this project.

An explanation of these different maximal absorbances may be that pepsin in fact digests the dyed fibrin, and the eluted solution contains dyed fragments of fibrin, absorbing at 570nm, whereas the eluted solution showing maximal absorption at 610nm is the dye, which has been eluted by the proteins in the fractions of this purification, thus masking true activity. Albumin shows the property of binding dyes (Travis *et al* 1973) and Bradford (1976) recently used utilized the principle of protein dye binding as a sensitive method for the quantification of microgram quantities of protein. The true activity of the fractions being masked by the protein eluting the dye would certainly explain the lack of inhibition by pepstatin and the greater than expected activities seen in the high molecular weight band from the SDS slab gel purification.

However, bearing in mind the criticism of the fibrin assay made at the commencement of the purification, justification could be found for proceeding with the purification using the newly devised assay. No other assay offered itself as a suitable alternative. Acid denatured haemoglobin shows activity, but only after eighteen hours incubation and there is a considerable risk of bacterial contamination during an incubation of this length at $37^{o}C$. The clot dissolving assay again shows activity, but it is difficult to determine the exact moment of clot dissolution, especially since the fibrin clots become transparent in an acid environment.

12.3.2 the APDT assay

N-acetyl-L-phenylalanyl-L-diiodotyrosine is a synthetic substrate for pepsin. The acid proteinase hydrolyses the substrate in 0.208M HCl, liberating diiodotyrosine which is then estimated by its reaction with ninhydrin.

Unfortunately, the majority of samples throughout this purification contained high levels of peptides, which masked the ninhydrin reaction, giving results of a too high optical density to be read spectrophotometrically. Precipitation and dialysis of the samples did not remove sufficient peptides and dilution of the samples would have diluted the activity. However, with one of the CM-cellulose fractions it was possible to obtain a reading and no difference was seen between the blank and the sample, indicating that there had been no hydrolysis of the synthetic peptide.

12.3.3 radial enzyme diffusion into skimmed milk containing agarose gel

Lowenstein and Ingild (1976) claimed that the technique is three hundred times more sensitive than photometric assays for pepsin and it was certainly found that 5µl of a pepsin solution of 10µg/ml was detectable with this technique. The assay relies on the ability of acid proteinase to digest casein.

The assay was first attempted at the lowest pH discussed in the paper, pH2.8, but at this pH, samples applied from the purification showed no digestion of casein. It was decided to take the pH of the reaction down to pH2.1. Still no activity was seen, but this may have been due to the inability of the pH2.1 buffer to penetrate the agarose gel, or it may be that the activity present does not have the same ability to digest casein as pepsin.

12.4 General discussion

Due to the uncertainty of the fibrin blue assay, the reliability which can now be placed on the calculated specific activities, purifications and recoveries throughout this project must be in some doubt. However, it could be expected that if the fibrin blue assay was measuring only protein, there would be no difference in the specific activities of different fractions, and this is clearly not so.

The proven presence of activity in the acid denatured haemoglobin and clot dissolving assays must point to the fact that some acid proteinase activity has been isolated during the purification process.

The fact that the activity isolated here was inactivated by pepstatin would seem to point to the activity belonging to the group of proteinases known as the carboxy proteinases. It has been found that the mode of inhibition of pepstatin is to block two carboxyl groups at the active site of the enzyme. This has the effect of inactivating the enzyme. Hence the name given to this group of enzymes would suggest that enzymes falling within the group have two essential carboxyl groups at their active sites: for example, pepsin is known to have two aspartyl groups.

Carboxy proteinases embrace a wide variety of acid proteinases, including pepsin, chymosin, gastricsin, proteases from microorganisms(especially moulds), proteinases from plants, mammalian lysomol proteinases such as cathepsin D and E, renin and proteinases in seminal plasma.

Evidence exists for the presence of more than one acid proteinase in human plasma and this work certainly confirms that, as act-

ivity has been isolated in a different fraction to the one which Ikemori *et al* (1975) followed.

Various acid proteinases have been found in the cellular components of the blood. The intracellular granules of platelets contain cathepsin A, which has the ability to digest denatured bovine haemoglobin at pH3.8 after a one hour incubation at $37^{o}C$, although it shows a maximum digestion between pH6.5 and 7.8. It also has the ability to digest fibrinogen, giving different breakdown products from plasmin (Nachman *et al* 1968).

Leucocytes, predominantly monocytes, contain cathepsin D, which shows a pH optimum of 3.6 and a molecular weight of 42,000 (Ishikawa and Cimasowi 1977). Red cells also contain cathepsin D (Reichelt *et al* 1974), probably membrane bound.

Lebez and Kopitar (1970) isolated a further acid proteinase from leucocytes, which they named cathepsin F. It has the ability to degrade fibrin and fibrinogen at a pH optimum of 3.5. Albumin was also digested at a pH optimum of 4.0. It also has the ability to digest fibrin at pH's 5.5 and 7.5 and albumin at pH7.5. This cathepsin F has a low molecular weight as it was found in the ultrafiltrate of an LSG60 Ultrafilter membrane filter and thus has a molecular weight of less than 15,000.

Plasma itself contains an acid proteinase enzyme, renin, which is produced by the kidneys. This enzyme is responsible for the conversion of renin substrate (angiotensin I) to angiotensin II, which is the most potent vasoconstrictor known and causes a rise in systolic and diastolic blood pressure. The conversion by renin of its substrate takes place in the lungs.

An increase of acid proteinase activity was seen in the plasma of the Swiss female patient of Ragaz *et al* (1976). She was on oral contraceptives. Renin activity is increased during oral contraceptive medication (Skinner *et al* 1969) and is totally inhibited by pepstatin and, to differing extents, by other active site directed inhibitors of carboxy proteinase (Inagami *et al* 1974).

The exact specificity of this enzyme will not be elucidated until further work has been done with a range of synthetic substrates and inhibitors of carboxy proteinases. Different carboxy proteinases vary in their ability to hydrolyse synthetic substrate and so there must be a slight variance in the active sites within the group. By following the rate of this isolated enzyme's reactions with various synthetic substrates, a pattern may emerge which would enable the enzyme to be classified.

After separation of the two protein bands seen in the SDS disc gel electrophoresis of concentrated CM-cellulose fractions, the majority of activity was seen in the 54,000 band, which showed only albumin precipitation lines. As discussed earlier, this molecular weight of 54,000 may in fact be low because of the problems of proteolysis within the sample, but it has been assumed for the purpose of this discussion that the 54,000 band is analogous to the 64,000 band of the other CM-cellulose fractions.

This protein appears homogeneous when subjected to Davies gels and when reduced with mercaptoethanol prior to SDS disc gel electrophoresis. The immunoelectrophoresis results point to the presence of albumin; the molecular weight is within experimental error of the molecular weight of albumin (68,000). However, when samples were

run side by side on a split SDS gel, the band containing activity ran ahead of the albumin band, thus indicating that the 64,000 molecular weight for the band activity is in fact correct.

It would seem from the results obtained from this purification that the activity must have digested the albumin and in some way attached itself to the degraded albumin molecule. This could explain the unexpected inability of the albumin contaminant to be bound in the affinity chromatography purifications.

13 Conclusions

During this purification, at least one enzyme which shows the ability to dissolve stable blood clots in an acid environment was isolated. It appears to have a molecular weight of 64,000 and seems in some way to be attached to a degraded form of albumin. Its activity against acid denatured haemoglobin was inhibited by pepstatin, which would suggest that it could belong to the group of carboxy proteinases. Activity against fibrin blue was not inhibited by pepstatin, which may be due to the inadequacies of the fibrin blue assay or to the presence of a second enzyme. Clearly the fibrin blue assay requires further investigation.

The exact physiological role of this enzyme is difficult to comprehend, and the pH optimum of 2.4 found *in vitro* could bear little relation to the situation *in vivo*. However, since the substrate of the enzyme is insoluble, it could be that charges on this substrate would influence the pH in the surrounding microenvironment of the enzyme, giving a localized pH lower than the physiological pH.

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