OBSERVATIONS ON A HUMAN ACID PROTEASE WITH FIBRINOLYTIC ACTIVITY

by

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DEDICATION

'And he pointed his pipe to the sofy
where his wife sat infusing the tea
And he said, "Yon's the bonniest trophy
that ever was won by McFee!"'

(Patrick Chalmers)

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ABSTRACT

An unusual bleeding disorder clinically resembling factor XIII deficiency was reported by Regaz et al (1976) in a female patient with a history of easy bruising since early childhood. In this patient post-operative recovery was also complicated by prolonged haemorrhage and delayed scar formation. Factor XIII levels, however, were normal and the only detectable abnormality was an increased activity of a pepsin-like protease in the plasma. The presence of an acid protease in normal human plasma capable of dissolving fibrin had been previously reported by Schanberge et al (1972), and Ikemori et al (1975).

Little information is available on the nature, function or normal levels of this enzyme and the work described here is:

(a) an assessment of an assay system developed for the enzyme (Pejhan and Kemp, 1985) and

(b) a report of the range of activities found in normal individuals and the significant variations associated with some conditions.

CHAPTER 1.

1. Introduction

CHAPTER 2.

2 General Methodology

ABBREVIATIONS

α_2^M	$alpha_2$-macroglobulin
EDTA	ethylenediamine tetra acetic acid
EACA	epsilon aminocaproic acid
MCA	monochloro acetic acid
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate

INTRODUCTION

1.1 PROTEASES

In view of the many important processes that are catalysed and regulated by specific proteases, the study of extracellular proteolytic reactions is of increasing and general interest. The characterisation of these reactions present difficulties which vary, depending on the reaction conditions and the degree of purity of the system under examination.

The invivo reaction is generally unsuitable for detailed enzyme kinetic investigation as the natural substrate often presents multiple sites of enzyme cleavage and the peptide products produced can change constantly, creating a mixture of secondary substrates and inhibitors (Knight 1977). Impure and complex mixtures such as native plasma or serum contain many proteins, zymogens and inhibitors capable of interacting either to amplify or to terminate a particular reaction of interest. Dilution of plasma or serum reduces the concentration of all protein species and may thereby initate a variety of artefactual reactions owing to the dissociation of normally inactive protein complexes or to the reduction of inhibition concentrations or both. Reactions limited to highly purified species may be associated with extensive autolysis of protease, promiscuous rather than limited proteolysis of substrates and a variety of other non-physiological hydrolytic events.

Proteases were originally divided into exopeptidases and endopeptidases, the distal ends of the peptide chains being cleaved by the former and the latter cleaving those bonds distant from the ends.

Barrett (1977) described proteolytic activity as the polypeptide initially being hydrolysed by endopeptidases then the removal of terminal amino acid residues by exopeptidases.

The endopeptidases are classified according to their essential catalytic groups and

are usually designated into one of the following classes serine, cysteine, aspartyl or metallo. Barrett and McDonald (1980) added a fifth class containing those proteases which do not fit into the previous four.

Aspartyl (also known as carboxyl or acid) proteases generally have their optimal pH below 6.0. Tang (1979) suggested that their zymogens can be divided into two groups, those that are irreversibly converted to active enzymes at acid pH by release of their NH_2 terminal amino acids and those that are activated by other proteases.

1.2 An overview of haemotasis

Before the beginning of this century very little was understood of the mechanisms of haemostasis. In 1905, Morowitz proposed a scheme for the mechanism of blood coagulation that remained essentially unchanged for thirty years. This basic concept was:

Thrombokinase + Calcium + Prothrombin = Thrombin

Fibrinogen + Thrombin = Fibrin

The 1930's renewed interest resulted in the discovery of several new clotting proteins, recognition of the importance of vitamin K, the discovery of the coumarin anticoagulants and of the heparin cofactor antithrombin III. In the 1950's haemophilia and Christmas disease were differentiated. Since the appearance in 1964 of the two papers (Macfarlane (1964), Davie & Ratnoff (1964)) linking blood coagulation to a linear sequence of proteolytic reactions (the "cascade or waterfall") much emphasis has been placed on the ordering of these reactions and on the chemical mechanisms of zymogen activation.

The serine proteases involved in coagulation differ in several essential respects, from the digestive proteases e.g. most require calcium ions for optimal function and in several reactions membrane surfaces and protein cofactors are also needed.

Coagulation may be activated through the tissue factor (extrinsic) pathway or via surface (intrinsic) activation of Factor XII. Clotting may also be initiated by

platelet activation of Factor XI (in the presence of collagen) or Factor XII (in the presence of Adenosine diphosphate) Walsh (1974). In the intrinsic pathway clotting is initiated by contact with a negatively charged surface. This causes a conformational change in the Factor XII molecule exposing the active enzyme site and thus activating Factor XI. The activation of these contact factors is enhanced by high molecular weight kininogen and prekallikrein. By the various reaction sequences and in the presence of calcium ions and platelet factor 3 the protease thrombin is formed, it cleaves the A_α and B_β chains of fibrinogen releasing fibrionopeptides A and B respectively. At the same time it activates Factor XIII (fibrin stabilising factor) which, is a calcium-dependent sulphydral type transglutaminase (Folk& Chung, 1973). The active enzyme stitches together neighbouring fibrin molecules by the formation of peptide bonds to form the ε - (γ -glutamyl) lysine cross-links of the fibrin polymer rendering it insoluble and resistant to proteolytic attack. There is evidence that this process of fibrin stabilisation may play a significant role in wound healing (Duckert et al, 1960).

1.3 FIBRINOLYSIS

Mammalian blood contains an enzyme system capable of dissolving blood clots, the fibrinolytic system. This plays a role not only in the removal of fibrin from the vascular bed (Astrup (1956), Sherry et al (1959), Fearnley (1973), Astrup (1978), Collen (1980), Marsh (1981)) but also in several other biological phenomena such as tissue repair (Astrup, 1978), malignant transformation (Reich, 1975), macrophage function (Reich, 1975), ovulation (Strickland, 1978), and embryo implantation (Strickland, 1978). Many proteolytic enzymes are able to digest fibrin, in particular serine proteases active around a neutral or slightly alkaline pH.

The term fibrinolysis relates to a more specific process in which liquifaction of the fibrin clot is caused by the splitting of only a few peptide bonds. It is the limited extent of this process that allows the organism to dissolve clotted fibrin without the risk of widespread proteolysis that distinguishes fibrinolysis.

Many maintain that the fibrinolytic system acts as the major defence against fibrin

deposition on the vessel walls and thus, arguably, is man's principle line of defence against thrombosis. Astrup (1956) proposed that coagulation and fibrinolysis normally exist in human plasma in a mutually compensating and balanced state. However, although there is no firm evidence that these compensating reactions occur systemically at a measurable level in normal plasma, most reviewers have maintained the view of the dynamic equilibrium between clotting and lysis. There is evidence that the balance between these two processes may be distinctly different in separate areas of a single clot, (Francis et al., 1984).

1.4 PLASMINOGEN ACTIVATORS

Much attention has been paid to plasminogen activators because of their important role in the fibrinolytic system. These enzymes convert the proenzyme plasminogen into the fibrinolytic enzyme plasmin. The activation of plasminogen is dependent on the cleavage of two specific bonds; an arginyl-valine (Arg 560 - Val 561) bond in the central position of the molecule resulting in a two-chain plasmin molecule connected by a single disulphide bond and a bond in the aminoterminal position of the molecule resulting in the release of a peptide with a molecular weight of approximately 5000 daltons.

The principle endogenous activator of plasminogen in blood is presumed to be present in the endothelium of blood vessels and is released "on demand" by, for example exercise, hypercoagulability and drugs. Venous endothelium contains more activator than aterial endothelium, (Todd 1959). At least two forms of this type of activator, extrinsic and intrinsic, are present and the former is enhanced by exercise while the latter is not, (Marsh & Gaffney, 1980). Intrinsic plasminogen activator is associated with Factor XII, (Hageman factor), prekallikrein (Fletcher factor) and high molecular weight kininogen activation.

An activator of plasminogen is present in all human organs except the liver (Astrup and Permin, 1947 ; Albrechtsen, 1957), although there is considerable variation in concentration between organs. High concentrations are found in the uterus,

adrenals and thyroid and low concentrations in the spleen and testis.

By "the fibrinolytic system" is usually meant that system in blood which involves the conversion of plasminogen to plasmin, but fibrinolysis can also be mediated through enzymes other than plasmin, (Plow and Edgington, 1975). It has also been suggested that plasmin may play only a minor role in the normal physiological expression of fibrinolysis (Moroz & Gilmore, 1976).

1.5 Fibrinolysis by enzymes other than plasmin

Perhaps the most important progress in our knowledge of the role of non-plasmin proteases in the pathophysiology of thrombosis should be ascribed to the neutral proteases of certain blood cells. Riddler and Burnhart (1964) demonstrated the participation of polymorphonuclear neutrophils in the process of fibrin dissolution.

Plow and Edgington (1975) demonstrated fibrinogen degradation by granulocyte neutral serine proteases. An elastase-like protease E.C. 3.4.21.11 was described by Janoff and Scherer (1968) and a chymotrypsin-like protease or cathepsin-G by Schmitt and Havemann (1977). The cleavage fragments are clearly distinguishable from those of the classical plasmin system with more potent anticoagulant properties. The release of these proteases has been observed in inflammation (Ohlsson, 1977 ; Saklatvala, 1980) and septicaemia (Egbring and Havemann, 1978), which resulted in a direct proteolytic degradation of several coagulation factors. This together with the possible anticoagulant properties of fibrinogen-split products may also be responsible for the marked haemorrhagic diathesis seen in septicaemia. The release of leucocyte neutral protease is markedly increased in some leukaemias and the subsequent proteolysis of platelet membrane structure may enhance the bleeding potential already occurring through depletion of platelet numbers.

Gottlob et al (1978) concluded that leucocyte proteases differed from plasmin in that less inhibition occurs in serum and they are less active towards the substrate casein. These enzymes are not inhibited by ethylene diamine tetra acetic acid, epsilon amino caproic acid or trasylol, and only weakly inhibited by α_2 macroglobulin.

Pucnwongsa and Chulavatnatol (1976) characterised an acid protease which had been

first described in human seminal plasma as pepsin and pepsinogen by Ludquist (1952). They found an acid dependency of activation of the proenzyme and postulated the enzymes proteolytic activity was a pre requis ite to sperm penetration through the cervical mucous. Recently at least five proteases have been identified in spermatozoa and three from seminal plasma (Barrett), several of these being active at low pH. It is known that platelets in their role of maintaining vascular integrity through plug formation can also activate as well as inhibit fibrinolysis. Booth et al. (1985) demonstrated an inhibitor released during aggregation inhibiting both tissue type plasminogen activator and urokinase but with no effect on plasmin.

Watada et al (1977) demonstrated a fibrinolytic enzyme in gel-filtered platlets that acted independently of the plasminogen/plasmin system. He concluded that the absence of this enzyme activity in platelet rich plasma was due to the presence of inhibitors.

Abery et al (1975) also concluded that a plasminogen/plasmin independent system was responsible for the high level of fibrinogen degradation products and unclottable fibrinogen in wound exudates. Gaffney et al (1976) showed not only β, γ-γ and α polymer present in the fibrin subunits of both venous and arterial thrombii but the presence of an unexpected subunit of molecular weight 33,000 which they identified as a degraded fragment of either β or γ chain produced, they postulated by some other proteolytic enzyme.

As described earlier, various plasminogen/plasmin independent enzyme systems have been shown to exist in the cellular and plasma compartments of the body most active at a neutral pH. There is sure evidence for the existance of aspartyl proteases in plasma and these have been shown to have a lytic action on fibrin (-ogen).

Shanberge et al (1972) investigated the variation in fibrin clot stability in acid media between individuals and found that fibrin clots from females, especially those on oral contraceptives, were more resistant to lysis in monchloro acetic acid. They demonstrated that this increased stability was not due to an increase in Factor XIII or fibrinogen. During the following year, Shanberge et al (1973) demonstrated an enzyme (molecular weight 44,000 optimal pH 2.0) which was found in lower concentrations in females especially those patients on oral contraceptive medication.

Ikemori et al (1975) when also examining clot stability in acid solution were able

to conclude that solubility in monochloracetic acid was not due to the concentration of Factor XIII but also that an enzyme was present which could be removed from the newly formed clot by washing free of serum before the addition of MCA when then became resistant to lysis. They postulated that this enzyme present in both plasma and serum was in fact pepsinogen since

(a) it was activated at pH 2.0

(b) the activity was destroyed by heating for ten minutes at 60 °C

(c) pepsinogen when added to monochloroacetic acid will dissolve well cross-linked fibrin clots

(d) in a purified form gave a reaction line (with slight cross over) in an immunodiffusion experiment with pepsinogen.

They also added that the very stable clots formed from a patient with pernicious anaemia was in fact due to the low serum pepsinogen level and not to an increase in clot stability as the clots would dissolve in acid media once washed and exposed to normal plasma.

1.6 Plasma acid protease

Ragaz et al (1976) reported an unusual bleeding disorder which at first was thought to be a Factor XIII deficiency. The clinical history revealed easy bruising since childhood, post operative haemorrhage with delayed wound healing and abnormal scar formation. The laboratory findings were normal except the Factor XIII screening test using 1% (w/v) monochloroacetic acid produced complete clot lysis within three hours but clots rdmained insoluble in 5 M urea.

When added to normal well cross-linked fibrin clots the plasma markedly enhanced their solubility and the lytic activity on denatured haemoglobulin was shown to be twice that of normal plasma.

Ragaz concluded that this patient (although taking oral contraceptives) had an increased pepsin-like protease activity.

Laing (1975) partially purified the plasma enzyme from a sample of normal plasma and that from the patient described by Ragaz et al (1976) and concluded, from the column chromatography elution pattern, that three acid proteases were present in the patients plasma but was uncertain if in fact they were different enzymes or forms of the same enzyme.

Taylor (1976) using dyed fibrin assay found the plasma enzyme to have ten times greater activity than pepsin although pepsin showed greater activity on denatured haemoglobin and concluded that the enzyme was not pepsin.

Law and Kemp (1977) reported that their semi-purified enzyme revealed two bands, a major band molecular weight 51,000 and a minor band of 86,000 on SDS gel electrophoresis. Immuno-electrophoresis showed contamination with albumin.

Law (1978) was able to further purify the enzyme and found the activity peak coincided with a molecular weight of 64,000, but was still in association with albumin which she concluded was in a partially degraded form and complexed to the enzyme. Law found the optimum pH to be 2.4 and its activity was inhibited by pepstatin, on this basis she postulated it to be an aspartyl protease.

Pejhan and Kemp (1984) compared assay systems using various substrates, namely denatured haemoglobin, acetyl-phenylalanyl-diiodo tyrosine as both of these are commonly used for pepsin assay (Ryle 1970), since the previous work of Ikemori et al (1975) and Ragaz and Kemp (1976) had suggested that the enzyme was a pepsin-like protease. Azocasein was chosen because of its known sequence homology between it and fibrinogen (casein is widely used in fibrinolytic enzyme assays e.g. that of plasmin) finally an assay system based on fibrin clot dissolution was developed. These results showed that although time consuming and cumbersome this was the most effective system tried to date and also indicated that the enzyme had some specificity for fibrin and was distinct from the pepsin/pepsinogen system as previously suspected (Ikemori et al 1975, Ragaz et al 1976).

Pejhan (1984) following further purification of the enzyme was able to pursue group specific inactivation studies and found inactivation with pepstatin and p-bromo-phenacyl-bromide. The enzyme was also shown to be heat labile but stable when stored

Pejham (1984) was also able to confirm Law's (1979) observation that the enzyme differed from pepsin in its course of isolation and was found in a different fraction to the one which Ikemori et al (1975) followed and had concluded was pepsin. Further dissimilarities became obvious to Pejham (1984) when the digestion products of fibrin were compared, when enzyme mobilities differed on both gradient and haemoglobin gels and when amberlite column elution occurred at different pH's.

1.7 AIM

The object of this project was to extend the work of Ragaz et al (1976) in order to estimate plasma acid protease levels in the normal population and to investigate selected sub populations and to pursue the investigation of the enzyme concentration in various body fluids and tissues with some limited characterisation.

CHAPTER 2

GENERAL METHODOLOGY

2.1 Sodium dodecyl sulphate (SDS) disc gel electrophoresis

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

Reagents

2.1.1 Chamber buffer

0.1 M Tris - H Cl, pH 7.4 containing 2.0% (w/v) SDS

2.1.2 Gel buffer

0.1 M Tris - H Cl, pH 7.4 containing 2.0% (w/v) SDS

2.1.3 Acrylamide: N', N' methylene bis acrylamide stock solution

5% stock solution

Acrylamide	9.5g
N' N' methylene bis acrylamide	0.5g

Volume made up to 200 ml with gel buffer

Stock solutions were kept at $4^{o}C$

2.1.4 Ammonium persulphate

1.2% (w/v) in distilled water, made up fresh for use

2.1.5 N, N, N', N' Tetra methyl ethylene diamine (TEMED)

0.25% (w/v) in distilled water, made up fresh for use

2.1.6 Staining solution

Coomassie brilliant blue R. 250.	3.6g*
Distilled water	454 ml
Methanol	454 ml
Glacial acetic acid	72 ml

The solution was filtered before use

* For reagent of 70% purity

2.1.7 Destaining solution

Distilled water	675 ml
Methanol	250 ml
Glacial acetic acid	75 ml

2.1.8 Tracking dye

0.05 g bromophenol blue in 100 ml distilled water

2.1.9 Standards used for molecular weight determination

Cytochrome C	(Sigma)
Myoglobin	(Sigma)
Carbonic anhydrase	(Sigma)
Ovalbumin	(Sigma)
Bovine serum albumin	(Sigma)
Phosphorylase b	(Sigma)

Standard stock solutions

1.5 mg protein was dissolved in 1.0 ml of chamber buffer in thin walled glass tubes and placed in a boiling water bath for two minutes, cooled and stored at -20°C until required.

Standard working solutions

25 µl of each standard stock solution was mixed with 0.25 ml of a solution of 8M urea/3% (w/v) SDS/3% (v/v) β mercaptoethanol in thin walled glass tubes and placed in a boiling water bath for two minutes. They were allowed to cool and stored in small, covered containers, (less air space for re-oxidation), at -20°C, until required.

25 µl of each standard was applied per gel.

N.B. The β mercaptoethanol was added to the solution fresh each time.

2.1.10 Gel preparation

19 ml of acrylamide/bis acrylamide stock solution was mixed with 1.0 ml of 1.2% (w/v) ammonium persulphate. 25 µl TEMED were added. The gel was immediately transferred by pasteur pipette into 5 mm x 80 mm glass tubes. The gels were layered with distilled water to prevent formation of a meniscus.

Once the gels were polymerised, the water was replaced by gel buffer, and the gels covered and stored at $4^{o}C$.

2.1.11 Sample preparation

The samples were applied to the gels in either a reduced or non-reduced form.

Preparation of a reduced sample

To reduce the sample, an equal volume of 3%(w/v) SDS/3% (v/v) β mercaptoethanol/8M urea was added and then placed in a boiling water bath for five minutes.

Preparation of a non-reducing sample

To the sample, an equal volume of 3% (w/v) SDS/8M urea was added, mixed and incubated at room temperature for five minutes.

2.1.12 Gel electrophoresis

Samples and standards were mixed with a drop containing equal volumes of glycerol and bromophenol blue (tracking dye), 5-25 µl was then applied to the gels. The gels were electrophoresed at a constant current of 5mA/gel from cathode to anode. The electrophoresis was continued until the tracking dye reached approximately 5 mm from the bottom of the gel, approximately $1\frac{1}{2}$-2 hours. The gels were removed from the tubes, the length of the gel and distance travelled by the tracking dye measured. Gels were stained for

60 minutes and then destained for 48 hours with 2-3 changes of destaining solution. The gels were scanned in a Beckman CDS 200 densitometer.

2.1.13 Molecular weight determination

$$\text{Mobility} = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye}} \times \frac{\text{length of gel before staining}}{\text{length of gel after staining}}$$

The mobilities of the standards were plotted against the log of molecular weight and the molecular weight of the unknown sample read from the graph.

2.2 Collection of venous blood

Blood was collected from an antecubital vein using a 21G $1\frac{1}{2}$ needle into a siliconised glass tube containing 32 gl^{-1}(w/v) tri sodium citrate, in the proportion of 9 volumes of blood to 1 volume citrate and mixed gently by inversion. The venepuncture was repeated if not a "clean" one, all blood was withdrawn with a minimum of venous stasis. The specimen was centifuged without delay, at 1200-1500 g for 15 minutes at $4^{o}C$. The plasma was removed and either stored at $-20^{o}C$ for future testing or at $4^{o}C$ if to be tested within one hour.

2.3 Fibrinogen estimations

The method used was that of Clauss (1957) as modified by Blut (1971). Citrated plasma collected as described in 2.2 was diluted 1:9 with Owrens barbitone buffer pH 7.35. 0.2 ml of this mixture was incubated for one minute at $37^{o}C$. 0.2 ml of a solution of thrombin 10 NIH units per ml was added, a stop watch was started, then stopped when a clot appeared. A standard curve was prepared using dilutions of a known fibrinogen concentration supplied by Boehringer Mannheim GmbH. The standard curve is shown in figure 1a. Control plasmas of known

fibrinogen concentrations were also obtained from the above company. The Owren's buffer contained 16 mgL^{-1} polybrene as a heparin inhibitor to ensure heparin upto a concentration of 2 units ml^{-1} would not interfere with the determination.

2.4 Fibrin estimation

The method was based on that of Ratnoff and Menzie (1951) modified by Dacie and Lewis (1975 a).

2.4.1. Estimation of residual fibrin in clots

Clots were dissolved in 1.0 ml of 2.5M NaOH by heating at 100°C (water bath) for 15 minutes. The solution was allowed to cool and the volume made up to 7.5 ml with distilled water, 3.0 ml 20% Na_2CO_3 added and the solution was mixed. 1.5 ml Folin-Cio calteau phenol reagent was added, mixed and incubated at room temperature for 20 minutes for the colour to develop. The absorbance was read at 650 nm.

A blank was prepared as above by omitting the fibrin clot.

A standard was prepared using 0.2 ml of 0.2 mg tyrosine ml^{-1} in 0.1 M HCl in place of the fibrin clot.

2.4.2 Calculation of the results

$$\frac{t}{s} \times v.s \times c.s \times \frac{1}{vt} \times 11.7 = \text{mg ml}^{-1} \text{ concentration of fibrinogen}$$

where t = absorbance of the test solution - blank

s = absorbance of the standard solution - blank

v.s = volume of standard in ml

c.s = concentration of the standard in mg ml^{-1}

vt = original volume used to make the fibrin clot

11.7 = conversion factor for liberated tyrosine to fibrinogen concentration

The standard curve was constructed using different volumes of

the 25% ammonium sulphate precipitate from the gel fractionation of plasminogen free bovine plasma substrate for the fibrin clot formation. The standard curve is shown in figure 1b. Standard fibrinogen solution was obtained from Immuno Limited*.

2.5 Fibrinogen/Fibrin degradation products assay (FDP)

The assay procedure was that used by Wellcome in their FDP assay kit. This uses antibodies raised to highly purified preparations of human fibrinogen fragments X, Y, D and E. Antibodies to other serum proteins have been removed by solid phase absorption and the specific antibodies to fragments D and E used to coat, by absorption, a suspension of latex particles in glycine saline buffer (pH 8.2).
2.0 ml of venous blood was collected as 2.2 into a glass tube containing 20 NIH units thrombin (to promote rapid and complete clotting) and 3600 N.F units soya bean trypsin inhibitor (to prevent the further invitro breakdown of fibrin). The tube was incubated at $37^{o}C$ for one hour. The specimen was then centrifuged at 2500 g for ten minutes, and a 1:5 and 1:20 dilution, with glycine saline buffer pH 8.2, of the serum made. A drop of each dilution was added to one drop of latex suspension on a glass plate (position 1 and 2 respectively) which was gently rotated for two minutes. A positive result in position 1 indicated that FDP were present in the original serum at a concentration in excess of 10 $\mu g ml^{-1}$ while agglutination in position 2 indicated the original concentration to have been greater the 40 $\mu g ml^{-1}$. Further dilutions were performed to give an accurate value to the nearest 10 μg concentration. A resting normal value was taken as being less than 10 μg FDP per ml serum.

*Immuno Limited, Sevenoaks, Kent

Fig. 1a.- STANDARD CURVE FOR THE DETERMINATION OF PLASMA FIBRINOGEN

A graph of plasma fibrinogen concentration versus the clotting time

Fig. 1a.- STANDARD CURVE FOR THE DETERMINATION OF PLASMA FIBRINOGEN

A graph of plasma fibrinogen concentration versus the clotting time

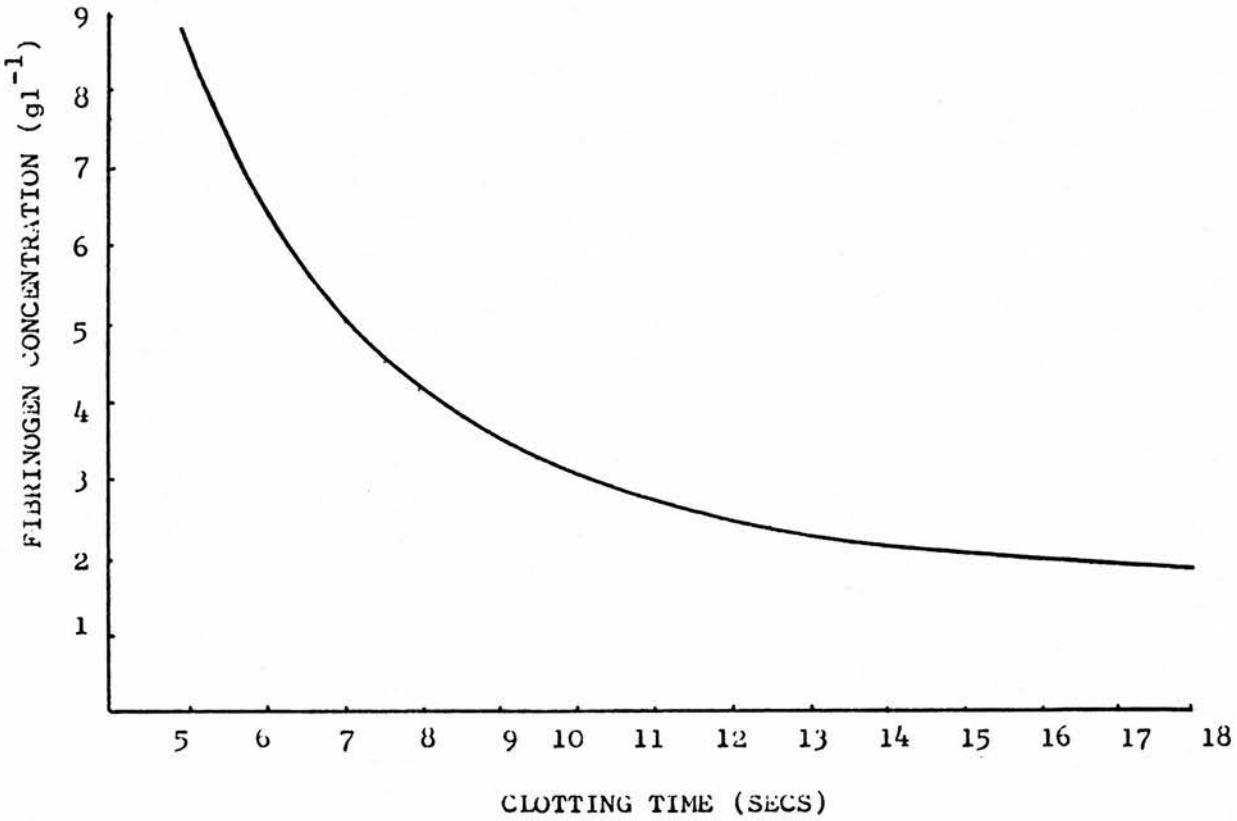
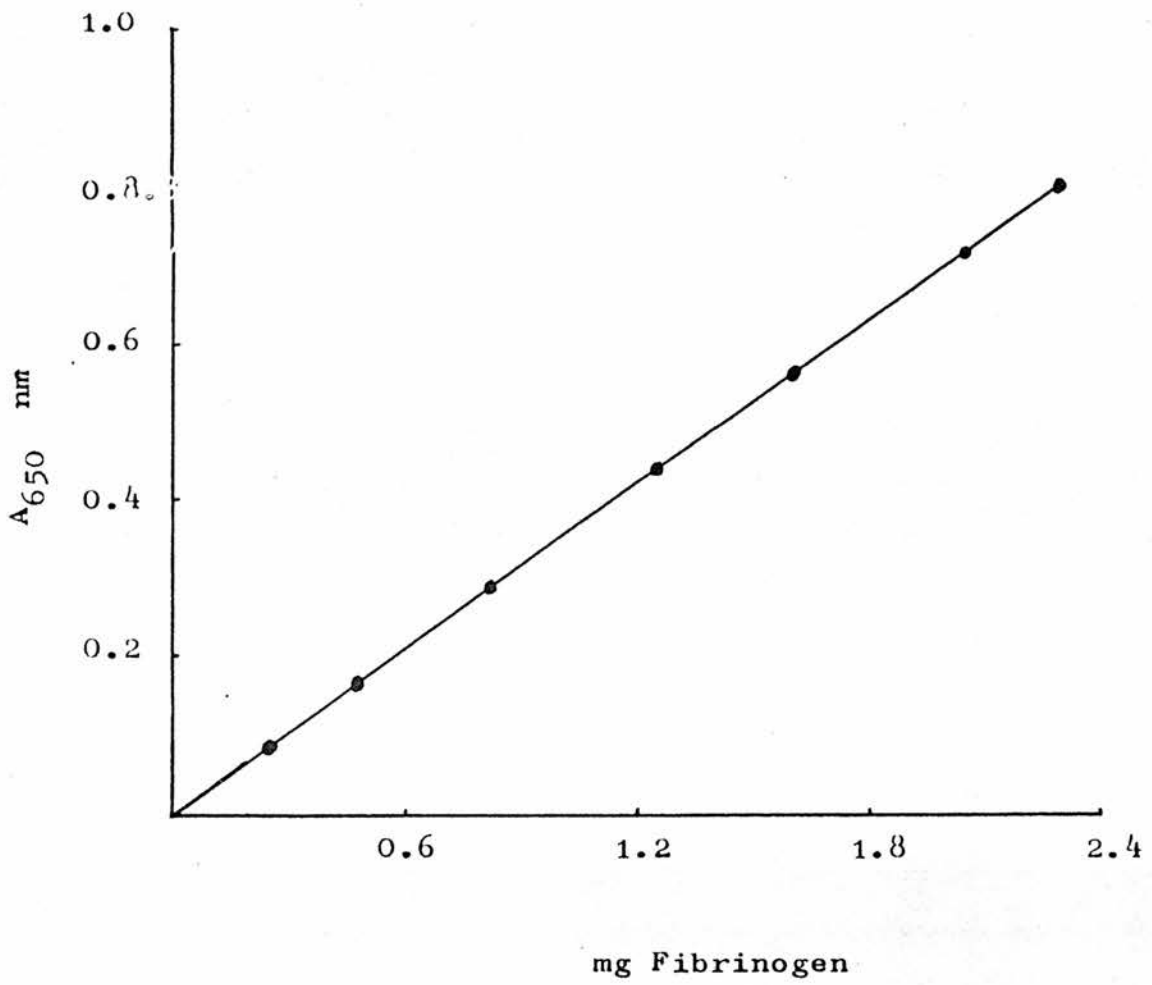


Fig. 1b. STANDARD CURVE FOR THE CLOT FIBRINOGEN CONTENT

A graph of variation of absorbance at 650 nm. versus the concentartion of fibrinogen used to make fibrin clot


1.0
0.8
0.6
0.4
0.2
A_{650} nm
0.6
1.2
1.8
2.4
mg Fibrinogen

2.6 Plasminogen assay

2.6.1 Plasminogen assay (Human)

Plasminogen was measured in a two step amidolytic assay employing a synthetic chromogenic substrate, according to the method of Soria (1976). In the first step, plasminogen is converted to an active form by streptokinase, which if present in excess forms on active plasminogen - streptokinase complex, which reacts similarly to plasmin with small substrates (Reddy, 1972). This activity was now measured spectrophotometrically by its action on the chromogenic substrate C.B.S 30.41 resulting in the release of p-nitro aniline, which is detected by its absorbance at 405 nm. A 1:20 dilution of plasma (test or control) was made with buffer pH 7.0 and incubated at 37°C for four minutes. 200 µl of 5,000 iu ml^{-1} streptokinase was added. The solution was mixed and incubated for exactly three minutes. 200 ul of 5 uM ml^{-1} chromogenic substrate was added and incubated for exactly sixty seconds. 200 µl glacial acetic acid was then added to stop the reaction, the solution was mixed well and the absorbance read at 405 nm. The plasminogen level was then extrapolated from a standard curve prepared from dilutions of a known 100% standard.

2.6.2 Plasminogen assay (Bovine)

Summaria et al (1974) found that bovine plasminogen (o.1 mg) could not be activated to plasmin even with as much as 100,000 units of streptokinase. However, the addition of a small amount of human plasminogen triggered the activation to plasmin.

To estimate bovine plasminogen, a known value of human

plasminogen was added, the mixture assayed as 2.6 and the human value substracted from the result.

2.7 Estimation of plasminogen activator

The method used was that of Astrup and Mullertz (1952). 0.2 ml 20 Iu ml^{-1} thrombin was pipetted into a plastic petri dish, containing 6 ml of fibrinogen. (0.1% (w/v) veronal/HCl buffer pH 7.8 ionic strength 0.15), swirled gently to mix thoroughly, keeping the dish flat on the bench to avoid bubble formation. It was allowed to stand on a level surface for 30 minutes.

A blood sample was collected with a minimum of venous stasis using a 21G needle into a plastic syringe (preferably when the patient had been resting and fasting) then transferred to a pre-cooled plastic tube containing 3.8% (w/v) tri soium citrate (1 volume of citrate to 9 volumes of blood) centrifuged at 2000 g for twenty minutes at $4^{o}C$. The plasma was transferred to a plastic tube, using a siliconised pipette, and stored at $-20^{o}C$ prior to testing. The euglobulin precipitate was prepared in a centifuge tube by adding 9 ml 0.025% (v/v) acetic acid to 1 ml plasma (pH 5.9 - 6.0), mixing and allowing to stand for ten minutes at $4^{o}C$, to precipitate the euglobulins. The tube was centrifuged at 2000 g for five minutes at $4^{o}C$, the supernatant was discarded and the tubes drained by inversion on absorbant paper.

A standard preparation, (a euglobulin precipitate from a pool of 10 normal plasmas), and the test plasma euglobulin precipitate were tested in parallel. Three drops, each 3 ul, of each euglobulin fraction were pipetted onto the surface of the plate, and incubated at $37^{o}C$ for eighteen hours in a vibration free incubator.

Two perpendicular diameters, of each of the 3 lysis zones, were measured

and the fibrinolytic activity was expressed as the mean of the six diameters, and then as a percentage of the pooled normal plasmas. The normal range was taken as being 10 - 15 mm.

Comment - The area of lysis gave a measure of the plasminogen activator activity plus plasmin and other protease activity. Plasmin/protease activity can be measured by parallel testing of plasminogen-free fibrin plates. (This method is the same as described above, but use glass petri dishes and heat to 80°C for forty five minutes to inactivate the plasminogen, remove the cover and allow to cool to room temperature.)

2.8 Prothrombin time of plasma estimation

The single most important test to detect deficiences or abnormalities of the extrinsic coagulation mechanism is the one stage prothrombin time (PT), first described by Quick, (1935). This test is sensitive to deficiencies of factors V, VII and X (levels below 0.2 - 0.25 units ml^{-1}) and to a lesser extent factor II (levels below 0.10 units ml^{-1}), or gross changes in fibrinogen.

The method was that known as Quick's one-stage technique. Dacie and Lewis (1975b). A phenol-saline suspension of human brain known as the British Comparative Thromboplastin *1 was used as the thromboplastin source. 0.1 ml plasma (collected as described in 2.2) was placed in a 75 mm x 10 mm glass tube at 37°C and 0.1 ml brain suspension added to it. After a delay of about sixty seconds 0.1 ml of pre-warmed 0.025 $moll^{-1}$ $CaCl_2$ were added and the contents mixed. A stop watch was started and the tube tilted continually, but gently, from the vertical to just short of the horizontal, so that its contents could be observed for the first signs of clotting at which time the watch was stopped. All tests were performed in duplicate. The normal range established in the United

Kingdom is 10 - 14 seconds. Normal and abnormal control plasmas with reference to the International Committee for Standards in Haematology were obtained from *1.

*1 Available from the National Reference Laboratory for anticagulant reagents and control, Withington Hospital, Manchester.

2.9 Thrombin clotting time of plasma estimation

This test provides information on the quality and quantity of clottable protein present. The test is affected by defects in the thrombin fibrinogen reaction other than fibrinogen deficiency e.g. the presence of heparin or FDP.

The method is that described by Dacie and Lewis (1975c). 0.2 ml plasma (collected as described in 2.2) was placed in a 75 mm x 10 mm glass tube at 37°C. After a delay of about sixty seconds, 0.1 ml 20 units ml^{-1} thrombin was added and the clotting time measured (as described in 2.8) and the nature of the clot observed. All tests were performed in duplicate. The normal range established in the United Kingdom is 14 - 18 seconds. Normal and abnormal controls were included with the tests and were obtained from Immuno Limited.*

2.10 Activated partical thromboplastin time of plasma estimation

This is the most important test used to screen for intrinsic coagulation defects. It is sensitive to the overall effect of the intrinsic coagulation factors i.e. factors I, II, V, X, VIII, IX, XI, XII, pre kallikrein and high molecular weight kininogen. The test is most sensitive to an abnormality occuring in the early stages of coagulation i.e. factors leading up to the generation of activated F X, and less sensitive to the latter stages i.e. prothrombin and fibrinogen.

The method used was a modification of MacPherson et al (1961) and Proctor et al (1961) as described by Dacie and Lewis (1975d) where the time consuming reactions associated with the contact phase of coagulation

are completed before the addition of calcium. Phospholipid is supplied to replace platelet factor 3 activity.

0.1 ml phospholipid solution (obtained from**) was added to 0.1 ml plasma (collected as described in 2.2) in a 75 mm x 10 mm glass tube followed by 0.1 ml kaolin (5 gl^{-1} kaolin BP in barbitone buffer pH 7.4) and left at 37°C for ten minutes. 0.1 ml 0.025 M $CaCl_2$ was added and the clotting time recorded (as described in 2.8). All tests were performed in duplicate. The normal range established in the United Kingdom is approximately 38 - 46 seconds. Normal and abnormal reference plasmas were obtained from *.

** Immuno Limited, Sevenoaks, Kent.

* National Reference Laboratory for Anticoagulant Reagents and Control, Withington Hospital, Manchester.

2.11 Factor XIII screening test

The method used was that described by Dacie and Lewis (1975). Both 5 M urea and 1% monochloracetic acid were employed as solvents in parallel tests.

0.2 ml test plasma (collected as described in 2.2) and control samples were placed in 75 mm x 10 mm glass tubes, at 37°C, and 0.2 ml 0.025 M $CaCl_2$ added. The tubes were shaken and left for thirty minutes for a clot to form. 3.0 ml 5 M urea (or 3.0 ml 1% monochloracetic acid) were now added, shaken and left for 16 hours at 37°C. The tubes were then inspected for clots. If F XIII was present in normal amounts, the clot in the test sample will still be present. A positive control i.e. a urea-soluble clot was made by adding 0.2 ml thrombin 20 NIH units ml^{-1} to 0.2 ml EDTA plasma (blood collected in the same way as above but substituting 1.5 mg di potassium ethylene diaminetetraacetic acid per ml of blood as the anticoagulant). Such a clot was soluble because of the lack of free Ca^{2+} ions needed for the action of F XIII.

2.12 F XIII assay

Both active factor XIII (subunit a) and factor XIII carrier protein (subunit s) were assayed by an immunoelectrophoresis technique.

Anti-sera to both subunits were obtained from Behring Ltd.* The technique used for both assays varied only in the type of anti-serum used.

0.1 g of agarose was dissolved in 10 ml Barbitone buffer, pH 8.6, 0.05 M by heating in a boiling water bath. The agarose was cooled to 50 °C in a water bath, 50 µl of anti-serum previously heated to this temperature was added.

The tube was carefully mixed and the agarose poured onto a glass plate, 80 mm x 80 mm, giving a gel thickness of 2.0 mm. Two centimetres from one end of the agarose plate equally spaced wells 3 mm in diameter and 10 mm apart were prepared. Each well was filled with 10 µl plasma dilution. Four standard dilutions of normal plasma in saline, 50%, 25%, 12.5%, 6.25% were added to the first 4 wells and the test plasma dilutions to the next 4 wells.

Electrophoresis was performed overnight (18 hours) at a constant voltage of 110 v, using Barbitone buffer pH 8.6, 0.05 M, with constant cooling. The plate was removed from the electrophoresis tank and excess moisture removed by placing beneath adsorbent paper and a 2 kg weight for 20 minutes. It was then dried in a current of warm air. The plate was immersed in Coomassie blue solution. (0.2 g Coomassie blue in 40 ml absolute methanol, 60 ml distilled water and 10 ml glacial acetic acid) and then washed in a solvent mixture of 40 ml absolute methanol, 60 ml distilled water and 10 ml glacial acetic acid until the back ground was clear. The

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* Behring Ltd, Salisbury Road, Hornslow, Middlesex.

plate was then dried in warm air.

The rocket heights were measured to the nearest 0.25 mm and a graph constructed by plotting the heights of the normal plasma dilutions against calculated subunit concentration.

The normal range for the a subunit was 55 - 144% and for the s subunit, 50 - 150%.

2.13 Screening test for functional abnormalities of fibrinogen

Fibrin monomer polymerisation FMP was assessed by the Francis and Armstrong (1982) modification of the technique used by Green et al (1976). 0.25 ml plasma (prepared as described in 2.2) was diluted with 1.0 ml 0.15 M NaCl, and 0.05 ml Reptilase added. The change in optical density at 350 nm was monitored for ten minutes. The test was then repeated by diluting a further 0.25 ml volume of plasma with 1.0 ml of a mixture containing equal volumes 0.15 M NaCl and 0.025 M $CaCl_2$. Reptilase (0.05 ml(was then added and the change in optical density recorded as detailed above. The optical densities at ten minutes were noted and the results, expressed as the ratio A^{350} (calcium)/A^{350} (saline). It had previously been

found (Francis and Armstrong, 1982) that the FMP ratio remained constant despite variations in fibrinogen concentrations between samples. Fibrinogen concentrations were, therefore, not adjusted before testing.

The normal range for fibrin monomer polymerisation ratios was taken as 2.4 ± 0.25.

2.14 Partial Purification of bovine fibrinogen

The method used was based on that of Lawrie et al (1979).

Bovine blood was collected at slaughter in a solution of 0.106 M Tri sodium citrate, 9:1 (v/v) blood to anticoagulant, with continual mixing. The blood was centrifuged at 1200 g - 1500 g at $4^{o}C$ for one hour. The clear plasma was applied to a lysine-sepharose column.

For preparation of plasminogen-free plasma

Approximately 3 litres of blood was processed at each purification to provide a large volume of substrate from a single source to achieve some standardisation within our assays.

2.14.1 Lysine Sepharose 4B

2.14.1.1 Coupling Lysine to sepharose

The method used was based on that of Chibber et al (1974).

40 g of sepharose was suspended in 100 ml of distilled water. 5% (w/v) cyanogen bromide was prepared in cold water ($4^{o}C$) in an efficient fume cupboard. The cyanogen bromide solution was then added to the sepharose and stirred gently. The reaction was allowed to proceed for ten minutes, while the temperature of the reaction was kept at 18 - $20^{o}C$ with the addition of ice and the pH of the reaction was kept constant at 11.0 by the addition of 5 M sodium

hydroxide. The reaction was stopped by washing the activated sepharose with 1 L of cold (4°C) 0.1 M sodium bicarbonate buffer pH 8.9 in a Buchner funnel. The activated sepharose was mixed with lysine (10 g in 25 ml distilled water pH 8.9) and stirred gently at 4°C overnight. 10 g glycine in 50 ml distilled water was added to the mixture and stirred gently at room temperature for two to three hours.

The lysine-sepharose was washed with 1 L of 1 M sodium chloride, 2.0 L of distilled water and then stored at 4°C in the presence of 1 drop of toluene.

2.14.1.2 Lysine sepharose column

A column of 18 cm x 1.8 cm was packed with lysine sepharose and equilibrated by pumping the neutral buffer, 0.05 M Tris HCl pH 7.5, through at a flow rate of approximately 30 ml/h. 200 ml plasma was applied to the column and then eluted with the Tris buffer, all fractions with an absorbance (280 nm) greater than 0.5 were pooled, labelled "plasminogen-free plasma" and mixed well.

The column was regenerated with 5 to 10 column volumes of

(i) Tris buffer made 0.5 M with NaCl.

(ii) 0.1 M ε-amino caproic acid.

(iii) 0.2 M ε-amino caproic acid in 0.05 M phosphate buffer containing 1.0 M NaCl.

2.14.2 Ammonium Sulphate fractionation

The plasminogen free plasma from step 2.14.1.2 was mixed well, then brought to 25% saturation by the addition of solid ammonium sulphate, with constant stirring at room temperature for thirty minutes. The suspension was then centrifuged at 2000 g for twenty minutes. The supernatent was discarded and the precipitate dissolved in one tenth the original plasma volume of 0.85% (w/v) NaCl and dialysed against 0.85% (w/v) NaCl for twenty-four hours at 4 °C with continual stirring and 3 changes of NaCl. Each batch prepared was tested for plasminogen by 2.6.2. The dialysed solution was frozen at -20 °C in suitable aliquots. Only plasminogen free substrate plasma was used for clot formation.

2.14.3 Fibrin clot formation

An aliquot from step 2.14.2 was thawed at 37 °C and mixed well. The dialysed fibrinogen was diluted with 0.85% (w/v) NaCl to give a final concentration of 5 - 6 mg ml^{-1} as measured by step 2.3. An aliquot was subjected to electrophoresis as step 2.15.5 to check the purity of the preparation. *

One volume of 0.05 M Tris/HCl, 0.02 M lysine, 0.1 M NaCl pH 9.0 was added to 9 volumes of fibrinogen solution.

0.6 ml of the sample was placed in a 75 mm x 12 mm glass tube in a 37 °C water bath. Clotting was initiated by the addition of 0.2 ml of pre-warmed (37 °C) 0.05 $CaCl_2$. The clot was allowed to form for one hour and then squashed using a glass rod. It was then incubated at 37 °C for four to five hours in its own serum. The clot was now washed 5 times with saline then left overnight in saline at 4 °C. To remove the salt the clot was washed and left in distilled water for at least three hours.

*The preparation was accepted as long as there was no evidence of degraded fibrinogen found by protein electrophoresis

A final check to ensure the clot was indeed plasminogen-free was undertaken by incubating a sample clot with 0.1 ml urokinase (200 units/ml in 9 g/l NaCl) at 37 °C. No lysis was observed after twelve hours incubation. A control clot produced by adding 0.2 ml 10 NIH units/ml thrombin to 0.2 ml citrated plasma showed complete lysis when exposed to urokinase after 15 minutes.

2.14.4 Assay for Acidic Fibrinolysis

The method used was based on that of Ikemori et al (1975). 2.6 ml of 1% (w/v) monochloro-acetic acid was added to the washed clot in a centrifuge tube , and has been described by Pejhan (1984). 0.2 ml test plasma was added and the mixture incubated at 37 °C for eighteen hours. A batch of 20 assays were run at the same time. The solution was centrifuged at 200 g for twenty minutes, the supernatent was removed and discarded. The remaining clot was washed with 0.85% (w/v) NaCl 3 times and the protein content of the remaining clot was assayed as 2.4. Enzyme activity was expressed as mg fibrin dissolved by 0.2 ml of plas ma in eighteen hours at 37 °C. (1 unit dissolved 1 mg fibrin in 18h at 37°C)

2.15 Clinical chemistry parameters

The following determinations were performed on the Hitachi 7.5 "Selective Chemical analyser".

All methods used were the "optimised standard methods" conforming to the recommendations of the "Deutsche Gesellschaft fur Klinishe Chemic".

2.15.1 Bilirubin

The method was the 2.5 dichlorophenyl diazonium method for the determination of total bilirubin of Wahlefield et al (1972) based on the test principle that total bilirubin was coupled with a diazonium compound to form the corresponding azobilirubin. Indirect bilirubin being liberated by a detergent.

2.15.2 Aspartate transaminase

The method used was that of Anon (1970) and was based on the following test principle.

α-oxoglutarate + L-aspartate $\overset{AST}{\rightleftharpoons}$ L-glutanate + oxaloacetato

oxaloacetate + NADH + H^+ $\overset{MDH}{\rightleftharpoons}$ L-Malate + NAD^+.

2.15.3 Alkaline Phosphatase

The method used was that of Harsanen et al (1967) and was based on the following test principle.

ρ nitrophenyl phosphate + H_2O $\overset{AP}{\rightarrow}$ phosphate + nitrophenol.

2.15.4 Glutamyl Transferaze (γ-GT)

The method used was that of Persijn and Van der Silk (1976) based on the following test principle.

L-γ- glutamyl-3-carboxy-4 nitroanilide + glycylglycine

$\overset{\gamma GT}{\rightarrow}$ L-γ glutamylglycylglycine + 5-amino-2-nitrobenzoate.

2.15.5 Serum protein electrophoresis

Electrophoresis was performed to separate the serum proteins into the five major fractions: albumin, $alpha_1$ globulins, $alpha_2$ globulins, beta globulins and gamma globulins. These fractions could then be further resolved into a total of ten or more bands if desired.

Routine electrophoresis was performed on the Beckman microzome equipment using a barbiton buffer pH 8.6. The bands were then quantitated by the Beckman CS 200 densitometer.

Individual quantition of the immunoglobulins IgA, IgM, IgG was estimated using the Beckman I.C.S. rate nephelometer.

2.16 Purification of plasma enzyme

2.16.1 Ammonium sulphate fractionation

The sample was brought to 25% and 75% saturation with solid ammonium sulphate. The suspension was stirred gently at 4 °C for one hour, it was then centrifuged at 10,000 g for one hour. The precipitate was dissolved in and dialysed against 0.9% (w/v) NaCl for four hours at 4 °C using four changes of 0.9 (w/v) NaCl. To prepare the 25-75% saturation another aliquot of the sample was first brought to 25% saturation with ammonium sulphate and then supernatant was brought to 75% saturation and treated as above.

2.16.2 Affinity Chromatography

Cibacon Blue-sepharose column

The method used was based on that of Virca et al (1978).

A 10 cm x 2 cm was packed with resin and equilibrated with 0.05 M Tris HCl pH 8.0. The sample from 2.16.1 was dialysed against the above buffer at 4 °C for sixteen hours then applied to the column. Fractions were eluted at a flow rate of 30 ml/hour, using the aforementioned buffer. A fraction was collected every four minutes until no protein was detected in the effluent at 280 nm.

The column was now washed with 0.1 M NaCl and the fractions collected as before.

2.16.3 Ion Exchange chromatography

DEAE - Cellulose 52 (Whatman)

The DEAE - Cellulose resin was precycled and degassed. A column of 10 cm x 2 cm was packed with the resin and equilibrated overnight with 0.05 M Tris HCl, pH 8.0.

The sample was dialysed against the above buffer at 4 °C for sixteen hours then applied to the column. Using this buffer, fractions were eluted at a flow rate of 30 ml/hour and collected every eight minutes until no protein was detectable in the effluent.

The column was now washed with 0.1 M NaCl and the fractions collected as before.

2.17 Patient selection criteria

Introduction - All specimens for plasma protease estimation on receipt in the laboratory were immediately centrifuged and separated. Those which were not to be tested within one hour were stored at -20 °C.

All specimens entering the scheme had an initial basic coagulation screen

for assessment of their general haemostasis. This consisted of a prothrombin time (2.8), a thrombin time (2.9) and an activated partial thromboplastin time (2.10) together with a factor XIII clot solubility screening test (2.11). All specimens showing either a significantly increased or decreased protease level were examined for any functional abnormality of fibrinogen (2.13). The assay for acidic fibrinolysis was performed as described by Pejham (1984). All experiments on both normal and abnormal populations were carried out using the same fibrinogen batch, all plasmas were assayed in duplicate.
For each batch of plasmas assayed, samples from previous batches were run as controls to ensure reproducability of results, if any disparity occurred then the experiment was repeated.
The constitution of a batch varied, being from a single population or from a variety of sources. Enzymic activity was expressed as mg fibrin dissolved by 0.2 ml of plasma in eighteen hours at 37°C. Results were expressed as a mean value (with the number of determinations in parenthesis) $\pm$ 2 standard deviations. Means were compared by a two-tailed t-test for unrelated samples. Patient selection was as follows.

2.17.1 A normal population

The most readily accessible of populations attending a general hospital in large numbers are the volunteers of the National Blood Transfusion Service (NBTS).
Donations are accepted from both male and female patients in the age range of 16 - 65 years. All donors were interviewed, a concise medical history was taken and any patients who did not fulfil the NBTS criteria for donation were excluded from the scheme if they had a history of heart disease, liver disease, malignancy, diabetes, glandular fever, syphilis, severe allergy, epilepsy, high blood pressure or malaria. All contacts with rubella, measles, mumps, chicken pox and shingles were identified and their donations used in plasma fractionation, but not in our survey. All female donors with a haemoglobin of less than 12 gdl^{-1} and males less than 14 gdl^{-1} were also excluded from donation. Those who had taken medical preparations within the last fourteen days were also excluded as were those on oral contraceptive therapy who were identified and excluded along with cigarette smokers and used in later experiments.

2.17.2 Diabetes

Specimens were obtained from those patients attending the Medical out patient clinic. Diagnosis was made by both clinical and laboratory findings of a polyuric patient with a fasting blood sugar greater than 15 $mmol^{-1}$ in the juvenile onset disease and greater than 9 $mmol^{-1}$ in the mature onset form. Diagnosis was confirmed by a glucose tolerance test. The severity and control of the condition was assessed by fasting sugar, fructosamine and glycosylated haemoglobin measurements using the methods of Johnson et al (1983) and Schnek and Schroeder (1961) respectively.
Subjects included in the study embraced a wide spectrum of the disease, from the well controlled patient (blood sugar less than 12 $mmol^{-1}$) to those in severe diabetic ketoacidosis (blood sugar greater than 30 $mmol^{-1}$).

2.17.3 Patients suspected of having a disorder of haemostasis

The above patients were referred to the laboratory for investigation because of a clinically suspected bruising or bleeding tendency. Initial presentation was varied and usually involved one of the following:- widespread ecchymoses, frequent epistaxis, haemarthoses or excessive pre and post operative blood loss. An extensive personal and family medical history was obtained including the use of any drug preparation. The basic coagulation screen as described in 2.8, 2.9, 2.10, 2.11, 2.12 was performed together with a full blood count, platelet count and platelet volume measurement and platelet distribution width calculations as performed by the Coulter Model S plus IV. Plasma correction experiments, factor assays and platelet aggregation studies were then performed as thought necessary.

2.17.4 Patients taking oral anticoagulant medication

The objective of anticoagulant therapy is to reduce the potential of the haemostatic mechanisms to form thrombii whilst maintaining adequate haemostatic function to prevent haemorrhage. The main drug used to obtain this state is coumarin and its derivatives of which warfarin (3-∝acetonyl benzyl 4 hydroxy coumarin - trade name Marevan) is the most widely used long acting form. Its use results in the inhibition of the synthesis of factors II, VII, IX and X by inhibiting the vitamin $K_1 \rightarrow K_1$ oxide pathway, (Suttie 1978).

The control of anticoagulant therapy is monitored using the prothrombin time (2.8) to obtain a ratio of between 2 and 4 times the normal control value, which is accepted as the optimum therapeutic range, (Lam-Po-Tang and Potter, 1975). The variation in drug doasge to achieve this is great with some patients taking as little as 0.5 mg/day and other upto 20 mg/day.

2.17.5 Assessment of liver function

Assessment of liver function was undertaken by measuring the following parameters.

Aspartate transaminase E.C. 2.6.11 (2.15.2) occurs in the hepatic cell cytoplasm and mitochondria, plasma levels are increased with liver cell necrosis and with the abnormal membrane permeability associated with acute hepato-cellular damage and cholestasis. A transient rise is observed 3 to 5 days following a myocardial infarction when levels can reach 4 to 5 x normal.

Alkaline phosphatase E.C. 3.1.3.1 (2.15.3) this enzyme is

found at the sinusoidal surface of the hepatocyte and in the microvilli of the bile canaliculi and portal and central veins. Increased levels are observed in cholestasis.

γ Glutamyl transferase E.C. 2.3.2.1. (2.15.4) a microsomal enzyme. The synthesis of which is induced by alcohol and drug ingestion, diseases of the biliary tree and both acute and chronic liver disease especially cholestasis. It is a sensitive indicator of liver damage in the suspected alcoholic.

Serum protein electrophoresis (2.15.5) demonstrates in chronic hepatocellular damage a decrease in albumin with a polyclonal rise in the γ globulins. In cholestatic disease a small increase in γ and larger increases in α and β globulins are observed.

Bilirubin - the measurement of bilirubin is most useful in determining whether the liver disease is of an obstructive nature where very high levels are seen.

Acute hepatitis, gallstones and any extra hepatic obstruction (e.g. carcinoma of the head of the pancreas) and severe cirrhosis produce elevated levels. The use of chlorpromazine and the contraceptive pill may produce a rise in serum bilirubin due to their competition for the carrier protein as steroid treatment does also.

2.17.6 Patients having taken an overdose of paracetamol

Subjects were selected for this study if following an overdose of this drug their serum paracetamol level was greater than 150 $\mu g ml^{-1}$ when tested within one hour after injestion.

The hepatic changes produced by overdosage of paracetamol appear to result from the accumulation of a highly reactive intermediate metabolite in the hepatocyte. This destroys the parenchymal cells which compose approximately 98% of the liver cellular

content. The function of these cells is detoxification, bilirubin conjugation, glycogen storage and the synthesis of proteins. The other main components of the liver are the bile ducts and sinusoidal endothelium.

2.17.7 Post blood transfusion

Ragaz et al (1976) noted when testing 3,000 samples that the acid protease levels were raised following "massive" blood transfusions. Twenty specimens were obtained, 10 from post-operative cases where transfusion was due to blood loss and 10 from patients with severe anaemia. Samples were only taken in cases where 4 or more units had been transfused. The anaemic cases were transfused with blood from which approximately 200 ml of plasma had been removed.

2.17.8 Estimation of protease levels in banked blood

It is the policy of the NBTS to store units of whole blood four four weeks at a carefully controlled 4 ^{o}C. The anticoagulant used to keep the red blood cell s viable is "citrate phosphate dextrose adenine - Formula 1". 63 ml of which is mixed at donation with 450 ml of venous blood taken from an ante umbital fossa vein.

Ten bags were set aside in the blood bank and samples for protease estimation were taken over their four week life in the bank.

2.17.9 Tissue extraction

Crude extractions were performed on tissue obtained during post

mortem examination. Tissue was first washed from membranes and debris roughly dried and weighed. 1 g of tissue was then thoroughly ground in a mortar and pestle using silver sand as an abrasive and 1 ml 0.9% (w/v) NaCl. The resultant tissue juice was filtered and 0.2 ml used as the enzyme source in the fibrin clot assay.

2.17.10 Oral contraceptive users

Shanberge et al (1972) reported that acid protease acitivity was markedly decreased in women taking oral contraceptive medication. 30 women, ages ranging from 20 - 35 years, who had been taking oral contraceptives containing 30 - 50 µg of 17-α ethyloestradiol and between 30 - 250 µg levonorgestrel (trade names Eugynon, Microgynon, Microval, Ovranette and Logynon) for at least three months, were included in the study. Plasmas were obtained from these ladies during days 4 - 21 of contraceptive usage. None of these individuals had taken any other medications for at least two weeks prior to the study.

2.17.11 Semen

Ruenwongsa and Chulavatnatol (1975) reported an acidic protease in human seminal plasma detected using acid denatured haemoglobin and N' N' dimethyl casein substrates.

Specimens of semen were obtained from men aged 19 - 45 years attending the laboratory for investigation of their possible infertility. Specimens were collected in glass jars after a period of three to five days abstinence. Specimens were assessed for potential fertility using the following parameters pH, volume, sperm

count, sperm motility and sperm morphology. Only those specimens falling within the accepted normal range of values were used in this study.

Semen specimens were also obtained from patients attending for a post vasectomy check, only those specimens showing no sperm were used in this part of the study.

CHAPTER 3

PRELIMINARY STUDIES ON THE COMPONENTS OF THE ASSAY SYSTEM

3.1 Substrates

General introduction to choice of substrate

In their preliminary investigations of the plasma enzyme Laing (1975) and Taylor (1976) used dyed fibrin as the substrate of choice. Dyed fibrin has its limitations namely the disproportionate release of the dye to enzyme concentration. The assay was improved by Law (1978) but this system remains in doubt as to its specificity and reliability in quantitation.

Pejhan (1984) assessed the use of various substrates including the natural substrate, other proteins and synthetic substrates. These included acid denatured haemoglobin, which although widely used in acid proteinase investigations was not an effective substrate for this enzyme. Although haemoglobin incorporated into polyacrylamide gel was both effective and sensitive this system is purely qualitative and relatively time consuming. Azo casein was not very suitable as a substrate for this enzyme due to its insolubility at acid pH.

N-acetyl-L-phenylalanyl-L-diiodotyrosine was not cleaved by the enzyme. Because of the large number of assays to be performed the fibrin clot assay was chosen as the method of choice as enzyme quantitation is generally reliable and the results are easily reproducible. The disadvantage of this substrate is its protein nature, which with its multiple sites of cleavage and its peptide products constantly creating secondary substrates and possible inhibitors, makes a study of the enzyme kinetics impractical. The use of a synthetic substrate, possibly one of the multitude of chromogenic compounds currently available, with one cleavage point would make a suitable alternative for further study of the plasma enzyme kinetics. These are currently being undertaken.

3.1.1 Fibrin clot formation - Two methods were considered.

(a) The original method as described by Pejham (1984) where the fibrin clots were "wrapped" on a glass rod as they formed.

(b) The clot was allowed to form in the bottom of a 75 mm x 12 mm glass tube for one hour, the serum was then expelled by squashing the clot with a glass rod.

Duplicate clots prepared as described were incubated for five hours in their own serum and then subjected to 5% SDS PAGE disc electrophoresis.

Results - From the photograph (figure 2a) of the gels it would appear that there is more cross linking in the "squashed" clots. Although a "squashed" clot is much smaller than a "wrapped" clot the squashing does not appear to adversely affect their performance as substrate, as this method produced a clot of more uniform surface area which did not dissolve in acid without the presence of enzyme as had previously been experienced with wrapping.

3.1.2 To determine the incubation time required to achieve optimum cross-linking of the clots

Twelve fibrin clots were prepared as described in 2.14.3. The clots were squashed and then left to incubate in their serum at 37°C. At intervals of sixty minutes, over a period of one to six hours, duplicate clots were removed and denatured with β mercapto-ethanol as described in 2.1.11. and then subjected to

SDS PAGE electrophoresis.

Results - Gaffney & Whittaker (1978) demonstrated that cross-linking by F XIII follows a definite time course. Firstly, the fibrin γ chains are crosslinked to give γ-γ dimers and as this process reaches completion cross-linking of α chains begins, leading to the formation of polymers (α^P) of high molecular weight. The β chain is not involved. Fig 2a illustrates the degree of cross-linking constituting the clots produced by the two techniques. Molecular weight markers were used as described (2.1.9) to identify the polypeptide chain compositions. As previously described by Pejhan (1984), the ideal clot which is resistant to lysis by monochloroacetic acid but susceptible to enzymic lysis, is achieved by obtaining a level of cross linking where complete γ-γ dimer formation but not too much α^P formation occurs. Without complete γ-γ dimer formation (i.e. if there is any monomeric γ left) then the clot will tend to dissolve to some extent in monochloracetic acid, this was found to occur when incubation was carried out for periods less than 3 hours. If there is too much α^P then the clot becomes resistant to enzyme lysis, this was found with prolonged incubation over 5 hours. From this experiment it appears that optimum crosslinking occurs after 4 hours incubation at 37°C, with progressively more α^P formation after this time. This result agrees with that of Pejhan (1984) and hence detailed results have not been included in the text.

3.1.3. Choice of substrate concentration

Initial experiments using plasma from a variety of human sources produced several results showing almost 100% proteolysis of the clot when using a substrate fibrinogen concentration of 4.0 mg.ml-1 as described by Pehjan (1984).

Because of this, it was felt increased levels of enzyme activity would be masked and surveys in various populationsmightproduce an apparent maximum value at the level of 0.4 units of activity, whereas by increasing the initial protein content more accurate results would be obtained.

Fibrin clots were prepared with a fibrin concentration ranging from 1.0 mg.ml^{-1} to 8.0 mg ml^{-1}. Plasma enzyme was added to the clots and the residual fibrin measured after 18 hours incubation at 37 ^{o}C. The enzyme activity of the plasma was obtained by subtracting the residual protein produced by the enzymic lysis from the initial clot total protein when measured by the Folin-Ciocalteau technique.

Results - A graph of units of activity against substrate concentration [S] was plotted (figure 2b). A straight line graph was obtained suggesting the enzyme activity was proportional to the substrate concentration i.e. that of first order kinetics [S] < 0.01 Km. A fibrin concentration of 6.0 mg. ml^{-1} was chosen for further clot formation.

Fig. 2a - SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS OF FIBRIN CLOTS FORMED BY

(a) Squash Technique

(b) Wrap technique

Fig. 2b - THE EFFECT OF SUBSTRATE CONCENTRATION

A graph of units of activity versus concentration of fibrin

Fig. 2a - SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS OF FIBRIN CLOTS FORMED BY

(a) Squash Technique
(b) Wrap Technique

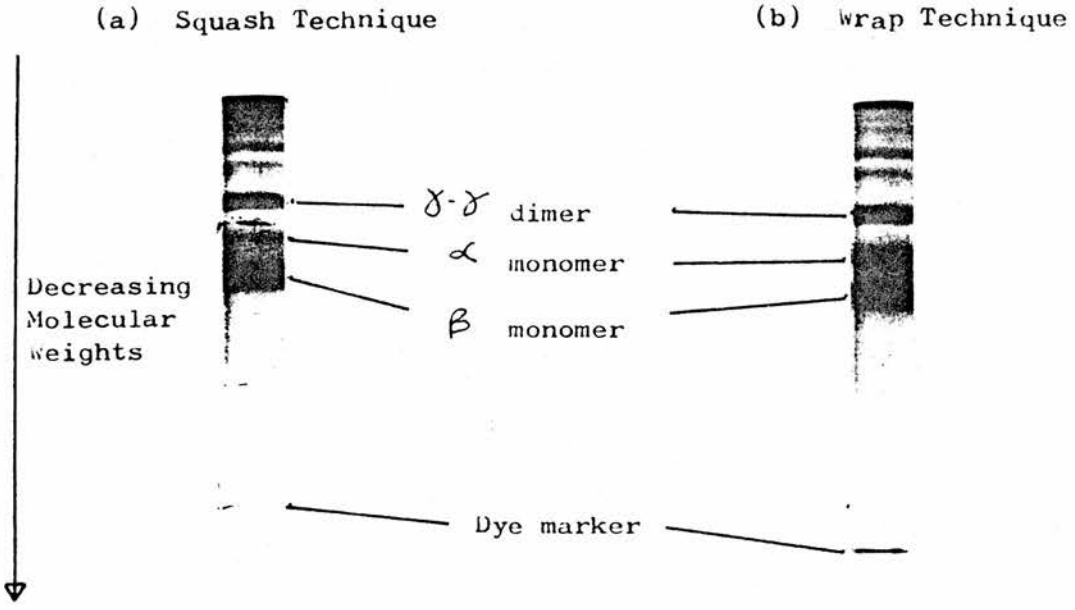
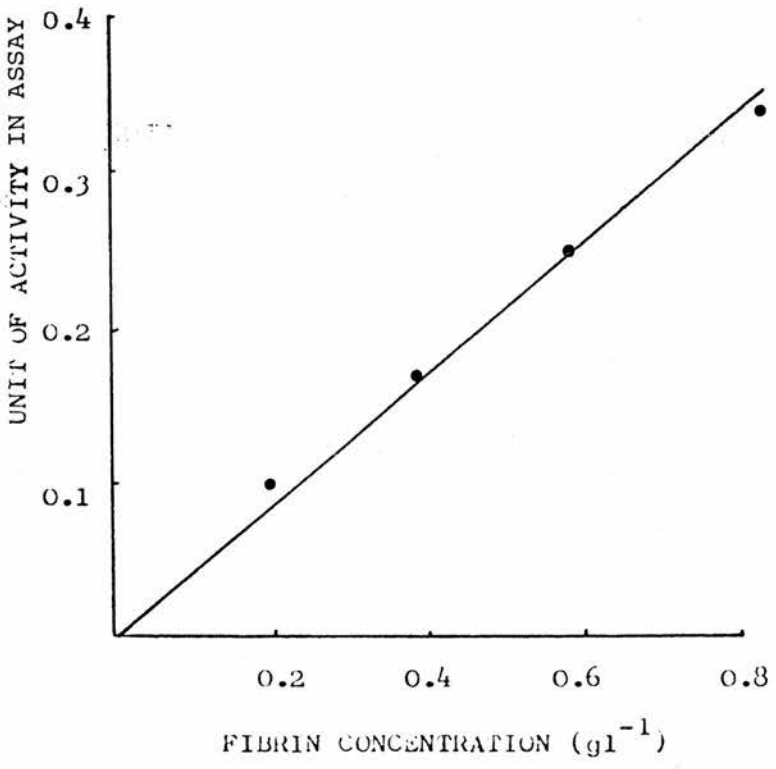


Fig. 2b - THE EFFECT OF SUBSTRATE CONCENTRATION

A graph of units of activity versus concentration of fibrin



3.2 Observation on the enzyme

3.2.1 Collection of specimens

3.2.1.1 Choice of container

Samples of venous blood were collected into anticoagulant as described in 2.2 and plasma put into either glass or plastic tubes.

Each specimen was divided into suitable aliquots, one was assayed for enzyme activity immediately, the rest stored at -20°C in their respective glass or plastic tubes. The enzyme activity was assayed after two weeks storage.

Result - no difference was found in enzyme activity obtained from collecting and storing plasma specimens in glass or plastic. It was concluded that the enzyme did not adsorb preferentially onto artificial surfaces and that specimens for future assay would be collected into glass collection tubes.

3.2.1.2 To observe the effects of various anticoagulants used in the collection of blood specimens on their enzyme activity

The activity of many enzymes is either reduced or abolished by the inhibitory effects of certain anti-coagulant substance used in the routine collection of blood specimens. Often this mechanism occurs by the inactivation of a necessary inorganic ion, activation of a multi enzyme sequence or adsorption of the enzyme onto a clot or foreign surface.

To examine the effect of several anticoagulant solutions

used in blood collection on their protease content five normal donors were selected, from each 5 ml of venous blood was collected into the following solutions.

(a) Sodium citrate 0.105M.

(b) Lithium heparin 14.3 U.S.P ml^{-1}.

(c) Ethylene diamine tetra acetic acid, Potassium salt.

(d) Sodium fluoride 2.5 $mgml^{-1}$, Potassium oxalate 2.0$mgml^{-1}$.

(e) A plain siliconised glass tube.

Results - All samples showed remarkably similar results except the oxalate/fluoride specimen which on initial investigation revealed a much lower activity. On further investigation this was shown to be artefactual when similar results were obtained to those with the other specimens. It was therefore decided that all specimens for protease estimation would be collected into citrate as the vast majority of specimens entering the laboratory are of this nature, although the other anticoagulants would be acceptable if only a limited volume of blood was available e.g. in paediatric cases.

3.2.1.3 The effect of storage on enzyme activity

A specimen from a single donor was collected, separated and the plasma aliquoted. Four tubes were left at room temperature (20°C) and ten at -20°C. The four tubes left at 20°C were tested for protease content at intervals of one to four hours and those at -20°C at intervals between one and twenty-four weeks.

Results - As can be seen from Figure 3a enzyme activity began to decline after one hour at 20°C then rapidly fell to approximately 50% of the original activity after four hours. Figure 3b

Fig. 3a - THE EFFECT OF STORAGE AT ROOM TEMPERATURE ON ENZYME ACTIVITY

A graph of plasma enzyme activity units versus hours stored at room temperature (20 °C)

Fig. 3b - THE EFFECT OF STORAGE AT -20 °C ON ENZYME ACTIVITY

A graph of plasma enzyme activity units versus weeks stored at -20 °C

Fig. 3a - THE EFFECT OF STORAGE AT ROOM TEMPERATURE ON ENZYME ACTIVITY

A graph of plasma enzyme activity units versus hours stored at room temperature (20 °C)

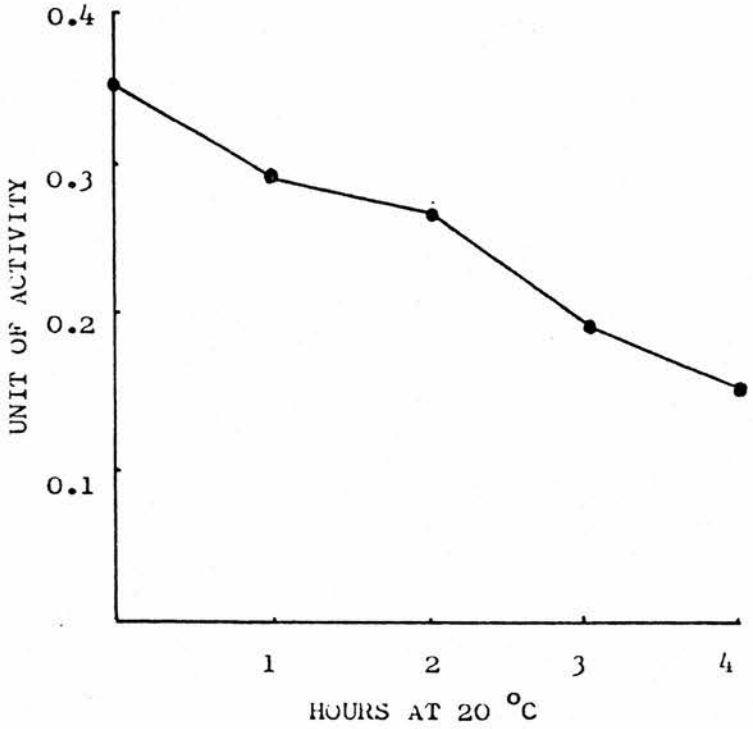


Fig. 3b - THE EFFECT OF STORAGE AT -20 °C ON ENZYME ACTIVITY

A graph of plasma enzyme activity units versus weeks stored at -20 °C

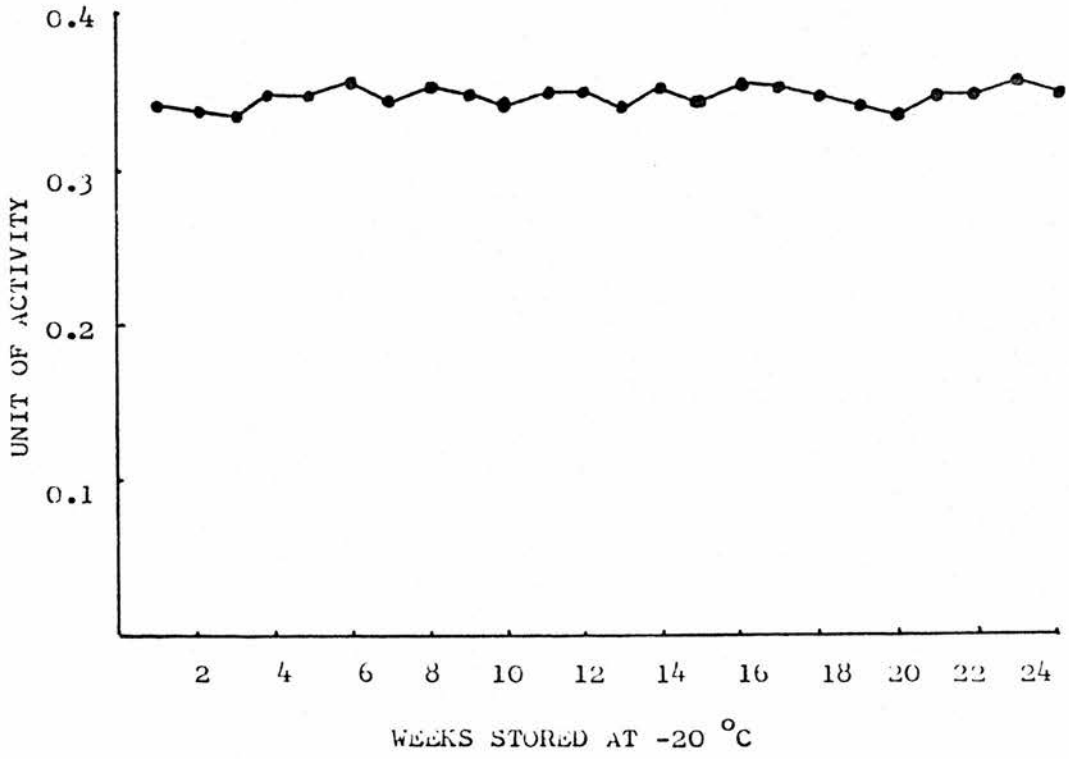
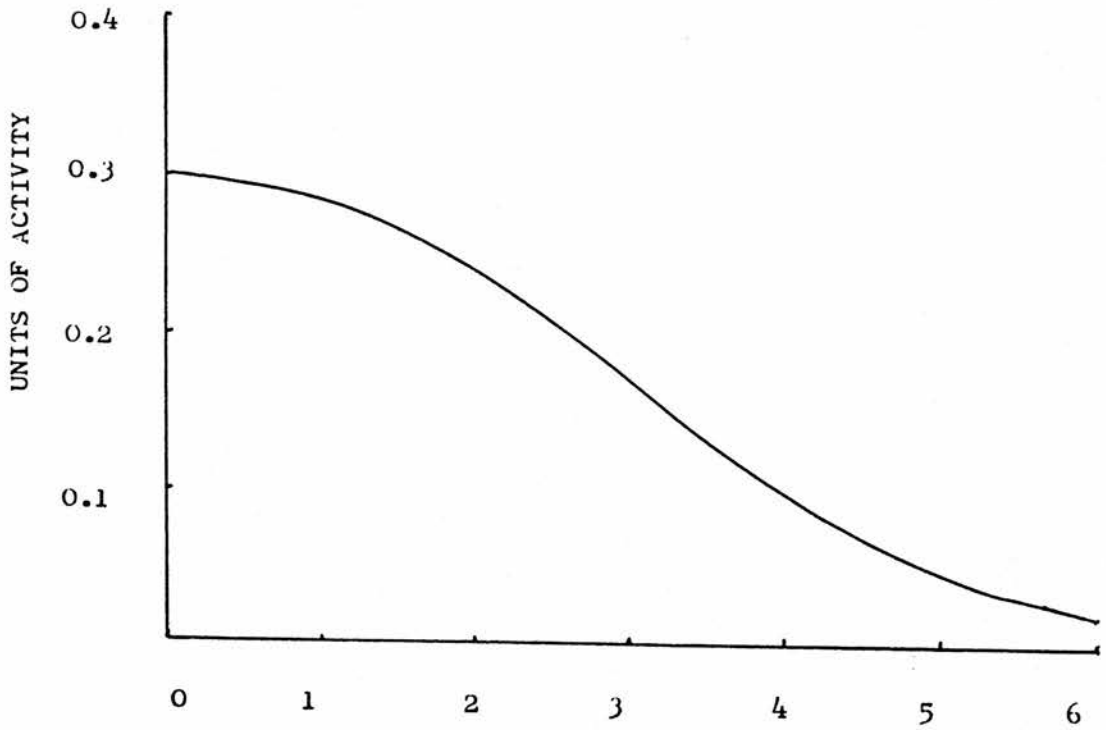


Fig. 4 - THE EFFECT OF REPEATED FREEZING AND THAWING ON THE PLASMA ENZYME ACTIVITY

A graph of units of activity versus a number of Freeze/Thaw cycles

Fig. 4 - THE EFFECT OF REPEATED FREEZING AND THAWING ON THE PLASMA ENZYME ACTIVITY

A graph of units of activity versus a number of Freeze/Thaw cycles



illustrated the activity observed when the specimen was stored at -20 °C for periods up to twenty-four weeks, as can be seen from the graph very little change in activity occurred. From the above results it was decided that specimens could be safely stored for long periods at -20 °C with no appreciable loss in activity but only if separated and frozen within one hour after venepuncture.

3.2.1.4 The effect of repeated freezing and thawing

Because the proposed investigation of plasma acid protease levels would involve initial testing, possible later verification and eventual partial characterisation on a particular specimen with an apparently elevated enzyme level it was thought expedient to observe the effect of repeated freezing and thawing on the enzyme level in a single specimen. A specimen was collected from a normal male donar and assayed for protease activity, the remaining plasma was stored at -20 °C. At seven day intervals the specimen was defrosted at 37 °C, thoroughly mixed and the assay repeated. This process was repeated over a period of six weeks.

Results - As can be seen in Figure 4 a single process of freezing and thawing had very little effect on the enzyme activity but each subsequent cycle reduced the activity until following five cycles the activity was approximately 25% of the original value. In practice the plasma enzyme was always assessed at the first defrosting of the specimen, further defrosting was only used when no duplicate specimen had been obtained and was used for enzyme characterisation and not for patient group comparisons.

3.2.2 Enzyme kinetics

3.2.2.1 Progress curve

Fibrin clots were incubated at 37°C in 1% (w/v) monochloroacetic acid in the presence of 0.2 ml plasma used:-

(a) neat

(b) diluted 1:2 with 0.9% (w/v) NaCl

to give "high" and "low" enzyme concentrations. At intervals of sixty minutes a pair of duplicate clots were removed and the amount of proteolysis estimated, this was continued over a period of twenty-four hours.

A graph of enzyme activity versus time was constructed, figure 5. As the results show, after a short lag period a linear relationship was maintained up to twenty hours as demonstrated by both enzyme concentrations. It was therefore decided to use an eighteen hour incubation period for all routine assays.

3.2.2.2 Effect of enzyme concentration

Fibrin clots were incubated in 1% (w/v) monochloroacetic acid in the presence of plasma used:-

(a) neat

(b) diluted 1:2 with 0.9% (w/v) NaCl.

specimens obtained as (a) and (b) were tested over a wide range of dilutions using a plasma volume from 0.01 ml to 0.19 ml made up to a total volume of 0.2 ml with 0.9% (w/v) NaCl. A graph was plotted of activity versus plasma enzyme concentration. Figure 6.

Fig. 5 - PLASMA ENZYME, PROGRESS CURVE

A graph of units of activity versus time incubated at 37 °C.

KEY - A Plasma enzyme neat.

B Plasma enzyme diluted 1:2 with 0.9% (w/v) Na

Fig. 5 - PLASMA ENZYME, PROGRESS CURVE

A graph of units of activity versus time incubated at 37 °C.

KEY - A Plasma enzyme neat

B Plasma enzyme diluted 1.2 with 0.9% (w/v) NaCl

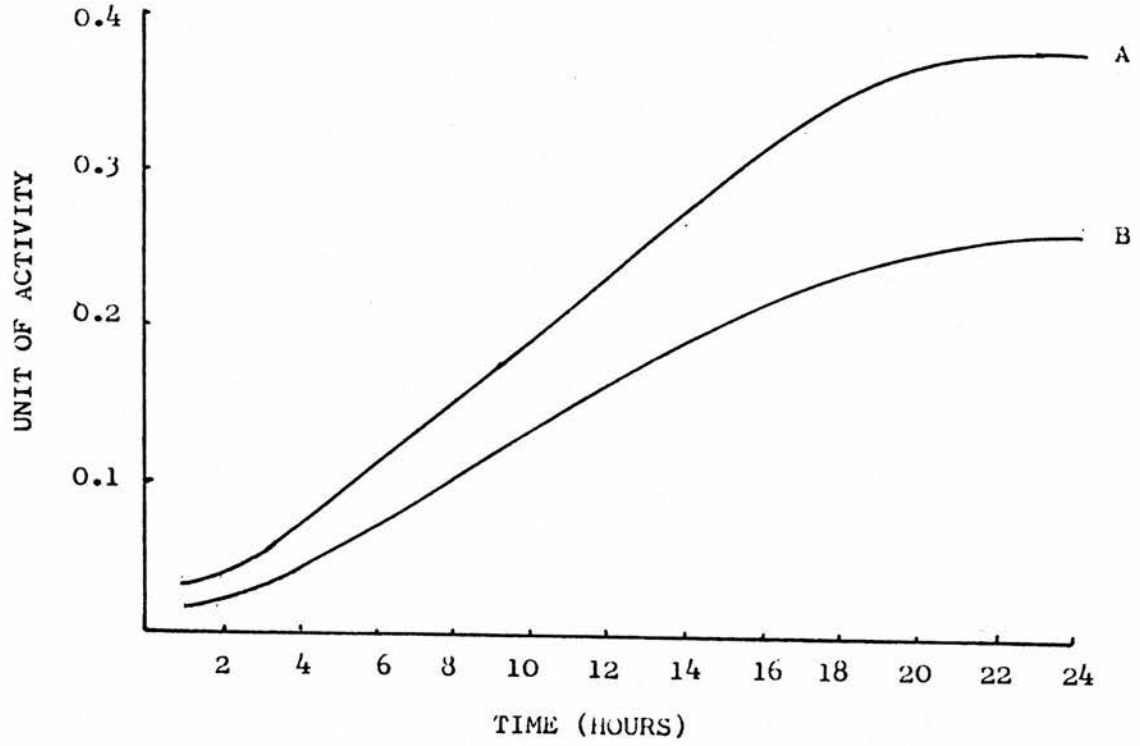


Fig. 6 - THE EFFECT OF PLASMA ENZYME CONCENTRATION

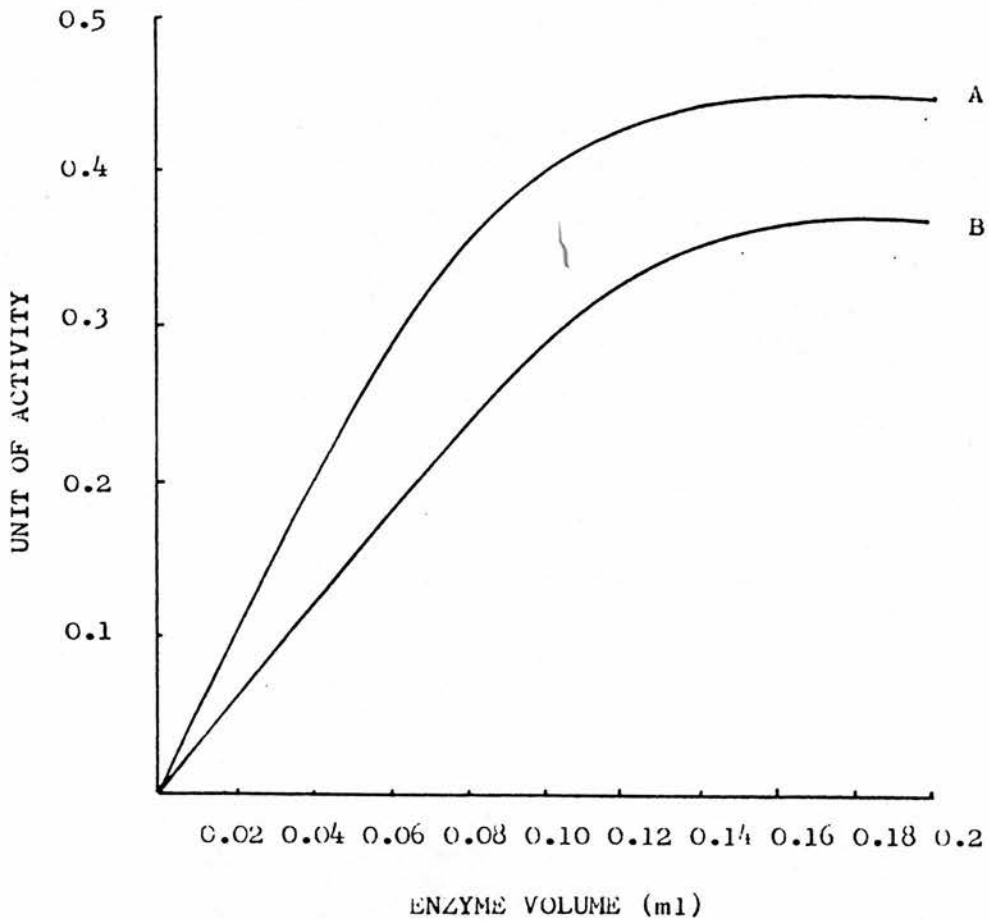
A graph of units of activity versus a range of plasma enzyme dilutions

Fig. 6 - THE EFFECT OF PLASMA ENZYME CONCENTRATION

A graph of units of activity versus a range of plasma enzyme dilutions

KEY - A plasma enzyme neat

B plasma enzyme diluted 1 : 2 with 0.9% (w/v) NaCl



As results show a linear relationship between enzyme activity and concentration was achieved, no inhibitory effect was observed as Pejhan (1984) had previously reported.

3.2.2.3 Plasma enzyme pH profile

Assays for acidic fibrinolysis were undertaken to obtain a pH profile of the native protease in its impure plasma form.

The buffer used was 1% monochloroacetic acid (w/v)/ NaOH and covered the range pH 1.5 - 6.0.

As the results show, (figure 7) the pH optimum for proteolytic activity of the plasma enzyme was approximately 3.0.

3.2.2.4 Plasma enzyme, the effect of temperature

Duplicate fibrin clots were incubated in the presence of 0.2 ml plasma enzyme and 2.6 ml 1% (w/v) MCA at varying temperatures between 5°C and 70°C for eighteen hours. The extent of the enzymes proteolytic activity at each temperature was then determined.

The results, figure 8 , show very little activity below 20°C and the activity then rises to a maximum at approximately 40°C. Activity was abolished above 60°C.

3.3 Observation of enzyme activity in selected populations

3.3.1 A normal population

Patient selection from the blood donor population was made as previously described (2.17 .1.). The accepted donors were further

Figure 7 PLASMA ENZYME, pH PROFILE

A graph of units of activity versus a range of pH values.

Substrate - fibrin clot.

Buffer - 1% monochloroacetic acid (w/v)/NaOH.

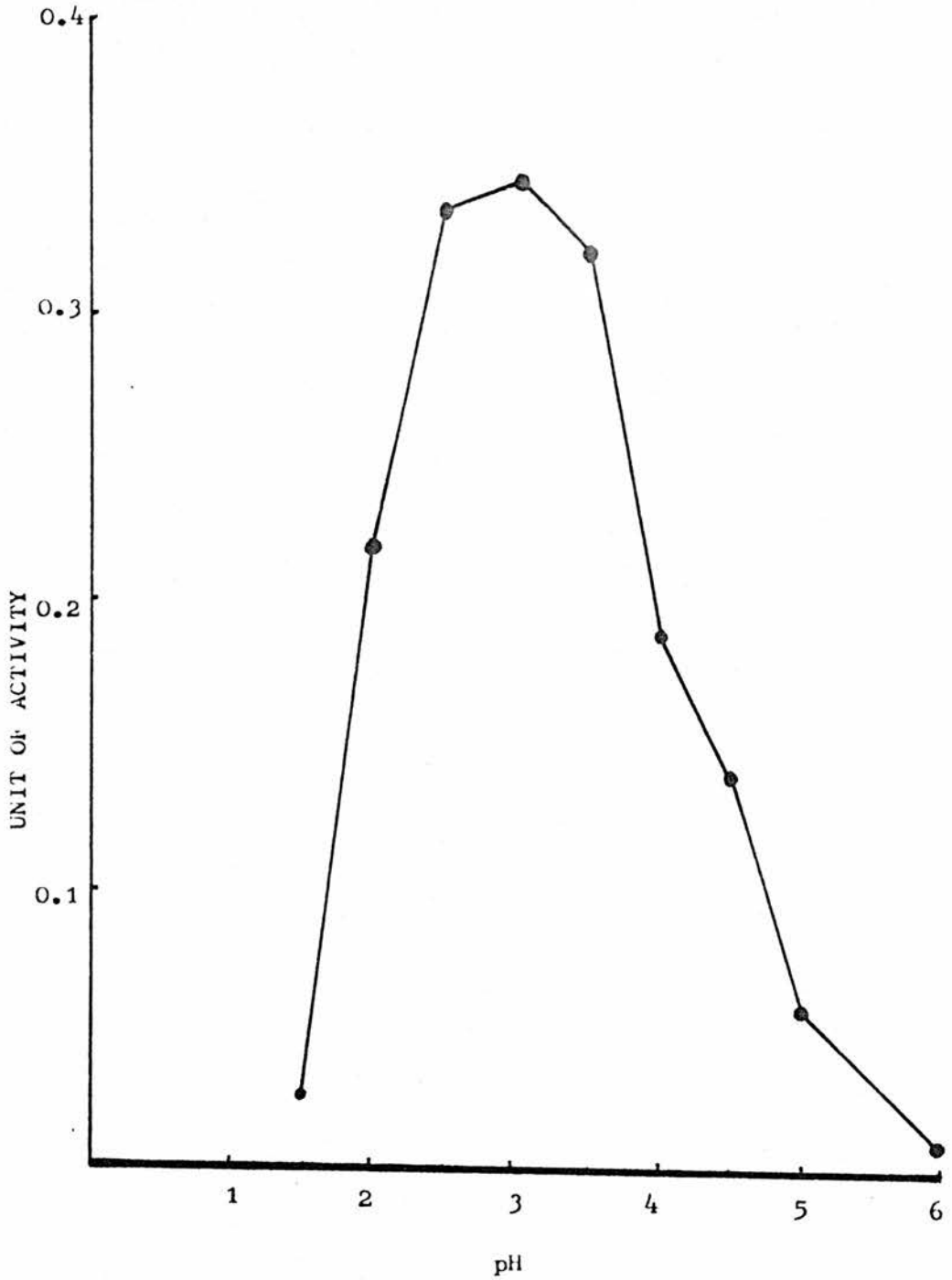
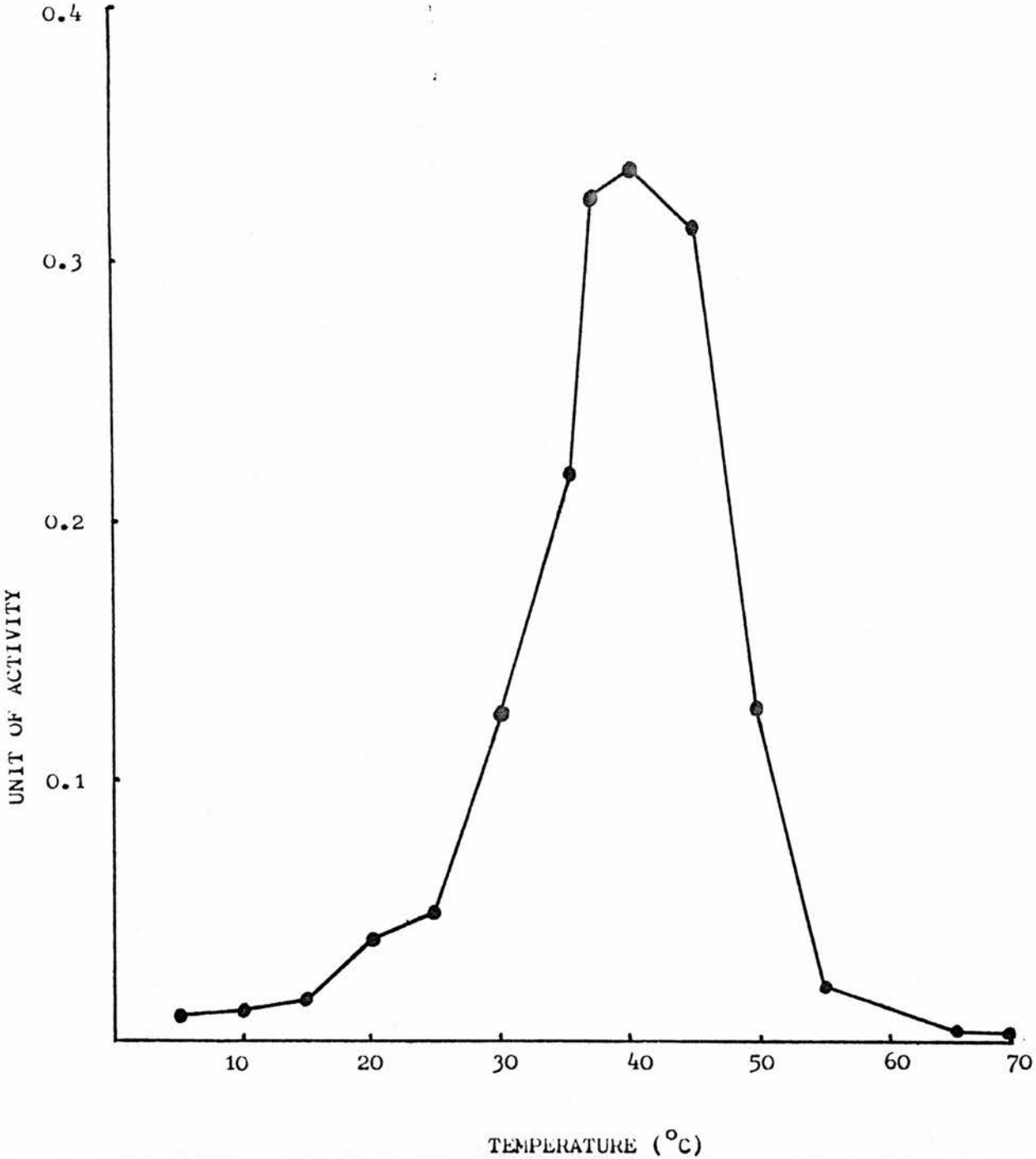

0.4
0.3
0.2
0.1
UNIT OF ACTIVITY
1
2
3
4
5
6
pH

Figure 8 THE EFFECT OF TEMPERATURE ON THE PROTEOLYTIC ACTIVITY OF PLASMA ENZYME

A graph of units of activity versus a range of temperature.


0.4
0.3
0.2
0.1
UNIT OF ACTIVITY
10
20
30
40
50
60
70
TEMPERATURE (°C)

subdivided by sex and each sex into the following age groups 16 - 20, 21 - 30, 31 - 40, 41 - 50, 51 - 60, 61 - 65 years. Female recruitment was regardless of the stage of the menstrual cycle in individual participants. Any effects of the cycle will therefore contribute to the random variation in results. All donations were taken from non-fasting subjects the majority about 5 - 6 pm. A total of 202 subjects, 112 male and 90 female were tested.

Results - For the normal population the mean value determined was 0.351 ± 0.158 (figure 9), for female subjects 0.346 ± 0.164 (figure10) and for male subjects 0.356 ± 0.154 (figure 11). Activity was found to vary little with age (figure 12), female figure 13 male figure 14. Although within the female 16 - 20 years age group the observed range of enzyme values was much greater.

3.3.2 Cigarette smokers

Patient selection was made as previously described (2.17 .1). Those blood donors who admitted to being cigarette smokers at their initial interview were identified. This group were sub divided into male and female and into those who smoked less than 5, 5 - 10, greater than 10 cigarettes per day. A total of 60 subjects were tested, 30 male and 30 female.

Results - For this population the mean value determined was 0.199 ± 0.104 for those who smoked less than 5 cigarettes per day, 0.193 ± 0.110 for 5 - 10 per day and 0.199 ± 0.082 for greater than 10 per day. As can be seen from figure 15 no significant difference was found between male and female subjects, but the mean value of acid protease levels measured in cigarette smokers was significantly less than that found in the normal population ($p < 0.001$)

Figure 9 THE DISTRIBUTION OF PLASMA ENZYME ACTIVITY WITHIN A NORMAL POPULATION

A histogram showing the distribution of enzyme activity occurring within 202 members of the Isle of Man Blood Donor population.

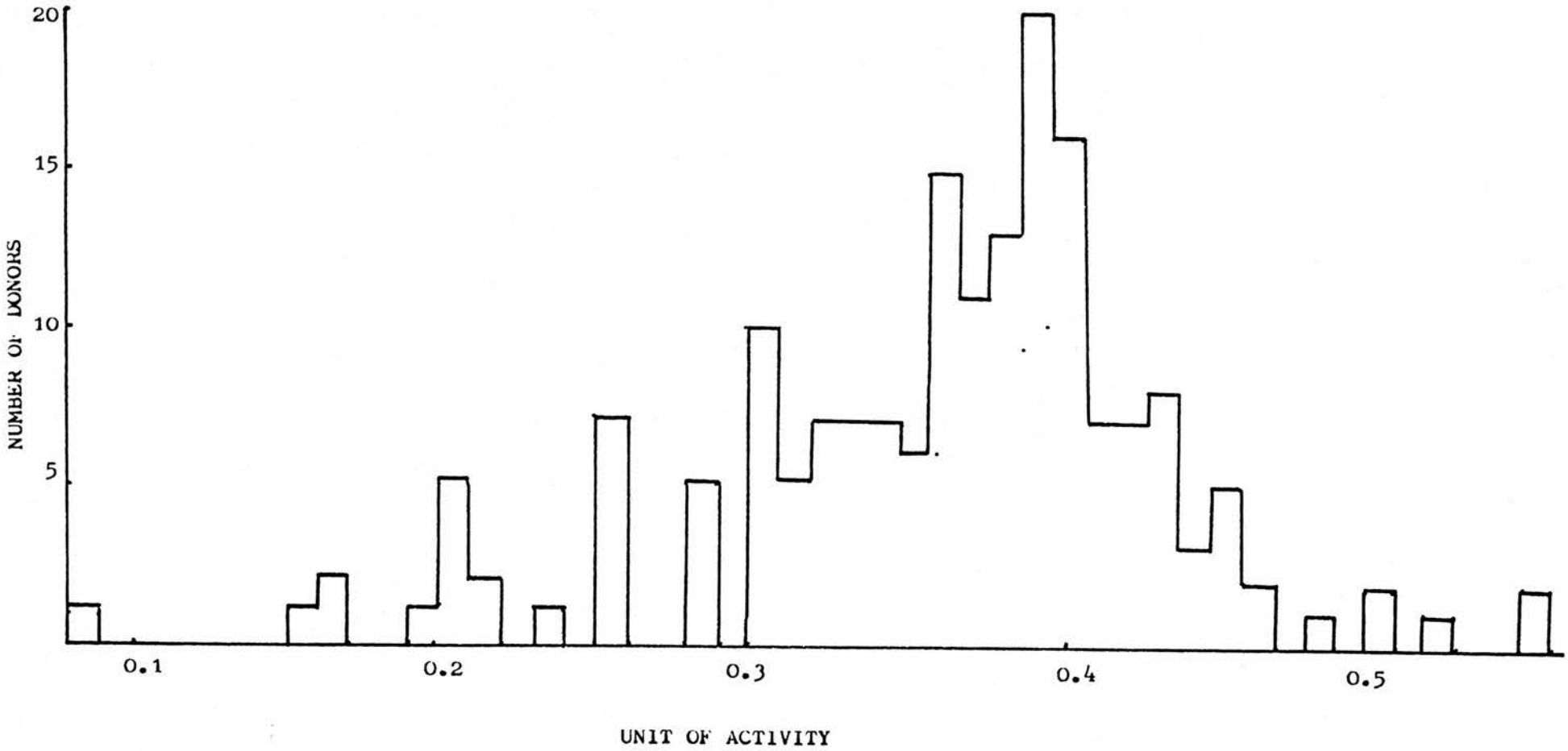

20
15
10
5
NUMBER OF DONORS
0.1
0.2
0.3
0.4
0.5
UNIT OF ACTIVITY

Figure 10 THE DISTRIBUTION OF PLASMA ENZYME ACTIVITY WITHIN A NORMAL FEMALE POPULATION

A histogram showing the distribution of enzyme activity occurring within 90 female members of the Isle of Man Blood Donor population.

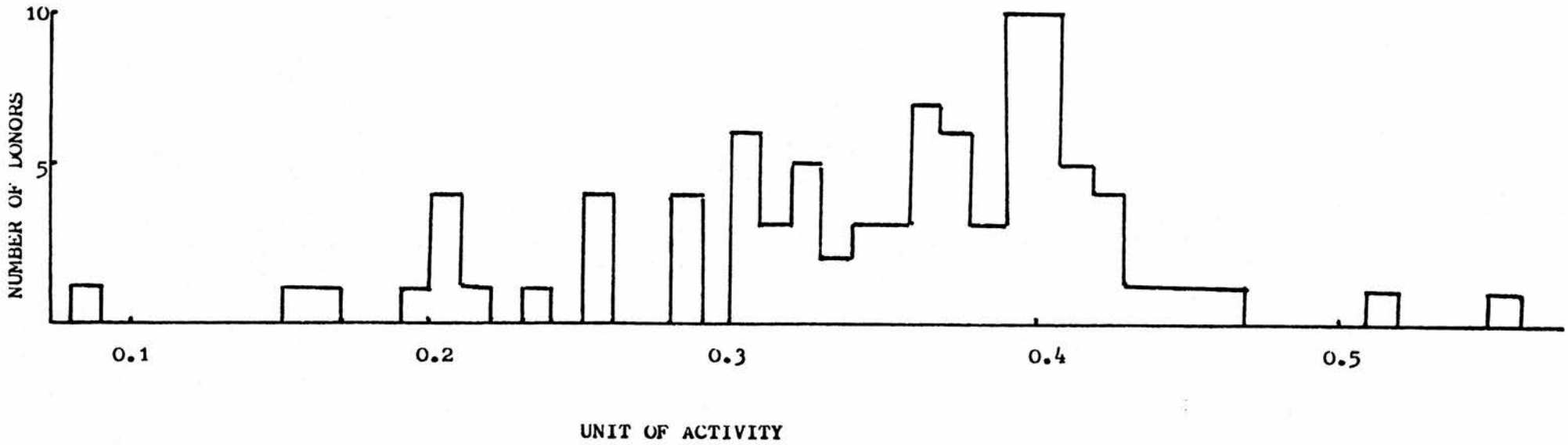

10
5
NUMBER OF DONORS
0.1
0.2
0.3
0.4
0.5
UNIT OF ACTIVITY

Figure 11 THE DISTRIBUTION OF PLASMA ENZYME ACTIVITY WITHIN A NORMAL MALE POPULATION

A histogram showing the distribution of enzyme activity occurring within 112 male members of the Isle of Man Blood Donor population.

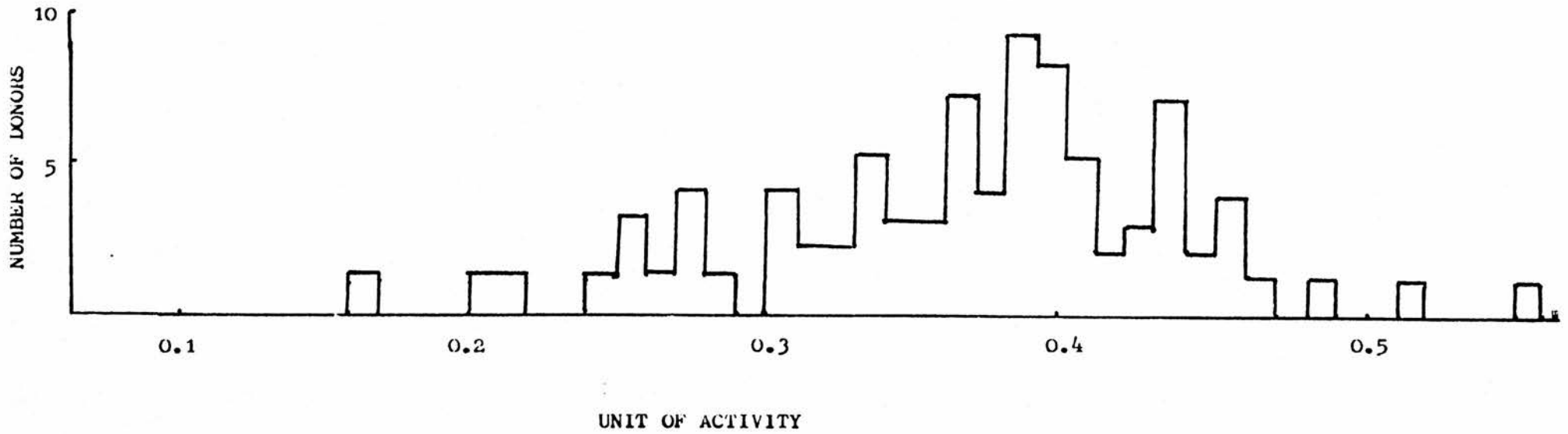

10
5
NUMBER OF DONORS
0.1
0.2
0.3
0.4
0.5
UNIT OF ACTIVITY

Figure 12 THE DISTRIBUTION OF PLASMA ENZYME ACTIVITY WITH AGE WITHIN A NORMAL POPULATION

A figure showing the distribution of enzyme activity with age occurring within 202 members of the Isle of Man Blood Donor population.

KEY

Mean

± 1 standard deviation

± 2 standard deviations

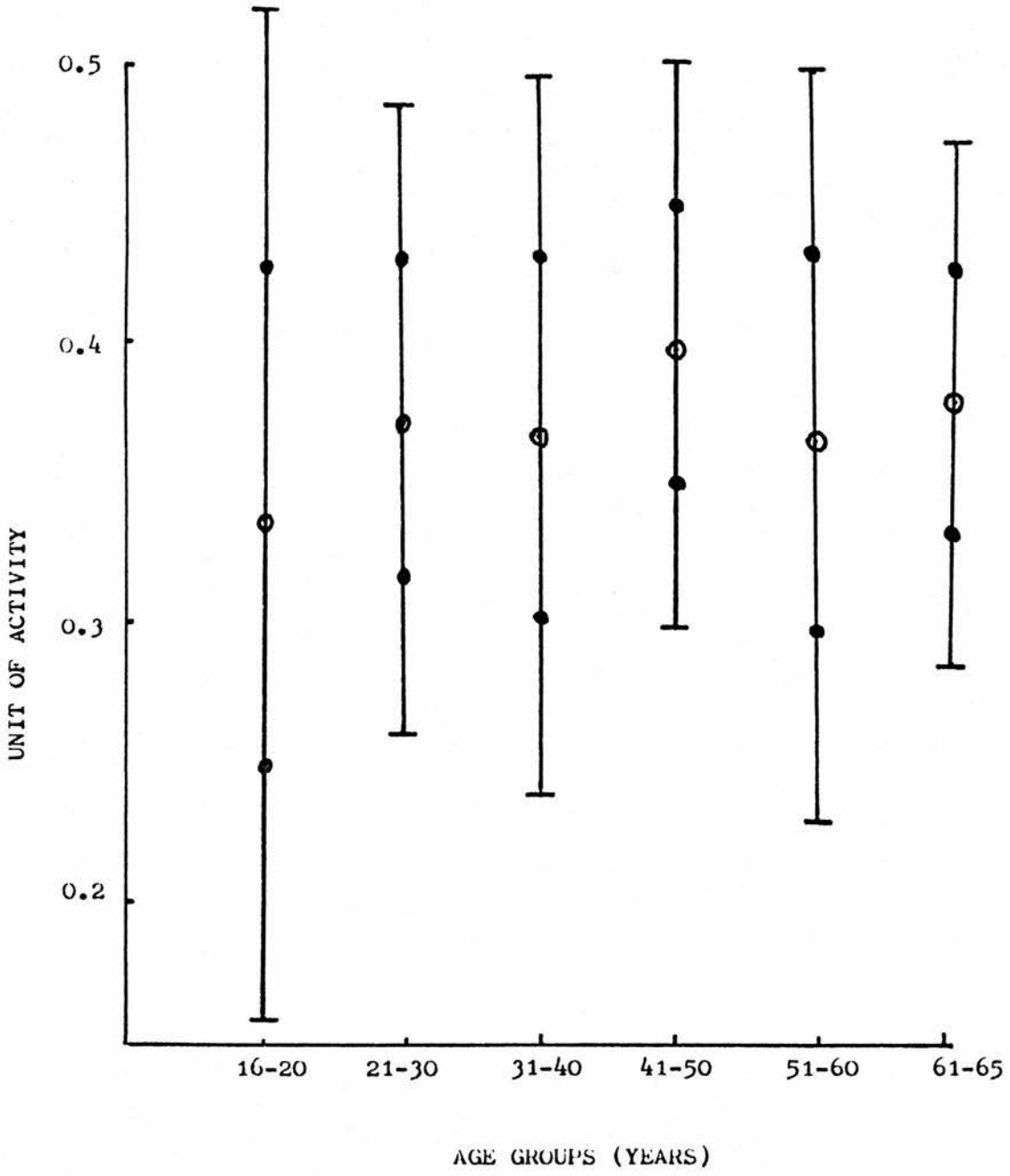

0.5
0.4
0.3
0.2
UNIT OF ACTIVITY
16-20
21-30
31-40
41-50
51-60
61-65
AGE GROUPS (YEARS)

Figure 13 THE DISTRIBUTION OF PLASMA ENZYME ACTIVITY WITH AGE WITHIN A NORMAL FEMALE POPULATION

A figure showing the distribution of enzyme activity with age occurring with 90 female members of the Isle of Man Blood Donor population.

KEY

Mean

± 1 standard deviation

± 2 standard deviations

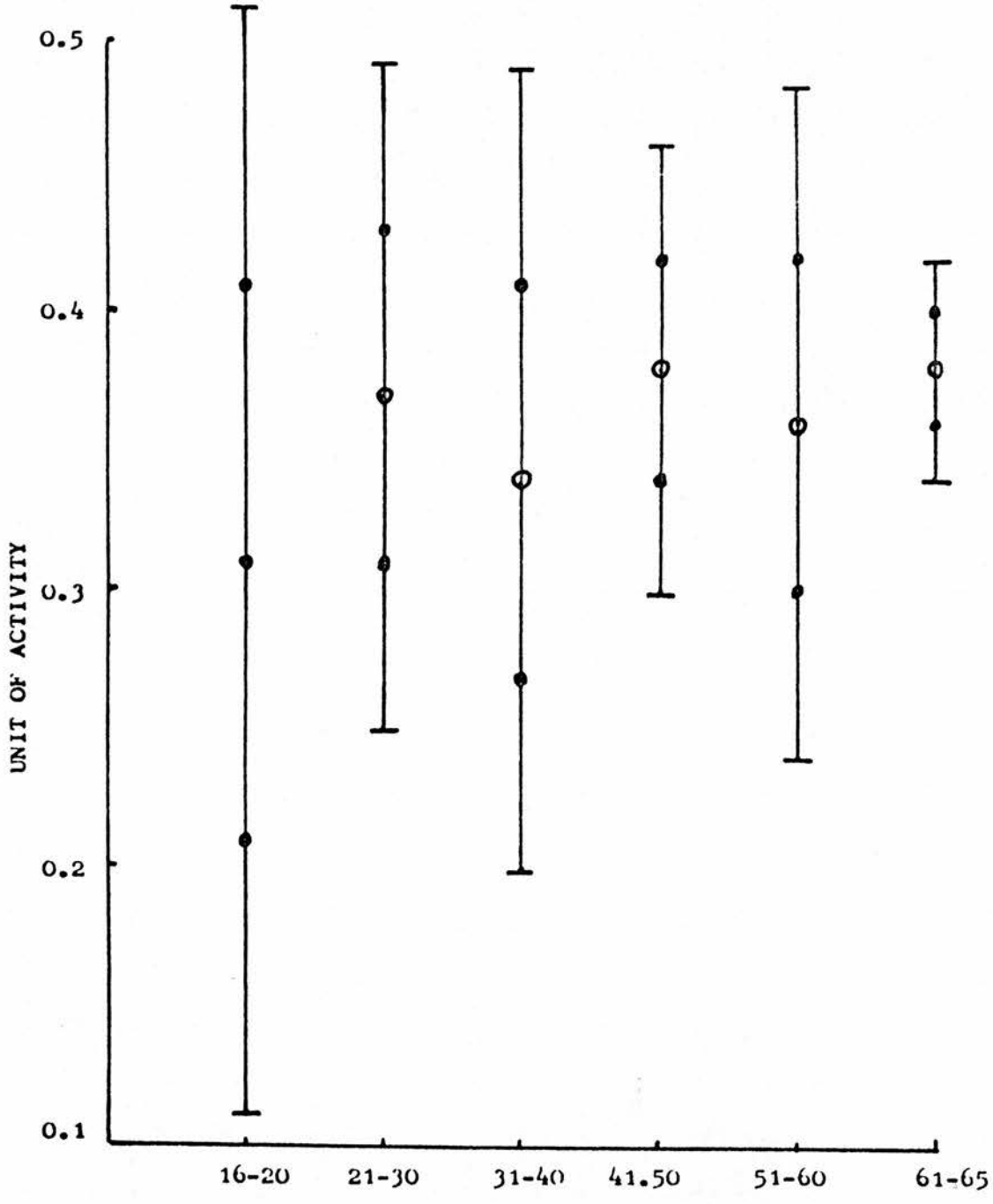

0.5
0.4
0.3
0.2
0.1
UNIT OF ACTIVITY
16-20
21-30
31-40
41.50
51-60
61-65
AGE GROUPS

Figure 14 THE DISTRIBUTION OF PLASMA ENZYME ACTIVITY WITH AGE WITHIN A NORMAL MALE POPULATION

A figure showing the distribution of enzyme activity with age occurring with 112 male members of the Isle of Man Blood Donor population.

KEY

Mean

± 1 standard deviation

± 2 standard deviations

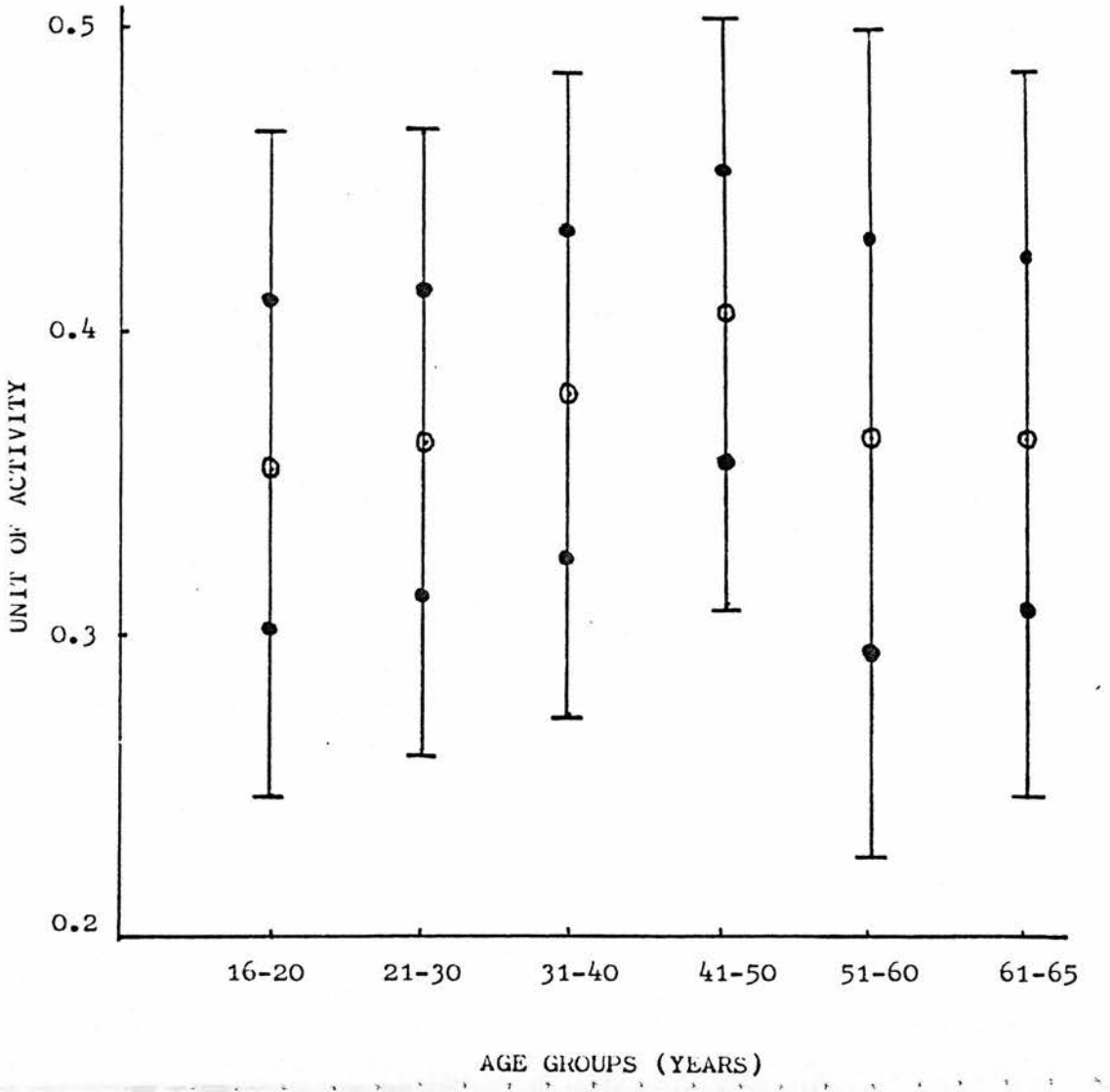

0.5
0.4
0.3
0.2
UNIT OF ACTIVITY
16-20
21-30
31-40
41-50
51-60
61-65
AGE GROUPS (YEARS)

Figure 15 THE RELATIONSHIP OF PLASMA ENZYME ACTIVITY TO THE NUMBER OF CIGARETTES SMOKED PER DAY

A figure showing the relationship of enzyme activity.

KEY

A General population

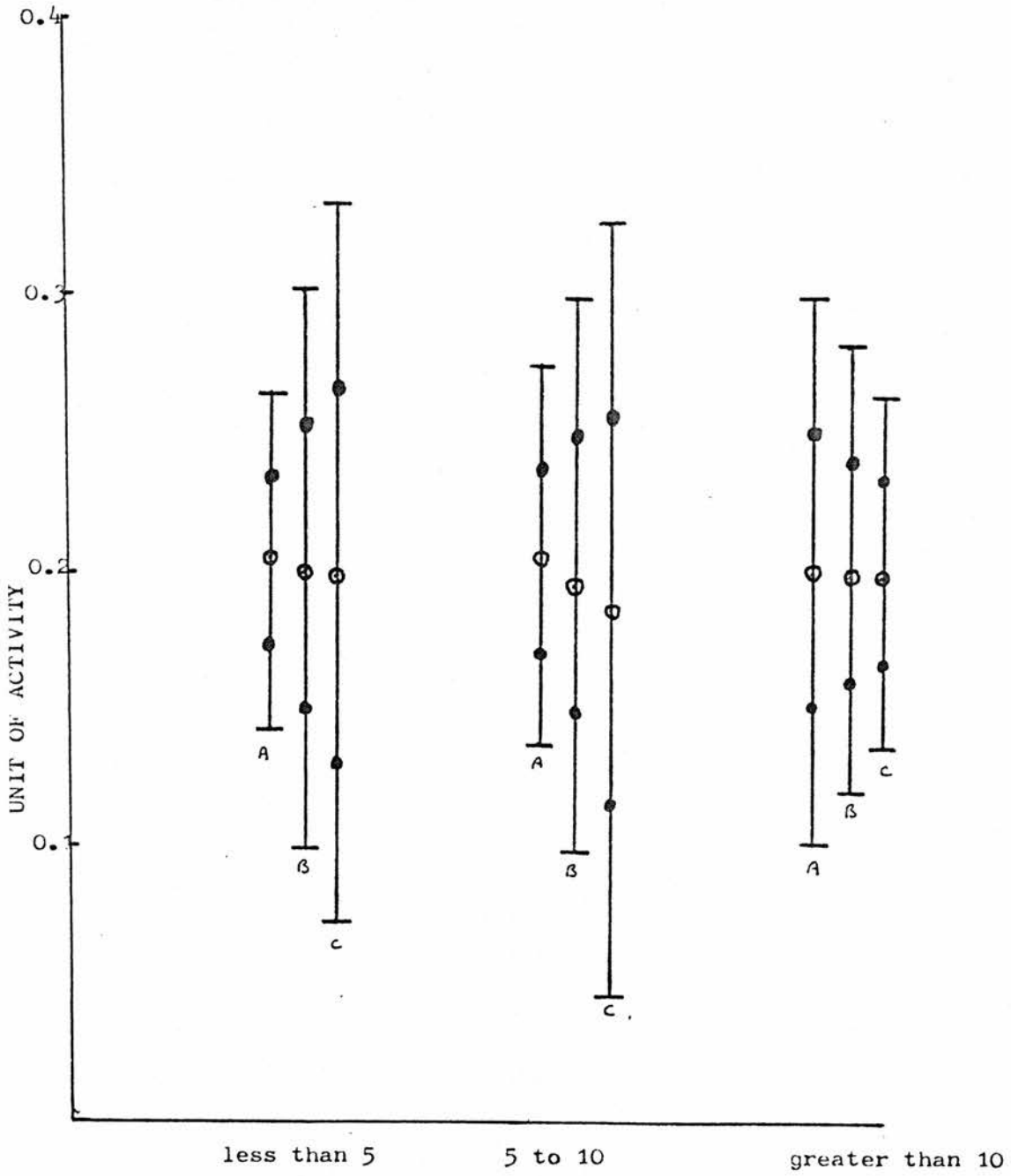
B Females

C Males

⊙ Mean

● ± 1 standard deviation

⊥ ± 2 standard deviations



THE RELATIONSHIP OF PLASMA ENZYME ACTIVITY TO THE NUMBER OF CIGARETTES SMOKED PER DAY

3.3.3 Pregnancy

Specimens were obtained from patients attending the obstetric out patient clinic. The patients were initially tested during the third trimester because of the ease of obtaining specimens. Initially 100 patients were examined over this ten week period. Because of the findings, studies were extended to include patients at 6, 10, 16, 24, 30 weeks duration of pregnancy, five subjects were tested at each stage. Note was made of the subjects parity and if they smoked. Several post partum specimens were also obtained to estimate protease activity over the days following delivery.

Results - Initial investigation of subjects in the third trimester revealed a mean activity 0.40 ± 0.07 $p < 0.001$. As can be seen from figure 16 further investigations revealed a gradual increase in protease activity from a mean (0.345 ± 0.04) not significantly different from that of the general population, observed at six weeks to maximum levels occurring in the third trimester. No decline in values was found up to the third day post partum, no difference was found between activities in primi para and multi para subjects. Cigarette smoking did not appear to cause a decrease in the protease activity when observed in second and third trimester patients ($m = .385$, $n = 4$).

3.3.4 Diabetes

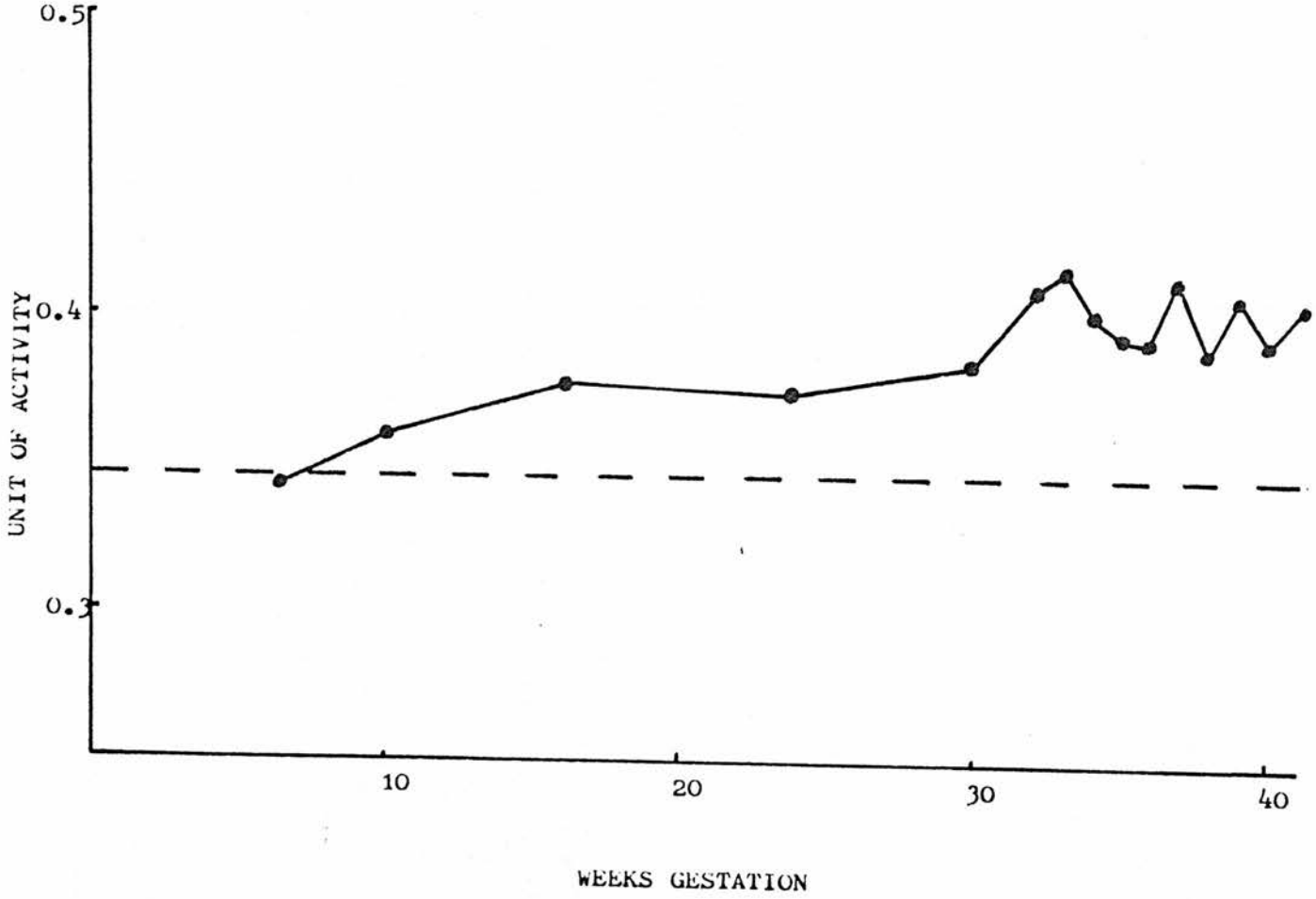
Patient selection was as previously described (2.17 .2) specimens were obtained from both hospitalised patients and those attending the medical out patient clinics for diabetic control.
The mean value determined using 20 subjects was 0.342 ± 0.144.
No significant difference in protease level was found in either the well controlled diabetic population or those in severe ketoacidosis.

Figure 16 THE RELATIONSHIP OF PLASMA ENZYME ACTIVITY TO DURATION OF PREGNANCY

A graph of units of activity versus the number of weeks gestation.

KEY

- - - Mean enzyme activity occurring in normal female population.


0.5
0.4
0.3
UNIT OF ACTIVITY
10
20
30
40
WEEKS GESTATION

3.3.5 Patients suspected of having a disorder of haemostasis

Patient selection was as previously described (2.17 .3). Cigarette smokers were excluded from the study as were those out patients who had taken any medication within the past seven to ten days.

The mean acid protease value determined using 48 subjects was 0.31 ± 0.2.

The range of values in this category is very wide and the overall mean is significantly less than normal ($p < 0.01$) probably reflecting the diverse nature of the symptoms of this group.

3.3.6 Patient taking oral anticoagulant medication

Patient selection was as described (2.17 .4). For protease estimation the patients were divided into groups according to their dosage, note was also made of their sex, age, smoking habit and drug use. The mean acid protease value determined using 60 subjects on Marevan therapy was 0.29 ± 0.16. The mean being significantly less ($p < 0.001$) than that in the normal population. No correlation was found between drug dosage and protease levels (figure 17) or in duration of drug treatment.

Because of the significantly reduced protease levels seen in this group it was thought possible that it could be in response to the underlying pathological condition. Several conditions with a similar history but where no drug treatment had been used were therefore investigated.

3.3.7 Deep venous thrombosis or pulmonary embolus

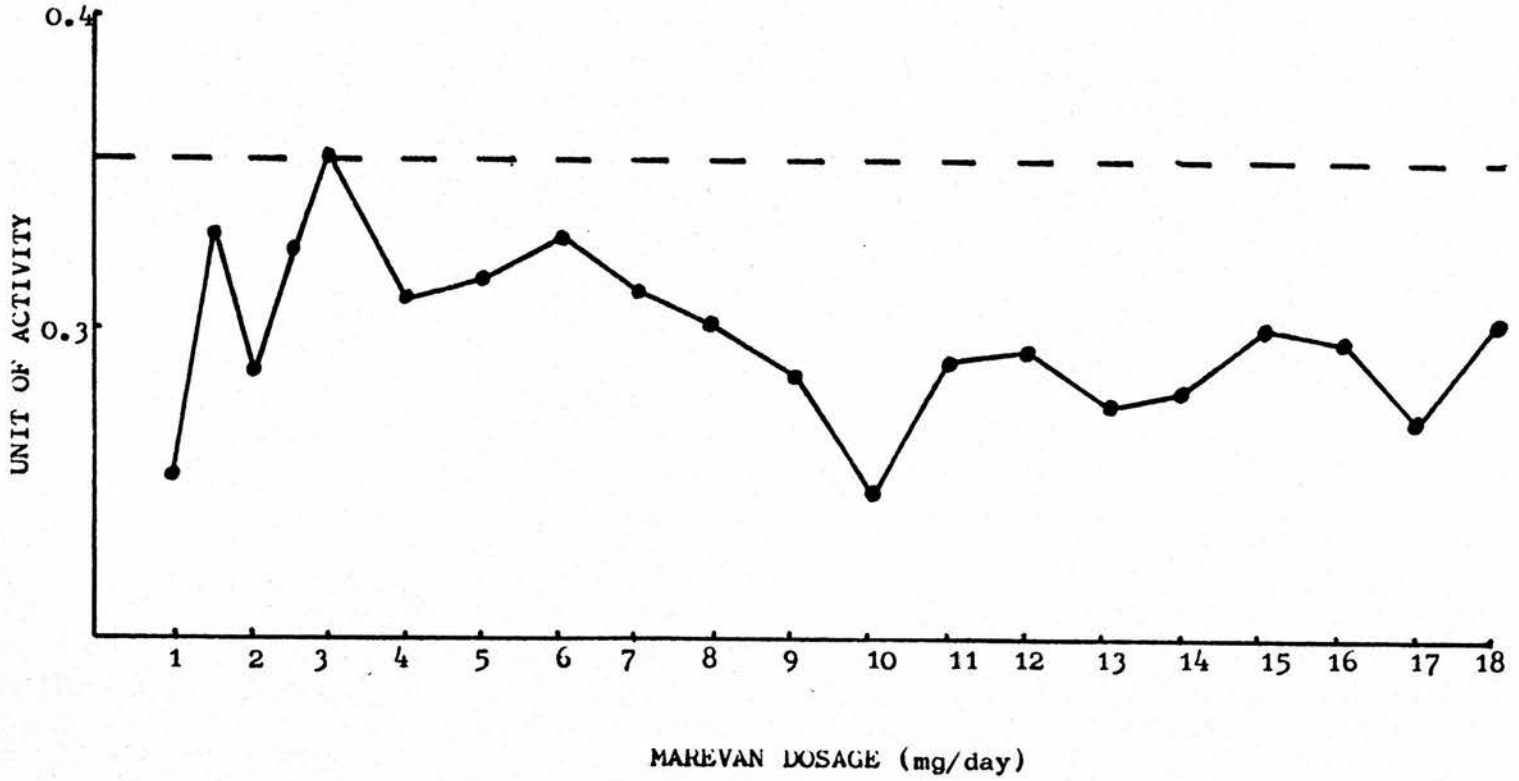
Plasma samples from patients clinically suspected of having one of the above conditions were examined for their acid protease level. Diagnosis of these conditions is not particularly easy

Figure 17 THE RELATIONSHIP OF PLASMA ENZYME ACTIVITY TO MAREVAN (3- acetonyl benzyl 4 hydroxy coumarin) DOSAGE (mg/day)

A graph of units of activity versus mg Marevan taken per day.

KEY

- - - Mean activity of population taking no drug therapy.


0.4
0.3
UNIT OF ACTIVITY
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
MAREVAN DOSAGE (mg/day)

and is often made after the exclusion of other conditions because of this specimens were often obtained up to 36 hours post episode.

The mean acid protease level, testing six subjects, was 0.228 ± 0.182 this result is significantly less ($p < 0.001$) than the level found in the normal population.

3.3.8 Alcohol abuse

In view of the fact that alcohol effects many enzyme systems especially those of the liver and because Marevan, which has its main effect on the liver, had been shown to produce a decrease in the plasma acid protease level it was decided to investigate the levels found in chronic alcoholics.

Samples were obtained from alcoholics, either hospitalised or those attending out patient clinics for chronic alcohol abuse. These patients had been diagnosed as such by both clinical judgement and the laboratory findings of elevated γ-glutamyl transferase (2.15.4) and elevated β globulin (2.15.5) level due to their abnormal liver metabolism. The mean protease level found in 29 subjects was 0.31 ± 0.18 which is significantly different ($p < 0.01$) than the mean for the normal population.

3.3.9 Abnormal liver function

Because of finding a significant reduction in protease activity in alcoholics and since many of these patients have abnormal liver function it was thought desirable to examine the protease activity in those patients with an abnormal liver function profile. It was thought this may give an in sight to an increase or decrease in production in association with specific cellular damage, possibly pointing to a site of synthesis of the protease. All specimens entering the laboratory for assessment of liver function (2.16.5)

were also examined for their acid protease content.

The mean acid protease activity found in 46 patients examined with abnormal liver function tests was 0.366 $\pm$ 0.226. As can be seen from TABLE 1, when the individual parameters were examined none were significantly different from the population mean. This is possibly explained by the biochemical parameter suggesting this population shows predominantly the obstructive type of liver disease and not a hepatocellular disease.

TABLE 1

LIVER FUNCTION TEST PARAMETER	NORMAL RANGE	NUMBER	MEAN VALUE	MEAN ACID PROTEASE ACTIVITY	$\pm$ 2 STANDARD DEVIATIONS
Total Bilirubin	Less then 17	8	99.5	0.371	$\pm$ .218
Aspartate Transaminase	Less then 37	13	77.2	0.359	$\pm$ .220
Alkaline Phosphatase	98-279	6	1159	0.338	$\pm$ .226
γ Glutamyl Transferase	Male 11-50 Female 7-32	16	347.7	0.366	$\pm$ .214
Globulins	26-30	22	34.6	0.351	$\pm$ .232
Albumin	36-52	3	27.4	0.374	$\pm$ .182

3.3.10 Patient having taken an overdose of paracetamol

Because of the association of decreased acid protease level in alcoholics it was decided to pursue the investigation of liver dysfunction further. A suitable model showing more specific cellular damage was chosen to be paracetamol overdosage. Patient selection was as described previously (2.17 .6).

The mean enzyme activity found in the 20 patients tested with serum paracetamol levels greater than 150 $\mu g ml^{-1}$ was found to be 0.342 $\pm$ 0.102. When severe parenchymal cell damage occurs in paracetamol poisoning aspartate transaminase is leaked into the

blood supply, as can be seen from TABLE 1 there appears to be no direct relationship between increased aspartate transaminase levels and acid protease activity.

3.3.11 Estimation of protease activity during oral contraceptive usage

Selection of participants for this study was as previously described (2.17 .10). Samples were obtained from 30 ladies during days 4 - 21 of the contraceptive pill cycle. Cigarette smokers were identified.

The mean activity found was 0.168 $\pm$ 0.062 which is significantly lower than the mean value for the "normal" population ($p < 0.001$). The variation of activity through the cycle of pill usage is shown in figure 18 (each point is only one observation). Cigarette smoking was shown not to significantly alter the level of activity (mean 0.174 $\pm$ 0.081 $n = 4$) , in this population. Two patients disclosed after the study, that they had ceased taking the medication 6 days and 9 days prior to the study, their results still showed markedly reduced values of 0.157 and 0.163 respectively.

3.3.12 Estimation of seminal fluid protease activity

Specimens were selected as previously described (2.17 .11). The patients were divided into two groups

(a) 10 patients with no sperm present

(b) 10 patients with normal seminal analysis.

The protease estimations were performed immediately on receipt of the specimen in the laboratory.

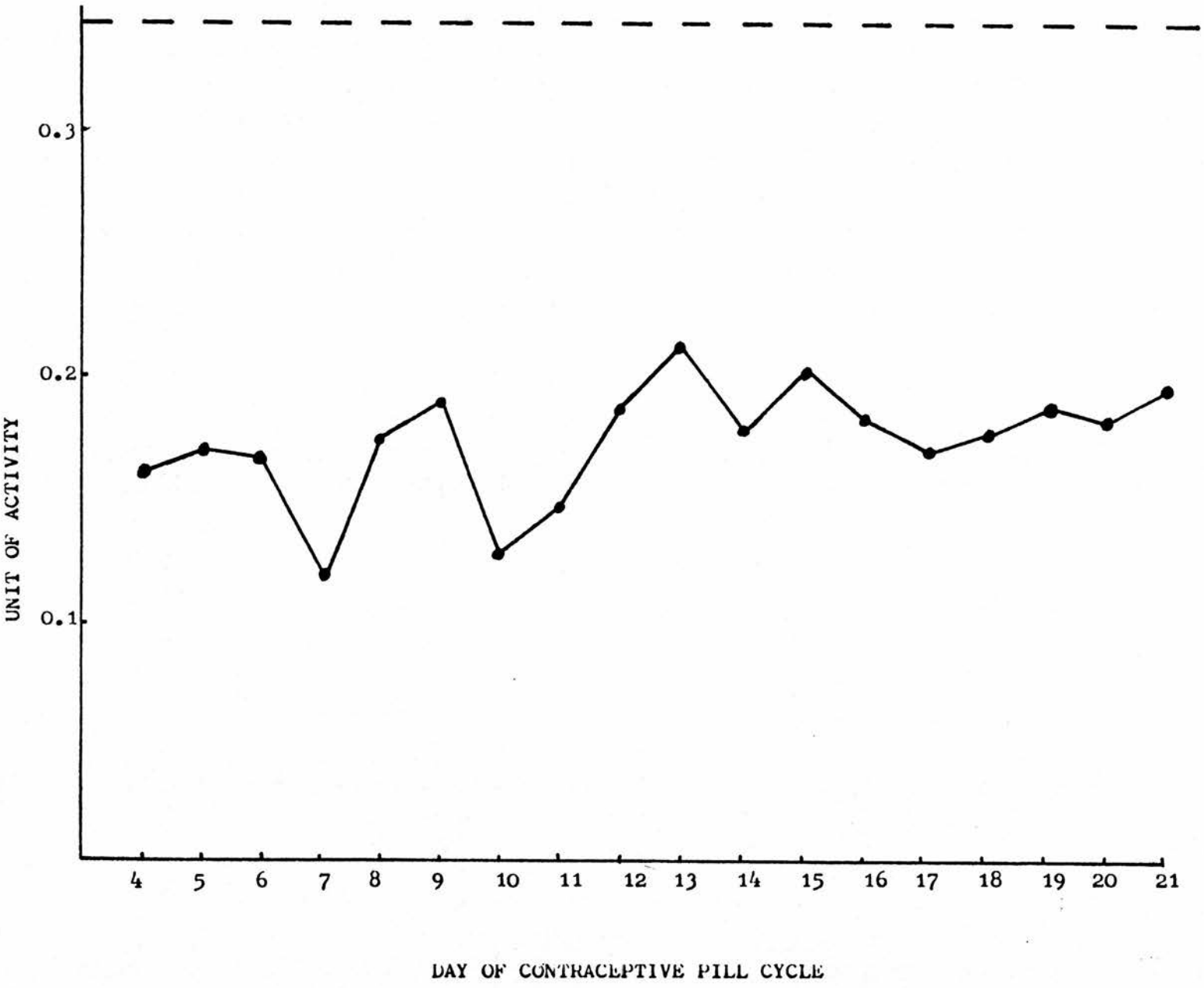
The mean acid protease activity found in normal seminal fluid was 0.579 $\pm$ 0.150 and in those with no sperm present 0.585 $\pm$ 0.128. These observations are significantly increased ($p < 0.001$) compared to the mean for plasma protease activity and agree with the observations of Ruenawongsa and Chulavatnatol (1975).

Figure 18 PLASMA ENZYME ACTIVITY FROM DAY 14 TO DAY 21 OF ORAL CONTRACEPTIVE USE

A graph of units of enzyme activity versus the number of days of contraceptive pill cycle.

KEY

- - - Mean enzyme activity occurring in female population not undergoing oral contraceptive medication.


0.3
0.2
0.1
UNIT OF ACTIVITY
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DAY OF CONTRACEPTIVE PILL CYCLE

3.3.13 Post blood transfusion

Patients were selected as previously described (2.17.7). These were divided into two groups:-

(1) those transfused with whole blood.

(2) those transfused with packed red cells.

Specimens for acid protease estimation were obtained within 24 hours following the last unit transfused.

The mean value determined for those patients transfused with whole blood using ten samples was 0.430 ± 0.10 and for those transfused with packed cells 0.433 ± 0.098 ($n = 10$).

Since the studies in the previous experiment confirmed Ragaz's observation that an increase in clot solubility was found following "massive" transfusion. It was decided to attempt to find the reason why - several suggestions could be made

(1) The acid protease existed in high concentration in banked blood.

(2) The protease was released in response to the bleeding.

(3) The protease was released as a result of trauma at the needle point.

(4) The protease was released during damage to the endothelium by the operative process.

(5) The rise in activity was hormonally induced due to fear, shock etc.

(6) Fresh frozen plasma is administered routinely for coagulation factor replacement in "massive" transfusion (usually 1 unit is given for every 4 units of whole blood) - does the fresh frozen plasma contain high levels of protease?

It was felt that it was possible to investigate points 1,2,4,5,6

3.3.14 Estimation of protease levels in banked blood

Samples were taken from bags stored as described (2.17.8). At the same time as assaying the supernatent plasma for acid protease

activity, lysates were made from the red blood cells by

(a) freezing and thawing the centrifuged red cells diluted with an equal volume of water

(b) "traumatic destruction" with an equal volume of water in a mortar and pestle.

These two methods were employed as it was thought that the protease may be in contact with the red blood cell membrane cytoskeleton and it was thought method (a) would preserve this intact and method (b) would disrupt the membrane and cellular organelles.

Result - The mean acid protease activity observed in testing ten bags of whole blood at intervals during their life in the blood bank can be seen in TABLE 2 .

Table 2

Storage time at 4°C	Stored blood plasma Mean acid protease activity	± 2 standard deviations
0 (at collection)	.385	.092
2 days	.399	.044
6 days	.432	.048
14 days	.416	.095
20 days	.365	.091
28 days	.347	.145

Haemolysates were made as described earlier, 10 samples were tested by each method, the mean acid protease activity found was:-

method (a) 0.748 ± 0.194

method (b) 0.790 ± 0.202.

As can be seen in Table 2 the acid protease activity of the supernatentplasma appears to increase over the first two weeks of storage, the activity then declines quite rapidly until the unit is discarded at four weeks.

Haemolysates made from red blood cells contain an acid protease acidity equal to approximately twice that found in plasma.

3.3.15 Estimation of protease levels following blood loss

Protease levels were estimated in plasma samples taken from ten patients who had suffered

(a) chronic blood loss over several months e.g. from a bleeding gastric ulcer

(b) acute blood loss over a period of hours, again ten patients were examined e.g. severe blood loss from bleeding oesophageal varisces, before blood replacement commenced. The mean protease levels found in each group were

(a) 0.374 ± 0.045

(b) 0.410 ± 0.069. As can be seen from these results the protease level following chronic blood loss is not significantly different from the population mean ($p < 0.5$) but that measured following acute blood loss appears to be significantly different ($p < 0.001$). Validation of this observation must now be performed to show that before blood loss such patients did not differ in their plasma acid protease activity from normal controls.

3.3.16 Estimation of protease activity following endothelium damage

Two conditions requiring transfusion were sought where

(a) minimal damage to the endothelium had occurred during transfusion

(b) major damage had occurred during the operative process.

(a) following transfusion due to anaemia where it was assumed there was little or no damage to the endothelium

(b) following transfusion due to the major vascular reconstruction involved in the dissection, and repair with a graft, of an aortic aneurysm.

Results - The mean acid protease value determined for group (a) using 10 subjects was 0.386 ± 0.050 and for group (b) the mean value using 10 subjects was 0.423 ± 0.09. As can be seen from the above results an elevation in protease activity was found in both groups. The increase observed in group (b) ($p < 0.001$) was approximately three times greater than that seen in group (a) ($p < 0.05$) when compared to the population mean.

Although it has been demonstrated here that protease levels are increased in conditions associated with endothelial damage the influence of certain other procedures must be taken into consideration. In such an operation, non-endothelial tissue is also damaged, all such patients undergo general anaesthesia plus infusion of blood and replacement fluids. Post-operative sedation and starvation is usual in such cases and it would be unusual for most patients not to undergo some form of emotional stress.

3.3.17 Estimation of the protease content of fresh frozen plasma

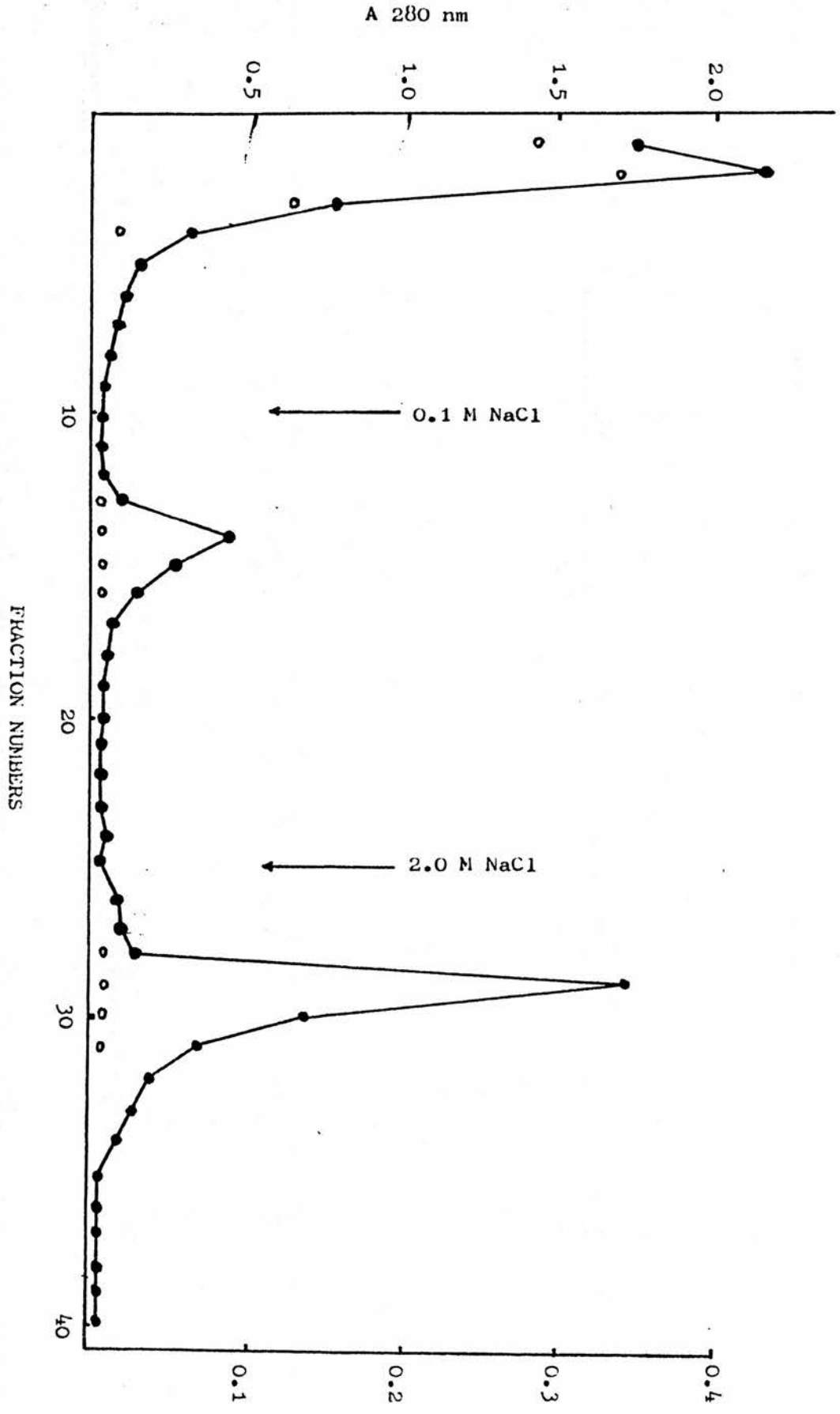
Fresh frozen plasma is prepared at donation where approximately 150 ml of plasma is removed from a unit of whole blood the plasma is stored at -20 ^{o}C until required for use. Fresh frozen plasma is used mainly for the correction of the coagulation factor deficit which occurs when large volumes of whole blood, which after storage generally have little or no functional coagulation factors, have been transfused.

10 units of fresh frozen plasma were defrosted and tested for their acid protease content, the mean activity per unit was 0.380 ± 0.060.

Figure 19 BLUE-SEPHAROSE COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

● absorbance at 280 nm plasma enzyme.

○ plasma enzyme proteolytic activity.


A 280 nm
0.5
1.0
1.5
2.0
10
20
30
40
0.1 M NaCl
2.0 M NaCl
FRACTION NUMBERS
0.1
0.2
0.3
0.4
UNIT OF ACTIVITY

3.3.18 Estimation of protease activity following exercise

Trace of plasminogen activator are present in normal plasma and increased amounts can be detected after exercise, emotional stress surgical operations and other trauma, adrenaline and nicotinic acid injection (Sawyer et al, 1960).

The mechanisms controlling the release of plasminogen activator from the endothelial cells have been reviewed (Cash, 1978) Parenterally administered adrenaline elicits a release of plasminogen activator. This may be caused by interaction with peripheral adrenergic receptor sites at the endothelial cell level but also in part by the central release of vasopressin like substances which stimulate plasminogen activator release (Cash, 1972).

It was thus decided to investigate the level of acid protease release induced by both strenuous and gentle exercise.

A "resting" specimen was obtained from a normal healthy male volunteer who then ran approximately one mile as fast as possible. Two further specimens were obtained from the same volunteer the following day, pre and post a leisurely cycle.

The protease values were found to increase from a resting level of 0.381 to 0.485 following the strenuous exercise and from 0.375 to 0.403 following the cycle.

3.3.19 Estimation of tissue acid protease content

Since previous observations had shown enzyme levels to be increased following disruption of the vascular endothelium it was felt pertinent to attempt some form of tissue extraction involving vascular tissue and samples from a selection of other tissues. These findings led to the following protease estimations after crude tissue extraction (2.17.9).

Results are detailed in Table 3. The activity found was corrected to the equivalent found in 0.2 ml tissue juice.

3.4 Purification of plasma enzyme

3.4.1 Blue sepharose column chromotography

Two plasma specimens which had previously been found to contain elevated acid protease activity (0.6 units and 0.58 units per 0.2 ml plasma respectively) were selected for partial purification, only 1.4 ml specimen I (SI) and 1.9 specimen II (SII) were available. Plasminogen was removed by passage through a lysine-sepharose affinity column (2.14.1.2), the eluates were tested for the presence of any residual plasminogen (2.6.1) none were found. The plasmas were brought to 25% - 75% ammonium sulphate cut (2.16.1) 1.3 ml SI and 1.5 ml SII from the above fractionation were dialysed against 0.9% (w/v) NaCl for two hours with four changes of NaCl. Both SI and SII were then dialysed overnight against 0.05 M Tris/HCl pH 8.0.

2.0 ml SI and 2.5 ml SII were applied to blue-sepharose columns as 2.16.2 to remove α_2 macroglobulin (Virca et al, 1978).

As the results show (figure 19) fractions that did not bind to the column contained proteolytic activity. The specific activities for SI (or SII) of the above steps are given in TABLE 4

Table 4 PURIFICATION OF PLASMA ENZYME

Procedure	Volume (ml)	Protein mg/ml A_{280}	Total Protein	Units enzyme/ ml	Total Enzyme	Specific activity
Blue-Sepharose column	2.0	4.58	9.16	17	34	3.7
DEAE cellulose	2.0	0.22	0.44	0.60	1.2	2.7

Table 3. Tissue acid protease activity (equivalent to 0.2 ml tissue juice)

Tissue	Protease activity
Liver	0.360
Lung	0.029
Kidney	0.052
Spleen	0.034
Atheroma - scrapings	0.102
Atheroma - plaques	0.082
Inferior vena cava	
(1) full thickness	0.367
(2) surface endothelium	8.48

3.4.2 DEAE cellulose

The fractions found to have enzyme activity following the Blue-sepharose column-chromatography were each pooled (SI, SII). 3.0 ml of each was applied to a DEAE cellulose column which had been equilibrated with 0.05 M Tris/HCl pH 8.0 buffer as 2.16.3. The samples were now eluted with 0.05 M Tris/0.05 M phosphoric acid pH 3.8.

Three similar protein peaks were observed with each sample. Sample I is illustrated in fig. 20 where the majority of the activity was found between pH 3.5 and pH 5.0 (and between pH 3.5 and pH 4.5 for specimen II).

3.5 Estimation of plasminogen-activator content of the partially purified acid protease

Human fibrinogen plates were prepared as described (2.7), 25 μl. of each of the samples SI and SII from the DEAE column fractions (3.4.2) were applied to the plates.

A euglobulin fraction was prepared from a donor plasma with elevated protease activity and also applied to the plates as was 25 μl. euglobulin fraction from a normal donor. The acid fibrinolytic activity was 0.06 units/ml for the euglobulin fraction and 0.6 units/ml for the partially purified preparation.

If plasminogen activator was present in any of these samples the plasminogen in the fibrin clot would be converted into plasmin and result in lysis of the plate.

After 24 hours incubation at 37°C the plates were examined for lysis, the euglobulin fractions from the normal plasma had produced an area of 160 mm^2 and from the donor sample showing elevated acid protease activity an area of 170 mm^2. The partially purified samples SI and SII produced lysis over an area of 42 mm^2 and 48 mm^2 respectively.

This test was repeated using plasminogen free fibrinogen substrate (a commercial reagent previously checked for the absence of plasminogen as described 2.14.3). Neither of the partially purified enzyme samples SI and SII showed an appreciable area of lysis. No lysis was observed with either euglobulin preparation.

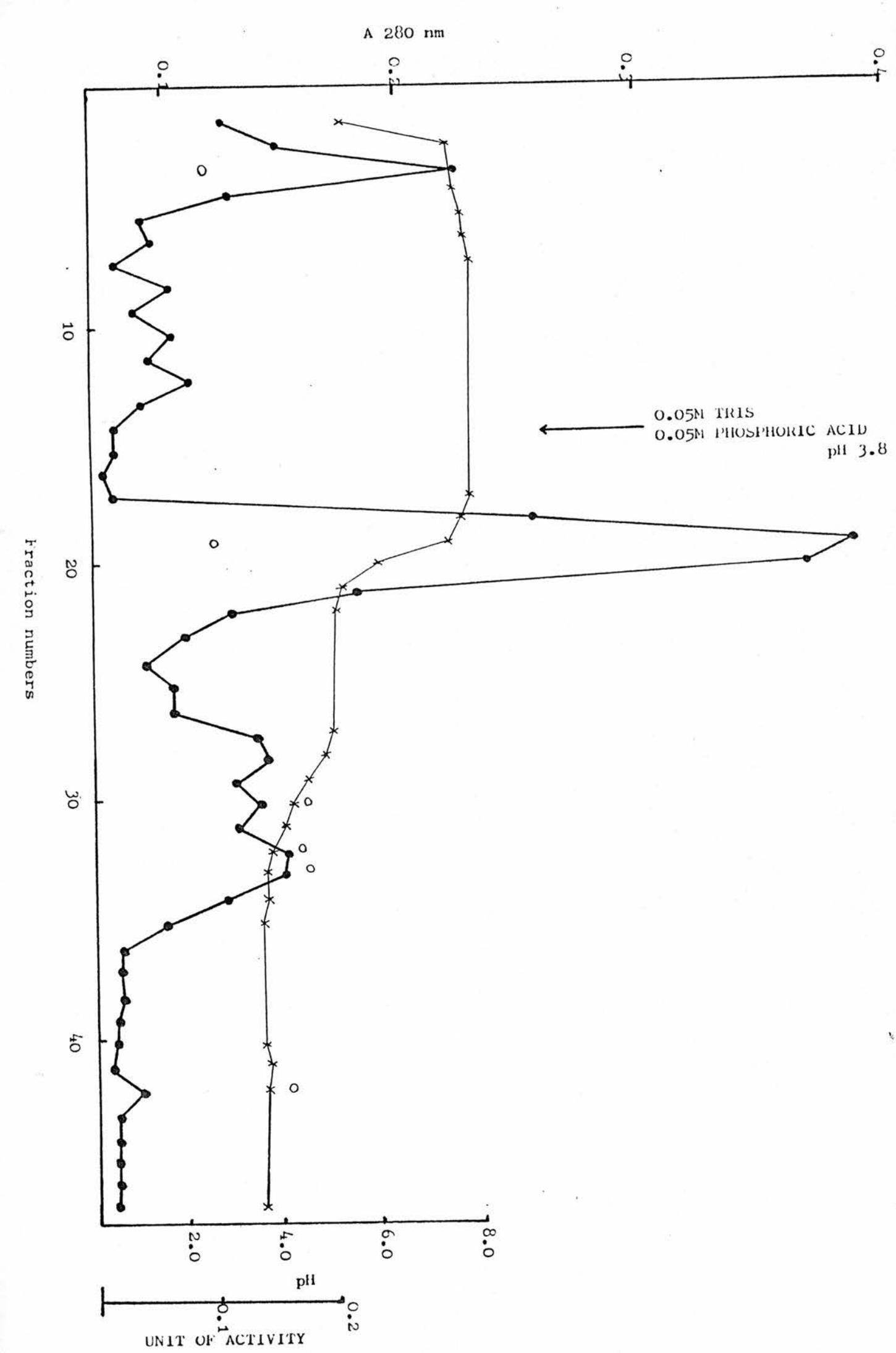
Initial investigations (3.5) have shown the partially purified DEAE fractions showing acid protease activity to contain only slight plasminogen activator activity.

Figure 20 DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PLASMA ENZYME

—●— absorbance at 280 nm plasma enzyme.

—x— pH of eluate.

o plasma enzyme proteolytic activity.


A 280 nm
0.1
0.2
0.3
0.4
10
20
30
40
Fraction numbers
0.05M TRIS
0.05M PHOSPHORIC ACID
pH 3.8
2.0
4.0
6.0
8.0
pH
0.1
0.2
UNIT OF ACTIVITY

CHAPTER 4

Discussion.

DISCUSSION

Aspartyl proteinases embrace a wide variety of acid proteinases present in nature, including pepsin, chymosin, gastricsin and renin, proteases in seminal plasma and spermatozoa, blood cells and tissues and those active in micro-organisms (especially moulds) and plants. Proteases have been identified in the cellular components of blood, erythrocytes, leucocytes and platelets. These 3 cell lines are observed in intimate association with fibrin deposits in two fundamental biological processes. Within the confines of the vascular compartment, leucocytes frequently accumulate in complex thrombi consisting of fibrin, platelets and erythrocytes, whereas in extravascular sites the migration of leucocytes to join platelets and red cells at sites of injury where fibrin has accumulated is a central feature of the inflammatory response,(Plow and Edgington, 1975).

Proteases with the ability to digest fibrinogen, pH optimum 6.8 (Nachman and Ferris, 1968) have been identified in the intracellular granules of platelets. This activity was termed cathepsin A.

Various activities have been reported as cathepsin D with the estimated molecular weight varying from 40,000 - 60,000. These include an enzyme bound to erythrocyte membranes and having a molecular weight of 55,000 with maximum activity at pH 2.8 against denatured haemoglobin. This enzyme was inactive against denatured albumin and the synthetic substrates specific for cathepsins A, B and C (Reichelt et al, 1974). The enzyme was also inhibited by dithiothreotol but not by ethylenediamine tetra acetic acid or metal ions. Cathepsin D has also been identified from human articular cartilage (Lapolsky et al, 1974).

In 1980, Barrett and McDonald reported the generally accepted molecular weight of

cathepsin D to be approximately 42,000. They concluded that cathepsin D was located in the cytoplasmic lysosomes and red blood cell membrane and that its optimum pH against its most sensitive substrate, haemoglobin, was 3.0.

Barrett and McDonald (1980) also reported the presence of another cathepsin in bone marrow and polymorphonuclear neutrophils, macrophages and platelets. They termed this activity cathepsin E, identifying it with a molecular weight of 100,000 and an activity peak at pH 3.5 against haemoglobin.

Pejhan (1984) concurred with Law (1979) that the enzyme observed was indeed not pepsin as originally concluded by Ikemori (1975). She based this conclusion on the difference in fibrin-clot digestion products, their differences in electrophoretic mobility on gradient and haemoglobin gels and that its elution from an amberlite column occurred at a different pH. In addition, fibrin clots proved to be more sensitive to this enzyme while pepsin was most active against haemoglobin.
The possibility that the enzyme observed was in fact renin was also excluded because of its higher pH optimum (6.5) and the fact that it was not bound by an Hb-Sepharose column at pH 3.5. Both bovine fibrin and haemoglobin proved to be acceptable assay substrates for the enzyme, whereas bovine fibrin is a weak inhibitor of renin and haemoglobin a potent inhibitor (Workman et al, 1974).

Neither Law (1978) nor Pejhan (1984) achieved a complete purification reporting two bands on SDS PAGE with molecular weights of 54,000 and 90,000. Law (1978) postulated that the activity was associated with the 54,000 component, but recognised that her preparations were contaminated with albumin which she was unable to separate.

The assay used in the present study was that established by Pejhan and Kemp (1984) as the most sensitive available at present for the demonstration of the plasma

protease. The technique is rather cumbersome and time consuming especially when assaying large numbers of samples, although results do indicate that the enzyme has some specificity for fibrin. The degree of fibrin cross-linking was critical in the present technique.

For maximum sensitivity it was important that the clot was not extensively cross-linked by factor XIII action. Conversely it was equally important that there was sufficient cross-linking to ensure that the fibrin remained insoluble in the mono-chloroacetic acid in the absence of enzyme. The cross-linking is dependent on the amount of factor XIII and prothrombin in the crude f ibrinogen preparation and these levels did vary from one batch of bovine plasma to the next. The extent of cross-linking could be decreased by shortening the incubation time following the addition of $CaCl_2$ or increased by decreasing the amount of pH 9.0 Tris/HCl /NaCl lysine buffer added to the crude fibrinogen. These two parameters were varied to achieve the desired degree of cross-linking which was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of fibrin reduced by 2-mercaptoethanol (Lawrie and Kemp, 1979). It was found that fibrin still possessing monomeric γ-chain was not sufficiently cross-linked while an $\alpha : \beta$ ratio of less than 0.25 was too resistant to proteolysis (Pejham and Kemp, 1984).

The above observations are similar to those of Sakata et al (1984) concerning the binding of plasminogen to cross-linked and non cross-linked fibrins becoming critical for the subsequent development of fibrinolysis.

The technique used has also been found to be very sensitive to the presence of NaCl. Maximum swelling of the substrate occurs at the assay pH, the presence of any NaCl in the surrounding medium increased the external ionic strength and therefore reduces the osmotic effect thereby limiting the number of bonds available for

proteolysis.

The plasma enzyme was not found to be adsorbed to artificial surfaces as demonstrated by no decrease in levels when collected into a plain glass bottle. This is in contrast to many proteases which are adsorbed rapidly to glass surfaces from whole blood (Brash and Lyman, 1969). Though often only transiently, as they are then released in a denatured form (Lee and Mairston, 1971). Surfaces that are hydrophobic adsorb more protein than do hydrophilic surfaces (Hoffman, 1974). In haemostasis factors XII, XI, VIII and fibrinogen show the greatest attraction. (Bagnall, 1978)

Heparins are a family of straight-chain anionic polysaccharides (glycosamino glycuronan sulphate esters) of highly variable molecular weight averaging 9000 - 15000 but extending from 4000 - 40000 (Johnson and Mulloy, 1976). Heparin inhibits the earlier serine proteases in the clotting cascade, together with its cofactor anti-thrombin III, it has been shown to inactivate factors XIa, IXa, Xa and thrombin. When used in the collection of specimens for plasma enzyme assay heparin at a concentration of 14.3 USP ml^{-1} appears not to inhibit the enzyme in its ability to digest fibrin clots. The plasma enzyme appears to be negatively charged as demonstrated by its strong binding to the DEAE cellulose column, heparin also has its functional groups negatively charged (possessing amino sulphate and carboxyl groups - Barrowcliffe et al, 1978). In this respect, the plasma enzyme is similar to most biological surfaces and cells such as endothelial cells bearing a negative charge (Copley et al, 1980) and to many clotting factors and to protein C.

Another anticoagulant, ethylene diamine tetra acetic acid, routinely used in the collection of blood inhibits both serine and metallo proteinases (Barrett, 1977) was observed to have no effect on the activity of the plasma enzyme when used in the collection of specimens. The plasma enzyme was found to maintain its activity

when stored for upto 24 weeks at -20 °C, but rapidly lost its proteolytic activity when stored at room temperature for more than 1 hour falling to 50% activity after 4 hours at this temperature. This could possibly be due to the action of natural inhibitors or adsorption onto suitable substrate molecules.

Only limited conclusions may be drawn from kinetic studies employing an enzyme in its impure form. The fact that the substrate used in this assay was protein presents many variables in the presence of multiple cleavage sites and the creation of secondary substrates and inhibitors as discussed in chapter 3.

When the effect of plasma enzyme concentration was studied, the graph of activity versus enzyme volume showed a linear increase in activity with increasing enzyme concentration followed by a plateau. When used at 50% concentration, the graph showed a similar levelling off - this plateau may be due only to substrate exhaustion, although the presence of a dissociable inhibitor cannot be excluded. If an inhibitor is present and it is of low molecular weight then it could possibly be removed by dialysis. The main purpose of this work was to extend the work of Ragaz et al (1976) using the fibrin clot technique described by Pejhan and Kemp (1984) in order to establish plasma acid protease levels in the normal population and to investigate selected sub-populations.

The results show that the range of values within the normal population is wide and shows a non-Gaussian distribution. Although the distribution is not normal, the distribution of the means of the samples taken from this population is normal since such a large number of samples (185) were examined to establish the range.

When comparing a selected sub-population with a normal population showing such a non-Gaussian distribution it is not always justified to use a parametric test of significance such as Students t-test. If the ratio of the variances of the two

populations to be compared was outwith the range of 0.25 - 4.0, then the non-parametric Mann-Whitney test was used.

When plasma samples from pregnant women were examined for acid protease activity, the level was found to be significantly greater ($p < 0.001$) than the levels found in the normal population. No increase was observed upto the 6th week, but this was followed by a steady rise to maximum levels in the 3rd trimester, no immediate decline in activity was found upto 3 days post partum. Certain physiological changes of pregnancy predispose to venous thrombosis. The overall effect of pregnancy is to increase the activity of many of the coagulation factors with the exception of factors XI and XIII and to lower slightly the coagulation inhibitors antithrombin III and anti Xa. Overall, the changes in the coagulation system during normal pregnancy are consistent with local activation resulting in a compensatory increase in the synthesis of fibrinogen and other clotting factors that exceeds the rate of consumption. Using electron-microscopy, fibrin deposition can be readily demonstrated in the walls of the spiral arteries supplying the placenta (Bonnar and Sheppard, 1977). Fibrinolytic activity in contrast decreases markedly during pregnancy remaining depressed during labour and delivery and rapidly returns to normal within 30 - 60 minutes of delivery (Bonnar et al, 1970).

Cigarette smoking was shown to significantly decrease ($p < 0.001$) the protease levels compared to the normal population although no relationship was found between the level of activity and the number of cigarettes smoked per day nor between male and female smokers. Jarzon and Nilsson (1975) found the fibrinolytic pathway to be stimulated by smoking and suggested this as an explanation for the observation by Handley and Teather (1974) that smokers apparently suffered no increased risk of venous thrombosis and pulmonary embolism. Meade et al (1976) found no increase in fibrinolytic activy, fibrinogen, factors V or VIII levels in his female smokers

but a significantly higher mean in his male smokers.

There is little doubt that some women taking oral contraceptives run an excess risk of thromboembolic disease and that the risk rises with increased oestrogen dose (Inman et al, 1970). Oral contraceptives have been found to significantly increase fibrinolytic activity (Meade et al, 1976) he also found increased levels of prothrombin and factors VII and V, although the mean antithrombin III level was significantly lower during oral contraceptive usage. A possible interpretation is that the increased fibrinolytic activity counteracts the potentially harmful effects of raised clotting factor levels. In this study, the use of oral contraceptives, was found to significantly decrease ($p < 0.001$) the plasma protease levels compared to normal - this is possibly due to adsorption of the enzyme onto any existing micro-thrombosis. The decrease was not affected by cigarette smoking, similarly Meade et al (1976) found the elevation in fibrinolytic activity observed in oral contraceptive users to be unaffected by smoking.

Schamberge et al (1972) found an increase in fibrin clot stability in patients taking oral contraceptives especially of the combined type which they explained in 1973 (Ikemori et al) as being due to their lacking a proteolytic activity present in non oral contraceptive users. They concluded that this increased stability reflected a decreased serum pepsinogen level.

When the local diabetic population was examined for acid protease activity, no significant difference was observed between established diabetics and the normal population. This differs with the coagulation system where most investigators have found the coagulation factors to be elevated. There is no such agreement about the fibrinolytic system, some workers have found an abnormally low activity (Fearnley et al, 1963), others, an abnormally high activity (Cash, 1969). Almer et al (1975) found a decreased release of plasminogen activator from endothelial cells

and vessel walls.

The levels found in patients on Marevan therapy are significantly less ($p < 0.001$) than those in the normal population. There was no significant correlation however between the drug dosage and the enzyme activity, this might suggest that the lower levels are a response to the underlying problem rather than the treatment. Marevan is essentially used as an antithrombotic agent. Plasma enzyme levels were estimated in two conditions where thrombosis had occurred namely pulmonary embolus and deep venous thrombosis. Levels in both these conditions were significantly less ($p < 0.001$) than in the normal population. It is possible, because the diagnosis of these two conditions is often made upto thirty-six hours after the incident, that the enzyme levels shortly after thrombus formation may have been even lower.

Significantly, lower levels ($p < 0.01$) were also associated with chronic alcohol abuse.

Many of the serine proteases and haemostatic factors associated with the coagulation system are also depressed in alcohol induced liver disease. These include factors II, V, VII, IX, X, XI, XII, XIII, Plasminogen and α_2 antiplasmin, whereas levels of plasminogen activator are increased during this period, acarboxy forms of factors II, VII, IX and X are found in the plasma of such cases, these cannot bind calcium and consequently cannot participate in normal haemostasis (Suttie, 1978)

In an effort to pin-point the possible hepato-cellular origin of the plasma enzyme patients with various forms of liver dysfunction were subjected to an array of liver function tests. Many of these tests are known to have a significant correlation with depletion of clotting factors in particular albumin levels show a significant relationship with levels of factor II ($p < 0.01$), factor VIII ($p < 0.01$),

factor V ($p < 0.05$), and antithrombin III ($p < 0.01$), (Lechner, 1977.) However, possibly due to such a wide range of pathologies, no significant differences in values were found between these and the normal populations in respect of the enzyme studied here. Paracetamol is known to be selective in its toxic effects on the liver, specifically damaging the parenchymal cells. Several cases of severe paracetamol poisoning were tested, but no significant difference from the normal population was observed. The liver enzyme aspartate transaminase is released into the circulation in severe paracetamol poisoning, no correlation could be formed between levels of transaminase and plasma enzyme. The above results would suggest that the plasma enzyme is not produced in the liver.

An unusual bleeding disorder clinically resembling factor XIII deficiency was reported by Ragaz et al (1976) in a female patient with a history of easy bruising since early childhood. In this patient, post-operative recovery was also complicated by prolonged haemorrhage and delayed scar formation. Factor XIII levels, however, were normal and the only detectable abnormality was an increased activity of a pepsin-like protease in the plasma. The presence of an acid protease in normal human plasma, capable of dissolving fibrin, had been previously reported by Schanberge et al (1972) and Ikemori et al (1975). Results within the group with haemostatic abnormalities of unknown aetiology confirmed the single finding of Ragaz et al (1976) that certain individuals can have an elevated level of protease associated with a long history of bruising and bleeding. The range of values within this category is, however very wide and the overall mean is lower than normal ($p < 0.01$). It is felt that this probably reflects the diverse nature of the clinical symptoms referred to the department for investigation, all subjects with histories suggestive of haemostatic disorder, but only a relatively low percentage in which an actual abnormality could be identified. Nevertheless, many subjects with significantly elevated protease levels were identified, the

TABLE - PLASMA ACID PROTEASE LEVELS OBSERVED IN VARIOUS "POPULATIONS"

Population examined	Number of subjects	Mean enzyme activity ± 2 standard deviations	Increased or decreased in activity compared to the normal population	significance compared to the normal population
Normal	202	0.351 ± 0.158		
Cigarette smokers	60	0.199 ± 0.104	decreased	$p < 0.001$
Pregnancies	125	0.40 ± 0.07	increased	$p < 0.001$
Diabetics	20	0.342 ± 0.144	no difference observed	-
Bruisers and Bleeders	48	0.310 ± 0.2	decreased	$p < 0.001$
Marevan therapy	60	0.290 ± 0.16	decreased	$p < 0.001$
Deep venous Thrombosis or Pulmonary embolism	6	0.228 ± 0.182	decreased	$p < 0.001$
Alcoholics	29	0.310 ± 0.18	decreased	$p < 0.01$
Liver disease	46	0.366 ± 0.226	no difference observed	-
Paracetamol overdose	20	0.342 ± 0.102	no difference observed	-
Oral contraceptive therapy	30	0.168 ± 0.062	decreased	$p < 0.001$

increase appears not to be transitory as it was possible to retest many of these individuals over a period of time. It was also possible in several cases to test other family members and no familial occurrence was found. A table showing the enzyme levels found in the previously described populations has been constructed - Table 5.

An acid protease with an optimum pH of 2.5 was first described in human seminal plasma as pepsin and pepsinogen (Lundquist and Seedorff, 1952). Ruenwongsa and Chulavatnatol (1977) purified the protease and its proenzyme proving it not to be pepsin or pepsinogen although its molecular weight and amino acid composition (335 residues) is similar to that of human pepsin (337 residues).

An acidic protease with a pH optimum of 2.8 has also been partially purified from the acrosomes of spermatozoa, (Polakoski et al 1973).

These proteases could hydrolyse both haemoglobin and N, N' dimethyl casein, but neither ovalbumin nor bovine serum albumin and were inhibited by both 1, 2 - epoxy-3 - (p-nitrophenoxy) propane and by p-bromophenacyl bromide. The levels found were significantly greater ($p < 0.001$) than those present in the blood plasma of the normal population. Ruenwongsa and Chulavatnatol (1977) concluded that the proenzyme was present in normal semen, activation occurring in the acid vaginal environment where the protease was an essential pre-requisite to sperm penetration through the cervical mucous.

In this study, acid protease activity was found in seminal plasma from both fertile and infertile men and in spermatozoal concentrates. The levels found in stored blood, both plasma and red blood cell lysates, were found to be significantly increased ($p < 0.01$ and $p < 0.001$ respectively). Protease activity following transfusion of both whole blood and concentrated red cells were also found to be significantly increased ($p < 0.001$) over the plasma levels of the normal population,

this confirms the observations of Ragaz et al (1976) who found increased clot solubility following "massive" transfusions. Ragaz et al (1976) postulated that this activity was possibly due to red cell cathepsin-D. Cathepsin-D has been shown to have activity toward fibrin clot (Pejhan, 1984), although it is less active than plasma enzyme toward this substrate. Plasma enzyme also differs from cathepsin-D as it has a molecular weightof 54,000 while cathepsin-D molecular weight is approximately 42,000. Obviously, further characterisation of the enzyme responsible for this fibrinolytic activity present in both red blood cells and plasma must be undertaken to exclude the possibility that cathepsin-D is responsible for this activity.

In an attempt to determine if the observation made by Ragaz et al (1976) of decreased clot solubility following transfusion was possibly due to the initial blood loss, two conditions were examined. Chronic blood loss showed no significant change in plasma enzyme activity than that in the normal population, whereas acute blood loss showed a significant ($p < 0.001$) increase in protease activity.

As suggested (3.3.15) this observation must now be validated by an experiment designed to ensure that the enzyme activity is normal until the acute blood loss occurs.

Damage to the endothelium as witnessed by major vascular reconstruction also showed a significantly ($p < 0.001$) elevated result. Although this experiment demonstrates that protease activity is significantly increased in association with endothelial damage other factors as described (3.3.16) could possibly interfere with this conclusion and a model demonstrating only endothelial damage must be sought.

Physical exercise is known to increase the release of several coagulation factors together with endogenous plasminogen activator and fibrinogen. During exhaustive physical exercise a very marked increase in the Euglobulin fibrinolytic activity was observed (Coller and Verstraete, 1978)

In this study, the plasma acid protease activity was seen to increase significantly ($p < 0.001$) during severe exercise from that of the normal plasma level.

Initial investigation into the acid proteolytic activity of various tissues revealed vascular surface endothelium to contain a much greater activity than any of the other tissues examined.

Previous observations in this study have suggested similarities in the release of the plasma acid protease and that of plasminogen activities.

The activators of plasminogen are distributed widely throughout the body being found in most body tissues and fluids including blood, urine, saliva, tears and cerebro-spinal fluid. With the exception of the liver, all human tissues contain plasminogen activators, (Davidson and Walker, 1981) and these are released during exercise, surgery, vascular disease and thrombotic states. Plasminogen activators have been isolated from polymorphonuclear neutrophils and red blood cell lysates, but not from platelets (Plow and Edgington, 1975). Activation of plasminogen can be achieved by a number of activator pathways usually classified into two groups.

(a) The intrinisic activators - It appears that 3 pathways exist, two of these require factor XII, one of these two involves prekallikrein (Fletcher factor) and high molecular weight kininogen (Fitzgerald factor), Kaplan et al (1976). The other intrinsic pathway is relatively unclear, but has been described in preparations of Hageman factor cofactor (Kluft, 1978).

(b) The extrinsic plasminogen activators are generally assumed to originate from the vascular endothelium - vascular activator. This activator is related to the activator released by post-mortem vessel profusion (Aoki and Von Kaulla, 1971).

Most estimates of plasminogen activators have been reported using non-specific methods which at best measure mainly extrinsic activator, but also measure undefined amounts of intrinsic activator. Kluft (1978) has shown it possible to define the proportion of plasma plasminogen activation due to extrinsic

and Von Kaulla, 1971)

Most esfimates of plasminogen activators have been reported using non-specific methods which at best measure mainly extrinsic activator, but also measure undefined amounts of intrinsic activator. Kluft (1978) has shown it possible to define the proportion of plasma plasminogen activation due to extrinsic activators by using C_1 inactivator to inhibit the intrinsic pathways. The extrinsic plasminogen activator is a serine protease, (2 chain molecular weight 31,000 and 33,000) connected by disulphide bridges, the small chain contains the serine active site. This enzyme is inhibited by phenylmethane sulphonyl fluoride (an inhibitor of serine and thiol proteases) in contrast to the acid protease upon which it has no inhibitory effect, it is also inhibited by dithiothreotol, but not by iodoacetamide, the trypsin inhibitor TLCK or epsilonamino caproic acid (Pepper and Allen, 1978).

A life long haemorrhagic disorder has been reported by Booth et al (1983), characterised by haemorrhage occurring after surgery, injury and dental extractions and finally by spontaneous intracerebral bleeding, due to excessive production of plasminogen activator and not by a deficiency of any known inhibitor of fibrinolysis as described by Aoki et al (1979).

Initial investigations (3.5) have shown the partial purified DEAE fraction to contain slight plasminogen activator activity on fibrin plates, but in view of the above observations that it is inhibited by phenylmethane sulphonyl fluoride, it seems unlikely that the plasma enzyme is either of the previously mentioned well characterised plasminogen activators but may well operate via plasminogen. Nevertheless, it will be of interest to see if the enzyme has any activity against the chromogenic substrates S-2444 (H-D-Val-Leu-lys-pNa) which is a sensitive and specific substrate for plasmin, plasminogen activator and streptokinase-activated plasminogen and S-2251. Both these chromogens are soluble and stable substrates being sensitive to very small amounts of the enzymes, S-2251 is sensitive to 10^{-11} moles of plasmin (Friberger et al, 1978). The pH optimum of plasma enzyme being approximately 2.5 - 3.5 makes it rather difficult to relate its role in physiological conditions, although it is not greatly different from the optimum pH's of the various cathepsins.

The possibility of a proenzyme has still to be excluded, indeed in the

DEAE-cellulose purification steps (fig. 20) an active fraction was found to elute much later than the main fractions. If this was a zymogen, it could be that it exists in this form at physiological pH only becoming active once incorporated into a thrombus where the pH within the clot may well be lower than that of the plasma.

Another explanation, of course, is that the pH profile observed in this study where a fibrin clot substrate has been used is in fact, largely due to the optimum swelling of the substrate at such a low pH making available a maximum number of susceptible bonds, It is possible the activity of the enzyme when observed against a non-protein substrate such as a chromogen may well spread from the low pH demonstrated to that approaching a more physiologically acceptable level.

Work is presently being undertaken to establish more sensitive techniques including the investigation of the chromogenic substrates S-2444 and S-2551. A method was investigated which utilised the incorporation of the non-mammalian enzyme urease within fibrin layers bound to a cellulose plate, the principle being that any fibrinolytic agent would release the urease which, being bound to a blue dye, would be measurable spectrophotometrically - unfortunately, non-specific dye release occurred with fibrin swelling at the acid pH.

These results agree with many current reports within the literature that the fibrinolytic system is a multitude of enzymes and proenzymes all acting in a great orchestra of proteolytic events concerned with the physiological dissolution of fibrin. Within this concept, the importance of acid proteases should not be ignored.

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