FIBRINOLYSIS BY BILE

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University of Cape Town

by

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for David, who helped with the shaping
and Marguerite, who gave it a frame

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FIBRINOLYSIS BY BILE

ABSTRACT

A protease has been found in the bile of 11 mammalian species investigated. The protease, given the tentative name of cholelysin, has been studied intensively in Abattoir ox bile. It makes up less than 10% of the ox bile protein, and is a potent fibrinolysin, as well as being active against the substrates α-casein, and the synthetic esters of tyrosin (ATEE) and arginine (BAEE). It is inactive against trypsinogen, chymotrypsinogen and plasminogen.

Isolation and purification of this protease from ox bile proved complex, and was finally achieved by an 8 step procedure which yielded a dry white powder, stable for 45 months (to date) at $4^{o}C$. Quality control of this procedure was effected by means of a fibrin plate assay, using chymotrypsin as a reference standard: the dose response curves of cholelysin and chymotrypsin were closely similar on the fibrin plate, enabling cholelysin (units/ℓ) to be substituted for chymotrypsin (mg/ℓ), in equivalent diameters of fibrinolysis.

Gradient elution by tris/NaCl from Whatman DE 32 produced four areas, or Peaks, of fibrinolytic activity of cholelysin, each with some differing characteristics against the various substrates. These complexities were not studied in detail, and a simplification of the procedure was discovered, using batchwise elution with tris buffer. Thereafter, only the first peak was studied (Peak I).

Studies on the inhibition of cholelysin were done using many known inhibitors, including serum of man, and 4 laboratory animals; serum of two patients with homozygous deficiency of α1-antitrypsin; α2-macroglobulin, soy bean trypsin inhibitor, and aprotonin. The serum studies were done with heated and unheated material; platelet-rich and platelet-poor plasma

were also studied. Serum was fractionated by paper electrophoresis in an attempt to discover the globulin fraction containing the inhibitor. No inhibition was found in the α-globulin fractions, and inhibition was maximal in the inter-α-globulin and β-globulin fractions. ATEE-esterase activity of cholelysin was inhibited by serum as stongly as fibrinolytic activity.

A limited series of studies of coagulation was done; cholelysin was only found to influence fibrinogen, being quite strongly fibrinogenolytic in vitro. A slight effect on ADP - induced aggregation of platelets was found at a low ADP concentration. Using a rat model originally devised for the study of the anticoagulant effect of ancrod, cholelysin was found to be weakly anticoagulant. The dose was low by comparison with ancrod, and the result approached, but did not reach, statistical significance.

The split products of plasmin and cholelysin digestion of stabilised fibrin were studied by polyacrylamide gel electrophoresis (PAGE), and these were found to be entirely different. The kinetics of the reaction between cholelysin and fibrin were studied by means of a new technique (the composite cuvette method) and by this method it was shown that cholelysin made a two-phase attack on the fibrin molecule. The attack was studied at 2, 10, and 30 minutes following commencement of fibrinolysis, and the biphasic nature of the attack was proved by a sharp increase in the number of reaction products present between the 2 and 10 minute samples. The molecular weight of fibrin split products by plasmin were shown to agree with those found in published work.

Finally, the molecular weight of cholelysin was estimated by PAGE and by column chromatography with and without SDS. It seems probable that the basic molecular weight is ~ 7 000, with a dimer of ~ 13 000 and a tetramer of ~ 28 000.

CHAPTER ONE

INTRODUCTION

Through the millennia of evolution, the mammalian body has evolved a complex system for immediate defense and repair of the tissues. The humoral elements of this are made up of four systems of zymogens - coagulation, fibrinolysis, complement, kallikrein-kinin which form an interlinking web to seal off injuries and then make a scaffold on which repair can take place.

In this web, the fibrinolytic system plays many roles, one of the more important of which is to maintain the integrity of small tube systems throughout the body. Thus, activators of fibrinolysis are found to be secreted from the lining cells of, or present in:

renal tract (Macfarlane and Pilling, 1942)
capillaries and veins (Fearnley, Revill and Tweed, 1952)
milk (Astrup and Sterndorf, 1953)
semen (von Kaulla, 1953)
tears (Storm, 1955)
saliva (Albrechtsen and Thaysen, 1955)
cerebrospinal fluid (Albrechtsen, Storm and Classen, 1958)
aqueous humour (Pandolfi, Nilsson, Robertson and Isaacson, 1967)
bile (Oshiba, Hata and Okamoto, 1969).

Intracellular activators of fibrinolysis have also been described, one of which is water soluble (Albrechtsen, 1957), the other extractable with potassium thiocyanate (Astrup and Stage, 1952).

All these activators activate the zymogen, plasminogen, present in the plasma, to plasmin. They are not themselves directly fibrinolytic.

The fibrinolysin described in the monograph differs from all enzymes described above, in being fibrinolytic in its own right. In this it differs from the activator of fibrinolysis described in bile by Oshiba, and listed above (Oshiba, Hata and Okamoto, 1969).

This substance was first observed during experiments in liver transplantation done by Dr Rosemary Hickman in the Surgical Research Laboratories of the University of Cape Town, which were designed to study the viability of the pig liver after a period of 'warm ischemia'. In these experiments, the pig liver was isolated from its circulation by clamps on the hepatic artery, portal vein, and hepatic veins. Because the pig does not readily survive occlusion of the portal vein, a bypass was led between the portal vein and the superior vena cava.

Ischemia of the liver obtained in this way was allowed to continue for up to 60'. During this time, various coagulation studies were carried out (fibrinogen, one stage prothrombin time, partial thromboplastin time, euglobulin lysis tiem). Of these, only the euglobulin lysis time showed a major change; there was a marked and rapid increase in fibrinolysis as measured by this test. At the end of the procedure, restoration of the normal circulation was followed by an equally rapid fall of the euglobulin lysis time to normal levels (Figure 1-1).

This lead naturally to a query as to the fate of the increased lytic activity: was it perhaps excreted into bile? A drop of bile was placed on a fibrin plate (Astrup and Müllertz, 1952) and a large zone of lysis developed. The stu-

dies described in this monograph grew out of this observation. They failed to confirm the presence of an activator of fibrinolysis, but instead, and serendipitously (Walpole, 1937), they revealed an unexpected new fibrinolysin, which has been given the tentative name of cholelysin (Figure 1-2).

Studies of cholelysin extended over some seven years, depending on the availability of staff, space and funding. These studies form the basis of this thesis.

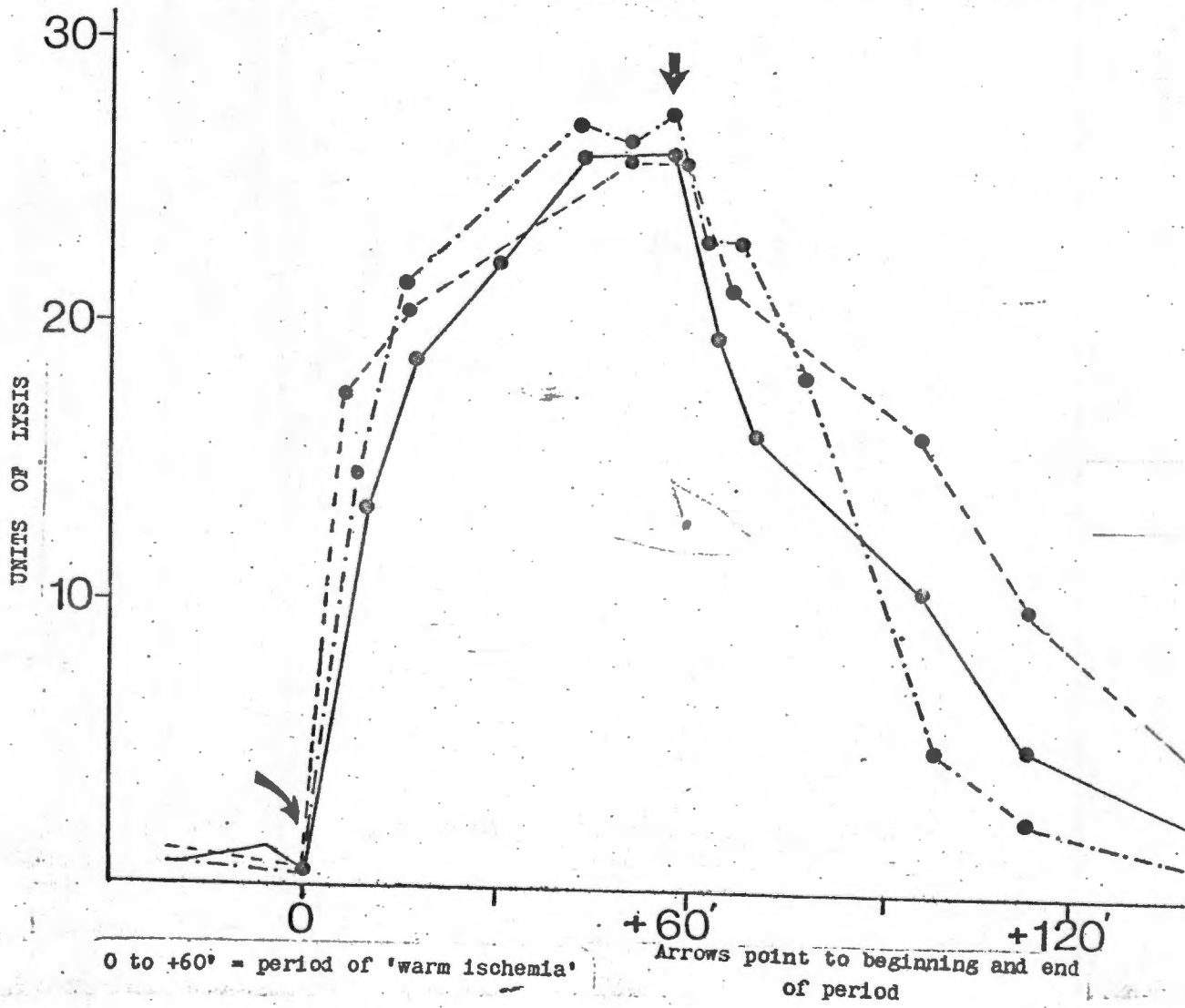


Figure 1-1: Fibrinolytic activity in the pig during the period of 'warm ischaemia'.

$$\text{Units of lysis} = \frac{1\,000}{\text{Euglobulin lysis time in minutes}}$$

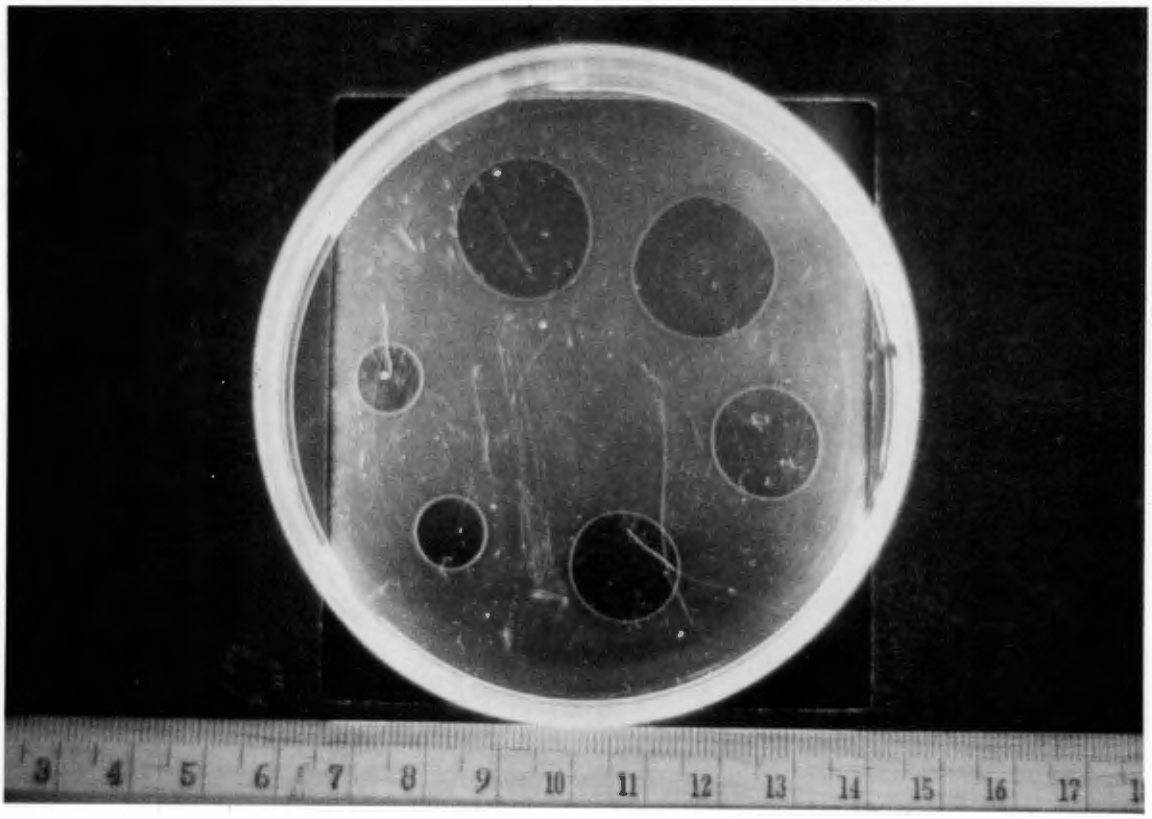


Figure 1-2: Fibrinolysis by three different preparations of bile fibrinolysin (in duplicate) on the standard fibrin plate.

CHAPTER TWO

THE PHYSIOLOGY OF FIBRINOLYSIS

2.1 At this stage, the process of fibrinolysis will be described, as it is understood to occur in man. This will form the background for some of the work described in later chapters.

Introduction

John Hunter (1812) first described the incoagulability of the blood folowing sudden death. The nature of this process remained obscure until Morawitz showed in 1906 that in such cases the blood lacked fibrinogen. He suggested that the fibrinogen had been digested by an enzyme.

The last 50 years have seen a steady expansion of our understanding of fibrinolysis, commencing with Tillet and Garner (1933) who showed that certain haemolytic streptococci produced a fibrinolytic agent, and followed up by Milstone (1941) who showed that this agent had a substrate (plasminogen) in plasma. Investigators too numerous to name have carried on this work. This account is based on reviews by Davidson (1977), Kaplan (1980) and Heimark, Kurachi, Fujikawa and Davie (1980).

2.2 Functions of the fibrinolytic system

These will be listed here without prolonged discussion. This study concerns a fibrinolytic agent that has only been shown to have local activity within the biliary

tree; thus it has no known relevance to fibrinolysis in the body as a whole.

(a) Dissolution of intravascular clot. This applies to sessile clot in veins or arteries, and to microclots occurring during disseminated intravascular coagulation (DIC). When fibrinolysis is less effective (eg pregnancy) or overwhelmed (eg meningococcal septicaemia), microclots may cause major occlusion of the microvascular circulation. Organ failure may follow (acute renal failure, gangrene).

(b) Fibrinolysis in small tube systems throughout the body: this was touched upon in Chapter One. The fibrinolytic system aids in maintaining patency in these small tubes. In the particular case of the capillaries, fibrinolytic activity is at all times higher than in general circulation, and is readily augmented by such stimuli as ischemia, anoxia, exercise, and pharmacological agents such as epinephrine, histamine, nicotinic acid and pyrogens. During any acute episode of defibrination, plasminogen activator activity increases sharply at capillary level (Ashford, Weinstein and Freiman, 1968). It is highly probable that similar considerations apply to other small tube systems; however, dynamic studies on these systems have not been reported yet.

(c) Wound repair. The stimulated macrophage may be a very active producer of plasminogen activator. Conceivably this plays a role in the remodelling of tissues during wound healing (Lind and Gordon, 1979).

(d) Metastasis. There is some evidence that implantation of a malignant metastasis in a blood vessel is more successful if fibrinolysis is increased (Salzbury,

White, Tsolakidis, McKinna and Griffiths, 1973).

(e) Cross-linking between enzyme cascades. There is suggestive evidence that plasmin may initiate complement sequence through C1q (Ruddy, Gigli and Austen, 1972) (Atkinson and Frank, 1977).

2.3 Activation of fibrinolysis

It has long been known that activation of the first factor of the intrinsic chain of coagulation (Factor XII) would lead to activation of

(1) coagulation
(2) fibrinolysis
(3) kallikrein-kinin system.

The activation of plasminogen was thought to proceed via activation of a plasminogen proactivator present in plasma, which was converted by activated Factor XII (XIIa) to plasminogen activator (Figure 2-1) (Fearnley, 1952).

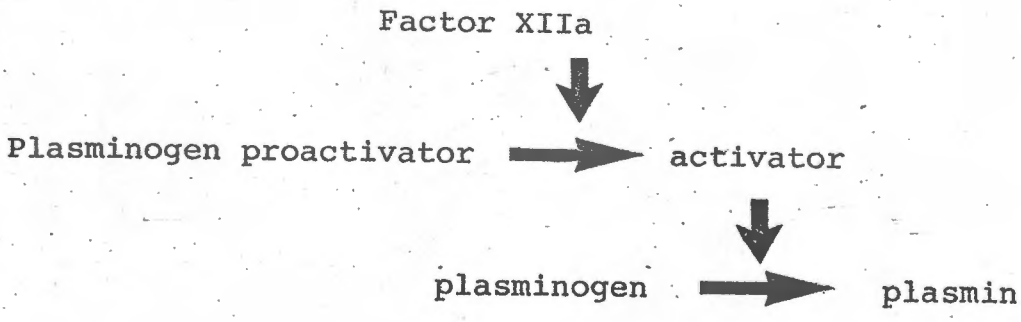


Figure 2-1: Early concept of the activation of plasminogen in plasma.

Later work has shown a more complex picture. One plasminogen activator present in circulating plasma - probably the major one - has been identified as prekallikrein (Mandle and Kaplan, 1977a) though other activators exist (Mandle and Kaplan 1977b). Initiation of the enzyme cascade leading to fibrinolysis can be attributed to Factor XIIa alone in the presence of kaolin (Heimark, Kurachi, Fujikawa and Davie op cit, 1980). The enzyme cascade proposed by these authors is as follows (Figure 2-2).

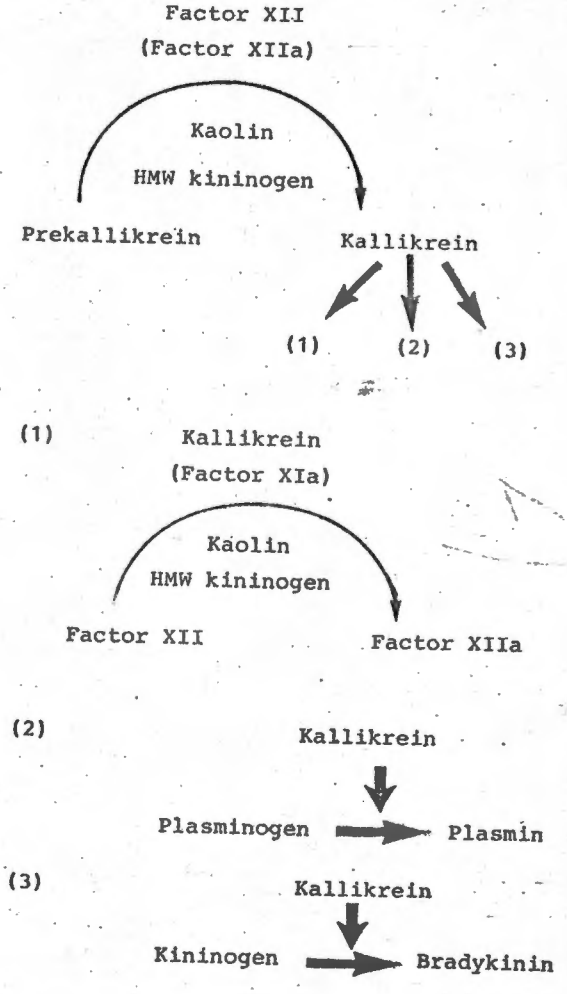


Figure 2-2: Generation of plasmin following interaction between kallikrein and Factor XII (from Heimark, Kurachi, Fujikawa and Davie, and Davie op cit, 1980).
HMW kininogen = high molecular weight kininogen, a catalyst in this reaction.

Other known activators are:

Factor XIa (weak)

Streptokinase derived from beta-haemolytic streptococci (Tillett and Garner, 1933; Milstone, 1941). This activates plasminogen to form a streptokinase-plasmin complex which itself acts as a powerful activator of plasminogen (Derenzo, Barg, Boggiano, and Buck [illegible], 1967).

Urokinase secreted by the kidney into the renal tubules, where it activates plasminogen to plasmin (Sgouris, Inman and McCall, 1960) (Lesuk, Terminiello and Traver, 1965).

Endopeptidases eg trypsin, thrombin and plasmin. These probably have little physiological effect, being strongly inhibited by circulating inhibitors. They may play a part in inflammation, but not otherwise (Sherry, 1977).

Tissue activators, referred to in Chapter One.

These enzymes all activate plasminogen to plasmin. It appears that physiologically this activation should be regarded as two phase (Sherry op cit, 1977 p 1297):

(i) sol phase: In circulating plasma, activator and free plasmin are rapidly inhibited by alpha 2-plasmin inhibitor and alpha-2-macroglobulin. A portion of the plasmin bound to alpha-2-macroglobulin is slowly released, and can exert a lytic effect on formed fibrin.

(ii) gel phase: Plasminogen is preferentially bound to fibrin formed. Activator diffuses in, and activates it to plasmin, which lyses the clot.

2.4 Inhibition of fibrinolysis

The major circulating plasmin inactivator is α2-plasmin inhibitor (Collen, 1976) (Moroi and Aoki, 1976). A specific antibody directed against this inhibitor neutralises virtually all inhibitory activity of plasma to activator-induced clot lysis (Moroi and Aoki op cit, 1976). The inhibitor complexes with plasmin, urokinase and trypsin on a 1:1 molar basis.

When α2-plasmin inhibitor is specifically removed from plasma, a slower reacting plasmin-neutralising ability remains, which is due to α2-macroglobulin. This inhibitor binds to plasmin, trypsin, chymotrypsin, thrombin, kallikrein, and elastase. These enzymes continue to show activity against small molecular weight substrates while so bound, but none against large proteins, and it has been suggested that they are sequestrated by the inhibitor, and are then protected against other inhibitors; while smaller substrate molecules still have access to the endopeptidase within the macroglobulin-enzyme complex (Barrett, Starkey and Munn, 1974).

Other circulating inhibitors have been found to have activity against plasmin, but it is doubtful if they play any major part in its inhibition. These are:

α1-antitrypsin, which reacts irreversibly with plasmin in a time-dependent and non-stoichiometric fashion.

C1 inhibitor which inhibits Factor XIIa and active fragments of XIIa. It also inhibits plasmin; variable amounts of this inhibitor may be precipitated along with plasmin during preparation of the euglobulin lysis time test, producing unreliable results unless strict attention is paid to methodology (Kluft and Brakman, 1975)

but little is known of the importance of antithrombin III as a plasmin inhibitor.

Inter-alpha-antitrypsin inhibits plasmin, but provides only a small contribution to the total antiplasmic activity of the blood.

Platelet inhibitor This is a rapidly acting inhibitor of uncertain significance.

Other inhibitors are undoubtedly present; they inhibit the activation of plasminogen but are not yet well characterised.

2.5 Fibrinolysis and fibrinogenolysis

Plasmin splits fibrinogen in a series of reactions that has been exhaustively studied (Marder and Budzynski, 1974). The molecular weight of fibrinogen is 340 000. The successive products of its digestion by plasmin are:

fragment	molecular weight
X	240 000
Y	155 000
D	83 000
E	50 000

The sequence of these reactions is shown in Figure 2-3.

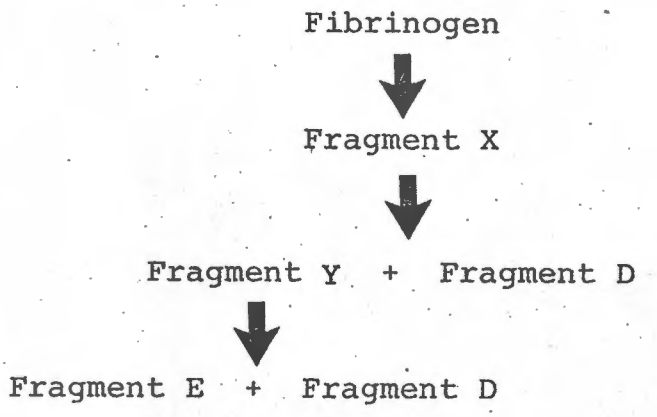


Figure 2-3: Sequence of digestion of fibrinogen by plasmin

Fibrinogenolysis and fibrinolysis

Plasmin splits both fibrinogen and fibrin. The structure of fibrinogen, and the degradation products of fibrinogen and fibrin, have been studied in detail. The following account is based on papers by Pizzo, Taylor, Schwartz, Hill and McKee (1973), Pizzo, Schwartz, Hill and McKee (1972), Budzynski, Marder and Shainoff (1974), Marder and Budzynski (1974), and Gaffney (1977).

2.5.1 Fibrinogen - structure

Fibrinogen is a soluble protein with a molecular weight of about 340 000. Its shape is uncertain, as biochemical data from fibrinogen conversion to fibrin are at odds with physical data from X-ray diffraction studies. It is produced by the liver, and has a biological half-life of ~ 48 hours, the mechanism of physiological breakdown being uncertain. The plasma concentration is 2-4 mg/ml.

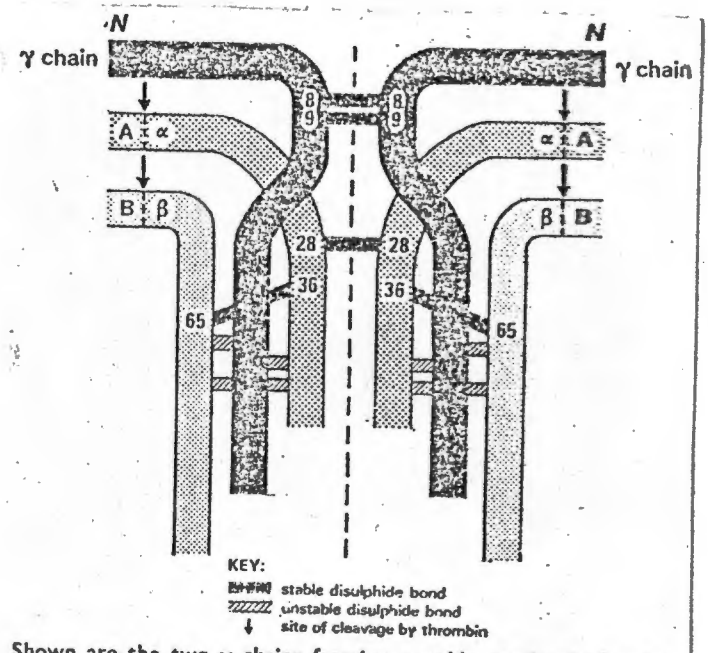
Fibrinogen is a dimeric molecule, each unit of the dimer

containing three individual polypeptide chains which are named Aα, Bβ, and γ. Their gross constitution is shown in Table 2-1.

Table 2-1: Gross constitution, and cross-linking of derived fibrin 'monomer', of human fibrinogen.

Polypeptide chain	Molecular weight	Fibrino-peptide	Polymerisation
A	67 000	A	polymer
B	58 000	B	-
	47 000	-	dimer

These chains have a complex interconnection at their N-terminal ends, forming the so-called 'disulphide knot'. This structure is shown in Figure 2-4 taken from Gaffney (1977 op cit, with permission). The γ chains form stable and symmetrical disulphide bridges, as shown; 11 of the 28 disulphide bridges of fibrinogen are contained in the disulphide knot.

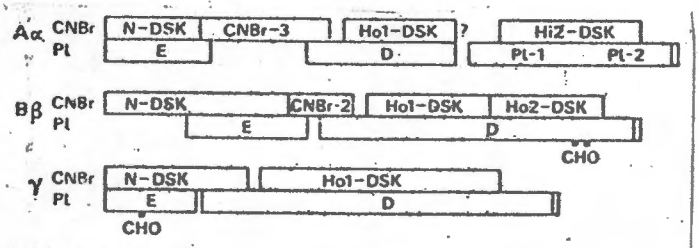


Shown are the two γ chains forming a stable covalently bonded structure onto which the other chains seem to be tied. The bonds between the two γ chains in positions 8 and 9, and those between the two Aα chains in position 28, are symmetrical and stable disulphide bridges. An additional stable disulphide bond is shown between the Aα position 36 and the Bβ position 65 in each of the fibrinogen subunits. (The remaining disulphide bridges are thought to be unstable and can undergo disulphide exchange (Blombäck *et al.* 1974))
This figure is adequate also to relate the two units of the whole fibrinogen dimer, since knowledge of the chemistry of the dimeric structure of fibrinogen is limited to the *N*-DSK domain
In this figure, also in fig. 4 and 5, the lengths of the lines representing the polypeptide chains are not proportional to their molecular weights

Figure 2-4: Schematic model of the N-terminal disulphide knot portion of the dimetric fibrinogen molecule.

The sulphur-containing amino acids cysteine and methionine have been of value in determining the primary and secondary structures of fibrinogen. The are 56-58 cysteine residues and about the same number of methionine residues. Cyanogen bromide fragmentation of the molecule yields a large number of peptides, which have been related to the dissimilar fragments produced by plasmin digestion, to build up a gross structure of the three chains. This structure is

shown in Figure 2-5 (from Gaffney op cit, 1977, with permission).



Abbreviation:
Hi2-DSK: a hydrophilic disulphide knot

Where applicable, the fragment to which each chain subunit belongs is indicated, e.g. the major hydrophobic disulphide knot (Ho1-DSK) is composed of three polypeptide chains that occupy the same regions of fibrinogen as the three subunits of fragment D. Peptide sequences in the CNBr- and Pl-mediated fragments account for nearly the total sequence of the three chains of fibrinogen, while those subunits with inter-chain disulphide bridges will help to establish the relative positions of the chains (see fig. 2). The approximate locations of the carbohydrate (CHO) side chains on the Bβ and γ chains are indicated

Figure 2-5 : Schematic representation of the locations of the major cyanogen bromide (CNBr)- and the plasmin (P1)-mediated fragments in the three chains of fibrinogen - Aα, Bβ, and γ: and of the major end products of plasmin digestion - fragments D and E.

2.5.2 Fibrinogen - coagulation

In the physiological situation, thrombin is the only enzyme capable of initiating coagulation through polymerisation of fibrinogen. Thrombin does this through two distinct mechanisms:

(1) conversion of fibrinogen to fibrin 'monomer'
(2) activation of Factor XIII (fibrin stabilising factor) in the presence of Ca^{++}.

(1) Thrombin cleaves fibrinopeptides A and B from the A and B chains of fibrinogen respectively. The original dimer $(A\alpha B\beta\gamma)_2$ then polymerises spontaneously to non-stabilised fibrin polymers $(\alpha\beta\gamma)_{2p}$. The resultant clot is readily lysed by plasmin.

(2) Thrombin activates Factor XIII to form stabilised fibrin in the presence of Ca^{++}. Activated Factor XIII 'stabilises' the fibrin clot by the formation of covalent isopeptide bonds between different fibrin molecules, crosslinking occuring between pairs of glutamine and lysyl residues. Two of these pairs are located close to the carboxy terminal ends of the γ chains, regions that are contained in the largest of the fragment D molecules. Four other pairs of crosslink sites are located at the carboxy terminal ends of the Aα chains. The Bβ chain does not polymerise.

Fibrin crosslinking involves rapid pairing of two γ chains from different fibrin molecules, and slower polymerisation of multiple α chains. A complex superstructure of covalent linkages results, which renders the clot insoluble in weak acid or 5 M urea (Marder and Budzynski, 1974).

2.5.3 Fibrinogen and fibrin - degradation by plasmin

2.5.3.1 Fibrinogen and non-stabilised fibrin

Plasmin produces virtually identical breakdown products from these two proteins, differing only in the presence or absence of fibrinopeptides A and B from the fragments. The complex breakdown sequence is shown in Figure 2-6 (from Marder and Budzynski, 1974, with per-

mission).

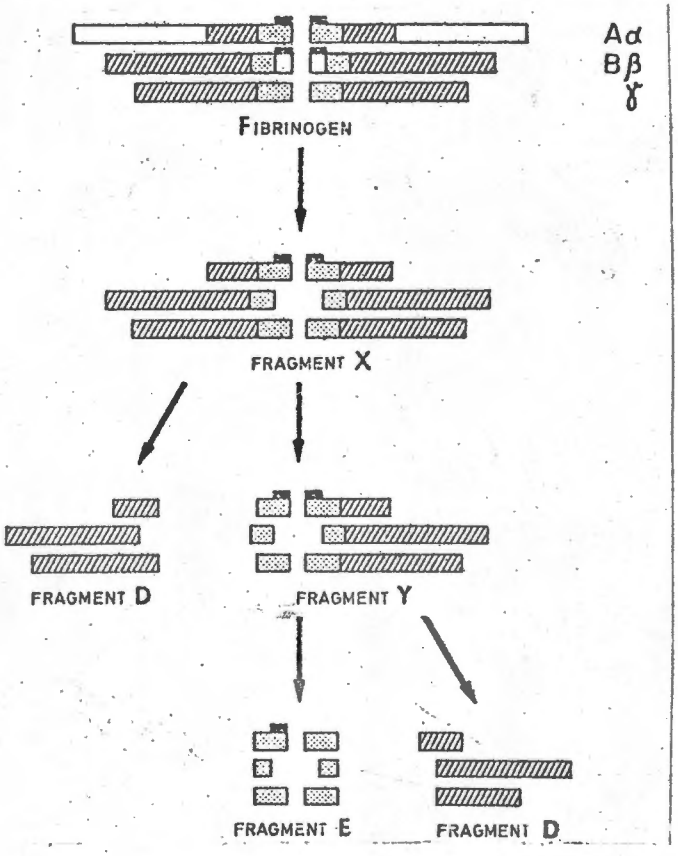


Figure 2-6 : The asymmetric pathway of plasmic degradation of fibrinogen. Fibrinopeptides A and B are noted in black; the portions that are first cleaved by plasmin are shown in white. The longitudinal position of the polypeptide chains indicates the junctional centre occupied by the NH_2-terminal portion (from Marder and Budzynski, 1974, with permission).

2.5.3.2 Stabilised fibrin

Stabilised fibrin is resistant to attack by plasmin. This appears to be due to the α chain crosslinking. The preferential attack by plasmin on fibrinogen and fibrin is shown in Table 2-6.

Table 2-6: Preferential attack by plasmin on fibrinogen and fibrin occurs as shown.

Fibrinogen / non-crosslinked fibrin		Crosslinked fibrin	
1	(Aα / (α	1	β or γ
2	(Bβ / (β	2	α - α Polymers
3	γ		

'The ability of crosslinking to transform highly labile carboxyl ends of the α chains into areas of high resistance to plasmin is quite dramatic. Indeed, the lysis of crosslinked α chains of fibrin seems to represent a rate-limiting step during digestion of fibrin clots' (Gaffney, 1977).

As a result of the crosslink bonds, digestion of stabilised fibrin produces products that are distinctive from those of fibrinogen or non-crosslinked fibrin. The most important of these distinctive products is a derivative of fragment D of approximately twice its size, called double-D or D-dimer. This D-dimer is linked by covalent bonds at the carboxy-terminal ends

of the γ chains. This is the major difference in structure between degradation products of fibrinogen and fibrin (Marder and Budzynski, 1974).

D-dimer is isolated as a single species, even after prolonged digestion of crosslinked fibrin by plasmin, with a molecular weight of 81 000, or about twice the weight reported for fibrinogen fragment D. After 18 hours of digestion of highly crosslinked fibrin by plasmin, sodium dodecyl sulphate and polyacrylamide gel electrophoresis showed the following products: D-dimer (γ-dimer) MW 81 000, β" fragment MW 45 000 and α" fragment MW 15 000 (Pizzo, Taylor, Schwartz, Hill and McKee, 1973).

The products of the formation of a fibrin clot have physiological functions. Fibrinopeptide A is chemotactic for neutrophils; fragment X clots slowly, crosslinks with fibrinogen and forms poor clots, while fragment D acts in a heparin-like way as an anticoagulant. Fragments D and E are commonly estimated by laboratory procedures that measure 'split products of fibrin(ogen)'. Further proteolysis produces peptide fragments having neither physiological nor immunological properties.

2.6 Fibrinolysis: the scope of this thesis

Studies reported in subsequent chapters deal with aspects of the primary fibrinolysin found in the bile of 11 mammalian species studied -man, baboon, dog, cat, mouse, rat, hamster, ox, pig, goat, sheep.

Chapter Three deals with methods of assessment of fibrinolysis, and gives reasons for choosing the fibrin plate test as the basic research tool for this study.

This fibrin plate is then studied in detail, and the nature of the dose-response curves that can be obtained on it are discussed. The nature of the dose-response curve of bile fibrinolysin (cholelysin) on the fibrin plate is presented, and a comparison made with the dose-response curve of chymotrypsin. Cholelysin is then quantitated in terms of chymotrypsin, which makes possible a quantitative assay in unit/ℓ. This assay is used in all subsequent work on cholelysin. The heated fibrin plate is discussed.

Chapter Four discusses bile - its composition, and the composition of gallbladder bile. The availability and purity of bile from various sources is presented, with reasons for standardising on ox bile from the Municipal Abattoir, Cape Town. Procuring and quality control of ox bile are discussed.

Chapter Five deals with the methods of purification of bile. Fibrinolysis by whole bile, and column chromatography of whole bile are briefly presented, followed by an account of ammonium sulphate precipitation of cholelysin, and more complex combined procedures of purification and concentration of cholelysin. Various fractions of bile -G 200, Peaks I, II, III and IV are described, and batchwise elution of Peak I. Molecular weights of the ammonium sulphate precipitate and of Peaks I, II, III and IV are estimated.

Chapter Six discusses substrates acted on by cholelysin. Those investigated are:

(1) endopeptidases trypsinogen, chymotrypsinogen, plasminogen
(2) α-casein

(3) ATEE

(4) BAEE

In the experiment studying the esterase activity of cholelysin on ATEE, simultaneous estimation of fibrinolytic and esterase activities was made. This produced evidence that different fractions of cholelysin might have differing esterase and fibrinolytic activities.

Chapter Seven discusses inhibitor of cholelysin. The major inhibitory substance studied was human serum. This potent inhibitory property was assessed in heated and unheated serum, and in serum from two patients with homozygous α-antitrypsin deficiency. An attempt was made, using paper electrophoresis of whole serum, to determine what fraction of serum was responsible for the inhibitory effect; this was compared with the inhibitory effect of the same serum on chymotrypsin. The serum of the rat, rabbit, guinea-pig and mouse was also studied for its inhibitory effect. Other substances studied were: α_2-macroglobulin, soy bean trypsin inhibitor and aprotonin.

Chapter Eight discusses the effect of cholelysin on routine coagulation and platelet function tests, and on the platelet count in the rat. An attempt is made to establish a biological half life, and a LD_{50} for cholelysin in the rat. An experiment was undertaken to establish if cholelysin had *in vivo* anticoagulant properties in the rat.

Chapter Nine discusses polyacrylamide gel electrophoresis (PAGE). PAGE of cholelysin with chymotrypsin control is followed by a study of the effects of mercaptoethanol and SDS on cholelysin. PAGE of the split

products of fibrin digestion by cholelysin and plasmin at 24 hours is compared..

Chapter Ten describes a new method for the study of enzyme kinetics at a sol/gel interface, termed the composite cuvette method. This method is used to study the kinetics of the cholelysin/fibrin reaction: the reaction is shown to occur in two phases, and this is proved by analysis of FSPC in the two phases by PAGE. Finally, a double reciprocal relationship is shown to exist between concentration of cholelysin and V, with stabilised fibrin as the substrate.

Chapter Eleven discusses molecular weights of cholelysin and of fibrin split products. Cholelysin was analysed by column chromatography with and without SDS. At least three separate forms exist, which are probably monomer (~ 7 000), dimer (~ 14 000) and tetramer (~ 28 000). However, the possible dimeric form exists in the presence of SDS 2 g/100 ml. Fibrin split products of digestion by plasmin are analysed and compared with the work of earlier authors; the results agree closely. The fibrin split products of digestion by cholelysin are analysed and are shown to be entirely different from those due to plasmin.

Chapter Twelve summarises this thesis, and discusses possibilities for future work on cholelysin.

Summary

Chapter Two the physiology of fibrinolysis is discussed in the light of recent advances. Activators and inhibitors are considered. The structure of fibrinogen and the nature of fibrinolysis and fibringenolysis are considered in detail.

CHAPTER THREE

THE ASSESSMENT OF FIBRINOLYSIS - THE FIBRIN PLATE

3.1 The assessment of fibrinolysis methods

Clinical situations usually require methods which will give a rapid assessment of fibrinolysis, and unfortunately these methods are all unsuitable for quantitation. The more accurate methods are either time-consuming, or both and have not found widespread acceptance in the clinical situation.

Clinical testing of fibrinolysis is usually done by:

(a) Dilute whole blood clot lysis time (Fearnley, 1965)

(b) Euglobulin lysis time (von Kaulla, 1963) (Chakrabarti, Bielawiec, Evans and Fearnley, 1968)

(c) Fibrinogen titre test (Sharp, Howie, Biggs and Methuen, 1958)

More accurate tests with widespread acceptance are:

(d) Fibrin plate test (Astrup and Müllertz, 1952). This is sensitive, precise, and reproducible. A large number of tests can be done in one day by one technician, and the plates are easy to prepare.

(e) Fibrin agar plate test (Wolf, 1969). The preparation of this test involves a heat degradation step for fibrinogen which is not easy to standardise; otherwise, this is a convenient and accurate method for the measurement of fibrinolysis.

(f) Radioactive isotope labelling of fibrinogen (Fletcher, 1964). This is extremely accurate, but is expensive and requires expensive equipment.

(g) Caseinolytic test (Johnson, Kline and Alkjaersig, 1969). This is the method recommended by the National Heart Institute of Bethesda, Md, USA. It is accurate and quick, but has the disadvantages of being cumbersome, thus limiting the number of tests that can be done in one day; and also non-physiological, so that its relationship to physiological events is unclear.

For the above reasons, the fibrin plate test was finally chosen as the basic research tool for this study.

3.2.1 The fibrin plate test

(a) Physiology

The fibrinogen used in standard techniques of preparation of the fibrin plate always contains both plasminogen and plasminogen activator. An unknown substance applied to the fibrin plate could therefore cause fibrinolysis by one of two methods:

(i) by activation of plasminogen activator or of plasminogen itself

(ii) by direct fibrinolysis.

These two possibilities are summarised in Figure 3-1.

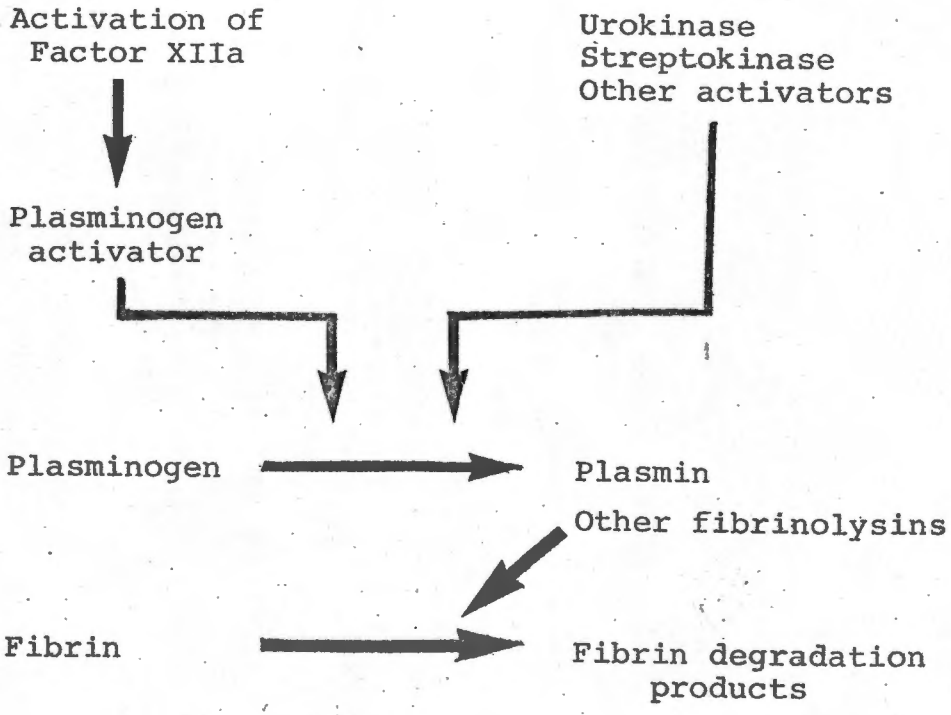


Figure 3-1: Pathways of fibrinolysis on the unheated fibrin plate.

If the fibrin plate is heated to 80°C for 45', all plasminogen and plasminogen activator activity is destroyed, while the fibrin of the plate suffers only minor heat degradation (Thomson, 1970). Thus, the heated fibrin plate may be used to exclude fibrinolysis requiring the intervention of either plasminogen or plasminogen activator. Figure 3-1 can then be redrawn as follows:

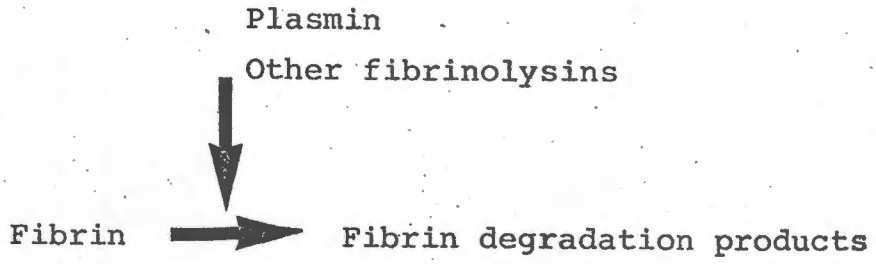


Figure 3-2: Pathways of fibrinolysis on the heated fibrin plate.

3.2.2 Materials and methods

Physiological conditions were adhered to wherever possible. The fibrinogen selected was > 90% clottable (Kabi human fibrinogen). Because higher concentrations of fibrinogen on the fibrin plate decrease sensitivity, the low physiological concentration of 2 mg/ml was chosen (plasma level 2-4 mg/ml). Calcium was added to the final concentration routinely used in laboratory coagulation techniques (M 0,025). After addition of the thrombin-calcium mixture to the fibrinogen solution in the Petri dish, careful slow rotation of the plate for 20 rotations was essential if reliable results were to be obtained.

The original fibrin plate technique described by Astrup and Müllertz (1952) was first attempted. For reasons that could not be established, this type of fibrin plate proved highly unstable, commonly undergoing spontaneous lysis within 24 hours of preparation. This made it unsuitable for periods of overnight incubation. Various modifications were tried; the technique finally adopted gave excellent four day stability at $4^{o}C$. The two methods are summarised in Table 3-1.

Table 3-1: Comparison of materials and methods used by Astrup with those used in this study.

	Astrup	King
Fibrinogen source	Bovine 80-90% clottable	Kabi human 90% clottable
Fibrinogen concentration	Usually 0,2% (2 mg/ml)	0,2% (2 mg/ml)
Thrombin source	Bovine	Bovine
Thrombin added	20 units	0,2 units
Calcium concentration	-	M 0,025
Type of Petri dish	Glass autoclaved	Plastic disposable presterilised (except heated fibrin plate
Period of incubation after formation of the clot	Not stated	2 hours at 37°C
Storage	Not stated	4 days at 4°C
Test incubation	18-20 hours at 37°C	21 hours at 37°C

3.2.3 Dose response curves on the fibrin plate

Two types of dose-response curve have been described on the fibrin plate (Haverkate and Traas, 1974). These are summarised in Table 3-2.

Table 3-2: Dose response curves on the fibrin plate.

Enzyme	Zone of lysis (x)	Concentration (y)	Equation of curve
(a) Plasmin	Log area	Log	Log y = Log x + k
(b) Trypsin	Diameter	Log	y = Antilog x + k
Chymotrypsin			
Papain			
Brinase			
Urokinase			
Bacterial-proteases			

Graph paper for (a): log/log
Graph paper for (b): semilog

Equal drop volumes are used, with a fixed period of incubation. If test and reference solutions plot in the same way, the test can be expressed in terms of the reference solution. Haverkate and Traas (op cit, 1974) suggested that the log/log curve generated by plasmin might be due to instability of plasmin under standard conditions of incubation: after 17 hours of incubation at 37°C, only 10-20% of plasmin activity remained, whereas 100% of chymotrypsin activity was retained. Trypsin and chymotrypsin have nearly identical curves, and are detectable at very low levels; Haverkate and Traas (op cit, 1974) give the following levels for lowest detectable concentrations:

trypsin	0,06	g/ml
chymotrypsin	0,20	g/ml
plasmin	0,003	U/ml

The difference between the dose-response curves of urokinase and plasmin remains unexplained.

Standardisation

This study commenced in 1969, and American National

Red Cross human plasmin was used as the standard from the commencement. It proved to be reliable; through 7 years of use, the standard test dose produced a zone of lysis of 13 ± 1 mm diameter. The later information from Haverkate and Traas (op cit, 1974) did not seem to necessitate changing to (say) a chymotrypsin standard.

3.2.4 Stability and reproducibility

Both these parameters were studied at intervals throughout this study. Results of stability testing on three separate occasions are given in Table 3-3 below.

Table 3-3: Doubling dilutions of human plasmin 0,1 U/ml on the fibrin plate in three different years, followed through 4 days of fibrin plate life. All results in mm diameter of zone of lysis. Full details in Appendix 3.

	Day							
Year	0	1	2	3	4	$\bar{X}$	s	SEM
1970	14,3	13,0	12,7	14,7	14,0	13,7	0,77	0,34
1972	13,3	13,7	13,7	12,7	12,3	13,1	0,62	0,25
1974	13,5	13,8	12,3	13,0	13,3	13,2	0,57	0,23
$\bar{X}$	13,7	13,5	12,9	13,3	13,2			

Reproducibility was also studied repea tedly. Shown here is a bile fibrinolysin preparation assayed on separate fibrin plates on one day in different years.

Table 3-4: Reproducibility of the fibrin plate assay, using a bile fibrinolysin, in three different years. All results in mm diameter of zone of lysis.

	Test								
Year	1	2	3	4	5	6	$\bar{X}$	s	SEM
1974a	26	25	27	28	25	25	26	1,26	0,51
1974b	12	12	11	14	12	11	12	1,10	0,45
1975	32	30	33	32	34	30	32,5	0,84	0,34

3.2.5 The heated fibrin plate

The physiology of this fibrin plate is discussed in 3.2.1 above (Figure 3-2, page 29). Agents causing lysis on this fibrin plate are primary fibrinolysins, and are differentiated fundamentally from those that trigger the lytic cascade at a more remote level. The results of this test using bile fibrinolysin are discussed in Chapter Three section 3.4 page 41.

3.3 The assay of bile fibrinolysin (cholelysin)

At this point it is necessary to anticipate some of the work reported in Chapter Five. All steps in the purification procedures required quantitation of the fibrinolytic effect present in eluates, precipitates, supernatants, etc. This was possible through comparison with the dose-response curve of chymotrypsin, and this work must be reported now to give intelligibility to later findings.

Cholelysin

The name cholelysin is proposed for the group of fi-

brinolysins studied in this work, and it will be used henceforth where relevant.

The dose-response curve of cholelysin proved to be very similar to that of chymotrypsin. Studies of doubling dilutions with five preparations are shown in Table 3-3 below.

Table 3-5: Fibrinolytic effect of various preparations of cholelysin Peak I (see 5.3.7) on the fibrin plate. Results in mm diameter of zone of lysis. Full details given in Appendix III. These results are shown graphically in Figure 3-3.

Dilution factor	Test No				
	1	2	3	4	5
1	37,2	35,0	28,5	25,3	20,5
2	33,0	31,3	22,0	19,0	15,0
4	28,8	26,3	16,3	14,0	10,0
8	23,6	21,0	9,0	9,3	5,8
16	19,2	16,7	(3,0)		

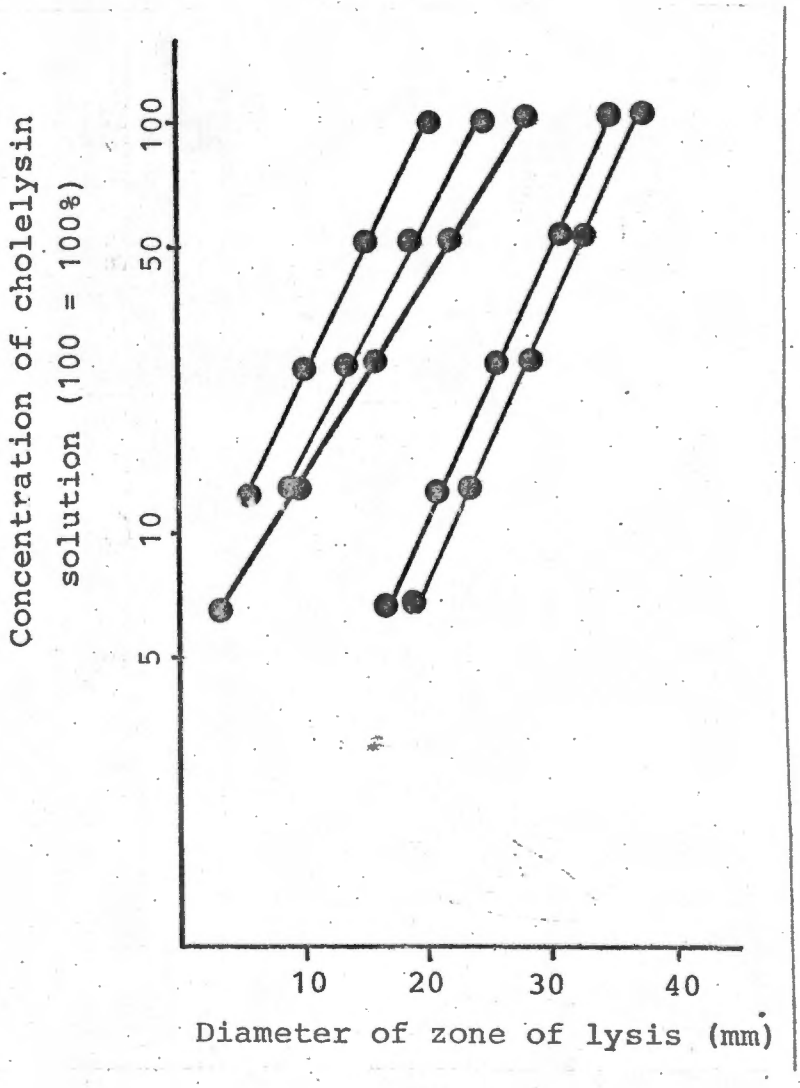


Figure 3-3: Fibrinolytic effect of five different preparations of cholelysin on the standard fibrin plate. Relative concentration plotted against diameter of zone of lysis (mm).

From Figure 3-3 it can be seen that cholelysin fell into the second category of fibrinolytic agents, giving a straight line on semi-logarithmic paper. The slope of these five curves over 1 log cycle is given in Table 3-6.

Table 3-6: Decrement of lytic diameter of cholelysin Peak I (various preparations) over one log cycle of dilution (extrapolated in the two cases where a full log cycle was not covered).

	Decrement in lytic diameter (mm) over one log cycle of dilution							
Expt	1	2	3	4	5	$\bar{X}$	s	SEM
(mm)	15	15,5	21	17	18	17,3	2,39	1,07

3.3.1 Quantitation of cholelysin

As discussed in 3.2.3 above, if two fibrinolysins plot in the same way, the one can be expressed in terms of the other. Both trypsin and chymotrypsin gave closely similar plots, but chymotrypsin was preferred because of the finding by Haverkate and Traas (op cit, 1974) that chymotrypsin had 100% stability under standard conditions of incubation on a fibrin plate, whereas trypsin showed a 40% degradation.

In Tables 3-7 and 3-8, and in Figure 3-4, the dose-response curve of chymotrypsin is shown, and is compared with the 5 dose-reponse curves of cholelysin shown in Figure 3-3.

Table 3-7: Fibrinolytic action of chymotrypsin on the fibrin plate. All results in mm diameter of zone of lysis.

Chymo-trypsin	Test								
mg/ℓ	1	2	3	4	5	6	$\bar{x}$	s	SEM
200	40	43	41	41			41,25	1,26	0,63
100	31	34	33	31	31	33	32,2	1,33	0,54
50	26	27	26	25	25	26	25,8	0,75	0,31
25	20	20	19	20	21	21	20,2	0,75	0,31
12,5	15	15	14	16	14	15	14,8	0,75	0,31
6,25	9	9	8	8	9	9	8,7	0,52	0,21
3,13	4	4	4	3,5	3,5	4	3,8	0,26	0,11

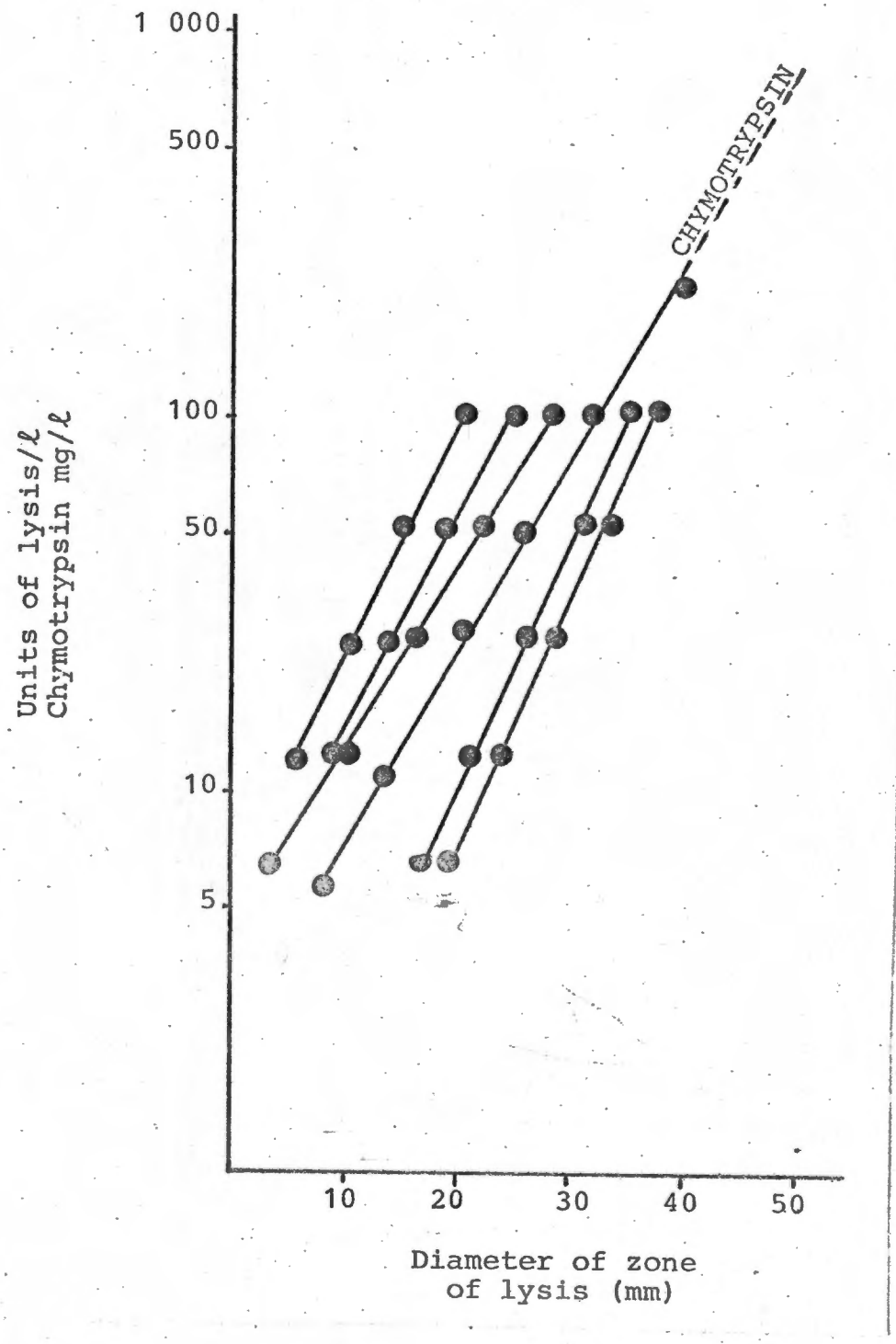


Figure 3-4: Dose-response curve for chymotrypsin on the fibrin plate. Results in mm diameter of zone of lysis. Cholelysin dose-response curves from Figure 3-3 plotted for comparison.

Concentrations of chymotrypsin above 200 mg/ℓ gave less accurate results, and the large zones of lysis were seldom truly circular. However, when results were averaged the curve held true up to 1 000 mg/ℓ. Above that point, the curve was extrapolated for the occasional very high concentration of cholelysin.

Table 3-8: Dose-response curve of higher concentrations of chymotrypsin on the fibrin plate. Results in mm diameter of zone of lysis.

Chymotrypsin concentration mg/ℓ	Zone of lysis (two diameters measured at right angles)		Mean
500	49 X 44	50 X 42	46,25
1 000	55 X 49	52 X 46	50,5

The decrements over one log cycle in mm of lysis for chymotrypsin and cholelysin Peak I are shown in Table 3-9.

Table 3-9: Decrement over one log cycle in millimeters of chymotrypsin and the mean of five curves of cholelysin Peak I.

	mm decrement over one log cycle
chymotrypsin	19
cholelysin Peak I	17,3 ± 1,07

Comment: These curves agreed closely enough for chymotrypsin to be used as a reference unit for cholelysin. Accordingly mg/ℓ for chymotrypsin was transformed into units/ℓ for cholelysin Peak I, and a table was drawn up (Table 3-10).

This was the standard reference table for this study.

Table 3-10: Activity of cholelysin expressed in units/ℓ of chymotrypsin equivalent. The equation of this curve is:

$y = \text{antilog } (0{,}0533x + 0{,}3)$ units

where x = diameter of zone of lysis in mm

y = concentration in units/ℓ,

where 1 unit = 1 mg chymotrypsin

Diameter of lysis (mm)	Units/ℓ	Diameter of lysis (mm)	Units/ℓ	Diameter of lysis (mm)	Units/ℓ
4	3,3	21	26,3	38	212
5	3,7	22	29,7	39	239
6	4,2	23	33,6	40	270
7	4,7	24	37,9	41	306
8	5,3	25	42,9	42	346
9	6,0	26	48,5	43	391
10	6,8	27	54,8	44	442
11	7,7	28	62,0	45	500
12	8,7	29	70,1	46	565
13	9,8	30	79,2	47	638
14	11,1	31	89,6	48	722
15	12,6	32	101	49	816
16	14,2	33	115	50	923
17	16,0	34	129	51	1 043
18	18,2	35	146	52	1 180
19	20,5	36	166	53	1 333
20	23,2	37	187	54	1 507

3.4 The heated fibrin plate

Cholelysin invariably caused lysis of a heated fibrin plate, proving that it was directly fibrinolytic. There was little or no difference between results on a heated and an unheated fibrin plate. Results using various fractions of cholelysin (see Chapter Four for details of purification of these fractions) are shown in Table 3-11 and are plotted in Figure 3-5.

Table 3-11: The effect of various preparations of cholelysin on heated and unheated fibrin plates. Presterilised glass Petri dishes used throughout. Results in mm diameter of lysis.

Enzyme		Fibrin plate					
		Unheated			Heated		
		1	2	Mean	1	2	Mean
Whole bile	1	40	44	42	44	42	43
	2	35	32	33,5	26	28	27
Ammonium sulphate precipitate	1	18	18	18	18	16	17
	2	36	32	34	37	32	34,5
Fractions (see Chapter IV)							
G 200		30	32	31	30	34	32
Peak I		16	19	17,5	15	19	17
Peak II		4	4	4	4	4	4
Peak III		7	6	6,5	5	6	5,5
Peak IV		10	11	10,5	10	12	11
Plasmin		14	14	14	13	14	13,5
Urokinase		14	14	14	0	0	0
Streptokinase		20	22	21	0	0	0

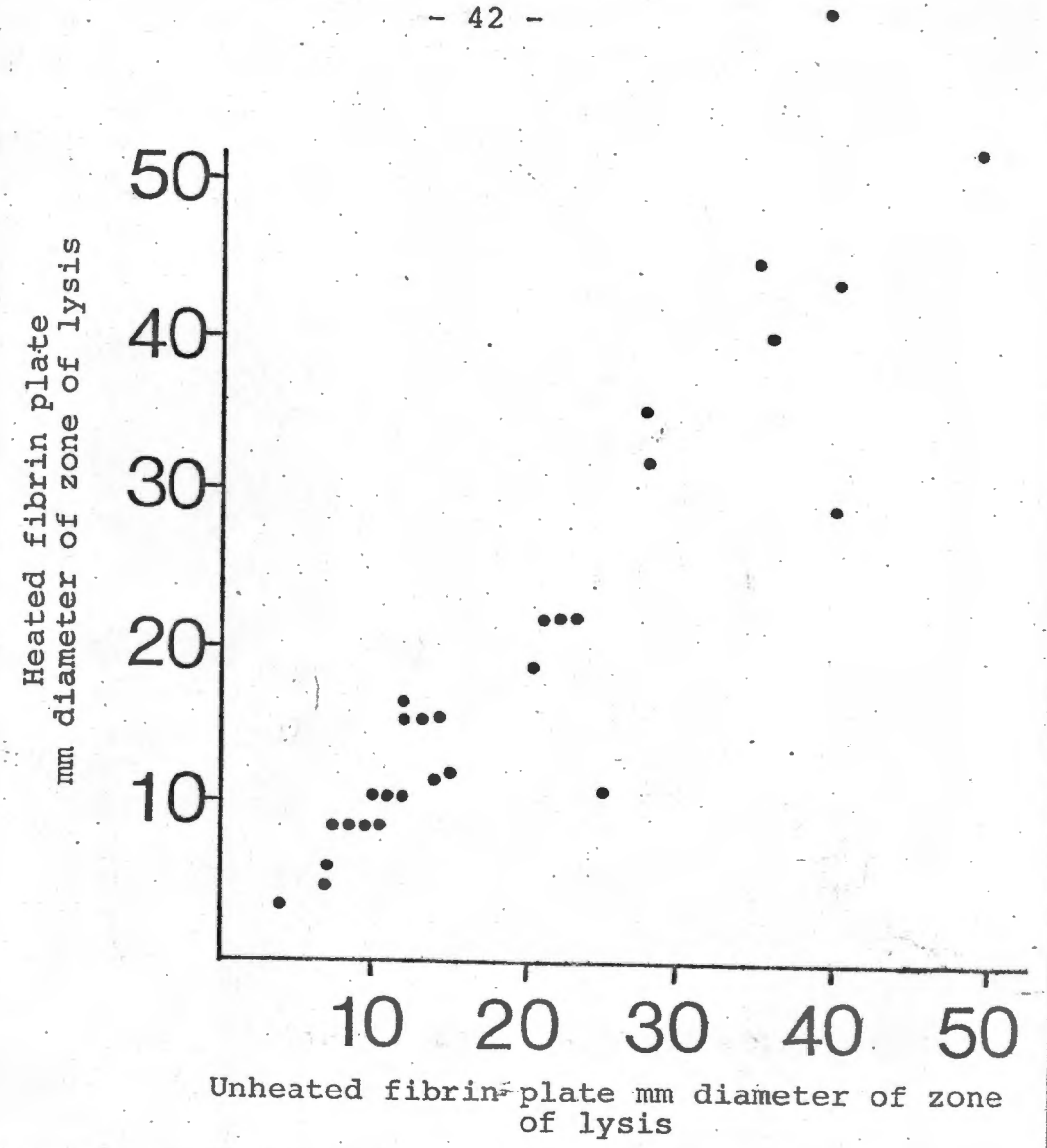


Figure 3-5: Fibrinolytic effect of cholelysin on heated and unheated fibrin plates compared. Several experiments are plotted here that were not listed in Table 3-11.

3.5 Summary

Methods of assessing fibrinolysis are briefly discussed. For scientific accuracy, one of the less rapid tests was preferred, and reasons are given for choosing the fibrin plate assay as the basic research tool for this study. The physiology and methodology of the fibrin plate are then discussed, and the nature of the dose-response curves to be found on it. It shows a high degree of sensitivity, and has excellent stabili-

ty when stored at 4°C, and reproducibility. The heated fibrin plate is a useful research tool for distinguishing fibrinolysins form activators of fibrinolysis. The dose-response curve of cholelysin is shown, and compared with that of chymotrypsin. A table of units/ℓ of activity is drawn up for cholelysin, using chymotrypsin as the reference standard.

CHAPTER FOUR

BILE

4.1 Bile is a complex mixture of substances. For the purposes of this study, the point of major importance was the composition of gall bladder bile, as nearly all studies were done on bile derived from the ox gall-bladder. The composition of mammalian bile does not appear to vary greatly from species to species, but I was unable to find data on ox bile. Figures for the composition of secreted bile and gall-bladder bile given here are quoted from

(i) Liver and biliary disease Eds R. Wright, K. Alberti, S. Karran and G. Millward-Sadler, (1979)

(ii) Handbook of Physiology: Section 6 - Alimentary Canal (1968) Vol 5 Chapters 110, 112, and 113.

Bile acids - cholic and chenodeoxycholic acids (ratio 2:1)

Cholesterol	0,5 g
Bilirubin	0,7 g
Protein	0,7 g
	0,03-0,3 g

are secreted per day.

However, figures for total protein/24 hours of 0,275 and 0,514 g respectively are given by Rosenthal, Kubo, Dolinski, Marino, Mersheimer and Glass (1965) for two patients with presumed normal livers on T-tube drainage for extra-hepatic obstruction. Of this, about 80% was albumin, and the remainder was not identified. Total

volume of bile per day is up to 1 litre, or 0,5 mg protein/ml bile. However, as the gall bladder concentrates bile up to 100 times, there could be significant quantities of bile proteins in it, and in this study we found raw bile to contain 2-6 mg protein/ml.

Electrolytes are present in approximately isotonic quantities with plasma, and are almost entirely removed during concentration in the gall bladder, while bile acids and cholesterol concentrate up to 100 times. Precipitation of insoluble cholesterol and lecithin is prevented by the formation of micelles or aggregates of bile salts and lecithin, forming 'liquid crystals' into which cholesterol is incorporated. Lecithin greatly increases the ability of bile salts to incorporate cholesterol, by acting as a polar lipid to swell the micelles into 2 molecule thick discs. It drops the number of bile salt molecules required to solubilise one cholesterol molecule from 35 to 10.

According to Wheeler (1968), the gall bladder exists because the secretion of bile is continuous, but its arrival at the intestine for its digestive function must be periodic. By concentration, it can accommodate about half the daily output of bile at any one time.

From the point of view of this study, everything except the protein content was irrelevant, and much time and effort was devoted to removel of the strongly charged bile salts, as will be discussed below. Bile pigments served as a marker for successful purification of bile, and Fouchet's test for bilirubin was used for quality control.

4.2 Availability and purity of bile

The first experimental work was done using human bile.

This was obtained from T-tube drainage of patients in the Department of Surgery. It was potently fibrinolytic, but was usually infected, so that many specimens were unsuitable for study. The supply of patients on T-tube drainage was erratic as well.

Other available species of mammal were therefore studied to discover a reliable source of bile. Fibrinolytic activity was found in the bile of 11 species: cat, dog, goat, sheep, guinea-pig, mouse, ox, pig, rabbit and rat - as well as man.

It was decided to standardise on ox bile from the Municipal Abattoir, Cape Town as this was available in large volumes under controlled conditions. The Director of the Abattoir, Dr Albertyn, was most helpful at all times.

Oxen are killed at the Abattoir by stunning followed by exsanguination. The carcasses are immediately processed. Bile was obtained directly from the gallbladders within 60' of death.

Ox bile was also processed in Pretoria by Dr T Haylett of the Council for Industrial and Scientific Research, acting on behalf of the South African Inventions Development Corporation. He produced a highly potent cholelysin solution for our biological experiments (Chapter Eight), but experienced difficulty with the gelling of the bile after ammonium sulphate precipitation, which he ascribed to mucoproteins present in the bile ? from winter feed in the Transvaal.

4.3 Quality control

Because the Director of the Abattoir, Dr Albertyn, kind-

ly allowed direct access to the oxen while being processed, most contamination was excluded. The Abattoir workers would hand us the excised gall-bladder, and this would be aspirated with a sterile needle attached to a 50 ml syringe, which was then emptied into a pre-sterilised glass container. Volumes of 5 - 10 litres were readily obtained in a short space of time.

All batches of bile were cultured for organisms, and were discarded if organisms were grown in significant quantity, or if any organisms found showed inherent fibrinolytic potential. Specimens from which 'occasional' colonies were grown were regarded as acceptable.

A batch would usually commence its processing in the laboratory on the same day, but occasionally samples would be left for 1 - 7 days at -40°C before work commenced. This did not affect the lytic potential of raw bile.

4.4 Summary

Human bile was used in the first series of experiments, but supplies were unpredictable, and often infected. A survey of available mammalian species was made. All showed fibrinolytic activity, and it was decided to standardise on ox bile from the abattoir. The procuring and quality control of ox bile are described.

CHAPTER FIVE

METHODS OF PURIFICATION OF CHOLELYSIN FROM OX BILE

5.1 Introduction

The complex micellar solution of bile was almost entirely redundant from the point of view of this study. All that was desired was a purified protein solution, but it took some years of experimentation to achieve this result, and it would be futile to retrace the many attempts made towards this goal. There were many blind alleys and many disappointments. Rather, in discussing the numerous techniques listed in Table of Contents, the emphasis will be on the positive result that led towards a successful solution, and the poorer methods will be discussed briefly in Appendix 5.

5.2 Bile

5.2.1 Column chromatography

The first method studied was column chromatography of raw human bile on sephadex G200 (fine). This gave several patterns of elution, three of which are shown in Figures 5-1, 5-2 and 5-3. Lytic activity was measured on the standard fibrin plate, and reported in mm^2, as at that time the semi-logarithmic nature of the dose-response curve had not become apparent.

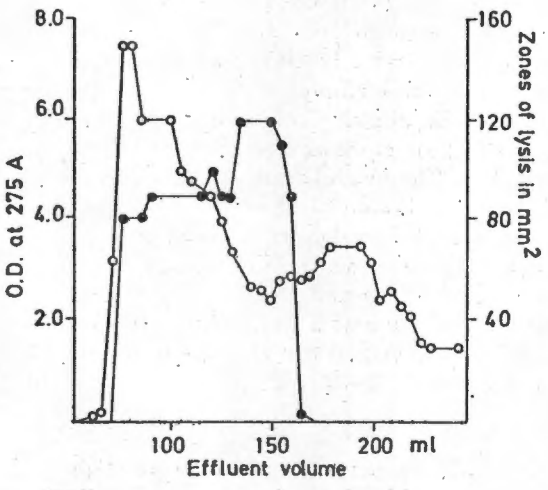


Figure 5-1:

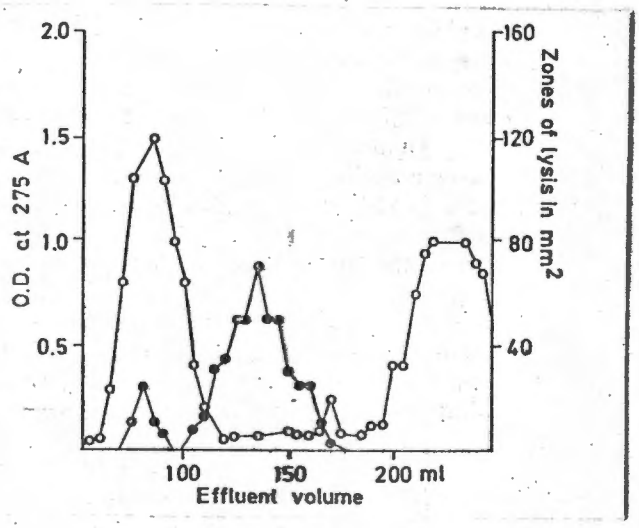


Figure 5-2:

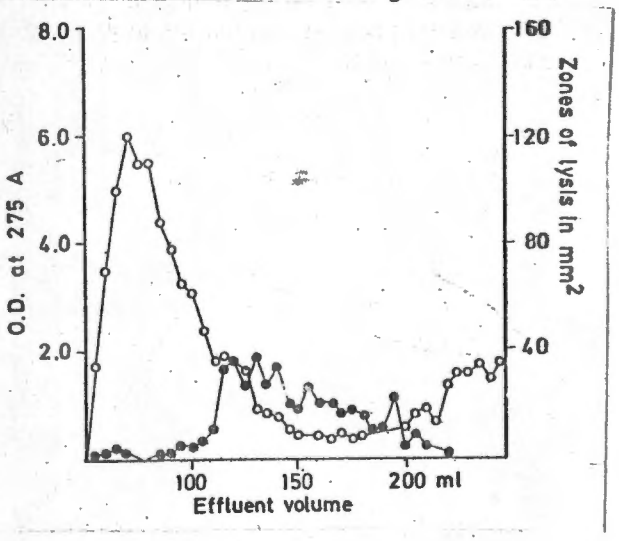


Figure 5-3:

Figures 5-1, 5-2 and 5-3: Profiles of elution of whole human bile from sephadex G200 (fine). Eluant tris/NaCl. Lytic activity ●—●—● Optical density ○—○—○ (King, 1972)

Comment on Figures 5-1, 5-2, and 5-3: the eluate was contaminated with bile pigments and bile salts, and the optical density of fractions at 275 nm bore no relation to their fibrinolytic activity. It was decided to test known constituents of bile for their lytic activity. Further methods of purification of the fibrinolysin(s) should be sought.

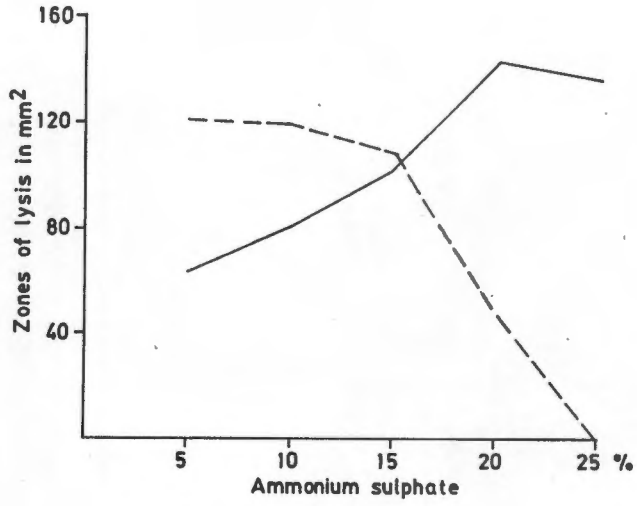
In Table 5-1, constituents of bile that were tested for their fibrinolytic potential are listed. They were available in pure form.

Table 5-1: The following were tested for fibrinolytic potential on the standard fibrin plate. No fibrinolysis was observed with any one.

sodium taurocholate	10 mEq/ℓ
sodium glycocholate	10 mEq/ℓ
bilirubin	5 mg/100 ml

5.2.2 Ammonium sulphate precipitation

Ammonium sulphate precipitated fibrinolytic activity from bile. The point of 100% precipitation varied over the whole range of trials from 8 to 45%. If a second precipitation was made after reconstitution of the precipitate, a lower concentration of ammonium sulphate might be effective. The first precipitation step gave concentrations of 2-4 X, the second of up to 50 times. A typical early experiment is shown in Figure 5-4.



——— Fibrinolytic activity of precipitate
----- Fibrinolytic activity of supernatant

Figure 5-4: Pattern of precipitation of fibrinolytic activity by ammonium sulphate from raw human bile in one typical experiment.

Comment: No sample of human or ox bile was found which failed to show 100% precipitation of lytic activity at ammonium sulphate concentration of 46% or lower. For this reason, an initial concentration of 50% W/V was decided on for later work.

Column chromatography of a once precipitated sample, which was then reconstituted, is shown in Figure 5-5. Such samples gave a more consistent pattern of elution.

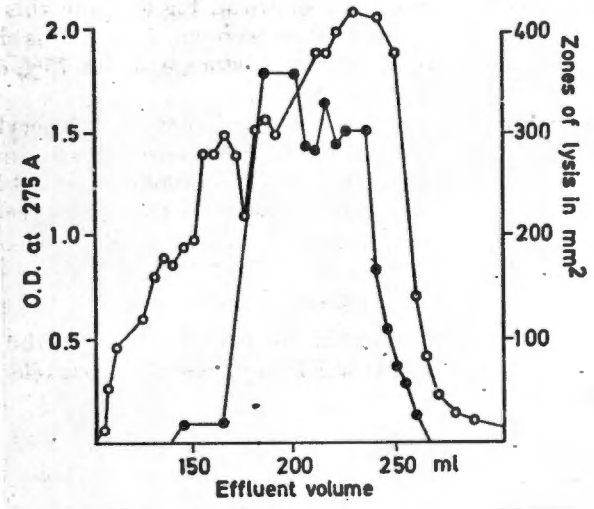


Figure 5-5: Elution profile of an ammonium sulphate precipitated concentrate of bile applied to sephadex G200 (fine) V_o = 85 ml $K_{av} = \pm 0,9$.

Finally, a sample of reconstituted concentrate, following one precipitation with ammonium sulphate, was applied to sephadex G50 (medium), and the elution profile recorded. Neutral sugar and protein (biuret) were also measured, but bore no relationship to fibrinolytic activity, as is shown in Figure 5-6.

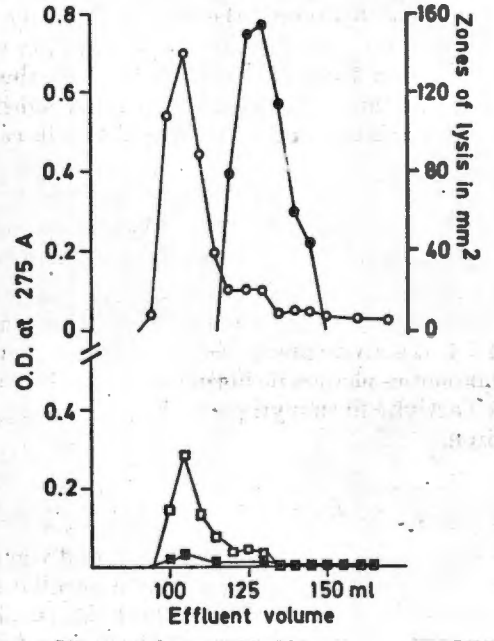


Figure 5-6: Elution profile of an ammonium sulphate precipitated sample of raw bile on sephadex G50 (medium). Neutral sugar □—□ and protein (biuret) ■—■ were estimated on the same fractions. V_o = 95 ml K_{av} = 0,3.

Comment: it was apparent that insufficient purification had yet been achieved, and other methods of precipitation of fibrinolytic activity from raw bile were sought. These were:

5.2.3 Acetone precipitation

5.2.4 Chloroform/methanol partition (Bligh and Dyer, 1959)

5.2.5 Polythylene glycol precipitation

5.2.6 Precipitation with various agents capable of causing cholestatic jaundice in man:

promazine, chlorpromazine, amitryptilene, trifluperazine (Clarke, Maritz and Denborough, 1972).

Unfortunately, these methods proved either ineffective, or unacceptably damaging to fibrinolytic activity. They are discussed briefly in Appendix 5.

5.3 Precipitated fraction

5.3.1 Redissolving of the ammonium sulphate precipitate at various levels of pH was studied at the commencement of this work. Tris buffer M 0,006 was used, and the following result obtained.

Table 5-2: Ammonium sulphate precipitate of bile, 50% W/V, redissolved in tris M 0,006 at various pH levels. Volume of added tris kept constant, and lytic activity tested on the fibrin plate. Results in mm diameter of zone of lysis.

	Test 1		Test 2		Test 3	
pH	mm	units	mm	units	mm	units
10,5	14	11,1	19	20,5	24	37,9
9,5	17	16,0	21	26,3	25	42,9
8,5	22	29,7	25	42,9	29	70,1
7,5	20	23,2	24	37,9	28	62,0
6,5	16	14,2	20	23,2	26	48,5
5,5	13	9,8	18	18,2		

Comment: following this result, the pH of tris buffer was standardised at 8,5 except where otherwise stated.

5.3.2 Reprecipitation with ammonium sulphate led to considerable concentration, and 25-50% loss of activity. This could have been acceptable, but unfortunately the bile salts and pigments formed a tarry deposit that interfered with further work. It was necessary to find more effective ways of removing them from solution.

5.3.3 An effective anion exchanger was sought, and found in sephadex A50. This produced marked clearing of bile pigments and salts, and the remainder of the unwanted substances, and at the same time absorbed fibrinolytic activity strongly.

5.3.4 The most effective method for desorbing fibrinolytic activity was found to be an acidification step. Used with sephadex, this did not produce marked loss of activity, while at the same time bile pigment concentration, and protein, fell sharply in the desorbed fraction. Following this, an ammonium sulphate precipitation step was incorporated. These steps, and their effects on bile, are shown in Table 5-3.

Table 5-3: Effects of adsorbtion with sephadex A50, desorbtion at pH 3, and subsequent precipitation with ammonium sulphate, on fibrinolytic activity, and protein and bilirubin content, of bile. Volume of redissolved solutions held constant through.

Fraction	Lysis mm diam	Units/ℓ	Protein mg/ml	Units/mg protein	Fouchet (bilirubin)
Raw ox bile	44	442	2,4	130	++++
sephadex A50 supernatant	4	3,3	0,6	2,8	+++
sephadex A50 desorbtion at pH 3	40	270	1,1	208	+++
ammonium sulphate supernatant	0	0	0,2	-	+++
ammonium sulphate precipitate redissolved	41	306	0,75	408	++

5.3.5 Adsorbtion on AG1-X.8

This is a synthetic compound. It is a strongly basic anion exchanger having quarternary ammonium active groups on a polystyrene framework.

It was tried instead of sephadex A50 because it is considerably cheaper, and easier to regenerate. It proved effective, but led to a somewhat lower recovery of fibrinolytic activity than when sephadex A50 was used.

5.3.6 Column chromatography

Because Fouchet's test was still positive after the ammonium sulphate precipitation step, molecular exclu-

sion was added for further purification. Sephadex G200 had proved effective in earlier experiments, and was used again. The eluted fractions off the column were pooled, and referred to as 'Fraction G200'. The effect on fibrinolytic activity, protein content, and bilirubin concentration, are shown in Table 5-4.

Table 5-4: Effects of column chromatography (on sephadex G200) of the fraction produced by the steps outlined in Table 5-3, on fibrinolytic activity, and protein and bilirubin content of bile.

	Lysis mm diam	Units/ℓ	Protein mg/ml	Units/mg protein	Fouchet
Fraction G200					
Experiment I	32	101	0,178	567	+
Experiment II	25,5	44	0,231	190	+
Experiment III	24	38	0,159	239	±

This fraction could be concentrated by ultrafiltration, but it was still contaminated, and the pattern of elution varied considerably, without clearcut relationship to optical density. One experiment is illustrated in Figure 5-7.

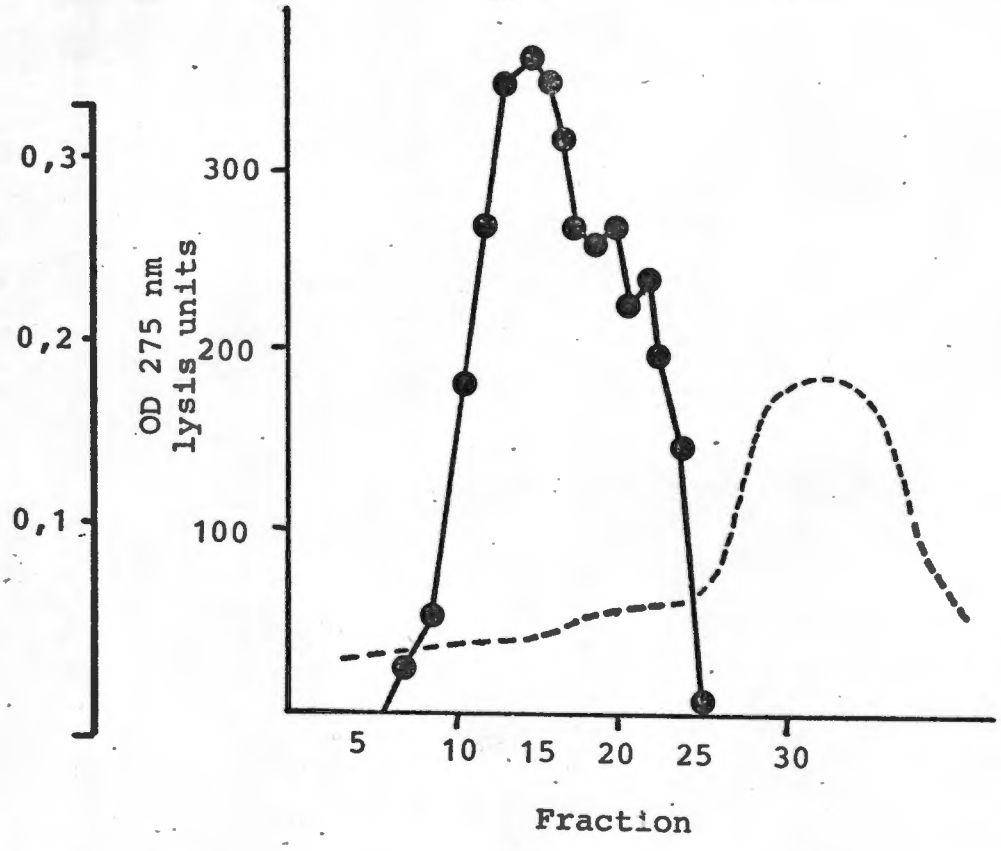


Figure 5-7: Production of Fraction G200. A sample of bile following redissolving of ammonium sulphate precipitate (section 5.3.4) was applied to sephadex G200. Fractions were tested for fibrinolytic activity, and for optical density at 275 nm.

This fraction is roughly comparable to the fraction shown in Figures 5-5 and 5-6.

5.3.7 NaCl gradient elution of 'fraction G200' from Whatman DE 32.

This step was introduced because 'fraction G200' showed a poor correlation between protein content and fibrinolytic activity, and still contained bilirubin. It was thought that gradient elution might also add information about the physical nature of cholelysin.

The pattern of gradient elution was variable. Four

peaks of fibrinolytic activity were usually seen. Of these, only the first peak was consistently present, though usually all four were seen. Peak 4 was most likely to be absent. These were designated Peaks I, II, III and IV, and were collected separately and concentrated by ultrafiltration for the work on substrates described in Chapter Six. In Figures 5-8, 5-9, 5-10 and 5-11 below, four typical patterns of gradient elution are shown. Fouchet's test was usually negative in the Peaks.

Figures 5-8, 5-9, 5-10, 5-11.
Fraction G200 applied to a Whatman DE 32 column, and eluted with a sodium chloride gradient. Conditions for all four experiments were the same:

Sample:	4 ml dialysed G200	
Column:	Whatman 1,5 x 45 cm	
Flow rate:	28 ml/hour	
Resin:	Whatman DE 32	
Buffer:	Tris M 0,006 pH 8,5	
Fractions:	3,2 ml	
Gradient:	M 0,006 - 0,05	40 ml
(Nacl)	M 0,05 - 0,10	40 ml
	M 0,10 - 0,15	40 ml
	M 0,15 - 0,20	40 ml
	M 0,20	100 ml

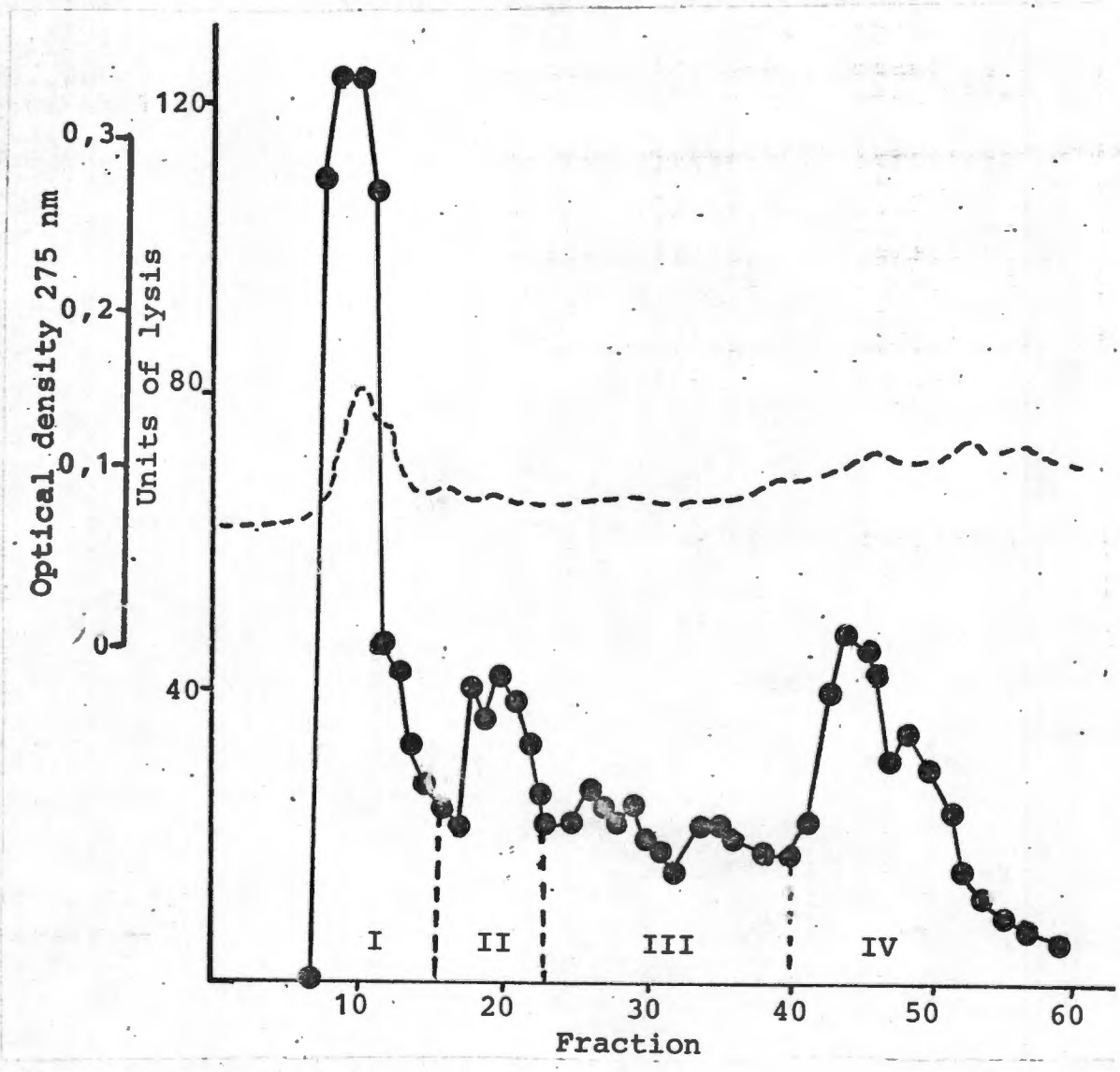


Figure 5-8: Pattern of gradient elution of cholelysin G200 from Whatman DE 32.

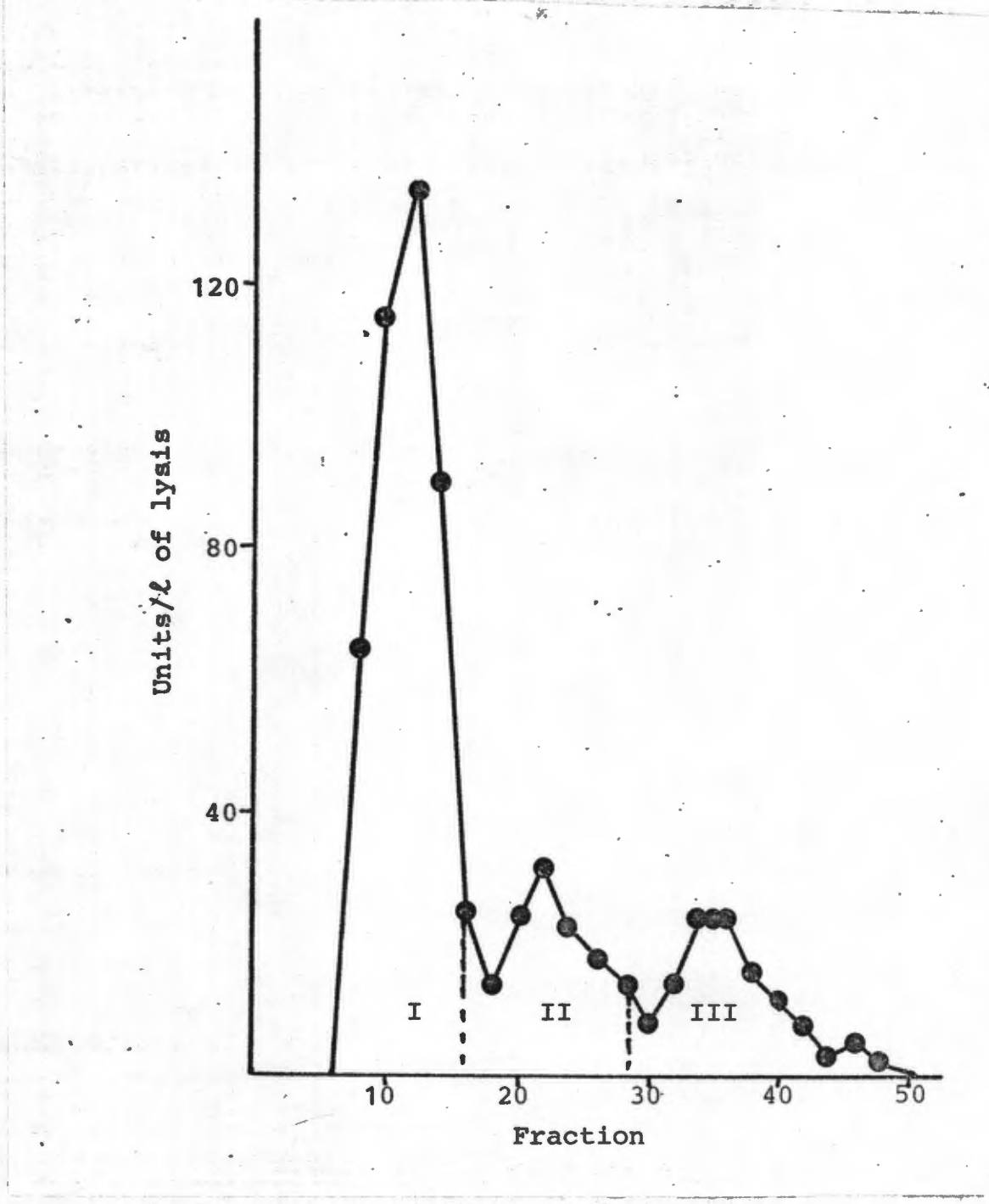


Figure 5-9: Pattern of sodium chloride gradient elution of cholelysin G200 from Whatman DE 32. Peak IV is absent.

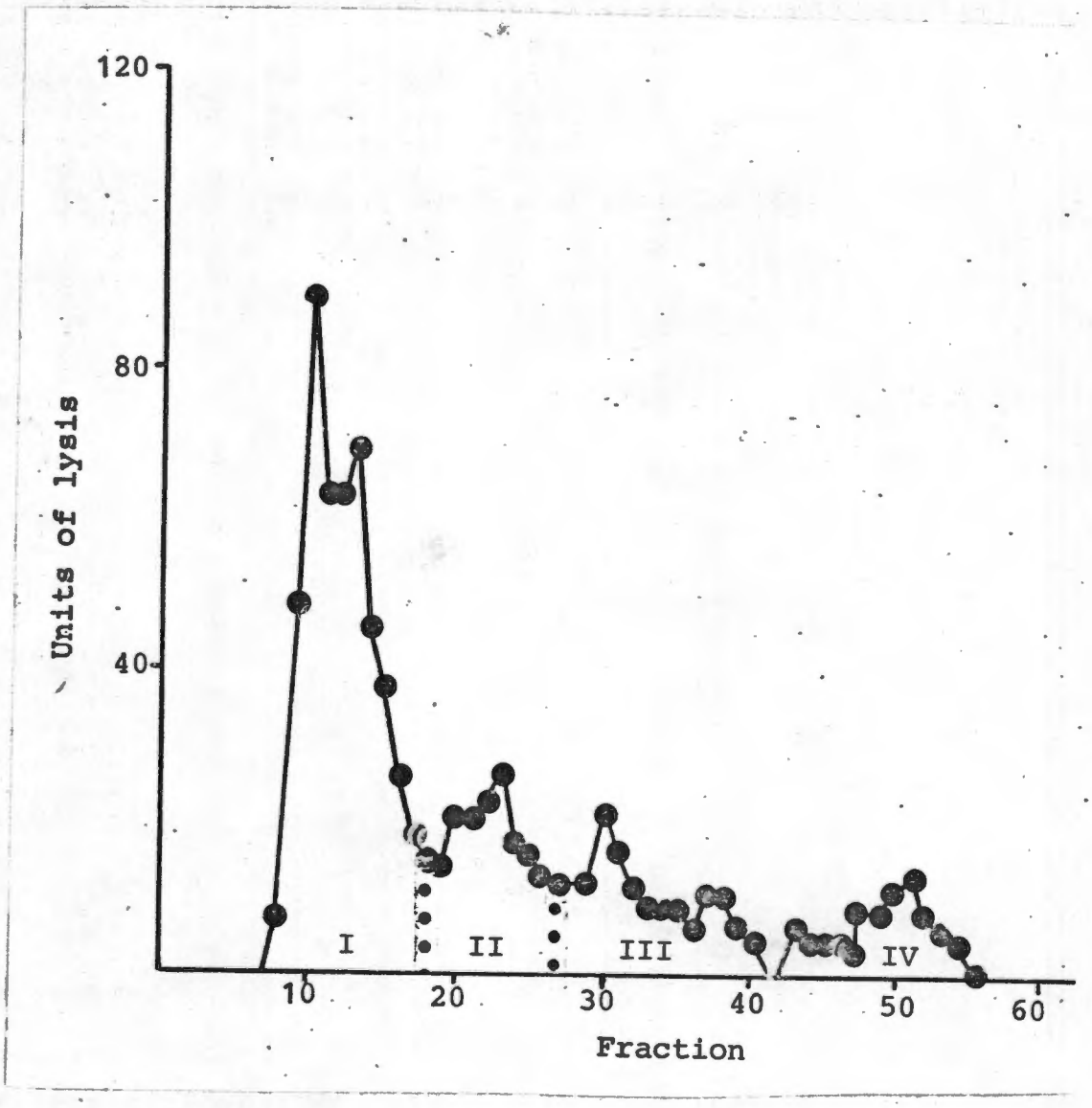


Figure 5-10: Profile of elution of cholelysin from Whatman DE 32, Method A. The four Peaks obtained are indicated in the graph.

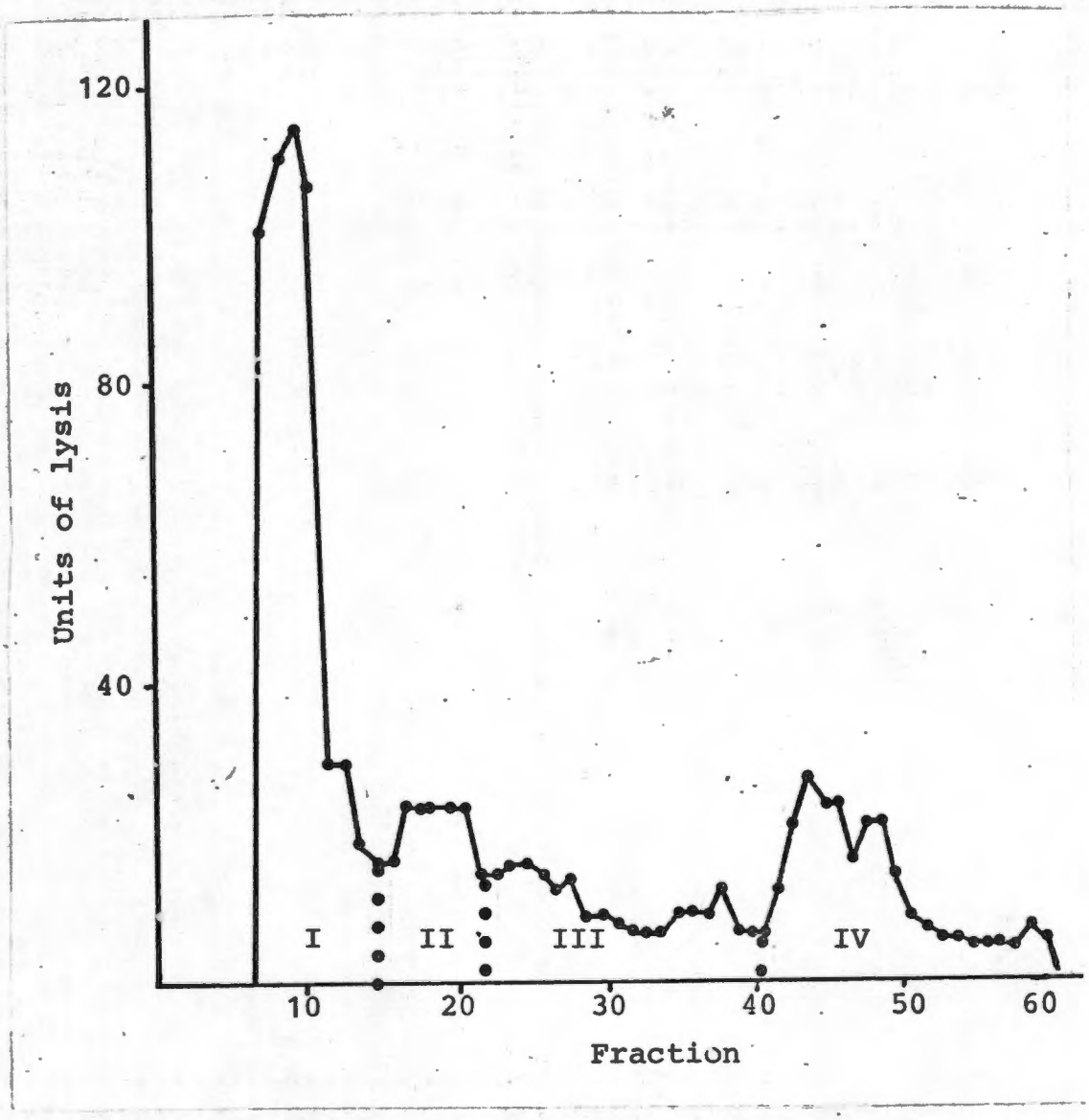


Figure 5-11: Profile of elution of cholelysin from Whatman DE 32, Method A. The four Peaks obtained are indicated on the graph.

Table 5-5: Results of the experiment shown in Figure 5-8 expressed in tabular form:

Peak	Fractions	Volume (ml)	Total activity units/ℓ	Total units	Protein mg/ml	Units/mg protein
I	8-15	25,6	636	16,28	0,055	296
II	16-22	22,4	235	5,26	0,012	438
III	23-38	51,2	276	14,13	0,032	404
IV	39-51	41,6	359	14,93	0,050	299

These results showed satisfactory reduction in protein content while retaining fibrinolytic activity. Higher values for units per mg protein were obtained on other occasions. Three sets of results are shown in Table 5-6.

Table 5-6: Results of NaCl gradient elution of cholelysin from Whatman DE 32 under conditions identical with those set out in Figure 5-8. Three experiments.

Peak	Lysis diam mm	Activity (units/ℓ)	Protein mg/ml	Units/mg protein
I	26,5	51,6	0,019	2 716
	26	48,5	0,055	882
	21	26,3	0,034	774
II	13	9,8	0,010	980
	7,5	5,0	0,016	313
	17	16,0	0,027	593
III	9	6,0	0,015	400
	11	7,7	0,023	335
	(No Peak III in third experiment)			
IV	12	8,7	0,020	435
	22,5	31,7	0,062	511
	6,5	4,5	0,035	129

5.3.8 Full flow sheets for sephadex A50 and for AG1-X8 are shown in Tables 5-7 and 5-8.

Table 5-7: Flow sheet for the purification and concentration of cholelysin from raw ox bile.

Method A

Ox bile 100 ml

1 Desalting with sephadex A50: 4 g added at 20°C. Stirring continuously for 30'.
2 Centrifuge - 15 000 rpm for 30 minutes at 4°C.

Supernatant retained at 4°C

Pellet

1 Wash with 50 ml Tris HCl pH 3,0
2 Centrifuge as above

Supernatant

Pellet (discard)

recombine supernatants

1 Make up volume to 100 ml with Tris buffer pH 8,5
2 Add ammonium sulphate 40 g
3 Centrifuge at 15 000 rpm for 15 minutes at 4°C

Supernatant (discard)

Pellet

1 Redissolve in Tris HCl pH 8,5
2 Dialyse against Tris HCl pH 8,5 overnight at 4°C - 1 litre changed once

Column chromatography on sephadex G200

All lytic fractions combined = Fraction G200

Sodium chloride gradient on Whatman DE 32

Peaks I II III IV

Dialyse overnight against Tris HCl buffer pH 8,5 at 4°C (dialysis fluid changed once)

Table 5-8: Flow sheet for the purification and concentration of cholelysin from raw ox bile.

Method B

Ox bile 2 litres

1 Desalting with AG 1X-8 160 g, stirring continuously by hand for 30 minutes
2 Centrifuge in basket centrifuge

Effluent — Precipitate - regenerate (see methods)

1 Add ammonium sulphate 50% W/V
2 Stir slowly for 30 minutes at room temperature
3 Centrifuge in basket centrifuge

Effluent (discard) — Precipitate

1 Dissolve in minimum volume of Tris HCl pH 8,5
2 Dialyse overnight against 30 litre Tris buffer pH 8,5, in boiled dialysis tubing approximately 4,5 cm diameter, changing dialysis fluid once

Whatman DE 32

1 Elute with 6 ℓ Tris buffer pH 8,5
2 Elute with 0,1 M NaCl in Tris buffer pH 8,5

Fractions were typically 200 ml. All fractions retained and tested for lytic activity.

3 Retain first elution (= Peak I)
4 Dialyse in Tris buffer pH 8,5 to remqve NaCl
5 Concentrate on Amicon filter
6 Assay for lytic activity and protein content
7 Flash freeze dry

This multiplicity of Peaks, and irregular elution from a column, led into greater complexity than seemed justified at this stage. This thought was strengthened by the discovery that Peak I could be eluted from Whatman DE 32 by tris buffer alone. Subsequently, a pulse of sodium chloride could be used to clear remaining activity from the column. When the procedure was carried out in this way, activity was fairly evenly divided between the two batches, though with runs on a large column more activity tended to remain in the second batch. Results of 6 experiments are shown in Table 5-9.

Table 5-9: Batchwise elution of cholelysin from Fraction G200 applied to Whatman DE 32. Tris M 0,006 pH 8,5 followed by NaCl 0,1%.
Two sizes of column: (a) 40 x 1,5 cm
(b) 72 x 14 cm

	Applied to column (units)	Recovery (units)	Recovery %	Tris eluate	NaCl eluate	Tris/NaCl ratio
(a)	2,3	2,46	107	1,26	1,20	1,05
	5,4	5,28	98	3,17	2,11	1,50
	4,7	4,42	94	3,29	1,13	2,91
(b)	105,2	137,3	131	39,1	98,2	0,40
	96,1	101,6	105	44,2	57,4	0,77
	58,2	58,2	100	25,8	32,4	0,80

These results, though variable, did indicate that sufficient Peak I could be obtained for experimentation, and subsequently this Peak was used for all experimental work except some of the substrate studies reported in Chapter Six. An example of batchwise elution is given in Figure 5-12.

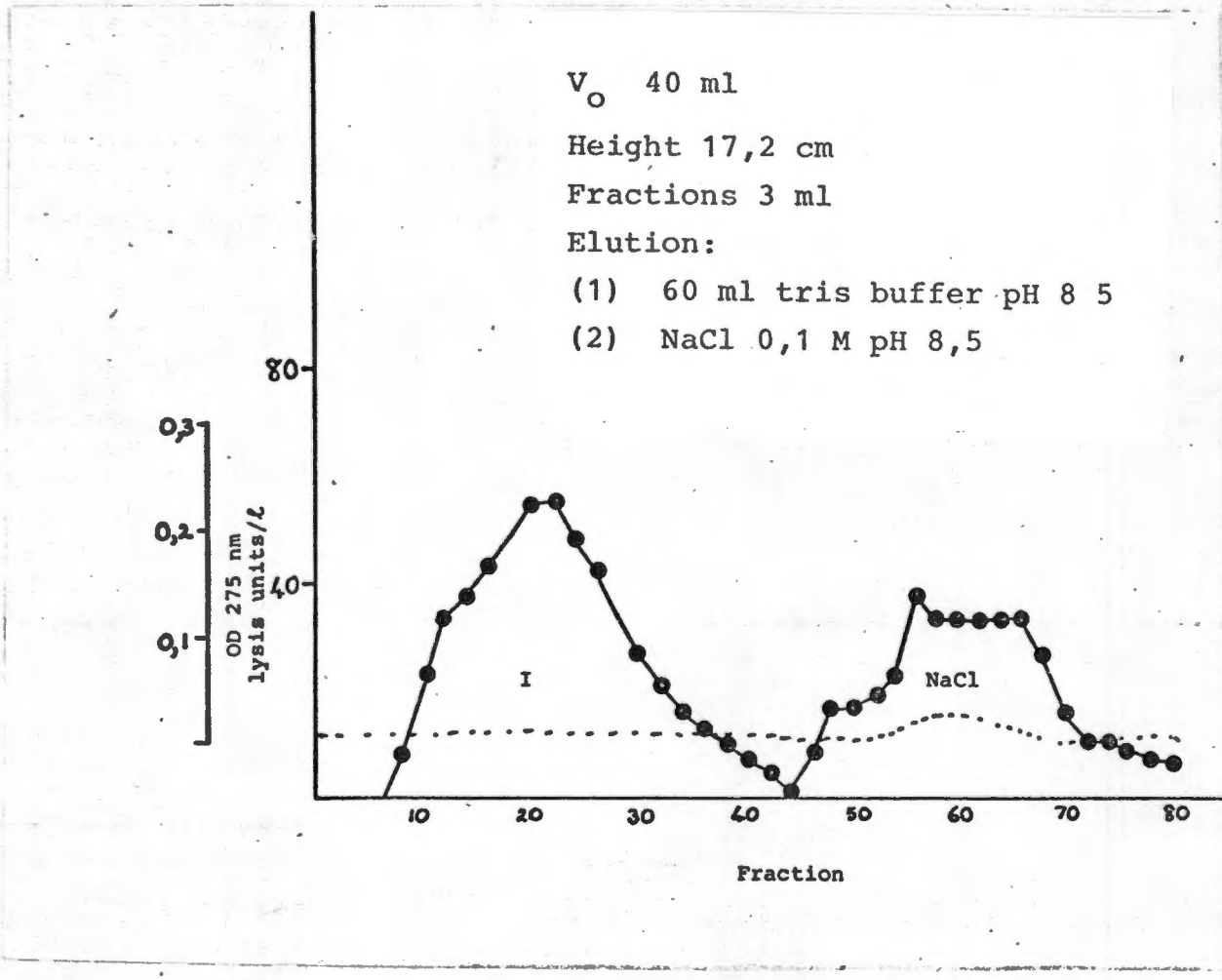


Figure 5-12: Batchwise elution of Peak I off Whatman DE 32.

5.3.9 Dialysis

5.3.9.1 Lowering of electrolyte and other chemical concentrations. Dialysis was resorted to, to remove unwanted chemicals from the solution eg ammonium sulphate, sodium chloride, potassium biphthalate; and for lowering of electrolyte concentration after ultrafiltration. The standard technique employed was to use double the volume of the solution to be dialysed, and to change this once over 24 hours at 4°C. Usually tris buffer M 0,006 pH 8,5 was employed. Dialysis led to an unacceptable loss of activity (up to 100%) until it was found that boiling the dialysis tubing first prevented this loss.

5.4 Concentration

5.4.1 Considerable concentration was possible using ammonium sulphate precipitation alone. A twice precipitated sample of bile could attain a concentration of lytic activity = ~ 20 x original sample, as the following table shows.

Table 5-10: Summarised experiment in which fibrinolytic activity was twice precipitated from raw bile by ammonium sulphate. It was found that low concentrations of ammonium sulphate might be sufficient for complete reprecipitation.

Stage	Volume	Lysis (mm diam)	Units/ℓ	Concentration
1	20 ml	20	23,2	1,0
2	10 ml	25	42,9	1,8 X
3	2 ml	44	442	19,1 X

Stages (1) Raw bile
(2) Ammonium sulphate added W/V 33%
(3) Ammonium sulphate added W/V 4%

However, there was heavy contamination from bile salts and pigments, and this procedure was unsatisfactory as a final method of purification.

5.4.2 Ultrafiltration

This technique was used extensively. It led to concentration of fibrinolytic activity of 2 - 15 times, with considerable loss of activity during the process. An Amicon ultrafilter was used, with UM 2 filter passing all molecules below 1 000 molecular weight. Results are shown in Tables 5-11 and 5-12.

Table 5-11: Effect of concentration by ultrafiltration on activity of cholelysin. Amicon with UM 2 filter. Peak I used in all four experiments.

Experiment	Units	ml	Total units	Concentration Post/Pre	
				ml	units
A	Pre: 0,0098	111	1,088		
	Post: 0,062	7,9	0,490	14,1	6,3
Loss			55%		
B	Pre: 0,0064	172	1,110		
	Post: 0,095	8	0,763	21,5	14,9
Loss			31%		
C	Pre: 0,0792	145	11,484		
	Post: 0,165	12	1,984	12,1	2,1
Loss			83%		
D	Pre: 0,0862	132	11,380		
	Post: 0,710	6	4,260	22,0	8,2
Loss			63%		

Protein concentrations were estimated in experiment D above, and are reported in Table 5-12.

Table 5-12: Protein concentration, and acitivity/mg protein during concentration of cholelysin Peak I by ultrafiltration.

	Protein mg/ml	units	Units/mg protein
Pre-concentration	0,0071	86,2	12 141
Post-concentration (22x)	0,1611	710	4 407
Increase	22,7 X		2,7 X

Comment: These experiments showed a considerable loss, with much variability. They also showed that one possibility was denaturation of the protein concerned (Table 5-12).

5.5 Stabilisation

5.5.1 Stabilisation by addition of calcium or magnesium

Earlier preparation with heavy contamination by other bile constituents showed much loss of activity with time. The addition of Ca^{++} or Mg^{++} in physiological quantities led to no improvement.

5.5.2 Freeze drying

This technique was used in an attempt to cut down loss of fibrinolytic activity during storage of products over months at $-40^{o}C$. It was found that there was an average loss of fibrinolytic activity of 50% at 6 months of storage at $-40^{o}C$.

Freeze-drying resulted in even greater loss of activity.

5.5.3 Flash freezing - freeze-drying

In this technique, the concentrated and purified specimen was frozen instantly in 2 ml aliquots in liquid nitrogen at -196°C, and then freeze dried in the usual way. Using this method, there was no loss of activity over 6 months, and this was the procedure which was standardised for later work.

5.6 Summary and flow sheet analysis

The procedure of purification, concentration, and stabilisation of cholelysin is described. The final method used the following procedures in succession:

	Protein mg/ml	Fibrinolytic activity (units/ℓ)	Units/mg protein
Raw ox bile	2-6	10-1500	-
Typical specimen of bile	2,4	442	130
Redissolved after sephadex A50 step	1,1	270	208
Redissolved after $NH_4(SO_4)$ step	0,750	306	408
Fraction G200	0,200	120	600
Peak I	0,034	26,3	774
Peak I after second elution off Whatman DE 32	0,007	86,2	12 141

The above figures are, of course, an average statement of a highly variable process. The maximum and minimum figures for the various steps are given in Table 5-13. 'Peak I after second elution' gave the highest figure for activity in units/mg protein recorded during this study.

Table 5-13: Recovery of fibrinolytic activity during processing of ox bile.

	Maximum	Minimum
Raw bile	100	100
Redissolved after $NH_4(SO_4)_2$ precipitate	91	69
Fraction G200	85	50
Whatman DE 32 (total activity)	85-90	42
Peak I	63-67	12

In practice, figures fell between the two extremes, and large scale recovery of cholelysin from ox bile was obviously possible.

CHAPTER SIX

SUBSTRATE SPECIFICITY OF CHOLELYSIN

6.1 Endopeptidases such as trypsin and chymotrypsin are active against many substrates. In this chapter, the activity of cholelysin against some of these substrates is described, and compared with that of chymotrypsin and trypsin. Plasminogen was added in a further attempt to find plasminogen activator activity of cholelysin. Substrates studied were:

(1) Trypsinogen, chymotrypsinogen, plasminogen
(2) Casein
(3) N-acetyl-L-tyrosine ethyl ester monohydrate (ATEE)
(4) N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE)

6.2 (1) <u>Trypsinogen, chymotrypsinogen, plasminogen</u>

These zymogens are activated by their own activated forms (Guyton, 1976). Because trypsinogen and chymotrypsinogen derived from the pancreas are physically in such close contact with the biliary tree, it was essential to establish if cholelysin was related to either of them in any way.

In these experiments, each active enzyme was applied to the fibrin plate in a 1/1 dilution in tris buffer pH 8,5, M 0,006, when tested alone. In experiments that involved mixing the active enzyme with a zymogen, the zymogen was substituted in equal volume for the tris diluent.

Table 6-1: Attempt to activate trypsinogen, chymotrypsin and plasminogen with cholelysin. All results in mm diameter of zone of lysis. Full details are given in Appendix 6 p 220 Strength of enzyme solutions used in this experiment was:
plasmingen and plasmin - 0,1 CTA U/ml
trypsinogen and trypsin - 1 000 U/ml
chymotrypsinogen and chymotrypsin - 1 000 U/ml
Fraction G200 was used throughout.

Blank value	Substrate added	Enzyme Cholelysin	Trypsin	Chymotrypsin	Plasmin
0	None	12,2	14,8	14,4	12,2
0	Trypsinogen	11,6	23,6	-	-
4	Chymotrypsinogen	11,0	-	22,2	-
0	Plasminogen	13,0	-	-	21,0

The left hand column - 'Blank value' - gave the lytic activity of each zymogen when tested separately. All available commercial samples of chymotrypsinogen had lytic activity, though the other zymogens were inert on a fibrin plate.

Comment: Cholelysin failed to activate any of these zymogens, proving its separate identity. It seemed possible that chymotrypsinogen interfered with the lytic activity of the cholelysin solution tested; the calculated combined lytic effect should have been about 14,8 mm, which is significantly different from the result observed. However, this observation was not followed up.

6.3 (2) α-casein

In 1968, the National Heart Institute, Bethesda, Md, USA, approved a caseinolytic assay for plasmin, plasminogen and urokinase in purified systems (Johnson, Kline and Alkjaersig, 1969). This described a reproducible system for the assay of plasmin in terms of the release of tyrosine from α-casein. Under the described conditions (see Methods), one unit of plasmin was defined as the amount which released 0,1 micro-equivalent of tyrosine/minute from a specified casein preparation. In terms of the spectrophotometric result, 1 unit of CTA plasmin/ml after 20 minutes of incubation with casein, caused an increase in optical density of the TCA-soluble supernatant at 275 nm of 0,300. This was approximately true of this experiment when done in Cape Town, though values tended to be slightly lower (see Table 6-2). This was ascribed to slight deterioration in the α-casein and/or the plasmin during their journey from the United States.

Table 6-2: Caseinolytic effect of plasmin in the standard system (Johnson, Kline and Alkjaersig op cit, 1969). These results were considered close enough to the published paper to accept the system for study.

	Increase in OD at 275 nm
1971	0,270
1972	0,290
1973	0,285

6.3.1 Cholelysin was also caseinolytic under the conditions described above. Results of 3 experiments are shown below in Figures 6-1, 6-2, and 6-3. Results of these experiments are summarised in Table 6-3.

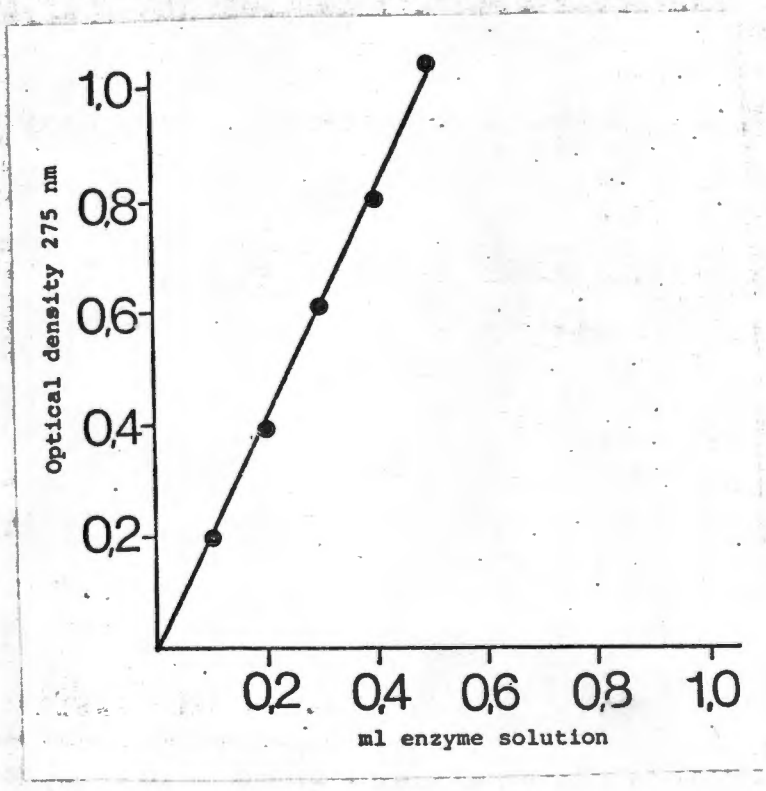


Figure 6-1: Caseinolytic effect of cholelysin Peak I.

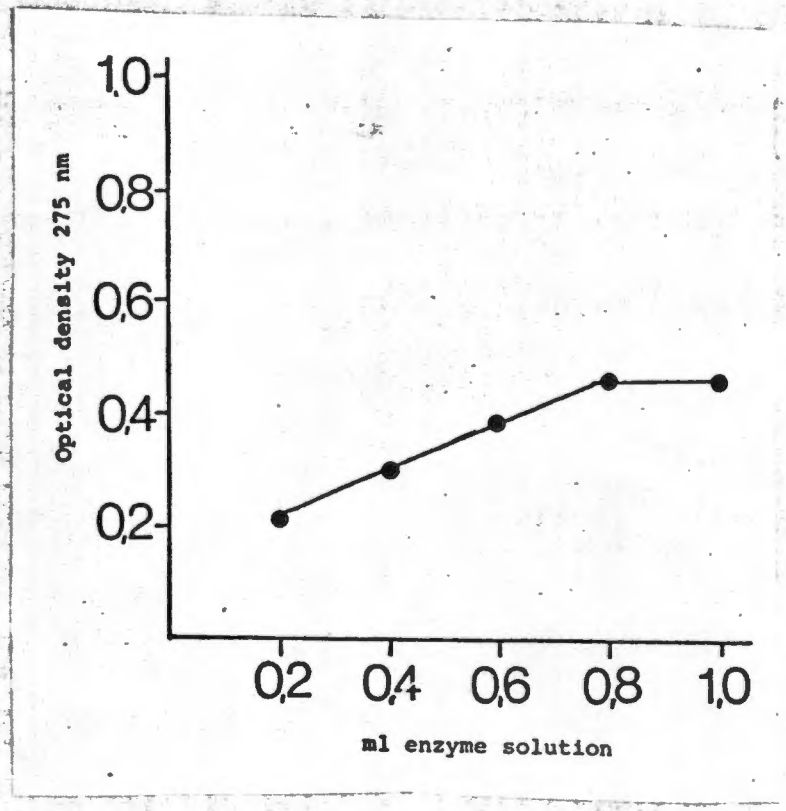


Figure 6-2: Caseinolytic effect of cholelysin Peak I.

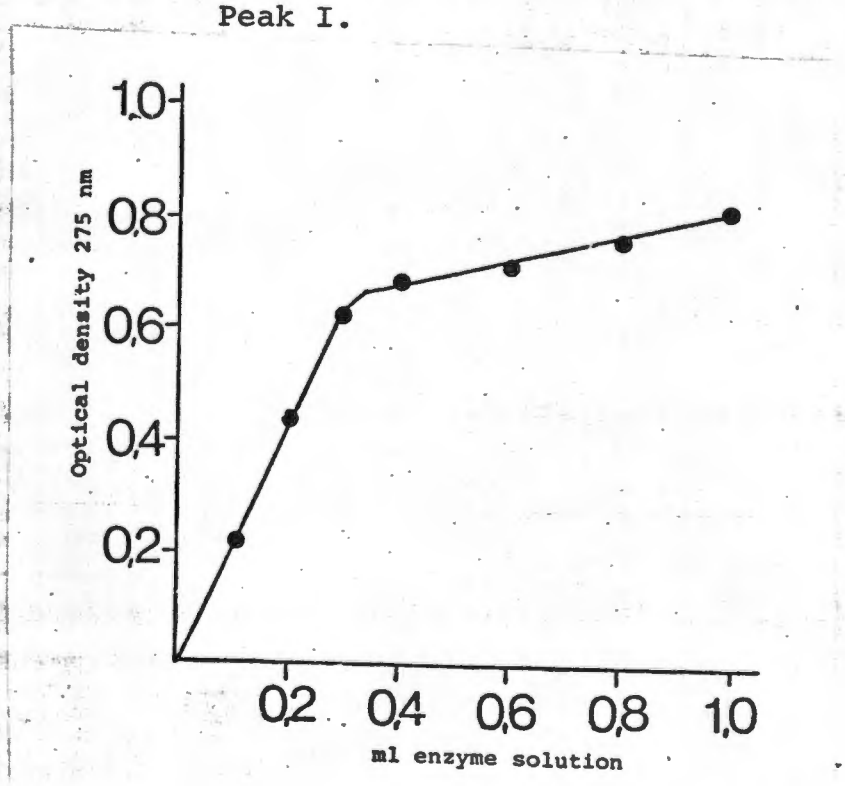


Figure 6-3: Caseinolytic effect of cholelysin Peak I.

6.3.2 Caseinolysis by Peaks II, III, IV

All fractions studied exhibited caseinolytic activity. This is shown in Figures 6-4, 6-5 and 6-6 below, and caseinolytic activity is expressed in terms of enzyme concentration in mg/ml in Table 6-4 below.

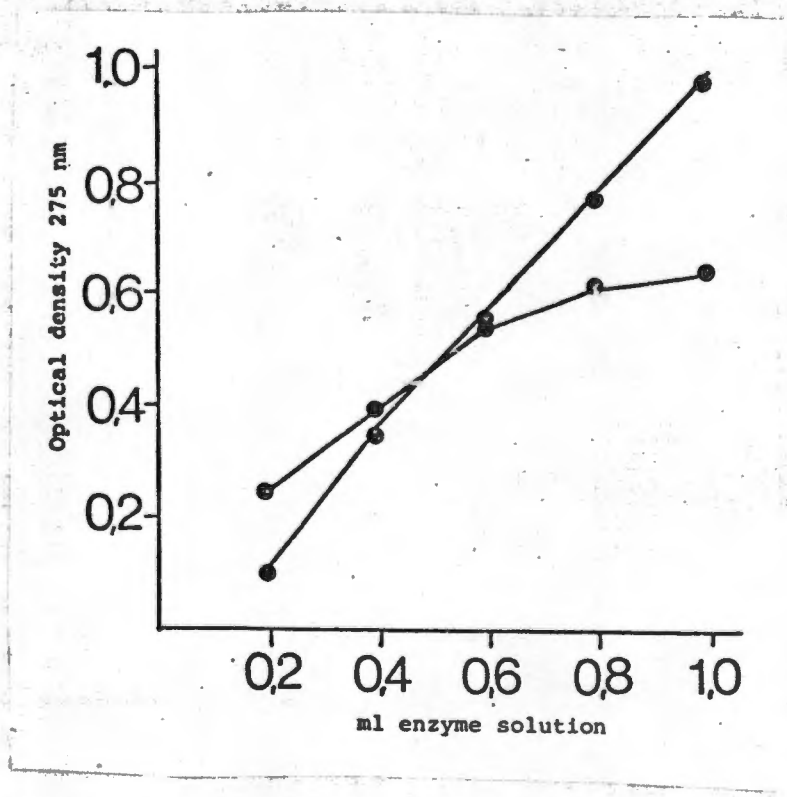


Figure 6-4: Caseinolysis by Peak II.

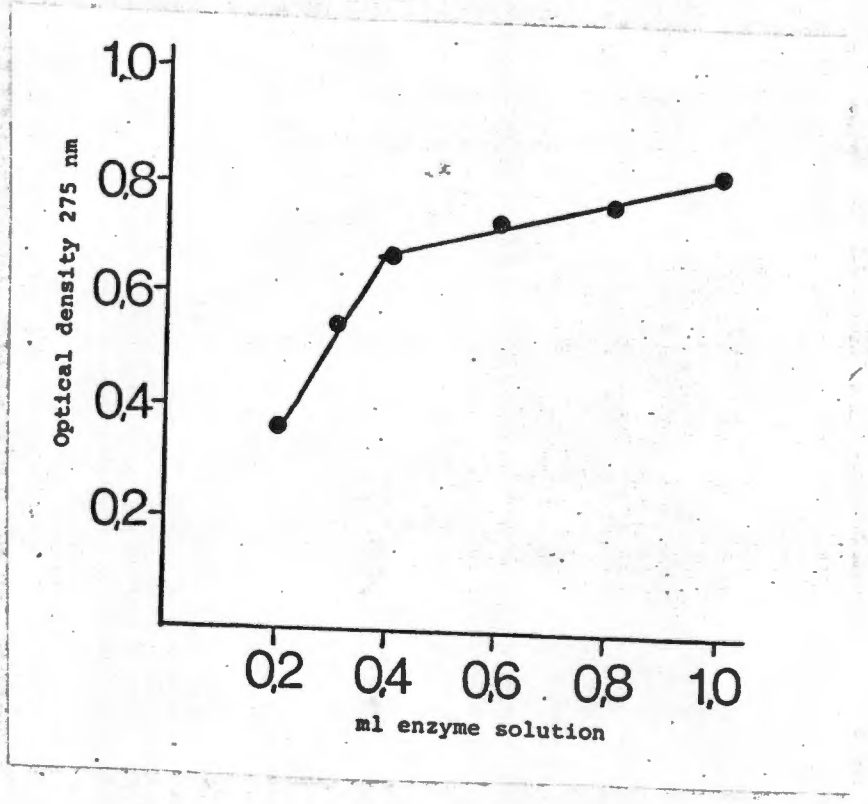


Figure 6-5: Caseinolysis by Peak III.

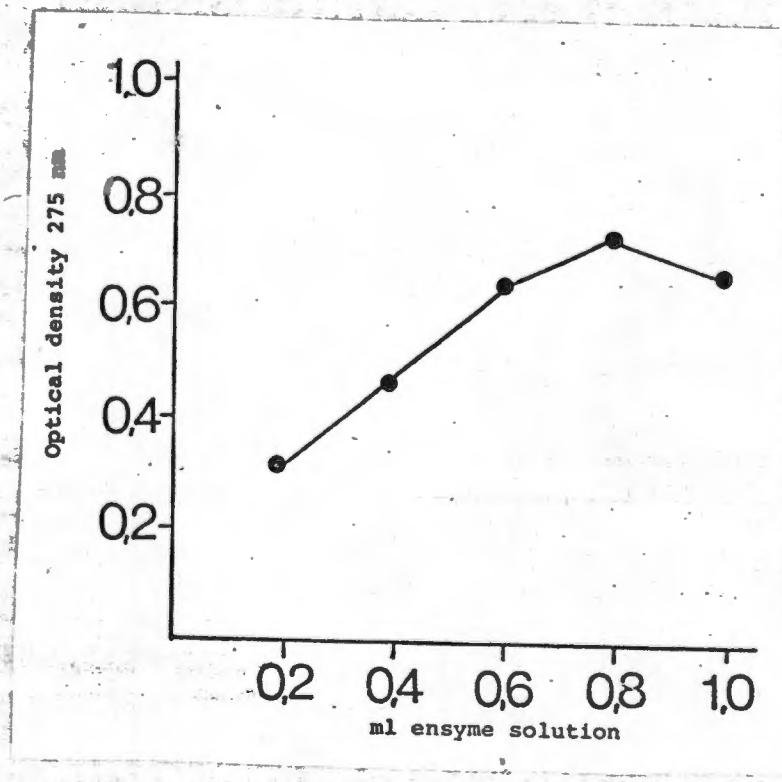


Figure 6-6: Caseinolysis by Peak IV.

Comment: There was a poor correlation between caseinolysis and protein concentration, and also between fibrinolysis and caseinolysis in the 3 experiments where combined results are available. Figure 6-7 shows these results graphically.

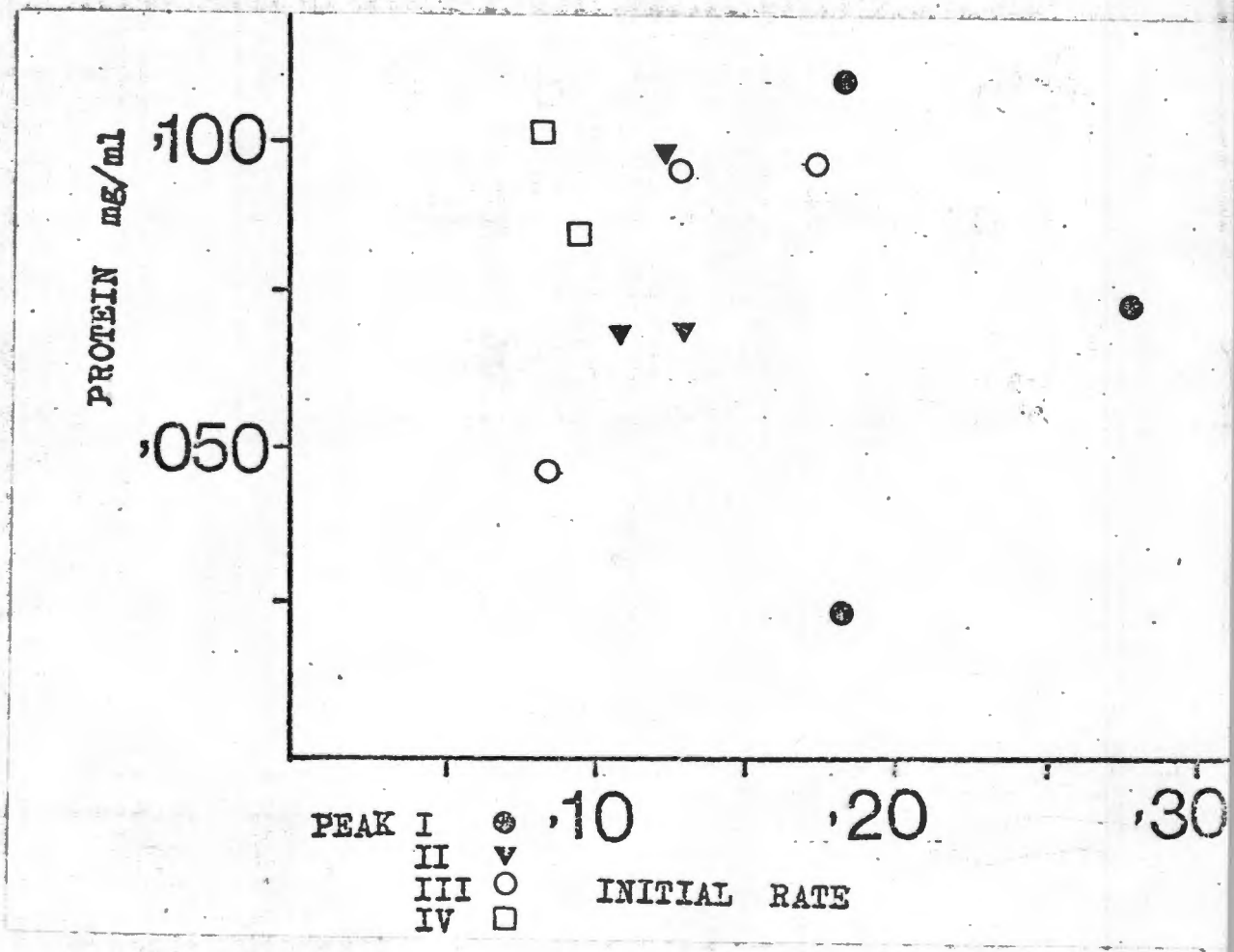


Figure 6-7: Relationship between protein concentration and caseinolytic activity of

Peaks I - ●
II - ▼
III - ○
IV - □

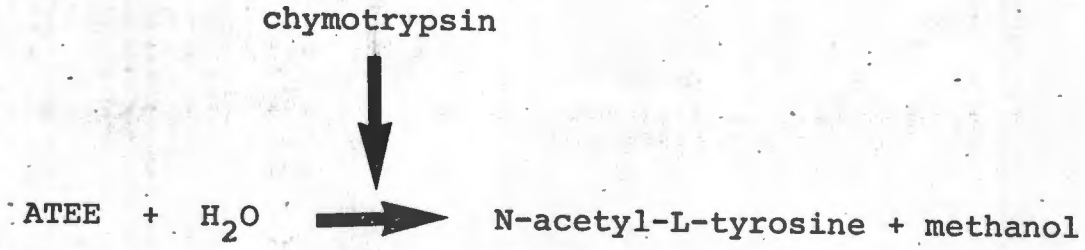
Table 6-3: Caseinolysis in terms of protein concentration of Peaks I, II, III, IV. Fibrinolytic activity is only available for Peak I.

A	B	C	D	E	F
Peak	Δ Activity*	Protein mg/ml^{-1}	Δ Activity/ mg protein	Lysis units/ℓ	D/E
I					
(Fig 6-1)	0,42	0,023	18,3	21	0,87
(Fig 6-2)	2,08	0,113	18,4	42,6	0,43
(Fig 6-3)	2,08	0,075	27,7	23,6	1,17
II	0,98	0,202	4,9		
(Fig 6-4)	0,76	0,069	11,0		
(Fig 6-4)	1,25	0,101	12,4		
	1,10	0,083	13,3		
III	0,41	0,048	8,5		
	1,01	0,070	14,4		
(Fig 6-5)	1,71	0,097	17,6		
IV	0,95	0,109	8,7		
(Fig 6-6)	0,085	0,086	9,9		

* Activity expressed as tangent of best slope on graph

6.4 (3) N-acetyl-L-tyrosine ethyl ester monohydrate (ATEE)

This substrate is used in an accepted technique for the assay of the chymotrypsins (Schwert and Takenaka, 1955). ATEE is hydrolysed at the ester linkage, causing a decrease of extinction at 237 nm. The reaction is formulated as follows:



The unit of enzyme is specified as that amount of enzyme causing a decrease of extinction at 237 nm of 0,0075 per minute at 25°C.

Technique The technique for this assay was taken from the Miles-Seravac manual (Miles-Seravac, 1972 pp 14-15). It was planned to measure ATEE esterase activity of cholelysin and also the effect of the known inhibitor(s) of cholelysin (see Chapter VII) when included in the reaction mixture. This made it necessary to modify the standard test method, for this allowed only 0,2 ml for added test substances.

The ATEE-esterase activity of cholelysin, as reported in section 6.4.1, was carried out in the standard way. However, for the experiments reported in 6.4.2 and in Chapter Seven (below) it was necessary to modify the standard test to make possible the inclusion of larger volumes of test material (either cholelysin or inhibitor) in the technique.

For these experiments, the following modification was made:

	ATEE mg/100 ml	Diluent (ml)	ATEE used (mg)	Volume for test (ml)	Unit of activity
Standard	25,2	3	0,757	0,2	0,0075/min
Revised	37,6	2	0,752	1,2	0,01/min

In the revised method, the unit of activity was altered, because conditions were no longer standard, and 0,01/min was more convenient than 0,0075/min.

6.4.1 Cholelysin was found to have esterase activity against ATEE. Peaks I, II, III and IV were assayed in separate experiments, which are shown in Figures 6-8 and 6-9.

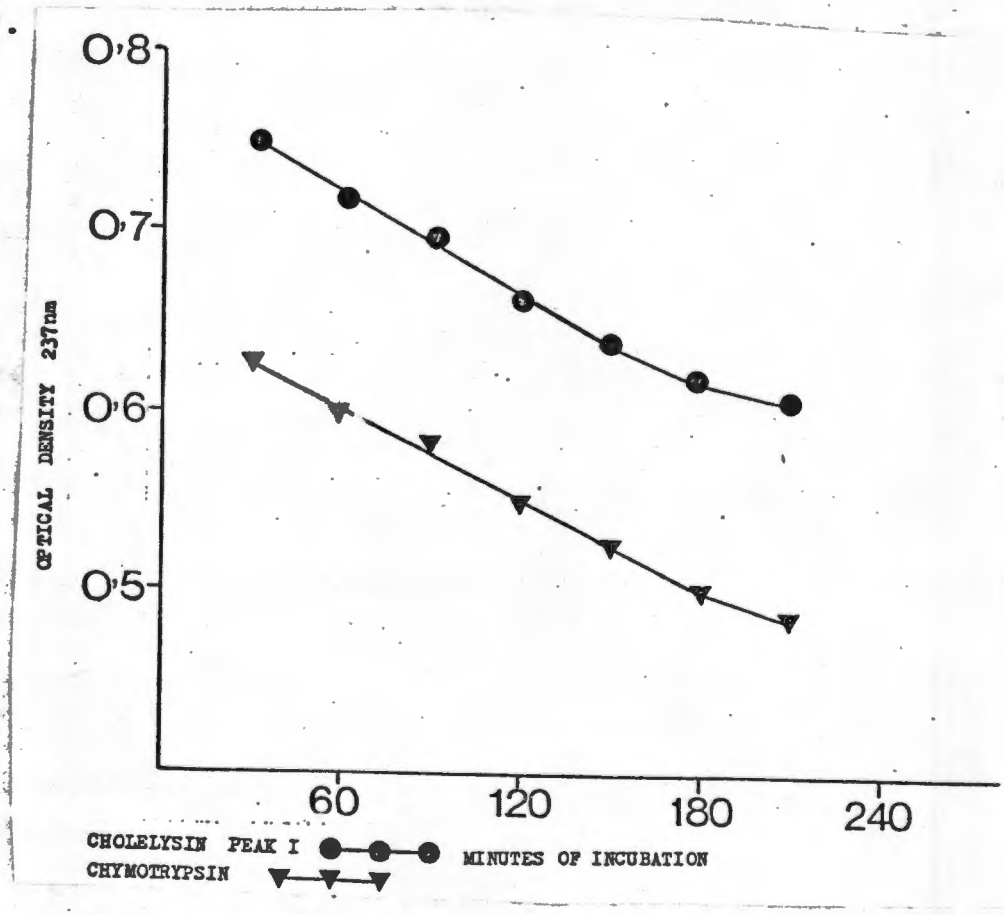


Figure 6-8: Esterase activity of cholelysin against ATEE. Chymotrypsin control is shown in Figures 6-10 and 6-11.

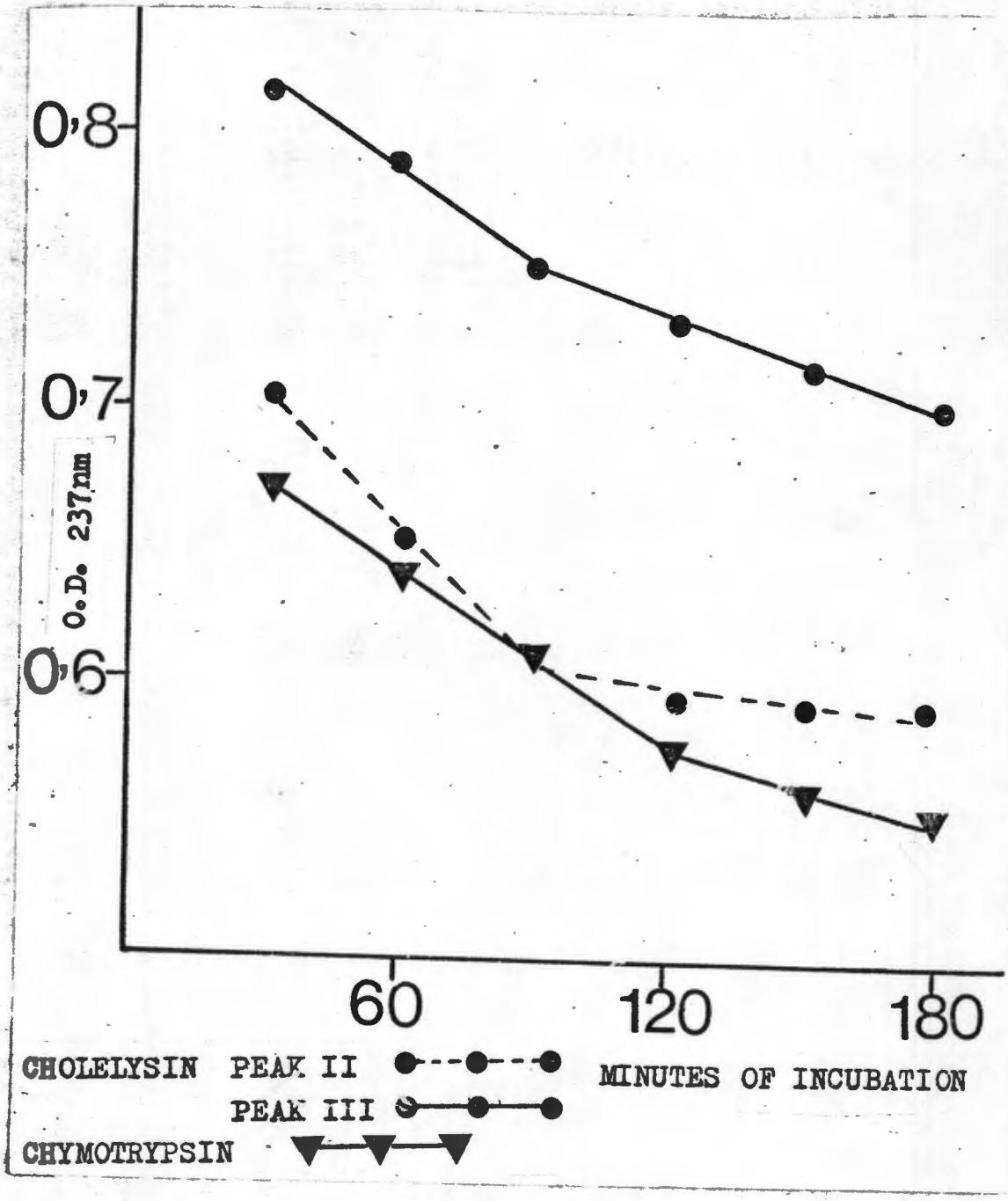


Figure 6-9: Esterase activity of cholelysin Peaks II and III against ATEE. Peak IV was inactive against ATEE. (Chymotrypsin control).

The experimental results shown in Figures 6-8 and 6-9 are set out below in Table 6-4.

Table 6-4: Esterase activity, protein in mg/ml^{-1}, and fibrinolytic activity of cholelysin Peaks I, II, III with chymotrypsin control.

Enzyme	Initial rate OD/min	Chymotrypsin units	Protein mg/ml^{-1}	Chymotrypsin units/mg protein		Lytic units	Chymotrypsin/ lytic ratio
Chymotrypsin							
(i)	0,0668	8,9	0,01	890	900		
(ii)	0,0682	9,1	0,01	910			
Peak I	0,0684	9,1	0,05	182		31,5	5,8
II	0,0903	12,04	0,235	51		11,7	4,4
III	0,0770	10,27	0,275	37		9,8	3,8

Note: Chymotrypsin control used at 10 units/ml concentration. Chymotrypsin (i) shown in Figure 6-9.

6.4.2 It was of interest to discover if esterase and fibrinolytic activities paralleled each other. Accordingly, simultaneous assay of these proteolytic activities was made on fractions of Peak I eluted off Whatman DE 32 in the usual way. The result is shown in Figures 6-10 and 6-11.

Table 6-5: Comparison of the fibrinolytic and esterase activities of cholelysin Peak I. In this table:

'Units/ml ATEE' are obtained from:

$$\frac{\text{Dilution factor X initial rate}}{0{,}01}$$

'ATEE/lysis ratio' is obtained from:

$$\frac{\text{Units/ml ATEE}}{\text{lysis units}}$$

Fraction	Dilution factor	Initial rate OD/minute	Revised Units/ml ATEE	Lysis mm diam	Lysis units	ATEE/lysis ratio
5	1	0,0320	3,20	9	6,0	0,53
10	1	0,0350	3,50	10	6,8	0,51
15	1	0,0362	3,62	13	9,8	0,40
20	1	0,0337	3,37	12	8,7	0,39
25	1	0,0394	3,94	14	11,1	0,35
30	1	0,0269	2,69	14	11,1	0,24
35	2	(0,2612)	52,24)			
	5	(0,1000)	50,00)			
	10	(0,0478)	47,80)			
		mean	50,01	25	42,9	1,17
40	20	0,0259	51,7	29	70,1	0,74
45	20	0,0299	59,8	29,5	74,6	0,80
50	20	0,0273	54,6	30	79,2	0,69
55	20	0,0271	54,2	29,5	74,6	0,73
60	20	0,0248	49,6	26,5	51,6	0,96
65	20	0,0230	46,0	26	48,5	0,95
70	10	0,0415	41,5	26	48,5	0,86
75	10	0,0371	37,1	22	29,7	1,25
80	5	0,0578	28,9	13	9,8	2,95
85	5	0,0450	22,5	8	5,3	4,24
90	5	0,0521	26,1	13	9,8	2,66
95	5	0,0650	32,5	20	23,2	1,40
100	5	0,0398	19,9	8	5,3	3,75

Fraction 35 was estimated at three different dilutions to confirm the large increase in esterase activity found at this point.

Table 6-5 is shown graphically in Figure 6-10 below.

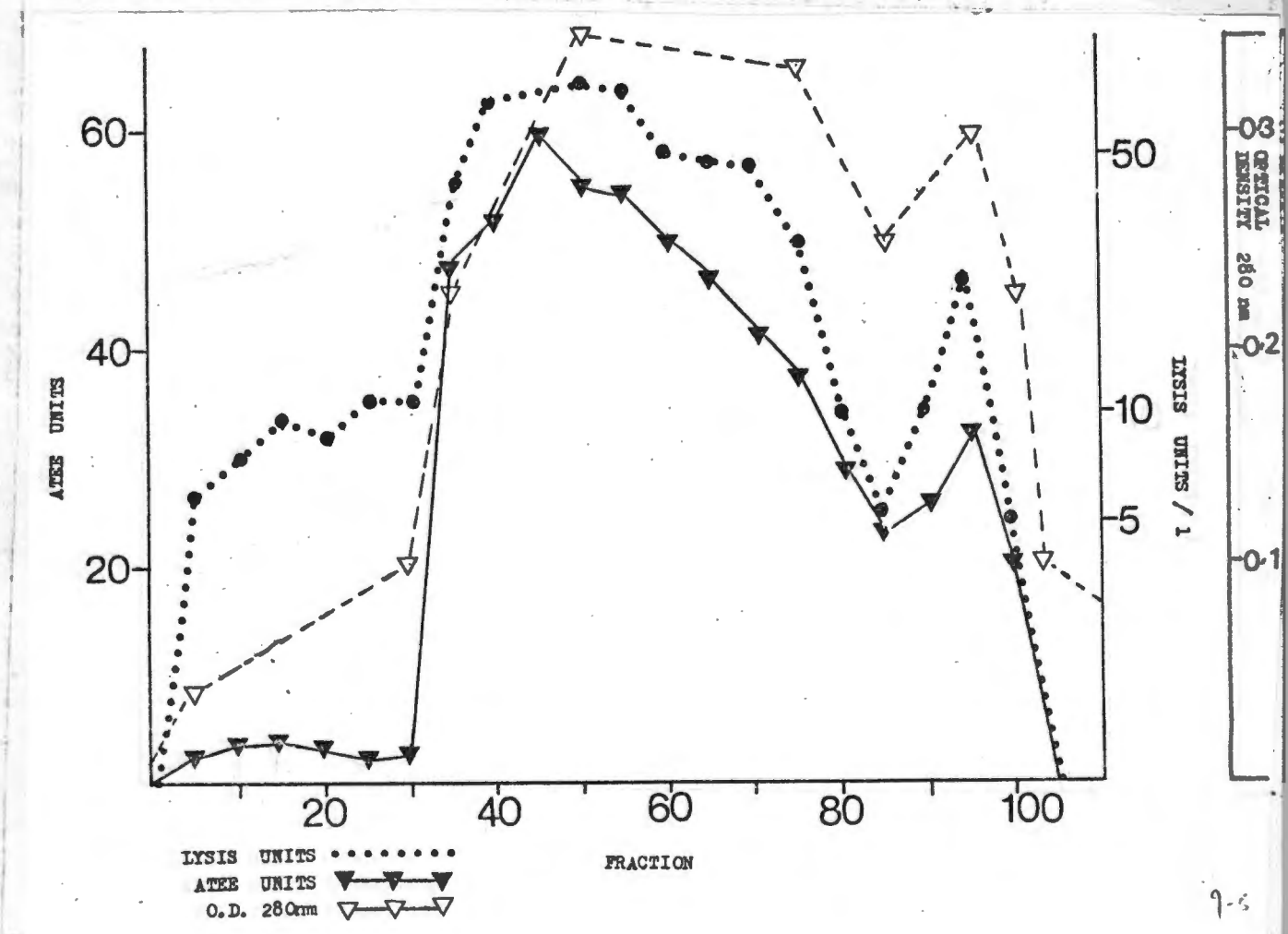


Figure 6-10: ATEE esterase and fibrinolytic activities of cholelysin Peak I estimated simultaneously. Whatman DE 32. Fractions reduced from 3,2 ml to 0,65 ml, and every 5th fraction estimated. Details as in Method A p66 for eluting of Peak I from Whatman DE 32. Optical density also shown.

From these results, it appeared that esterase and fibrinolytic activities were found together, but that there was a dissociation in the relative activities present; fractions 5 - 30 showed a different ATEE/lysis ratio from fractions 40/100. This point is shown graphically in Figure 6-11 below.

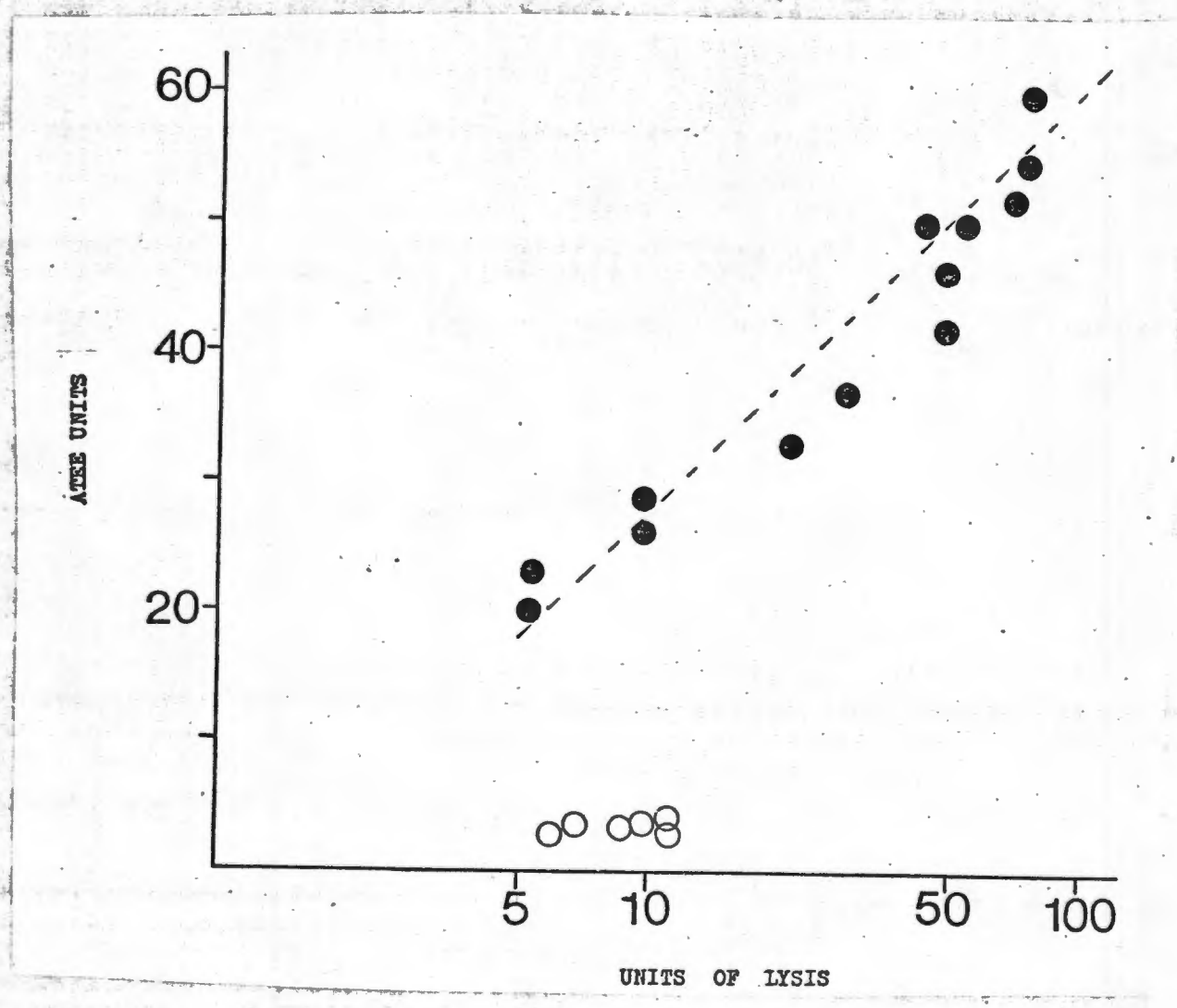
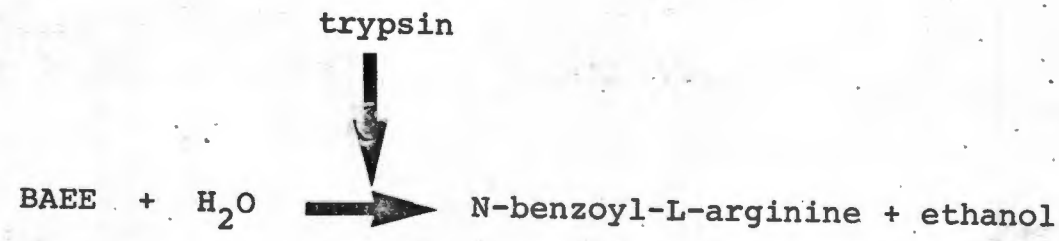


Figure 6-11: Esterase and fibrinolytic activities of experiment shown in Table 6-5 and Figure 6-10, plotted against each other.
Fractions 5 - 30: ○ ○
Fractions 35 - 100: ● ●

Comment: this interesting result suggests that there are two forms of cholelysin in Peak I, and that the more slowly eluting form has a higher esterase/fibrinolysis ratio. Furthermore, the two activities in this second form bear a semi-logarithmic relationship to each other, though the significance of this relationship has not been pursued further.

6.5 (4) N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE)

This substrate was also of interest, as it is used in an accepted method of assay for trypsin (Schwert and Takenaka, 1955). BAEE is hydrolysed at the ester linkage, causing an increase of extinction at 253 nm. This reaction is formulated as follows:



The unit of enzyme is specified as that amount causing an increase of extinction at 253 nm of 0,003 per minute at 25°C. The technique was taken from the Miles-Seravac manual (Miles-Seravac, 1972 pp 48-49). Trypsin standard gave 101% of expected activity. Standard assays are shown in Figures 6-12 and 6-13.

6.5.1 Cholelysin was found to have esterase activity against BAEE. Peaks I, II, III and IV were assayed in separate experiments. The results are given in Table 6-6, and are shown graphically in Figure 6-12.

Table 6-6: Esterase activity, protein in mg/ml^{-1}, and fibrinolytic activity of cholelysin Peaks I, II, III and IV on BAEE, with trypsin control.

Enzyme	Initial rate OD/min	Trypsin units	Protein mg/ml^{-1}	Trypsin units/mg protein	Lytic units	Trypsin/lytic ratio
Trypsin						
(i)	0,035	11,7	0,02	585		
(ii)	0,038	12,7	0,02	635	610	
Peak I	0,040	13,3	0,20	66,5	42,9	1,55
II	0,008	2,7	0,10	27	11,8	2,29
III	0	0	0,048	0	11,8	-
IV	0	0	0,109	0	8,7	-

Peak I used in this experiment had an unusually high protein content. The results of these experiments with various substrates are summarised in Table 6-7.

Table 6-7: Summary of observations on substrate activities of cholelysin studied here.

Substrate	Cholelysin Peaks				
	I	II	III	IV	G200
Casein	+	+	+	+	
ATEE	+	+	+	0	
BAEE	+	±	0	0	
Plasminogen					0
Trypsinogen					0
Chymotrypsinogen					0
Peak I/trypsin ratio	66,5/610 = 0,1				
Peak II/trypsin ratio	27/610 = 0,04				
Peak I/chymotrypsin ratio	182/900 = 0,2				
Peak II/chymotrypsin ratio	51/900 = 0,06				
Peak III/chymotrypsin ratio	37/900 = 0,04				

Note: (a) Peak I/trypsin ratio is derived from the fourth column of results in Table 6-6 'Trypsin units/mg protein'.

(b) Peak I/chymotrypsin ratio is derived from the fourth column of results in Table 6-4, 'Chymotrypsin units/mg protein'.

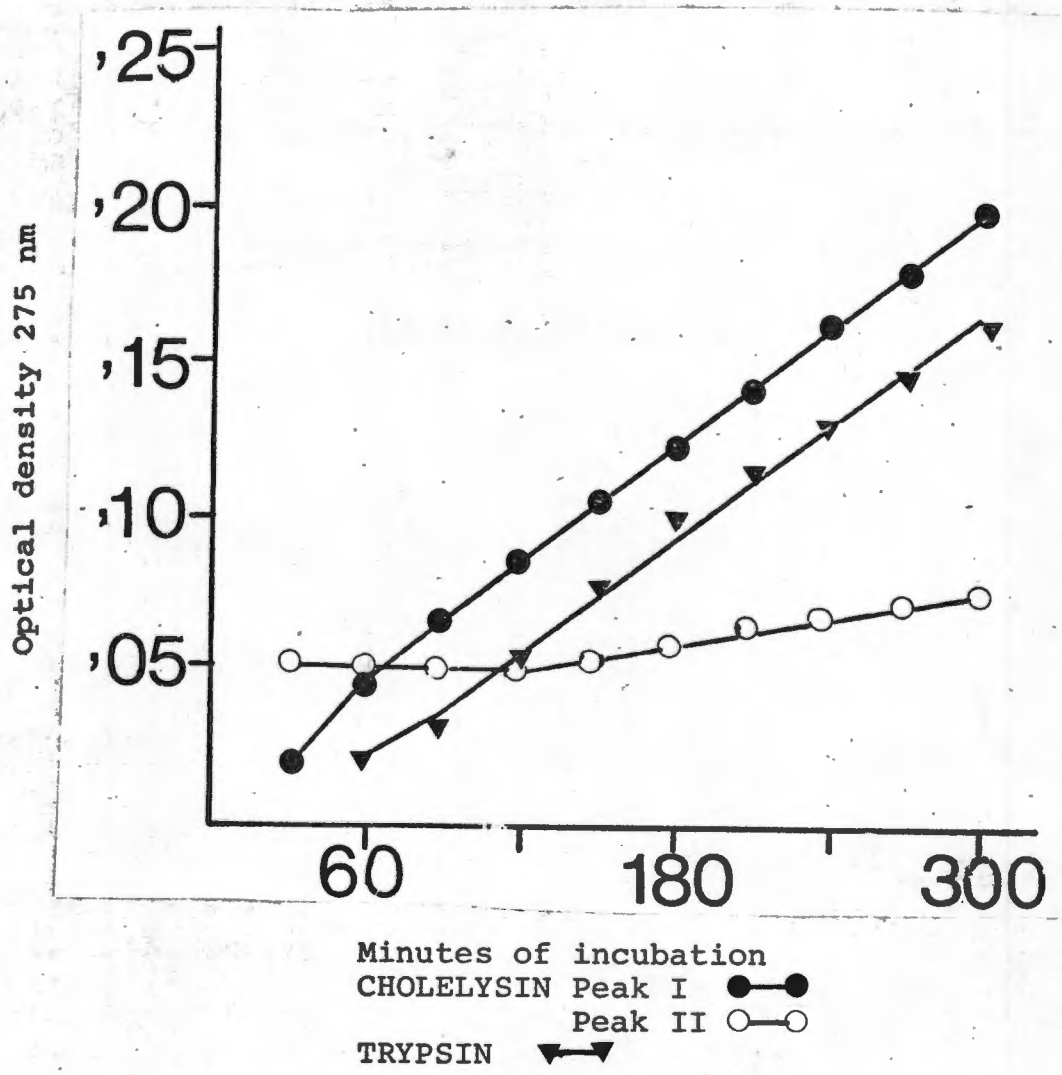


Figure 6-12: BAEE esterase activity of cholelysin Peaks I and II, with trypsin control. Peaks III and IV were inactive. Trypsin (i) shown.

The experimental results from which Figure 6-12 was derived are set out in Table 6-6 .

6.6 Discussion

1 All four peaks of cholelysin are proteolytic.

2 However, each peak shows apparently distinctive specificity for the two synthetic substrates.

(i) Peak IV hydrolyses neither the tyrosine-containing group of ATEE nor the arginine-containing group of BAEE. Its caseinolytic activity must thus be due to some specificity other than for these two commonly encountered amino acids.

(ii) Peak III shows some tyrosine specificity, but no activity against the arginine peptide bond. It is therefore chymotrypsin-like in this respect.

(iii) Peaks I and II both show broader specificity, hydrolysing both the tyrosyl and arginyl residues, though Peak II is less active against the latter substrate and is possibly thus distinguishable from Peak I.

(iv) Both Peaks I and II show a lower relative esterase activity against the two synthetic substrates than trypsin and chymotrypsin. However, the protein content of the Peak I used in the BAEE experiment was usually high for the lytic activity show, and it is probable that a preparation with higher specific lytic activity might have equalled or surpassed chymotrypsin in terms of BAEE esterase activity.

(v) The two forms of cholelysin demonstrated in section 6.4.2, with sharply different esterase/lytic ratios, were of particular interest. It is hoped to study this phenomenon further.

6.7 Summary:

Substrate specificities for cholelysin are presented. Trypsinogen, chymotrypsinogen, and plasminogen were studied, and alpha-casein, ATEE and BAEE. All four Peaks showed different specificities against the synthetic substrates ATEE and BAEE. Peak I had two forms with differing ratios of ATEE and lytic activities when measured simultaneously.

CHAPTER SEVEN

STUDIES ON THE INHIBITION OF CHOLELYSIN

7.1 Introduction

Proteases active against the substrates discussed in Chapter Five above have many naturally occurring inhibitors. It was of interest to study possible inhibitors of cholelysin, and these studies are reported in this chapter.

7.2 Substances studied for inhibitory effect were:

(1) Human serum on cholelysin
(2) Human serum on ATEE-esterase activity of cholelysin
(3) Serum of rat, rabbit, guinea-pig and mouse on cholelysin
(4) Alpha-2-macroglobulin
(5) Soy bean trypsin inhibitor
(6) Aprotonin ('trasylol')

All results are shown in abridged form. Full details are given in Appendix 7.

7.3 Experimental design

All experiments on inhibition which were done by mixing a potential inhibitor of cholelysin with a cholelysin fraction were done as follows:

Control of the same cholelysin fraction used in that specific experiment, diluted 1/1 with tris buffer pH 8,5 M 0,006.

Test: Control + inhibitory substance in equal volumes. Both read as mm diameter of zone of lysis after 21 hours

of incubation at 37°C. Results were then transformed into units from the scale in Chapter Three p 40.

7.4 Human serum

Human serum contains many potent inhibitors of triggered enzyme systems of the blood such as coagulation, fibrinolysis, complement, and kallikrein-kinin. Those relevant to fibrinolysis are listed in Chapter Two. It was of interest to discover if cholelysin was also inhibited by one or more of them.

7.4.1 Experimental design: Blood was taken from 10 healthy adults into glass test tubes and incubated in a waterbath at 37°C for 60 minutes. The tubes were then centrifuged at room temperature, and the sera were pipetted out and stored at -40°C until used. Storage did not exceed 30 days. 'Heated serum' was heated to 56°C for 30 minutes before testing. Through the courtesy of the Department of Chemical Pathology, Groote Schuur Hospital, α-1 antitrypsin was estimated on all sera studied.

7.4.2 Unheated serum. Testing for inhibition was carried out by the method described in 7.3 above. The percentage inhibition was calculated from the formula:

$$\frac{\text{Control (units)} - \text{test (units)}}{\text{control (units)}} \times 100$$

Every test was run in duplicate, and the results in mm diameter of zone of lysis were averaged before converting into units of lysis. The 50% inhibitory point was estimated from a plot of individual results for each serum on graph paper. Results are shown in Table 7-1 and Figures 7-1 and 7-2.

Table 7-1: Inhibitory effect of human serum on the lytic action of cholelysin. Control measured in 1/1 dilution in tris buffer M 0,006. Test serum introduced in place of tris buffer, and lytic effect measured. Full workings are shown in Appendix 7.

Dilution of serum	Serum sample Inhibition (%)												
	1	2	3	4	5	6	7	8	9	10	$\bar{x}$	s	SEM
1/20	83	89	100	84	100	100	100	100	100	90	95,1	6,59	2,08
1/40	54	72	72	69	59	87	78	79	85	70	72,5	10,45	3,3
1/80	37	45	57	70	25	48	51	33	0	33	46,9	15,47	4,89
1/100	30	10	34	10	30	41	41	25	48	9	27,8	14,14	4,47
1/160	5	0	20	0	30	30	25	15	29	3	15,7	12,74	4,03
1,320	0	0	0	0	15	3	15	9	15	0	5,9	6,94	2,19
α1-antitrypsin (mg %)	350	450	410	370	330	450	410	280	310	330			
50% inhibition	1/45	1/70	1/85	1/90	1/50	1/78	1/80	1/60	1/100	1/95	1/70		

When the mean values of these 10 experiments were plotted, they gave the result illustrated in Figure 7-1. The 50% inhibitory point was estimated from a plot of results from each serum on semi-logarithmic paper.

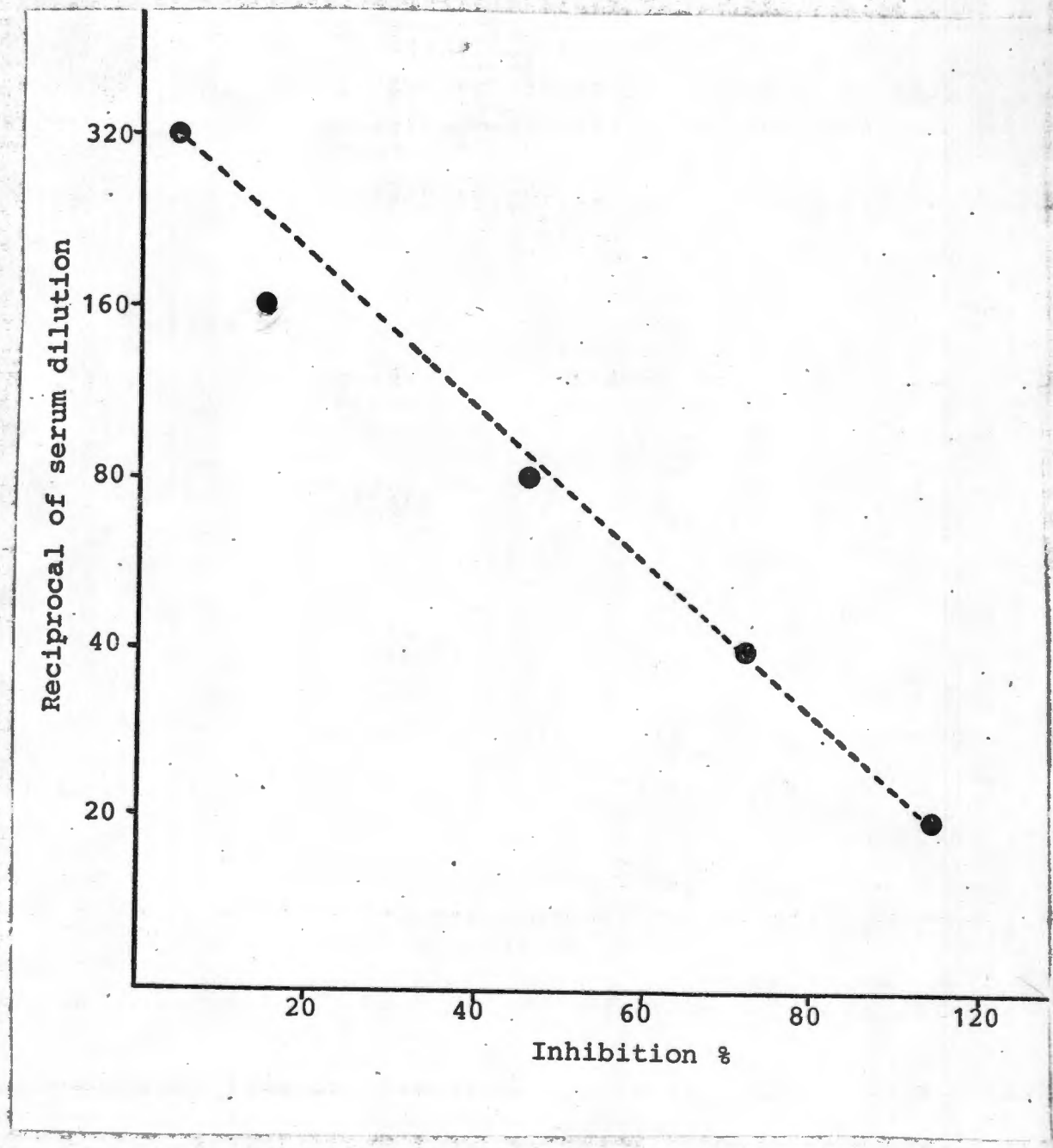


Figure 7-1: Inhibitory effect of human serum on lytic action of cholelysin. Mean of 10 experiments plotted (from Table 7-1).

The α-1-antitrypsin content of these sera did not have a meaningful relationship to the 50% inhibition point. This is brought out in Figure 7-2, which plots these two values against each other.

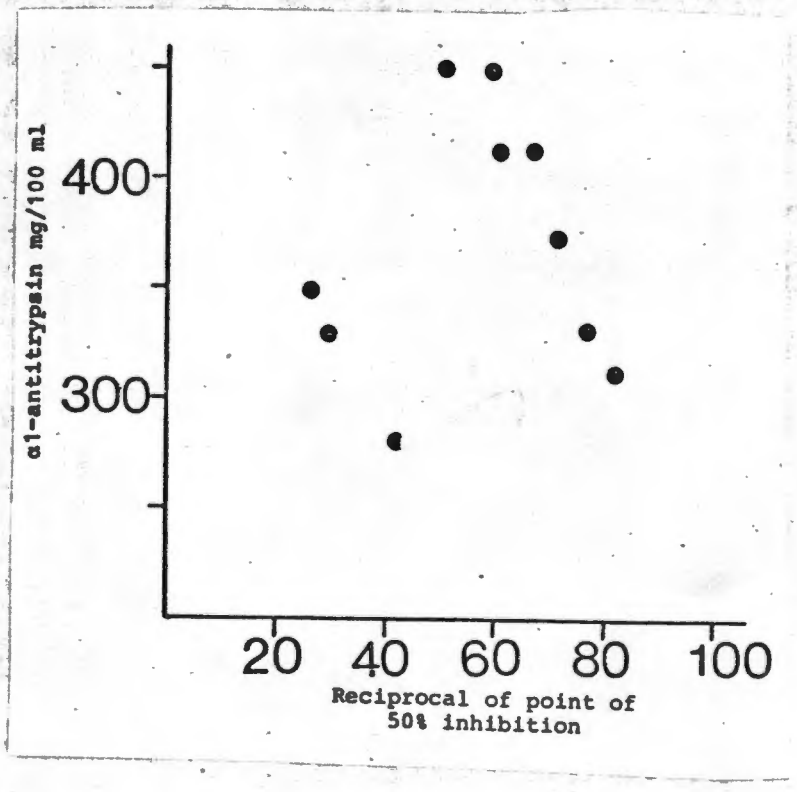


Figure 7-2: α-1-antitrypsin content of 10 sera plotted against the reciprocal of their point of 50% inhibition of the lytic action of cholelysin.

From these results, it can be concluded that a potent inhibitor or inhibitors of the lytic action of cholelysin exist(s), which probably bears no relationship to their α-1-antitrypsin content.

7.4.3 Heated serum

Serum heated to 56°C for 30 minutes was then tested for its inhibitory effect on the lytic action of cholelysin.

Results of 3 tests are shown in Table 7-2.

Table 7-2: Effect of heating human serum to 56°C for 30 minutes on its inhibitory potential against the fibrinolytic action of cholelysin.

	Inhibition (%)					
Serum number	3		8		10	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
Dilution						
1/20	100	100	100	80	90	100
1/40	72	72	79	70	70	82
1/80	57	30	33	38	33	72
1/100	34	15	25	38	9	64
1/160	20	5	15	29	3	9
1/320	0	0	9	20	0	0

Comment: From this experiment it was apparent that heat degradation of the inhibitor(s) of cholelysin in serum had not occurred. In fact, it was possible that inhibition had been slightly increased in the case of sera 8 and 10.

7.4.4 α-1-antitrypsin deficient serum

It was of interest to compare the inhibitory effect of these deficient sera on the lytic potential of cholelysin, with that of normal sera. Through the courtesy of Dr B Novis, Respiratory Clinic, Groote Schuur Hospital, sera were obtained from two patients with homozygous α-1-antitrypsin deficiency. These sera contained 27 mg and 18 mg/100 ml respectively (Normal 250-450 mg%).

Inhibition was tested in the usual way, and gave the re-

sults shown in Tables 7-3 and 7-4.

Table 7-3: Inhibitory effect of serum congenitally deficient in α-1-antitrypsin. Mean value of 10 controls (from Table 7-1) included for comparison.

Control: 16 15 14 Mean 15 mm = 12,6 units/ℓ

Test dilution	Lysis (mm)	Units	Inhibition %	Control %
1/20	0	0	100	95,1
1/40	0	0	100	72,5
1/80	0	0	100	46,9
1/100	0	0	100	27,8
1/160	8	5,3	58	15,7
1/200	10	6,8	46	
1/320	12	8,7	31	5,9
1/640	14	11,1	12	

Table 7-4: Inhibition of a second serum congenitally deficient in α-1-antitrypsin (homozygous). α-1-antitrypsin content 18 mg %. Control on this occasion was new.

Control: 15,15 16 Mean 15,3 = 13,1 u/ℓ

Test dilution	Lysis	Units	Inhibition	Control inhibition
1/10	0	0	100	100
1/20	11	7,7	42	38
1/40	13	9,8	26	17
1/100	14,5	11,8	10	0

Comment: The experiment in Table 7-3 suggested a more powerfully inhibitory effect of α-1-antitrypsin defi-

cient serum. This was not born out by the second experiment. However, the result shown in Table 7-3 led to a series of experiments to fractionate serum by electrophoresis, in an attempt to locate the inhibitors present.

7.4.5 Paper electrophoresis of normal human serum was carried out to establish which zone or zones were involved in the inhibition of cholelysin. Chymotrypsin was included for comparison. Paper was preferred to gel techniques, because of the high serum load that could be applied to paper, and for the ease with which it could be divided into strips for assay of fibrinolytic activity.

Experiment

Whatman filter paper No I was used in strips 5 cm wide. 0,2 ml of serum was applied 2 cm on the anode side, and run for 19 hours at 140 V and 4°C. The electrode buffer was 0,83 M barbitone pH 8,6.

Serum source: healthy adult male (No 8 of Table 7-1)
The procedure adopted was as follows:

(1) two strips of paper were used
  (i) contained 10 ul of serum diluted to 0,2 ml with electrode buffer, for staining and identity of bands
  (ii) contained 200 ul serum for test.

(2) the strips were dried at room temperature. Staining of strip (i) was with 1% amidoschwarz in 10% acetic acid and 90% methanol (ii) unstained.

(3) Strip (ii) was cut into longitudinal strips as is shown in Figure 7-3.

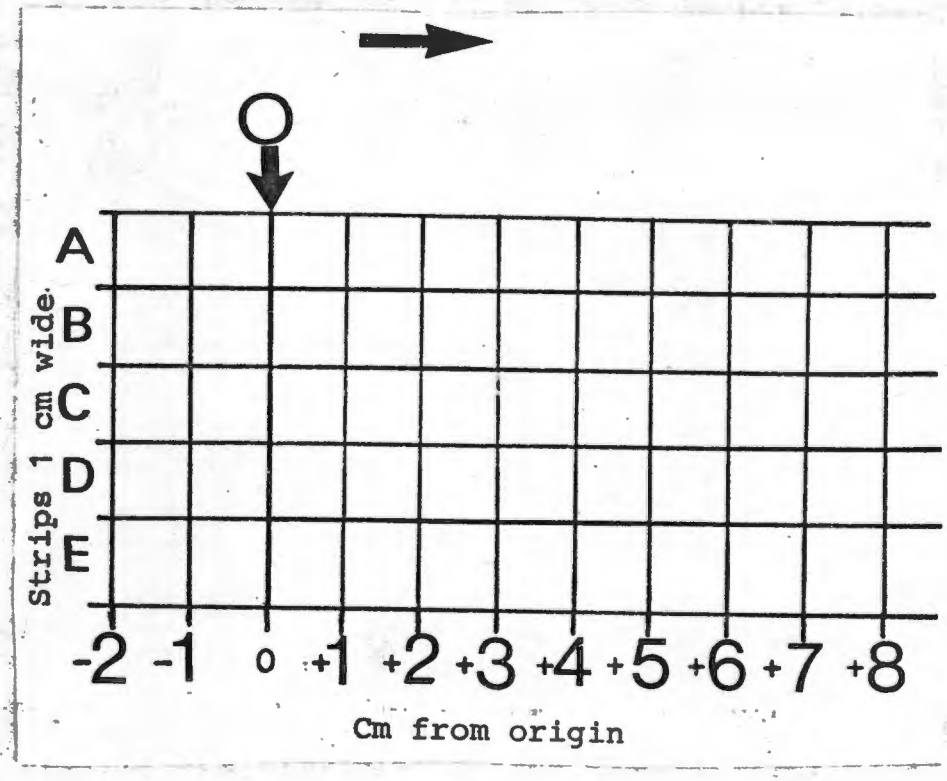


Figure 7-3: Partition of a paper strip for studies of inhibition of cholelysin by portions of serum separated by electrophoresis. o = origin.

After an electrophoretic run, the paper strip was dried at room temperature. Strips A and E were cut off and stained. Strips B and D were cut longitudinally and transversely, and applied directly to the fibrin plate. B was further cut into 0,5 cm strips before application. Onto each strip was pipetted either 0,1 or 0,2 ml of cholelysin Peak I, and the fibrin plate was incubated overnight in the usual way. Paper strips were lifted off the fibrin plate, and underlying zones of lysis were measured, and transformed into units: results for strip B were multiplied by 2 before recording them. Strip C was applied in 0,5 cm strips, and chymotrypsin solution 80 mg/ℓ, 0,1 ml, was pipetted on to each strip. The result was read directly in chymotrypsin mg/ℓ and

recorded.

Figure 7-4 is a photograph of the paper strip used for location of globulin bands, and of stained strips A and E.

Figure 7-5 shows the result of this experiment.

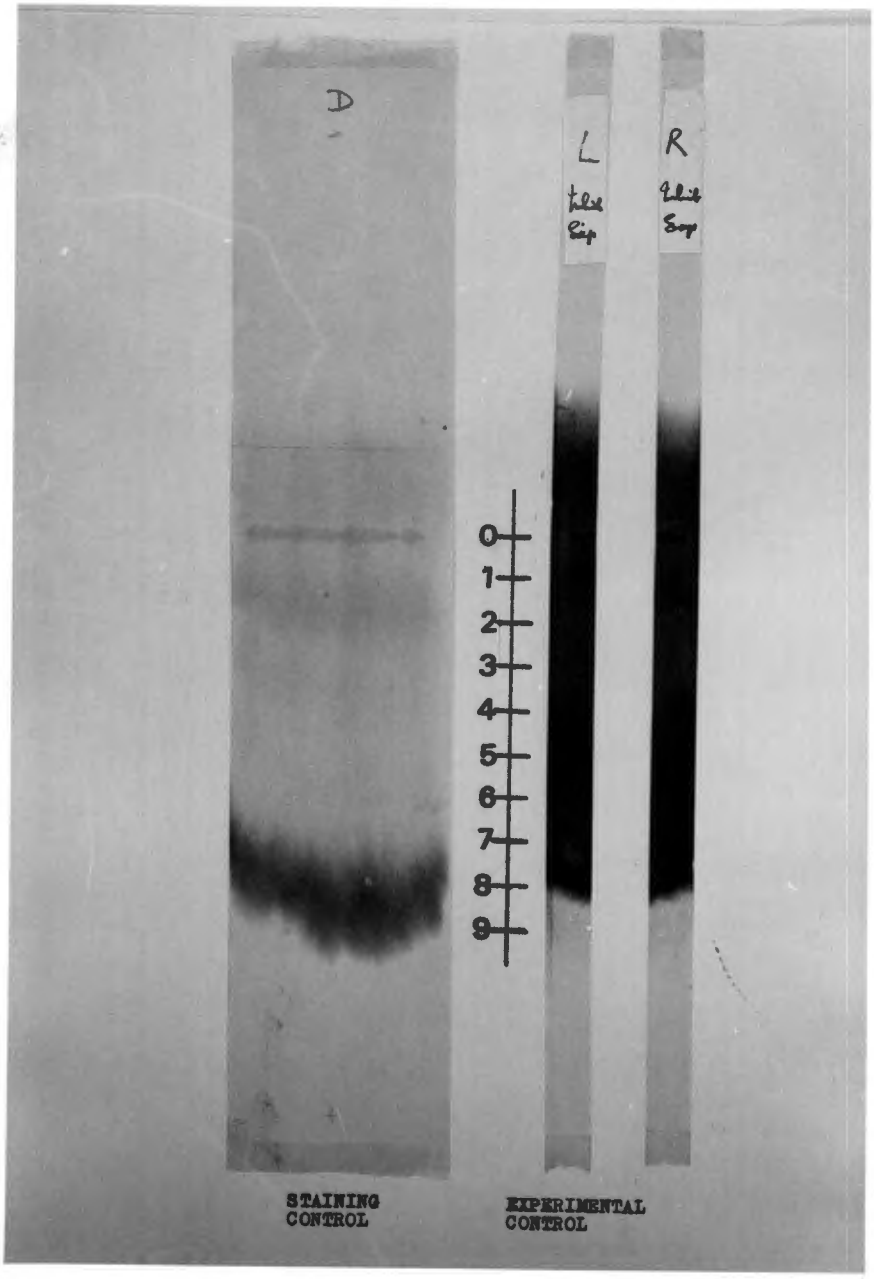


Figure 7-4: Staining and experimental controls prepared as described above. In experimental control, 'L' = Strip D; 'R' = Strip B } in Figure 7-3

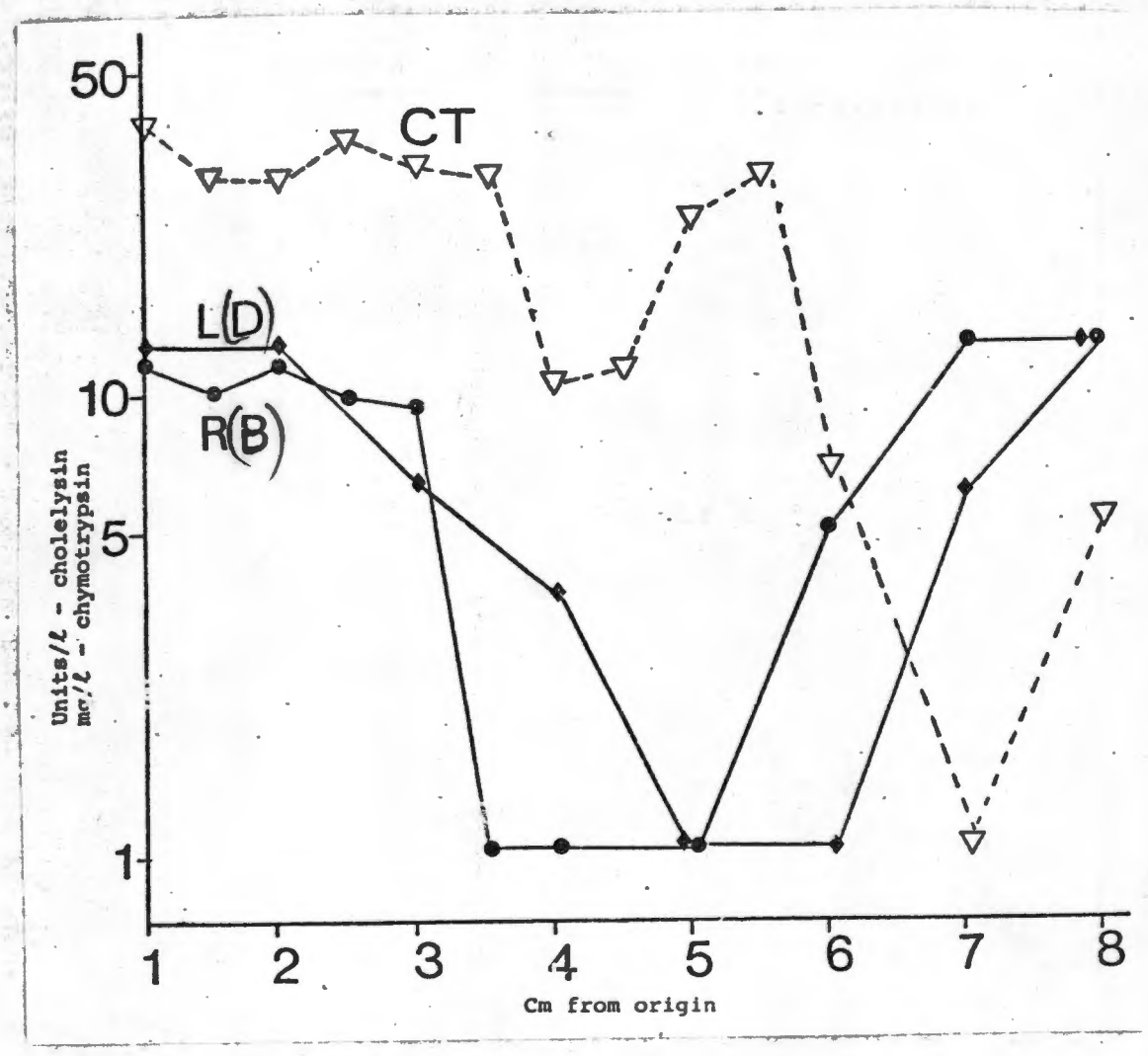


Figure 7-5: Serum inhibition of cholelysin 22,8 units/ℓ and of chymotrypsin 80 mg/ℓ. In this experiment, 0,1 ml cholelysin applied to strip B (0,5 cm partitions) and 0,2 ml cholelysin to strip D (1,0 cm partitions). Strip C divided into 0,5 cm partitions, and 0,1 ml of chymotrypsin solution applied to each partition. Results in units of lysis (cholelysin) or mg/ℓ (chymotrypsin). Results from strip B in units were multiplied by 2, before plotting on graph.

Figure 7-5. From Figure 7-4 it is apparent that:

zone 7-8 = albumin
6-7 = α-globulins
5-6 = inter α-globulins
4-5 = β-globulins

Inhibition is maximal in the β- and inter α-globulin areas, and is absent in the α-globulins. This provides further evidence that inhibition is not due mainly to either α-1-antitrypsin or to α-2-macroglobulin. Chymotrypsin shows a different pattern of inhibition, in the β-globulin and α-globulin (α-1-antichymotrypsin) areas.

7.4.6 Platelet-rich and platelet-poor plasma possessed inhibitory potential similar to that of serum derived from the same plasma. See Appendix 7.

7.5 Effect of serum inhibitor of cholelysin on ATEE-esterase activity

In this experiment, dilutions of normal human serum were added to the incubation mixture used in the modified ATEE-esterase test for cholelysin esterase activity. Because there were two variables (serum and cholelysin) the experimental arrangements were somehwat complex; they are described in detail in Appendix 7. The results are listed in Tables 7-5 and 7-6 and are plotted in Figures 7-6 and 7-7.

Table 7-5: Fibrinolytic activity of cholelysin Peak I, and inhibitory effect of human serum at various dilutions on it, assayed before use of this serum for the experiment detailed below in Table 7-6 and Appendix 7. Experimental design for assay of inhibitory effect of serum as set out in 7.3.

Cholelysin fibrinolytic effect control:

28 27,5 27 mm Mean 27 = 54,8 units

Serum dilution	Lysis		Mean	Units	Inhibition %
	1	2			
20	5,5	6,0	5,75	4,0	85
50	17,5	24,0	20,75	25,4	54
100	24,5	24,5	24,5	40,4	26
200	26,5	25,5	26,0	48,5	11
500	27,0	28,0	27,5	58,0	0

Table 7-6: Inhibitory action of human serum on ATEE-esterase activity, and fibrinolytic activity, of cholelysin Peak I. See Appendix for details.

Serum dilution	ATEE esterase activity (units)	% inhibition ATEE esterase activity	% inhibition fibrinolysis
20	0	100	85
40	2,7	93,1	
50	8,7	79,4	54
80	18,1	57,1	
100	22,4	47,9	26
200	32,6	22,7	11
400	37,5	11,1	
500	40,9	3,1	0

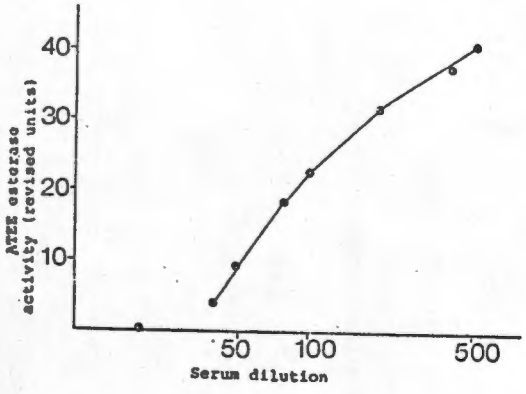


Figure 7-6: Graph of serum inhibition of ATEE-esterase activity of cholelysin.

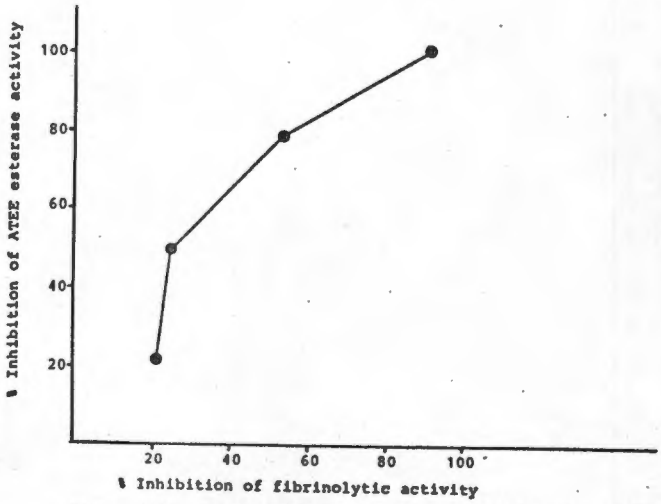


Figure 7-7: Relationship of inhibition of ATEE esterase activity, and fibrinolysis to each other, at similar serum dilution.

7.6 Inhibitory effect of serum of rat, rabbit, guineapig and mouse. It was decided, in conjunction with the South African Inventions Development Corporation, to study the anti-coagulant effect of cholelysin in an animal model. In view of the marked inhibitory effect of human serum on the fibrinolytic action of cholelysin, it was necessary to study the inhibitory effect of serum from possible laboratory animals. These studies are reported here.

Experimental design followed the plan described in 7.3 and 7.4.1 above. The results are described in Table 7-7. Only unheated serum was studied.

Table 7-7: Inhibitory effect of serum of rat, rabbit, guinea-pig and mouse against cholelysin. Full workings shown in Appendix 7.

Dilution	Rat	Rabbit	Guinea-pig	Mouse	Human (Table 7-1)
	Inhibition %				
1/1	100	100	100	100	100
1/2	100	100	100	100	100
1/4	89	81	100	95	100
1/10	51	45	56	36	100
1/100	0	17	0	0	27,8
1/1 000	0	0	0	0	0

Conclusion

This brief series of experiments showed that all 4 species possessed serum inhibition against fibrinolysis by cholelysin, but that they were (in single tests) less inhibitory than the average of 10 experiments with human serum. The cholelysin for most of these experiments was supplied through the courtesy of the South African Inventions Development Corporation and Dr T Haylett of the Council for Scientific and Industrial Research, Pretoria.

7.7 Alpha-2-macroglobulin

This inhibitor of many circulating activated enzymes was of interest, as it is known to bind plasmin. The plasmin-macroglobulin complex retains slow fibrinolytic activity (Harpel, 1977). A small quantity was obtained through the courtesy of Dr Potgieter, Department of Immunology, Natal Blood Transfusion Service, Durban, in the form of a 10% solution in tris buffer pH 8,0. It was not electrophoretically pure.

As expected, it was weakly fibrinolytic, and this effect had to be allowed for in calculating the inhibitory effect of the macroglobulin on cholelysin Peak I. This was done by altering the standard formula for the calculation of inhibition.

$$\frac{\text{Control (units)} - \text{test (units)}}{\text{Control (units)}} \times 100 \quad \ldots\ldots\ldots\ldots \quad \text{A}$$

to:

$$\frac{\text{Control (units)} - (\text{test units} - \text{macroglobulin lytic activity} \times 100))}{\text{Control (units)}} \quad \ldots\ldots \quad \text{B}$$

The sample supplied by Dr Potgieter had a lytic potential of 10 mm diameter zone of lysis = 6,8 units. Thus, equation (B) above was altered to:

$$\frac{\text{Control (units)} - (\text{test units} - 6{,}8)}{\text{Control (units)}} \times 100$$

It was assumed that this lytic potential was still expressed, in experiments in which macroglobulin solution was mixed with cholelysin, and the mixture incubated on the standard fibrin plate.

The following fractions were tested: G200 - Peaks I, II, III and IV.

Results are shown in Table 7-8.

Table 7-8: Inhibitory effect of macroglobulin on cholelysin fractions. Equation (B) on page 111 was used, to derive 'Inhibition %'.

Cholelysin fraction	Control mean	Control units	Test mean	Test units	Inhibition %
G200	28	62	18,8	20,3	78
Peak I	30,5	84,4	26	48,5	51
Peak II	19,5	22,2	15,8	13,7	69
Peak III	17,5	17,1	15	12,6	66
Peak IV	10,5	7,3	11	7,7	88

7.8 Soy bean trypsin inhibitor (SBTI)

This broad spectrum protease inhibitor is known to inhibit plasmin, trypsin, and chymotrypsin. It was therefore tested for inhibitory activity against cholelysin fractions. The routine method was followed, but as it proved somewhat ineffective, a 24 hour period of incubation was added to the study. Thus, incubation of SBTI and cholelysin fraction in equal volumes at 20°C took place (a) for 20 minutes
(b) for 24 hours.

Results are shown in brief below, with full details shown in Appendix 7.

Table 7-9: Incubation of SBTI and cholelysin for 20' at 20°C.

Cholelysin fraction	SBTI % 0,0005	0,001	0,002	0,004	0,006	0,008
G200	52	76	78	91	90	91
Peak I	55	55	68	82	96	90
Peak II	49	55	60	78	94	78
Peak III	77	80	81	100	100	100

Table 7-10: Incubation of SBTI and cholelysin for 24 hours at 20°C.

Cholelysin fraction	SBTI % 0,002	0,004	0,006	0,008
G200	84%	88	93	95
Peak I	83	91	96	96
Peak II	76	82	85	100
Peak III	100	100	100	100

Comment: Incubation for 20 minutes produced erratic results. However, incubation for 24 hours produced more marked, and more consistent results. Peak IV was 'out of stock' at the time that these experiments were carried out.

7.9 Aprotonin ('Trasylol')

This is a polypeptide derived from bovine lung tissue. It is an inhibitor of many proteolytic enzymes including:

trypsin
chymotrypsin
kallidinogenase
plasmin
plasminogen activator (Martindale, 1977).

Ampoules containing 10 000 units (Bayer) were available.

Table 7-11: Inhibitory effect of aprotonin 2 000 units on cholelysin fraction tested by pre-incubation at 20°C for 60 minutes.

Cholelysin fraction	Control mean	Control units	Test mean	Test units	Inhibition %
G200	27	54,8	11	7,7	86
Peak I	35	146,4	0	0	100
Peak II	20	23,2	20,4	7,2	69
Peak III	16	14,2	0	0	100
Peak IV	10	6,8	0	0	100

The effect on Peak I, the major cholelysin fraction used in this study, was of particular interest. This particular preparation contained 0,105 mg/ml of protein as determined by Folin. Martindale (1977) gives the following inhibitory effect of aprotonin on trypsin: 500 ng trypsin inhibited by 1 unit of aprotonin. Thus 500 x 2 000 ng = 1,0 mg of trypsin would be inhibited by the 2 000 units of aprotonin used in this study.

In Table 7-11 it was shown that 2 000 units of aprotonin gave complete inhibition to 0,1 ml of cholelysin Peak I with an activity of 146,4 u/ℓ, or a chymotrypsin equivalent of 0,015 mg, setting up a upper limit to the quantity of aprotonin required for full inhibition of this Peak. However, fraction G200 and Peak II both showed some resistance to the inhibitory action of aprotonin under the same conditions. As shown, these fractions had far lower lytic activity than the Peak I preparation tested, suggesting that there is another cholelysin with greater resistance than Peak I. As the Peaks are all derived from fraction G200, this result is logically acceptable. Further study of this aspect would be interesting.

7.10 Discussion and summary

(i) The human serum inhibitor of cholelysin discussed in this chapter has been shown to be potent, with an average 50% inhibitory point at a dilution of 1/70 (average of 10 sera studied).

(ii) This inhibitor is fully expressed in two sera from patients with homozygous deficiency of α-1-antitrypsin, and this point was confirmed by electrophoretic fractionation of normal serum on Whatman filter paper, where the inhibition was shown to be in the inter-α, and β-globulin areas.

(iii) Serum inhibitory for the fibrinolytic action of cholelysin was shown to be inhibitory for the ATEE-esterase activity of the same preparation.

(iv) α-2-macroglobulin, soy bean trypsin inhibitor and aprotonin were all shown to be inhibitory of cholelysin. In the case of α-2-macroglobulin and aprotonin, there was some suggestion that various fractions of cholelysin might have differential resistance to the inhibitory action studied. This was not followed further.

CHAPTER EIGHT

BIOLOGICAL STUDIES

8.1 Introduction

The potency of cholelysin as a fibrinolytic agent led to considerations of its use therapeutically. A limited set of investigations of the effect of cholelysin on routine coagulation tests was undertaken, and an attempt was made to determine the LD_{50} and the biological half life of cholelysin in the rat.

8.2 Routine coagulation studies - Experimental design

The following routine tests were done, with and without cholelysin.

Partial thromboplastin time (PTT)
One stage prothrombin time (PT)
Thrombin time (TT)
Fibrinogen assay (Fib)
Fibrin split products by latex particle agglutination technique (FSP)
Platelet aggregation studies: collagen
ADP
adrenaline

See Method 7 for references.

In all cases, the test was done in 1/1 dilution with barbitone buffer pH 7,4 as a control, and then with cholelysin substituted for the barbitone buffer.

Some studies were repeated after 1 and 2 hours of incubation of cholelysin with substrate at 37°C.

8.2.1 Routine coagulation studies

Results are shown in Table 8-1.

Table 8-1: Effect of cholelysin 75 u/ℓ in equal admixture with plasma, on the PTT, PT, TT and fibrinogen assay tests. Effects of 60 and 120 minutes of pre-incubation at 37°C are also shown. Clotting times in seconds.

	PTT seconds			PT seconds			TT seconds			Fibrinogen mg/100 ml		
Pre-incubation (min)	0'	60'	120'	0'	60'	120'	0'	60'	120'	0'	60'	120
Control	47	47	48	15	15	15	15	16	16	250	255	245
Cholelysin 38 u/ℓ	44	44	45	15	14	14,5	16	16	16	255	178	60

The experiment reported in Table 8-1 showed that cholelysin had little effect on the PTT, PT and TT, all of which are plasma tests. However, there was an obvious fibrinogenolytic effect, which was investigated further. This is reported in Table 8-2.

Table 8-2: Fibrinogenolytic effect of various concentrations and incubation times of cholelysin. Results in fibrinogen mg/100 ml.

Cholelysin concentration (u/ℓ)	60	45	30	15	0
Fibrinogen concentration (mg/100 ml)	54	108	162	216	270
Period of pre-incubation (minutes)	Fibrinogen mg/ml				
0	41	102	168	220	270
60	8	68	130	180	270

Comment: This fibrinogenolytic effect was only demonstrated in purified fibrinogen solution. Experiments using whole plasma showed no fibrinogenolytic effect, probably because of the strongly inhibitory effect of plasma, reported in Chapter Seven.

8.2.2 Platelet aggregation studies

Routine aggregation studies were performed, using cholelysin 75 u/ℓ mixed in equal volumes with platelet rich plasma 600 000/ul. These studies are shown in Figures 8-1, 8-2, and 8-3.

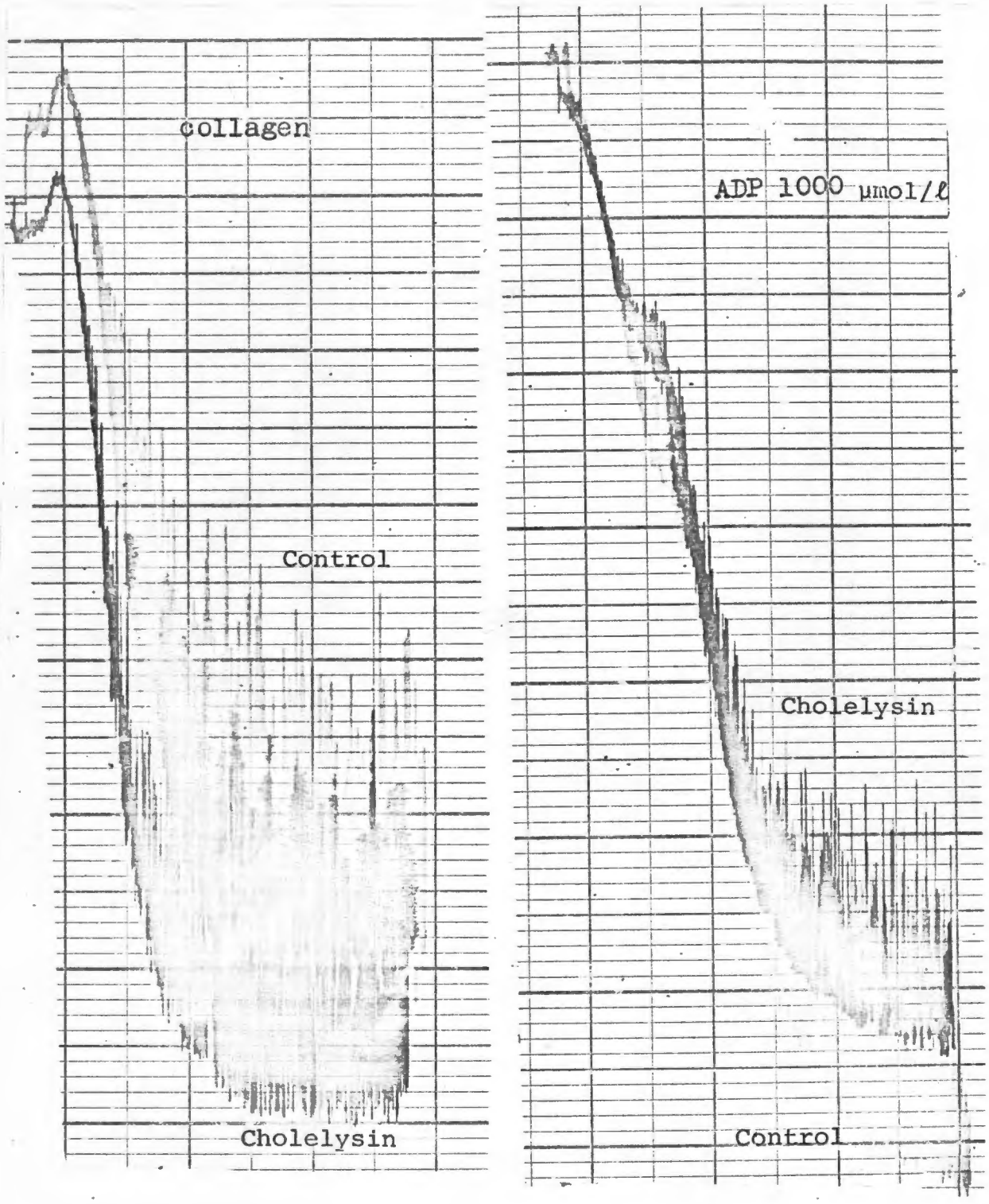


Figure 8-1: Left hand graph: routine platelet aggregation with collagen 1 mg/ml. Upper graph: control. Lower graph: cholelysin. Right hand graph: routine ADP aggregation study with ADP 1 000 µmol/ℓ final concentration. Upper graph: cholelysin. Lower graph: control.

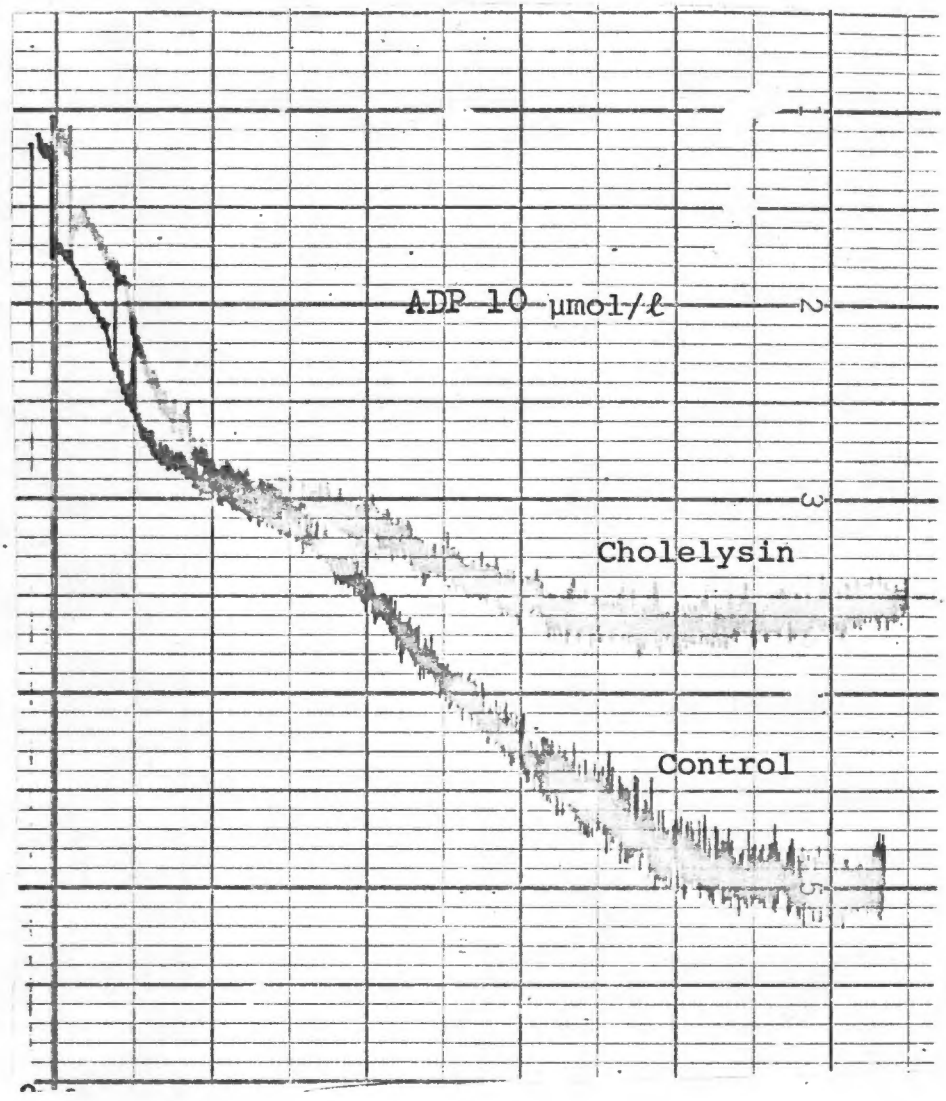


Figure 8-2: Routine platelet aggregation study with ADP 10 µmol/ℓ final concentration. Upper graph: cholelysin. Lower graph: control.

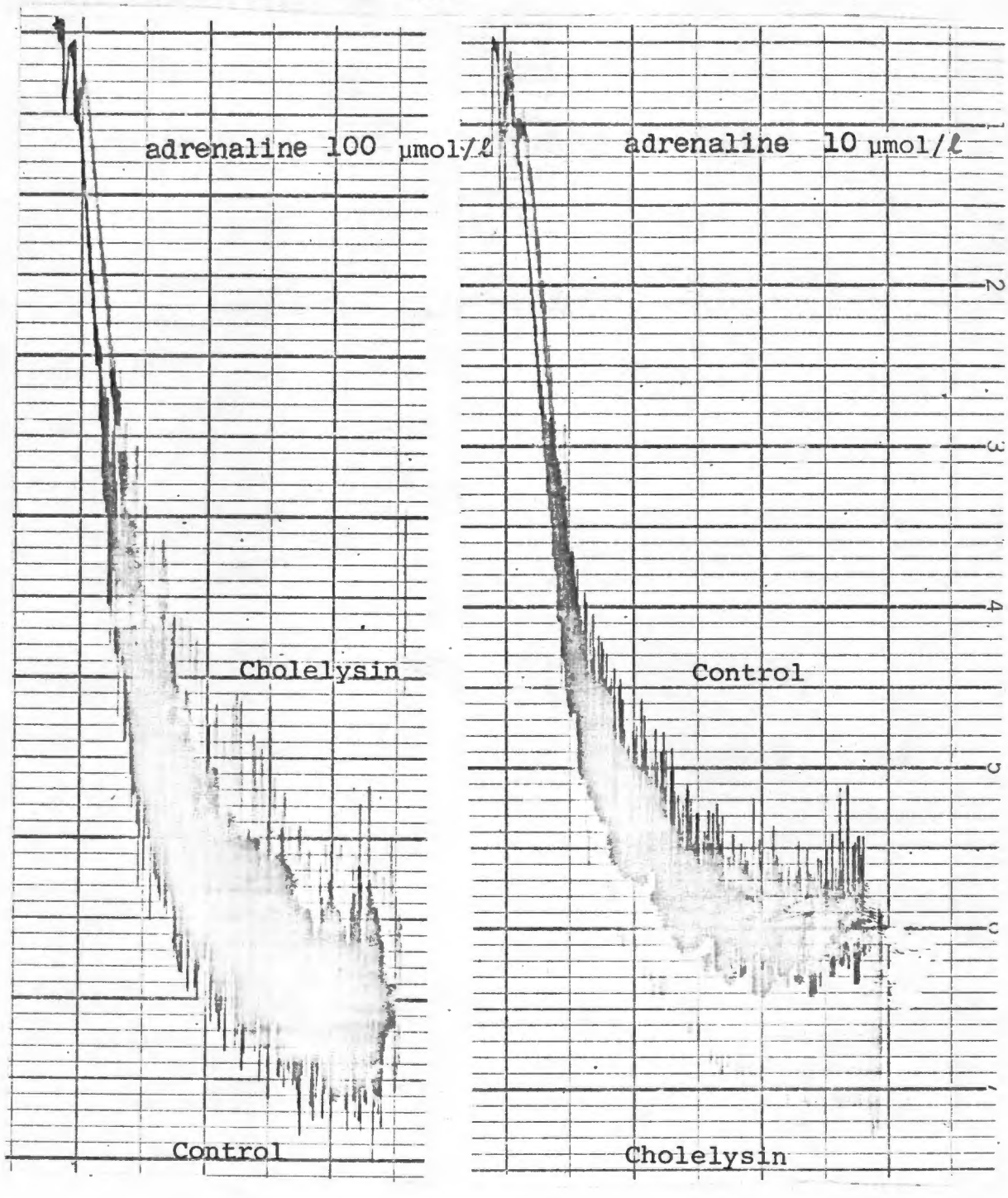


Figure 8-3: Routine platelet aggregation studies with adrenaline 100 and 10 µmol/ℓ. Left hand graph: adrenaline 100 µmol/ℓ final concentration. Upper graph: cholelysin. Lower graph: control. Right hand graph: adrenaline 10 µmol/ℓ final concentration. Upper graph: control. Lower graph: cholelysin.

Comment: collagen and adrenaline aggregation of human platelets were not influenced by cholelysin at the concentrations tested. However, there was probably an inhibitory effect on ADP-induced aggregation at 10 µmol/ℓ, though not at 1 000 µmol/ℓ. These experiments did not indicate a major effect on platelet function, and were not followed further.

8.3 LD_{50} - Experimental design

Six Wistar rats weighing between 200 and 250 g were chosen for this experiment. They were cannulated with a silicone cannula of external diameter 1 mm, in the jugular vein. The cannula was filled with heparin saline 200 u/ml. 0,5 ml of blood was aspirated, and 1,0 ml of cholelysin solution was then injected over 3 minutes. The cholelysin solution was in normal saline, 553 u/ℓ, protein concentration 82 µg/ml. The cannula was withdrawn and the rats observed. None died over 14 days. They were then killed. Autopsy showed no significant lesions.

3.4 Biological half-life: experimental design

The same model was chosen as in 8.3. At the end of the cholelysin infusion, blood was aspirated from the cannula into heparin saline 200 u/ℓ, equal volumes of both. The mixture was centrifuged at 12 000 x G for 10 minutes at 4°C, and the plasma plated on a standard fibrin plate in the usual way. Results are shown in Table 8-3.

Table 8-3: Biological half-life of cholelysin in the Wistar rat. Three rats tested. Results in units/ℓ.

Test	Rat No 1	2	3
Rat plasma with heparin 100 u/ℓ	0	0	0
Normal saline with heparin 200 u/ml	0	0	0
Cholelysin (total units)	0,553	0,553	0,553
2 minutes	10,6	8,2	6,5
10 minutes	0	0	0
25 minutes	0	0	0

8.5 Cholelysin as in vivo anticoagulant: rat model

The rat model is described in Method 22. The method was originally described by Chan (1967, 1958) and was used by him to study the anticoagulant effect of the snake venom ancrod ('arvin'). The basic method established a rate of thrombosis of the rat carotid artery following the chronic implantation in it of a polyethylene catheter. Ancrod was then injected into a series of rats; they were given doses 2-4 hours after the arterial implantation, 8 hours later, and agains at 24, 32, 48, and 56 hours after the completion of the operation. The results of these series of experiments are shown in Table 8-3.

Table 8-4: Anticoagulant effect of ancrod in the rat (from Chan, 1970).

No days postop	No animals		% occlusion		p	% red thrombus		p
	Control	Test	Control	Test		Control	Test	
2-3	19	19	63,2	36,8	>0,05	47,4	16,8	<0,05
4-5	13	11	76,9	36,4	>0,05	69,2	9,1	<0,01
7	6	6	100,0	50,0	<0,05	100,0	33,3	<0,05
Total	38	36	73,6	38,9	<0,01	63,1	16,7	<0,001

Comment: this series of experiments showed a clear cut anticoagulant effect of ancrod under these conditions in the rat. It wa decided to select this method for its relative simplicity, to study the anticoagulant effect of cholelysin; an important consideration was the relative scarcity of cholelysin concentrate, as at the time only small scale purification was being effected. Fortunately we were assisted by the South African Inventions Development Corporation, who kindly arranged with Dr T Haylett of the Council for Scientific and Industrial Research to manufacture larger quantities for us. He experienced some difficulties with ox bile (see Chapter Four) but was able to supply us with sufficient Peak I concentrate for a limited series of experiments.

8.5.1 Because of the short supply of cholelysin, and also because of the limited time available each day for experimentation, the method of Chan was modified as follows (Table 8-5).

Table 8-5: Comparison of the rat model of Chan and the model adopted for this study, to study the anticoagulant effect of cholelysin.

	Chan	This study
Rat	Sprague-Dawley	Wistar
Weight	180-250 g	200-250 g
Anaesthetic	Intraperitoneal pentobarbitone	Ether
Anticoagulant	Ancrod	Cholelysin - 3 ml of ~ 500 u/ℓ = ~ 1,5 units
Route of administration	Tail vein	Jugular
Time of administration (hours postop)	2-4, 10-12, 24, 32, 48, 56	Immediate
Duration of each injection	Single push	3 hours by slow pump
Day of killing for study	0, 3, 5, 7	0, 3, 5, 7

Comment: it is evident that the anticoagulant load on each animal would be far lower than was the case with ancrod, for the following reasons:

(1) Ancrod does not possess a naturally occurring antibody. Cholelysin has a potent antibody or antibodies.

(2) Ancrod was given in multiple injections, cholelysin only in one injection.

8.5.2 Results are shown in Tables 8-6 and 8-7 and Figure 8-4.

Table 8-6: Anticoagulant effect of cholelysin in rat model.

No of days postop	No animals		% Occlusion		% Red thrombus	
	Control	Test	Control	Test	Control	Test
0	20	19	35	10	20	5
3	18	20	67	20	50	10
5	16	14	69	57	63	29
7	10	10	100	100	100	70

For these experiments, significance is given in Table 8-7.

Table 8-7: Levels of significance for experiments shown in Table 8-6 (results for ancrod from Table 8-4 in brackets).

% Occlusion	$0,05 > p > 0,20$	$(< 0,01)$
% red thrombus	$< 0,05$	$(< 0,001)$

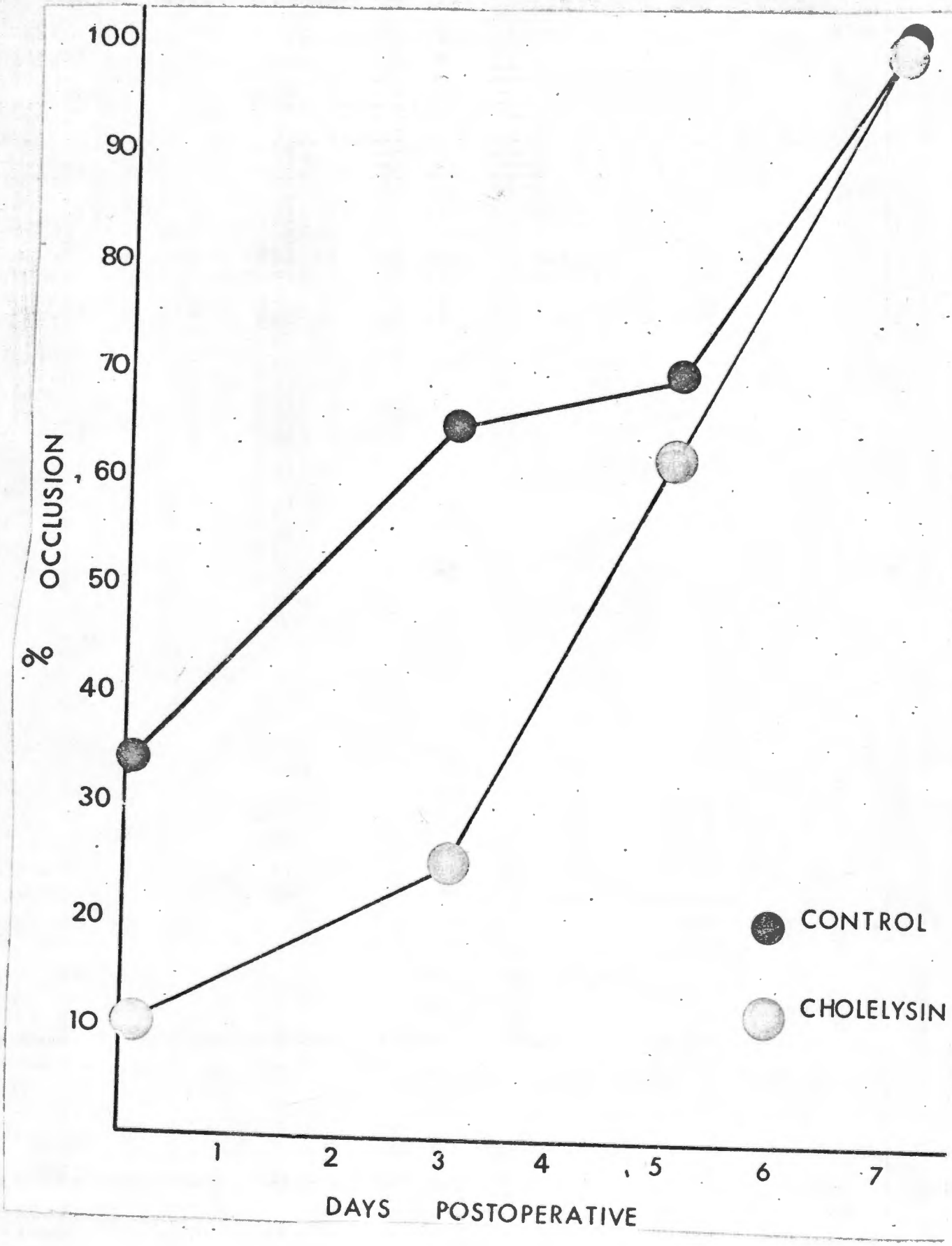


Figure 8-4: Anticoagulant effect of cholelysin in a rat model. ~ 20 Rats used for each point on the graph. This result gives $0,05 > p > 0,20$.

Comment: In view of the marked inhibitory potential of rat plasma for the fibrinolytic action of cholelysin, it is perhaps surprising that any kind of positive result was obtained. But is was not a striking result. The starting point for further investigation of the anticoagulant effect of cholelysin should be an attempt to saturate the inhibitor *in vivo* and *in vitro*, before proceeding to biological studies of the type described in section 8.4.

8.6 Summary

The effects of cholelysin on the partial thromboplastin time, one stage prothrombin time, and thrombin time tests are described. There was a slight shortening of the partial thromboplastin time test. A fibrinogenolytic effect was observed. After 60 minutes of incubation at 37°C cholelysin 60 u/ℓ had destroyed the clottability of 85% of the fibrinogen tested. Platelet aggregation studies were performed, and cholelysin appeared to interfere with the aggregating effect of ADP 10 µmol/ℓ. However, ADP 1 000 µmol/ℓ, adrenaline 10 and 100 µmol/ℓ, and collagen 1 mg were not interfered with.

The anticoagulant effect of cholelysin was tested in an *in vivo* rat model originally used to assess the anticoagulant effect of ancrod. The result approached, but did not reach, statistical significance, as $0,05 > p > 0,20$.

CHAPTER NINE

POLYACRYLAMIDE GEL ELECTROPHORESIS

9.1 Introduction

Polyacrylamide gel electrophoresis (PAGE) is an invaluable method for the rapid and accurate separation of proteins. The basic principle of the method is the formation of a lattice of polymerised acrylamide, which may range in final percentage concentration from 2 to 50%. Molecules of a test substance may be separated electrophoretically in this gel through the operation of two major factors:

(1) size of molecule in relation to size of pore in the lattice of polymerised acrylamide. With 2% gels, molecules of 800 000 molecular weight may migrate freely; with 40% gels, molecules as small as 20 000 molecular weight are retarded.

(2) charge on molecule in relation to potential difference applied. According to the conditions chosen, it is possible to alter the basis of separation i e: a small molecule migrating in a large pore gel can be separated on the basis of charge on the molecule alone. On the other hand, a large molecule migrating in a gel which restricts its movement is separated on the basis of size.

The charge difference between molecules may be eli-

minated by using sodium dodecyl sulphate (SDS). SDS has a hydrophobic chain with a sulphated hydrophilic tip. The hydrophobic chain embeds in the test protein, leaving the sulphate anion exposed. This causes the entire molecule to assume a negative charge, which effectively eliminates the differential charge effect from the process of separation by PAGE. SDS may partly of wholly denature a protein.

The choice of a single concentration of polyacrylamide will restrict separation of proteins to a small range of molecular weights. If a range of molecular weights is anticipated, they may be separated simultaneously by the use of a linear gradient gel. In such a gel, a gradient of polyacrylamides is produced from 2 to 40% or more.

Oligomers and polymers in the protein under investigation may be minimised or eliminated by the use of mercaptoethanol and urea.

(i) mercaptoethanol reduces disulphide bonds. If the test molecule is a polymer with disulphide bonding, the use of mercaptoethanol will reduce this to monomer. At the same time, if mercaptoethanol denatures the protein, this will give some indication of its chemical structure.

(ii) Urea used in high concentration before or during electrophoresis eliminates oligomers which might be due to hydrogen bonding; this enables the minimum molecular weight of a protein to be determined.

9.2 Experimental design

Early work with ammonium sulphate precipitated bile suggested a molecular weight for cholelysin of ~ 23 000 (Figure 5-6, where column chromatography on sephadex G50 gave a K_{av} of 0,3). Later work, reported in Chapters Six and Seven, suggested that more than one type of cholelysin might exist. For this reason, a linear gradient gel was selected, with a final range of polyacrylamide concentrations from 2,1 to 41% (see Methods for details).

Samples of test material were applied to the gel mixed with either SDS, or SDS + mercaptoethanol. Electrophoresis was run at a constant current of 2mA (~9V) per tube for 5 hours.

Chymotrypsin was used as a reference marker for cholelysin. Ribonuclease, chymotrypsinogen and lactate dehydrogenase were used as reference markers for fibrin split products.

This technique was used for several purposes:

(1) To estimate the molecular weight of cholelysin, correlating fibrinolytic activity with stained band on the gel. This involved an assessment of the effect of SDS and mercaptoethanol on the fibrinolytic activity of cholelysin.

(2) Separation of the split products of stabilised fibrin. Plasmin and cholelysin were compared Fibrinolysis was also inhibited by SBTI after varying periods of time, and the resultant split products analysed by PAGE.

9.3 Cholelysin Peak I on PAGE

Two preparations of Peak I were used:

(1) designated A1, with activity equivalent to 270 µ/ℓ (40 mm)

(2) designated B1, with activity equivalent to 199 µ/ℓ (37,5 mm).

100 ul of each, in 1/1 dilution in glycerol, was applied to the polyacrylamide gel = 50 µl of Peak I solution, giving a total applied to the gel for A1 of 0,0135 units and for B1 of 0,00995 units.

Chymotrypsin was used in a concentration of 1 mg/ml, diluted 1/1 with glycerol and a total of 0,1 ml applied = 0,05 mg.

Table 9-1: Experiment to determine characteristics of cholelysin Peak I by PAGE. Chymotrypsin control. Under the column heading 'destination' - 'Frozen' means freezing in liquid nitrogen, 'Plated' means: 1,5 mm thick sections of the gel were made and applied to a fibrin plate for incubation in the usual way (see Table 9-2).

Tube No	Destination	ul applied	Sample applied
1	Stained	100	A1 + B1 + SDS + glycerol 1/1
2	Stained	100	B1 + mercaptoethanol + SDS + glycerol 1/1
3	Stained	100	A1 + SDS + glycerol 1/1
4	Stained	100	Chymotrypsin standard
5	Plated	100	A1 + B1 +SDS + glycerol 1/1
6	Plated	100	B1 + mercaptoethanol + SDS + glycerol 1/1
7	Plated	100	A1 + SDS + glycerol 1/1
8	Plated	100	Chymotrypsin + glycerol
9	Frozen	50	Chymotrypsin standard
10	Frozen	100	A1 + SDS + glycerol 1/1
11	Frozen	50	A1 + SDS + glycerol 1/1
12	-	-	-

Table 9-2: Results of fibrinolytic assay of cholelysin after PAGE. Chymotrypsin standard. 1½ mm thick cuts of each gel applied to a fibrin plate which was incubated for 21 hours at 37°C. Every second section was plated. Total volume of gel = 2 ml; 60 cuts were made = 0,033 ml per cut. Results calculated as follows:

(1) assume that each unplated gel had the activity of the section above it, except in the case of the last active cut.

(2) volume per cut 0,033 ml = 0,000033 ℓ. 'Units per gel section' derived from units X 0,000033. Unplated sections of gel, and extrapolated values, shown in brackets.

	(A1) Gel 1 (Cholelysin)			(B1) Gel 2 (Cholelysin)			Gel 4 (Chymotrypsin)		
Cut No	mm diam	units/ℓ	Units per gel section	mm diam	units/ℓ	Units per gel section	mm diam	mg/ℓ	mg. per gel section
2-40	0			0			0		
42	20	23	0,000759	0			0		
(43)	(20)	(23)	(0,000759)	0			0		
44	21	26	0,000858)	0			0		
(45)	(21)	(26)	(0,000858)	0			0		
46	10	6,8	0,000224	21	26	0,000858	26	48,5	0,001601
(47)	0			(21)	(26)	(0,000858)	(26)	(48,5)	(0,001601)
48	0			10	6,8	0,000224	11	7,7	0,000254
(49)	0			0			(11)	(7,7)	(0,000254)
50	0			0			10	6,8	0,000224
(51)	0			0			(10)	(6,8)	(0,000224)
52	0			0			10	6,8	0,000224
(53)	0			0			(10)	(6,8)	0,000224
54	0			0			10	6,8	0,000224
Total units			0,00346			0,00194			0,00483
Total units applied to gel			0,01350			0,00995			0,05000 (mg)
% loss			74			81			90

9.3.1 Results

The stained gels are shown in Figure 9-1.

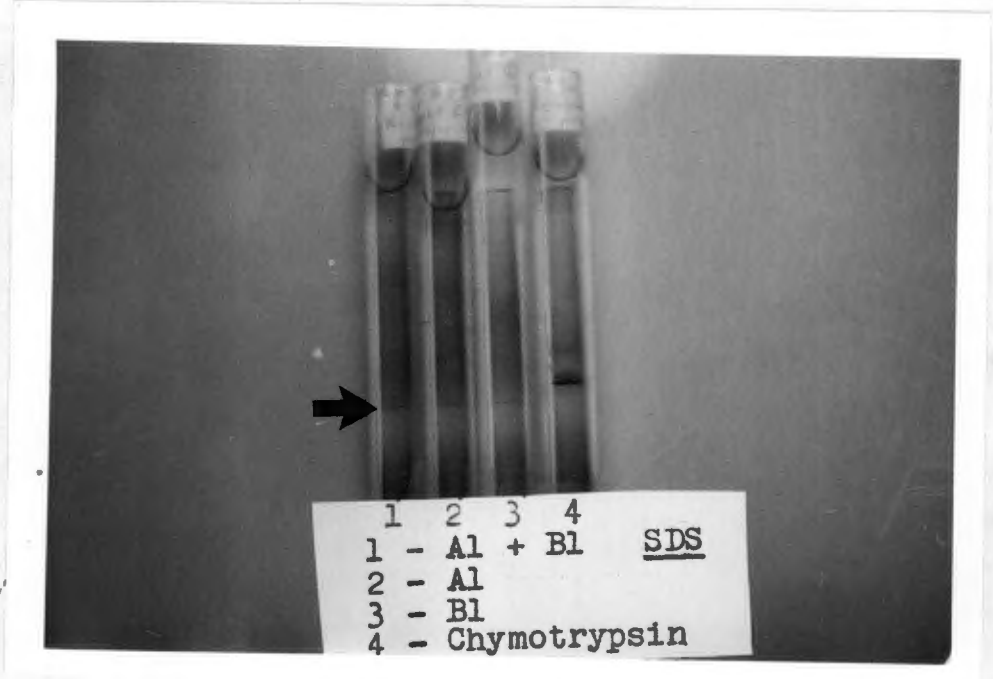


Figure 9-1: PAGE of two preparations of cholelysin, designated A1 and B1.

In the Figure tubes 1, 2, 3 and 4 refer to tubes of the same number in Table 9-1. Thus

1 = A1 + B1
2 = B1
3 = A1
4 = chymotrypsin

The arrow points to the faintly staining cholelysin band. All cholelysin tubes contained SDS to a final concentration of 2 g/100 ml. Tube 2 contained mercaptoethanol 2 g/100 ml in addition to SDS.

The fibrinolytic activity of gel sections from equivalent tubes was measured as described under Table 9-1. The results are shown in Table 9-2 and in Figure 9-2.

The results of the plated gel sections are shown graphically in Figure 9-2.

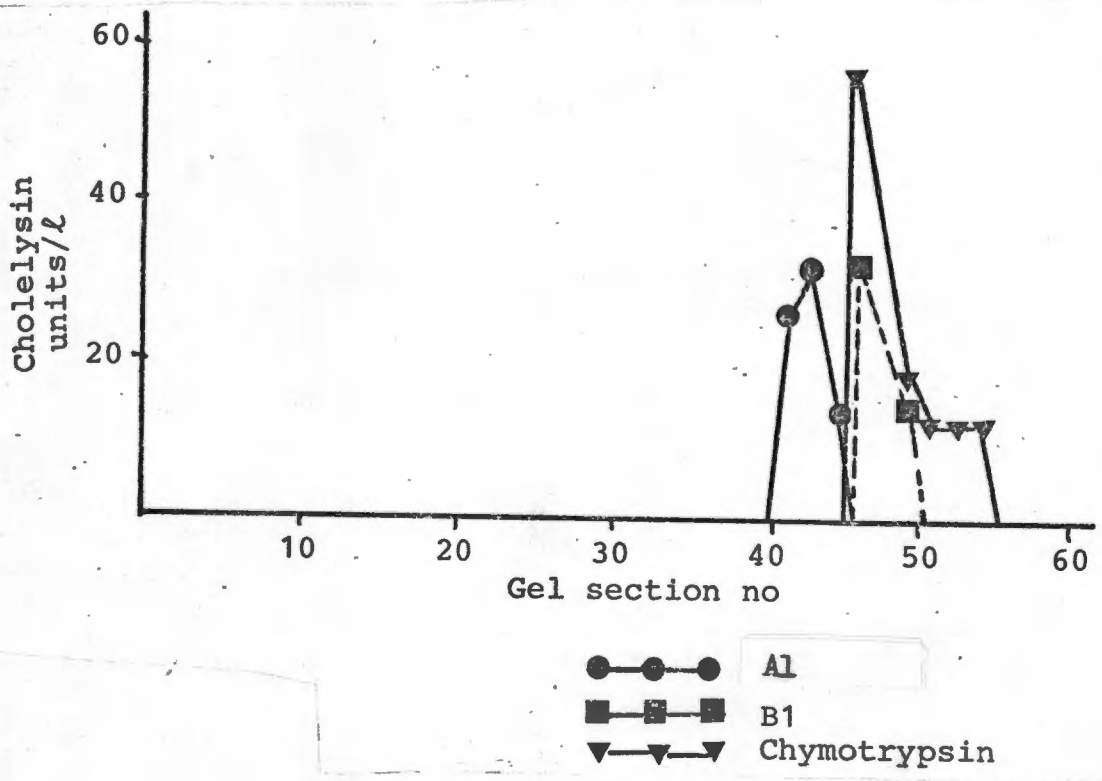


Figure 9-2: Fibrinolytic activity of two preparations of cholelysin Peak I and chymotrypsin standard obtained following PAGE. Alternate gel sections plated. Results from Table 9-2.

Comment on this experiment:

There was satisfactory resolution of protein bands by PAGE with SDS as is shown in Figure 9-1. However, there was considerable loss of fibrinolytic activity, greater in the specimen (B1) with mercaptoethanol, and greater yet in the chymotrypsin control. It was evident that the molecular weights of the two preparations of cholelysin examined were close to that of chymotrypsin (~ 13 000).

It was necessary to examine the effects of (1) SDS and (2) mercaptoethanol on the fibrinolytic activity of Peak I.

9.4 Effects of SDS and mercaptoethanol on fibrinolytic activity of Peak I.

9.4.1. SDS

In an experiment to be shown in Chapter 11, cholelysin Peak I + SDS 2 g/100 ml was run through a sephadex G50 column. The cholelysin preparation had an activity of 38,7 mm = 232 u/ℓ. 2 ml were loaded on the column = 0,464 units. The effluent contained a total fibrinolytic activity of 0,225 units = 48% recovery, within the range of 50-85% recovery from sephadex G200 reported in Table 5-13. Thus, under these conditions SDS had slight or no inhibitory effect on the fibrinolytic action of Peak I.

9.4.2 Mercaptoethanol

Two experiments were done to investigate the effect of mercaptoethanol on cholelysin. In the first experiment, cholelysin was diluted 1/20 with sample application buffer containing mercaptoethanol 0,3 mg/100 ml. The mixture was left at room temperature for 30 minutes and 21 hours, and then plated on a fibrin plate in the usual way. The results are shown in Table 9-3.

Table 9-3: Effect of mercaptoethanol on fibrinolytic activity of cholelysin. Cholelysin diluted 1/20 with sample application buffer containing mercaptoethanol 0,3 g/100 ml and left for varying periods of time at room temperature before incubating on the tandard fibrin plate. Results in mm diameter of zone of lysis and in units. Inhibition calculated as set out in section 7.4.2.

Control: 20, 20, 20, 20: mean 20 nm = 23,2 units

Incubation	Lysis mm diam	Units	Inhibition %
0	17,5	17,1	26
30 minutes	13,5	10,5	55
20 hours	0		100

In view of this result, a more elaborate experiment was carried out to establish the inhibitory potential of mercaptoethanol on the fibrinolytic activity of cholelysin. In a further experiment, the cholelysin-mercaptoethanol mixture was dialysed against tris buffer to test if the fibrinolytic activity of cholelysin had been irreversibly destroyed by mercaptoethanol. The results of these experiments are shown in Tables 9-4 and 9-5.

Table 9-4: Inhibitory effect of various concentrations of mercaptoethanol on the fibrinolytic activity of cholelysin. Cholelysin C1 diluted 1/20 with sample application buffer containing various concentrations of mercaptoethanol in g/100 ml. Mixture incubated on standard fibrin plate after 0 and 30 minutes of incubation. Inhibition calculated as set out in section 7.4.2.

Control (mm diamter of zone of lysis: 19, 18,5, 19, 18,5: Mean 18,75 = 19,1 units/ℓ.

Mercapto-ethanol % (mg/100 ml)	1	2	mean	units	Inhibition %
	Incubation 0 minutes				
0,3	16	16	16	14,2	26
0,2	16,5	16	16,25	14,7	23
0,15	16,5	16,5	16,5	15,1	21
0,1	17,5	17,5	17,5	17,1	10
0,05	17,5	18,0	17,75	17,6	8
0,01	18,0	18,0	18,0	18,2	5
	Incubation 30 minutes				
0,3	11	11	11	7,7	60
0,2	12	12,5	12,5	9,0	53
0,15	13	13	13	9,8	49
0,1	16	15	15,5	13,4	30
0,05	16	16	16	14,2	26
0,01	18	18	18	18,2	5

Table 9-5: Effect of dialysis of mercaptoethanol-cholelysin mixture on fibrinolytic activity of cholelysin. Cholelysin 34,5 mm (140 u/ℓ) diluted 1/20 in sample application buffer containing mercaptoethanol 0,3 g/100 ml and both dialysed for 48 hours against tris buffer M 0,006 pH 8,5 with one change of dialysis fluid. Control diluted 1/20 in sample application buffer containing no mercaptoethanol. Result in mm diameter of zone of lysis and in units/ℓ.

Specimen	Lysis mm diam		Units/ℓ		Inhibition
	1	2	mean	units	
Control	11,0	11,5	11,25	7,9	
Test	0	0	0		100

It was concluded that mercaptoethanol produced irreversible denaturation of cholelysin Peak I.

9.5 Fibrinolysis

9.5.1 Introduction

Fibrinolysis was discussed in section 2.5. For ease of reference, this discussion is briefly recapitulated here.

Thrombin splits fibrinopeptides A and B off the intact Aα and Bβ chains of the fibrinogen molecule. The resultant fibrin 'monomer' polymerises spontaneously, through weak H^+ bonding, to form non-stabilised fibrin,

which is readily lysed by plasmin.

Thrombin also activates Factor XIII (fibrin stabilising factor). Factor XIIIa, potentiated by Ca^{++} (Thomson op cit, 1970) causes glutamyl-lysyl transpeptidation of the α and γ chains of non-stabilised fibrin, forming α polymers and γ dimers. This procedure is 95% complete after 60 minutes; increasing opacification of the clot accompanies the transpeptidation, and the resultant fibrin clot contains only stabilised fibrin. These processes are illustrated in Figures 9-3 and 9-4.

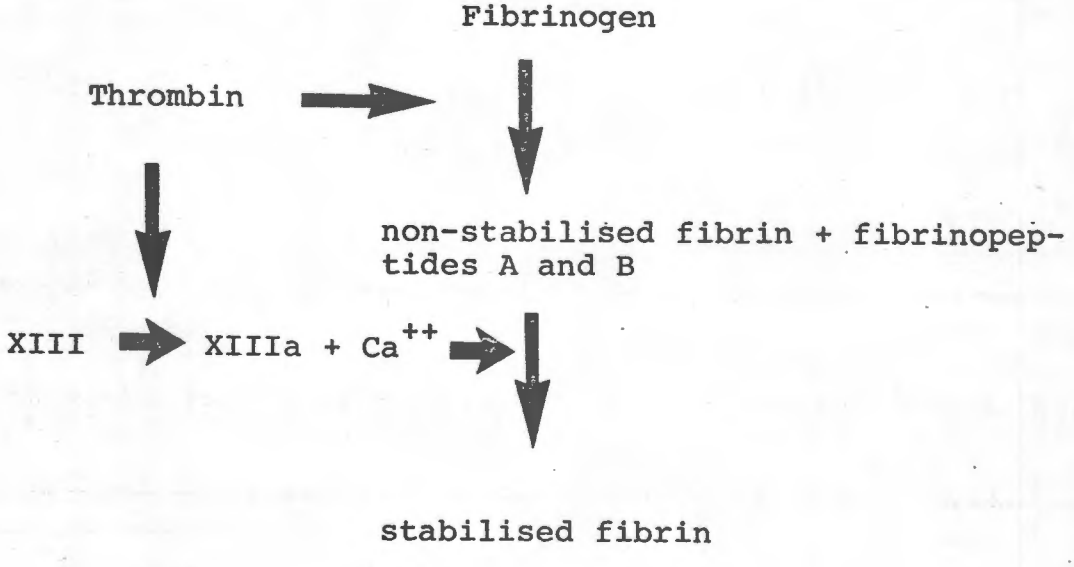


Figure 9-3: Formation of non-stabilised and stabilised fibrin under the influence of thrombin and Factor XIII.

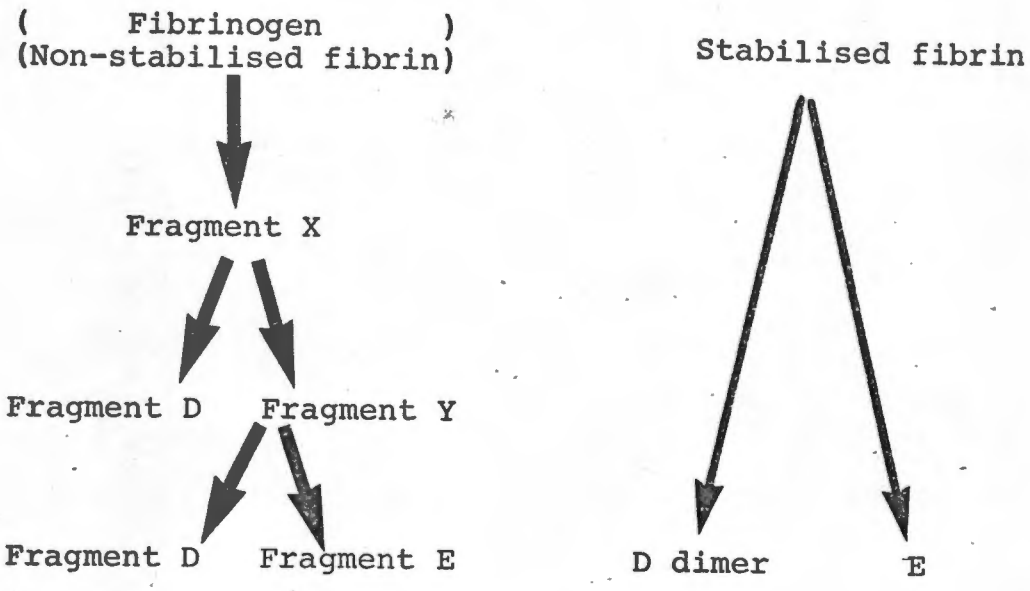


Figure 9-4: Comparison of the split products of plasmin digestion of fibrinogen and non-stabilised fibrin, and of stabilised fibrin. The major differerence is the presence of D dimer in the split products of stabilised fibrin. The split products of non-stabilised fibrin differ from the split products of fibrinogen only in the absence of fibrinopeptides from the X, Y and E fragments.

9.5.2 Fibrinolysis by cholelysin and plasmin

The technique adopted for the manufacture of fibrin plates for this study produced stabilised fibrin. These plates alone were used during investigation of fibrinolysis by cholelysin and plasmin.

9.5.2.1 Experimental design

Polyacrylamide gel electrophoresis (PAGE with a linear gradient) was used, for resolution of the wide range of molecular weights of fibrin split products that was anticipated (Stead, 1981).

Split products of fibrin digestion by cholelysin and plasmin after 24 hours incubation were prepared as described in Method 18. In brief, soluble fibrin products were aspirated from a standard fibrin plate and the fibrinolysin/fibrin reaction was quenched with SBTI. The split products were then mixed with reducing agents in sample application buffer to give a mixture with final concentration of:

SDS	2 g/100 ml
mercaptoethanol	2 g/100 ml
urea	6 M.

The mixture was loaded on the gel to give a total load of 10-20 ug of protein and run at 2 mA and ~ 9 V per tube for 5 hours. Lactate dehydrogenase, chymotrypsinogen and ribonuclease were used as molecular markers. Staining and destaining were carried out as described in Method 18.

9.5.2.2 Results are shown in Figure 9-5.

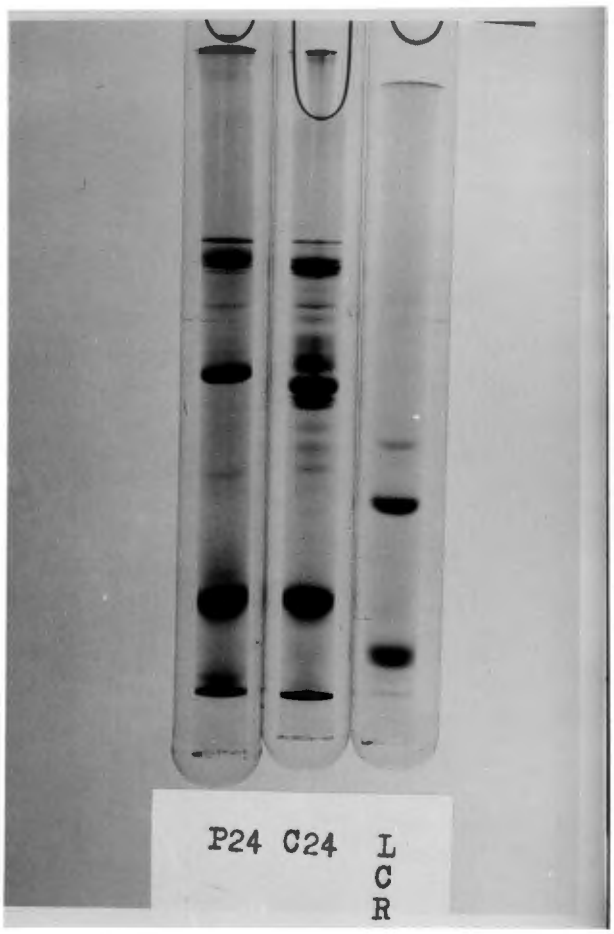


Figure 9-5: Split products of plasmin and cholelysin digestion of cross-linked fibrin at 24 hours. 'P24' = Plasmin 'C24' = cholelysin products. L = lactate dehydrogenase; C = chymotrypsinogen; R = ribonuclease.

9.5.2.3 Discussion

From this figure, it was evident that the split products of cholelysin and plasmin differed completely. Cholelysin appeared to have a wider range of substrates than plasmin. Because cholelysin produced very rapid appearance of lysis on the fibrin plate, a clearing zone usually being visible within 3-5 minutes, it was desirable to study the kinetics of the cholely-

sin/fibrin reaction more closely. These kinetics are described in Chapter Ten.

9.5.3 Summary

Cholelysin with chymotrypsin control, and split products of fibrin digestion by cholelysin and plasmin were studied by PAGE. Cholelysin was studied with SDS added, and with and without additional mercaptoethanol. Only a single band was obtained by PAGE, which stained poorly by standard techniques. The molecular weight appeared close to, but not identical with, that of chymotrypsin. Simultaneous assay of fibrinolytic potential of gel sections was done; this showed that lysis and stained bands coincided for cholelysin and chymotrypsin.

Split products of plasmin and cholelysin digestion of cross-linked fibrin were entirely different.

CHAPTER TEN

SPECTROPHOTOMETRIC STUDIES - ENZYME KINETICS

10.1 Introduction

The rapidity with which fibrinolysis by cholelysin occurred on a fibrin plate led to a desire to study the kinetics of this reaction. By taking advantage of the adherence of the fibrin gel to surfaces, and of the solubility of fibrin degradation products, a new method was devised to follow enzyme kinetics. This was termed the composite cuvette method for the analysis of enzyme kinetics.

10.2 The composite cuvette method for analysis of enzyme kinetics.

10.2.1 Principles

(1) The substrate must be in gel or solid phase, and one or more degradation products must be soluble.

(2) A clear cut difference on the scanning spectrophotometer must be shown between substrate, and the degradation products of the reaction.

(3) The substrate must be able to occupy half of the light path inside the cuvette when held in the vertical position.

(4) The enzyme solution is then poured in, and the reaction followed at the wavelength of maximum difference.

In the case of a fibrin substrate, the preparation of the composite cuvette takes advantage of the fact that a fibrinogen solution can be made to clot slowly if low concentrations of thrombin are added. Clotting fibrinogen solution, as prepared for the standard fibrin plate (Method 10, solution after Step 3) took 2 - 3 minutes to begin clotting, and was thus entirely suitable.

10.2.2 Experimental design

(1) Spectrophotometric scanning
(2) Preparation of composite cuvette
(3) Study of enzyme kinetics

10.2.2.1 Spectrophotometric scanning

For crosslinked fibrin, the cuvette was filled with clotting fibrinogen solution, and was then incubated for 2 hours at 37°C. For fibrin split products of cholelysin digestion (FSPC), the cuvette was filled with lysed fibrin aspirated from a fibrin plate incubated with cholelysin for 24 hours. The reaction was quenched with SBTI (see Method 18).

The spectrophotometric scans performed are listed in Table 10-1.

Table 10-1: Composite cuvette. Spectrophotometric scans and reference standards.

Scan	Substance scanned	Reference standard
A	Fibrin	Air
B	FSPC	Air
C	Fibrin	FSPC
D	FSPC	Fibrin

10.2.2.2 Results

The first three scans are shown in Figures 10-1, 10-2 and 10-3. Scan D gave no clear cut peak (not shown).

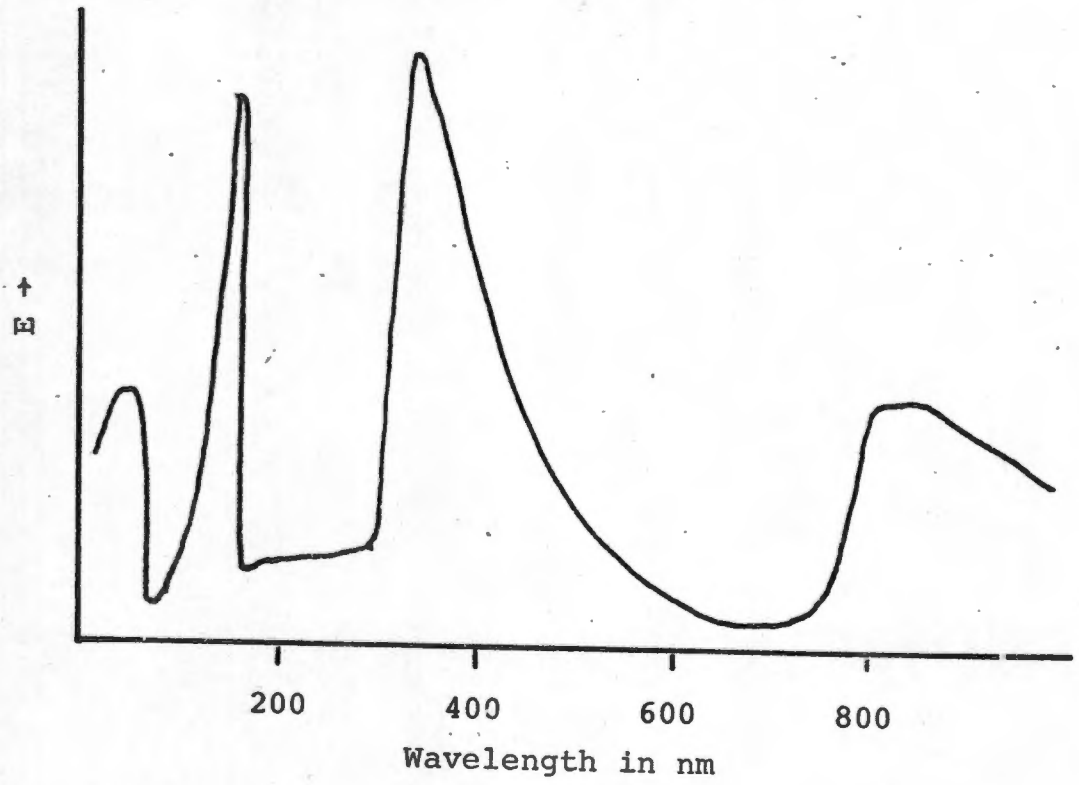


Figure 10-1: Spectrophotometric scan of fibrin with air as reference standard, Scanning rate 100 nm/minute. Span 0,5A = 0,50/ℓ". Chart speed 0,5"/minute.

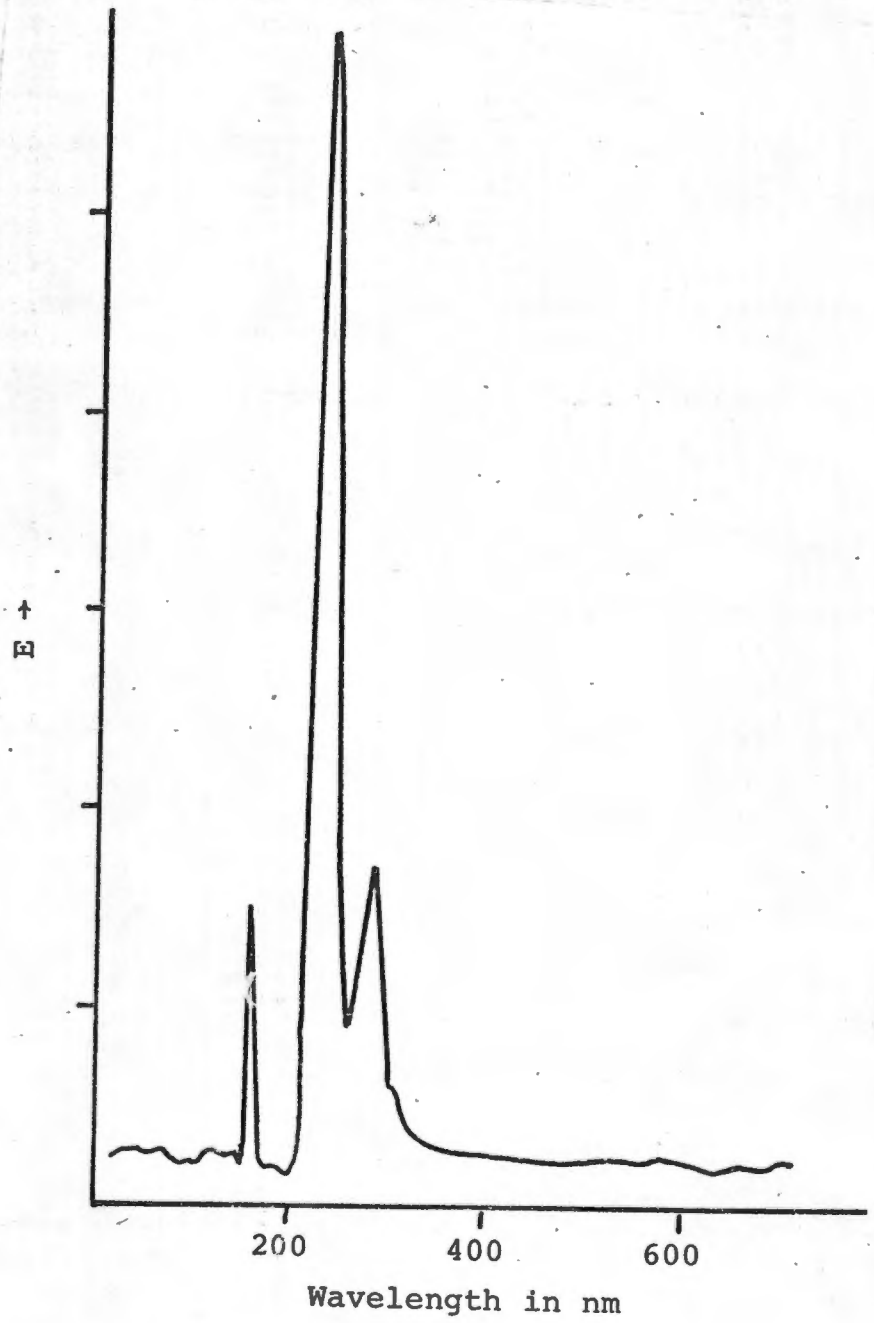


Figure 10-2: Spectrophotometric scan of FSPC with air as reference standard. Scanning rate 100 nm/minute. Span 0,5A = 0,050/1". Chart speed 0,5"/minute.

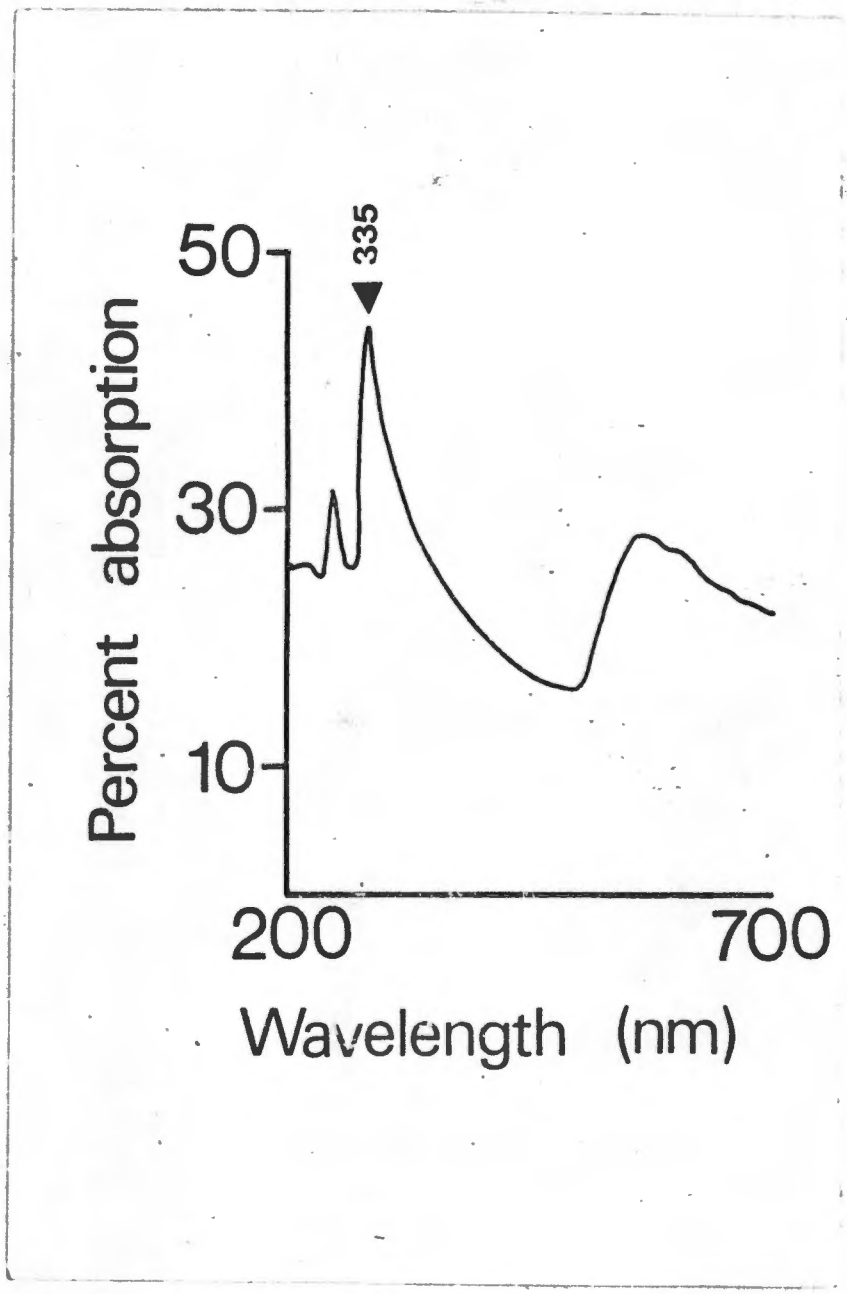


Figure 10-3: Spectrophotometric scan of fibrin clot, using FSPC as the reference standard. Scanning rate 100 nm/minute. Span 0,5A (0,050/1"). Chart speed 0,5"/min.

Comment: The peak shown at 335 nm was due to fibrin. Thus, digestion of fibrin by cholelysin in the composite cuvette resulted in a decrease of extinction at 335 nm. When FSPC were scanned, using fibrin as a reference standard, no sharp peak was found.

10.2.3 Preparation of composite cuvette

A quartz cuvette was half-filled with clotting fibrinogen solution prepared as for use on the standard fibrin plate. The cuvette was capped, laid on its side (with a side towards the light path within the spectrophotometer upwards) and incubated at 37°C for 2 hours. A fibrin clot formed which was firmly adherent to the cuvette. The cuvette was removed from the incubator, the cap taken off and cholelysin or plasmin solution poured in. This was now a composite cuvette. The technique is illustrated in Figure 10-4.

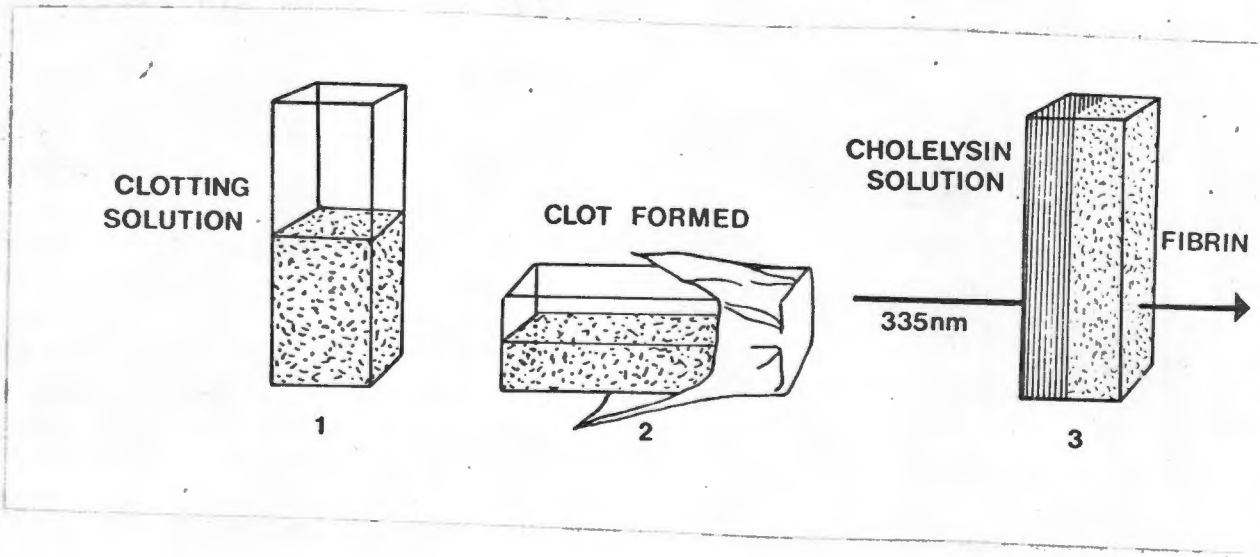


Figure 10-4: Method of preparing a composite cuvette for study of the kinetics of the cholelysin-fibrin reaction. The figure 335 nm refers to the wavelength at which the reaction was followed in the spectrophotometer.

10.2.3.1 Study of enzyme kinetics

The composite cuvette was placed in a thermostatically controlled spectrophotometer at 37°C, and the decrease of extinction at 335 nm followed on a recorder.

10.2.3.2 Results are shown in Figures 10-5, 10-6 and 10-7.

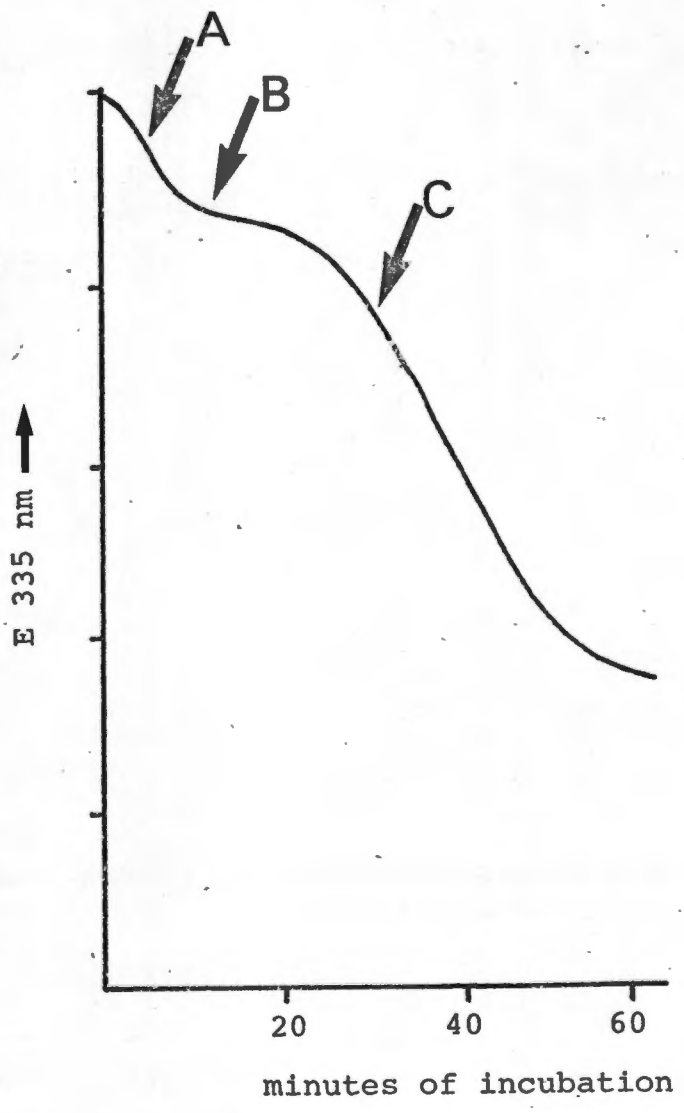
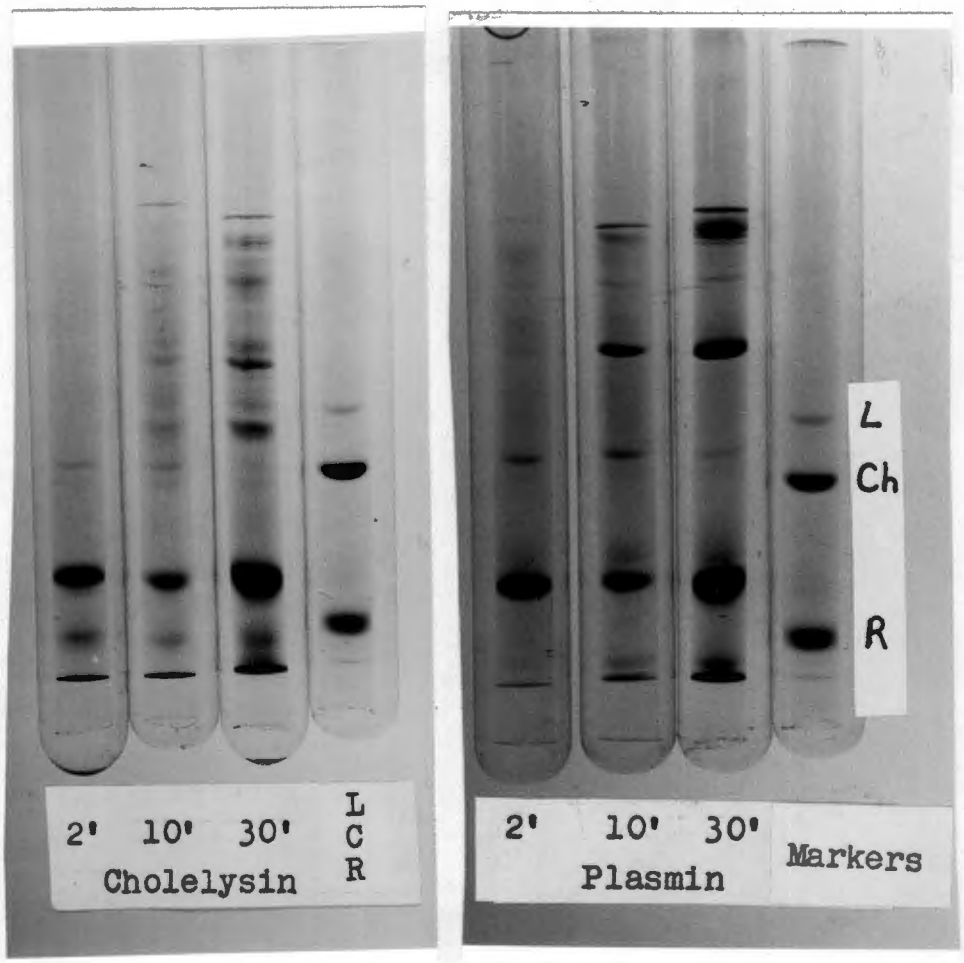


Figure 10-5: Composite cuvette. Cholelysin-fibrin reaction. Stabilised (crosslinked) fibrin 2 mg/ml. Cholelysin 110 u/ℓ. Two phases of relatively rapid decrease of extinction (A and C) are shown, separated by phase B where the rate of change plateaus.

Comment: The graph of the cholelysin-fibrin reaction shown in Figure 10-5 suggested that there was a two-phase attack on the fibrin molecule by cholelysin. This hypothesis was tested by subsampling from the composite cuvette (liquid phase) and carrying out PAGE on the samples. As a control, plasmin 0,2 units/ml was used in the composite cuvette instead of cholelysin, and subsampled at the indicated times.



Figures 10-6 and 10-7: Comparison of the split products of cholelysin and plasmin digestion of stabilised fibrin. Molecular markers (from above downwards) L = lactate dehydrogenase C = chymotrypsinogen R = ribonuclease.

Comment on Figure 10-6: This figure demonstrated the correctness of the hypothesis that cholelysin made a two-phase attack on the stabilised fibrin molecule. It is evident that only two products were present at 2 minutes, and many more at 10 minutes. On the other hand, the plasmin digest contained many products at the outset, which increased in density but not in number up to the 30 minute point.

Figures 10-5 and 10-6 are shown superimposed in Figure 10-8.

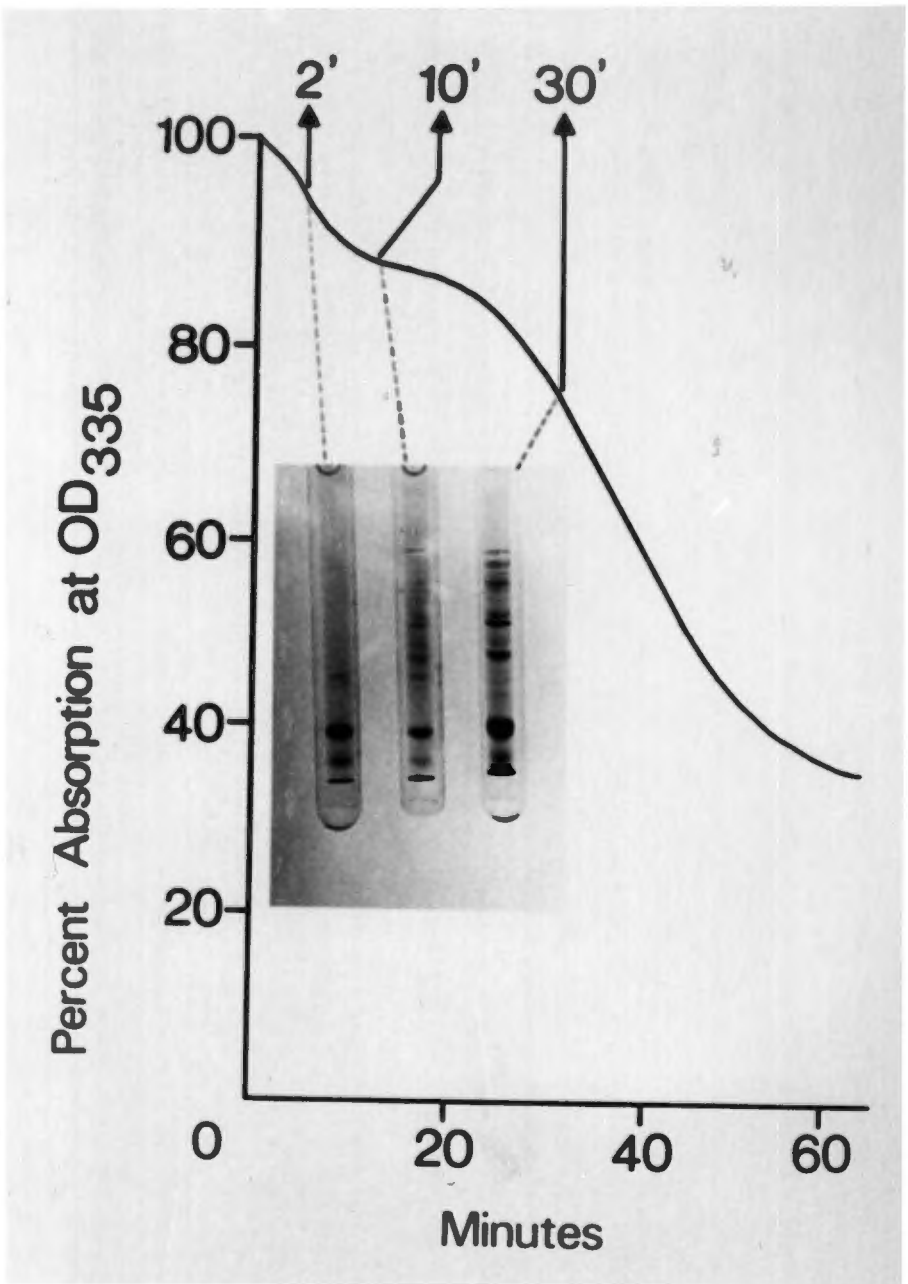


Figure 10-8: Superimposition of Figures 10-5 and 10-6 to show time relationship of generation of split products by cholelysin from stabilised fibrin.

10.2.4 Discussion

This interesting result showed that the composite cuvette method was a valid technique for following the cholelysin-fibrin reaction. Beyond using the method for obtaining early split products of plasmin digestion of stabilised fibrin, no attempt was made to study the plasmin/fibrin reaction further.

However, advantage was taken of this method to study kinetics of the cholelysin/fibrin reaction. These experiments are described in 10.3.

10.3 Kinetics

Both substrate and enzyme were used in different concentrations in the composite cuvette method, in an attempt to establish a Lineweaver-Burk relationship (Lehninger, 1975). Unfortunately, the results of varying the concentration of substrate were anomalous, and require further study. They will not be reported here.

Varying the concentration of the cholelysin solution produced a clearcut change in the velocity of the reaction in the second phase (phase C in Figure 10-5). This is shown in Figure 10-9.

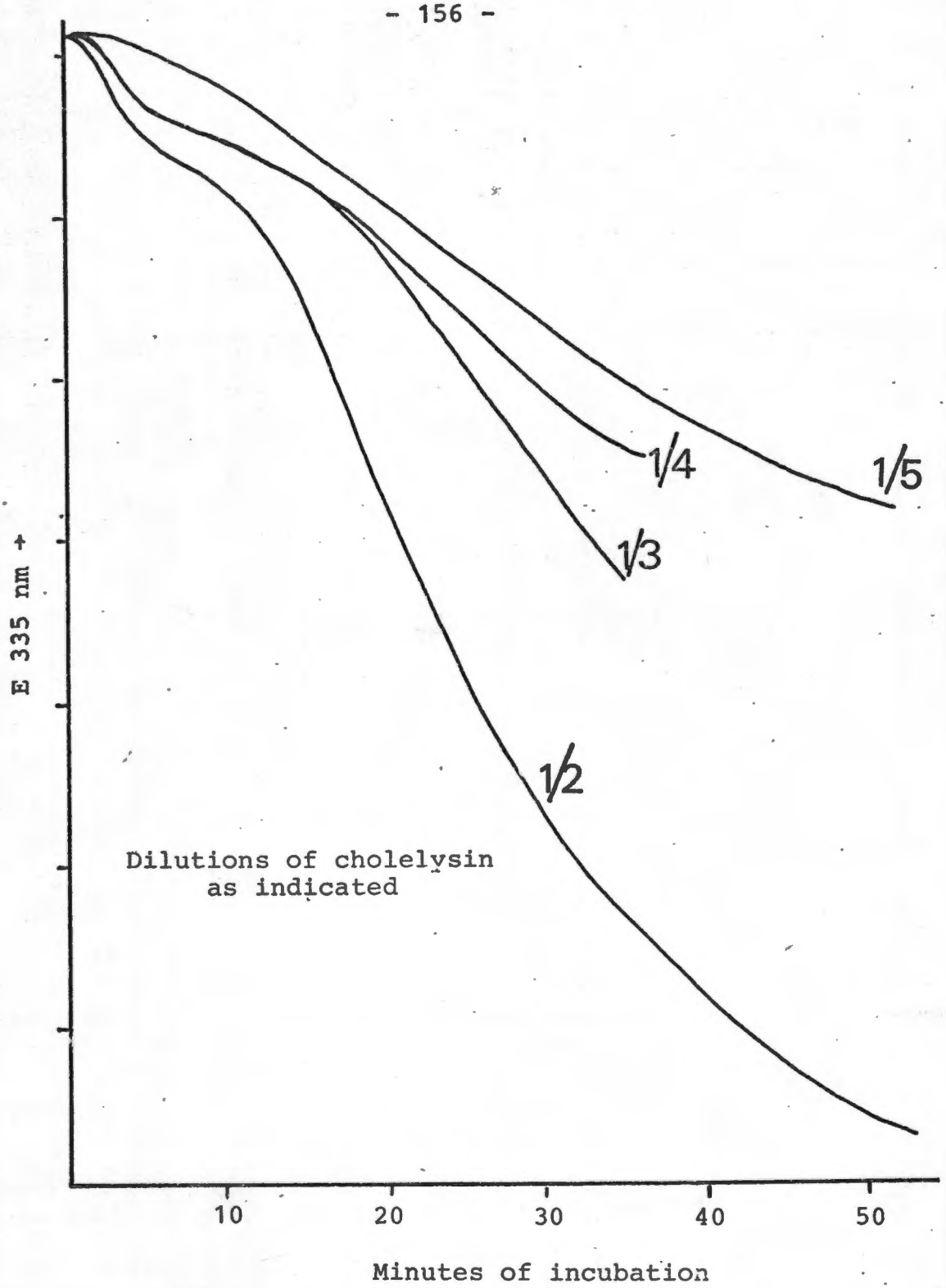


Figure 10-9: Superimposition of four graphs of variations in V with concentration of cholelysin. Substrate: stabilised fibrin 2 mg/ml. Enzyme: Cholelysin 1/2 = 140 u/ℓ, 1/3 = 93 u/ℓ, 1/4 = 70 u/ℓ, 1/5 = 56 u/ℓ. This Figure is analysed overleaf.

Comment on Figure 10-9: this Figure was analysed for relationship between velocity of reaction in first and second phases (Phases A and C) and relative enzyme concentration. A clearcut relationship was found for phase C; the results are shown in Table 10-2, and graphically in Figure 10-10.

Table 10-2: Analysis of kinetics of fibrinolysis by cholelysin in terms of relative concentration of enzyme vs V. The steepest portion of the second phase of each curve was selected, and Tan θ estimated.

Concentration of cholelysin	θ	Tan θ (V)	$\frac{1}{\text{Tan } \theta}\left(\frac{1}{V}\right)$
1/2	66,5	2,30	0,435
1/3	53	1,33	0,752
1/4	44	0,97	1,031
1/5	38	0,78	1,282

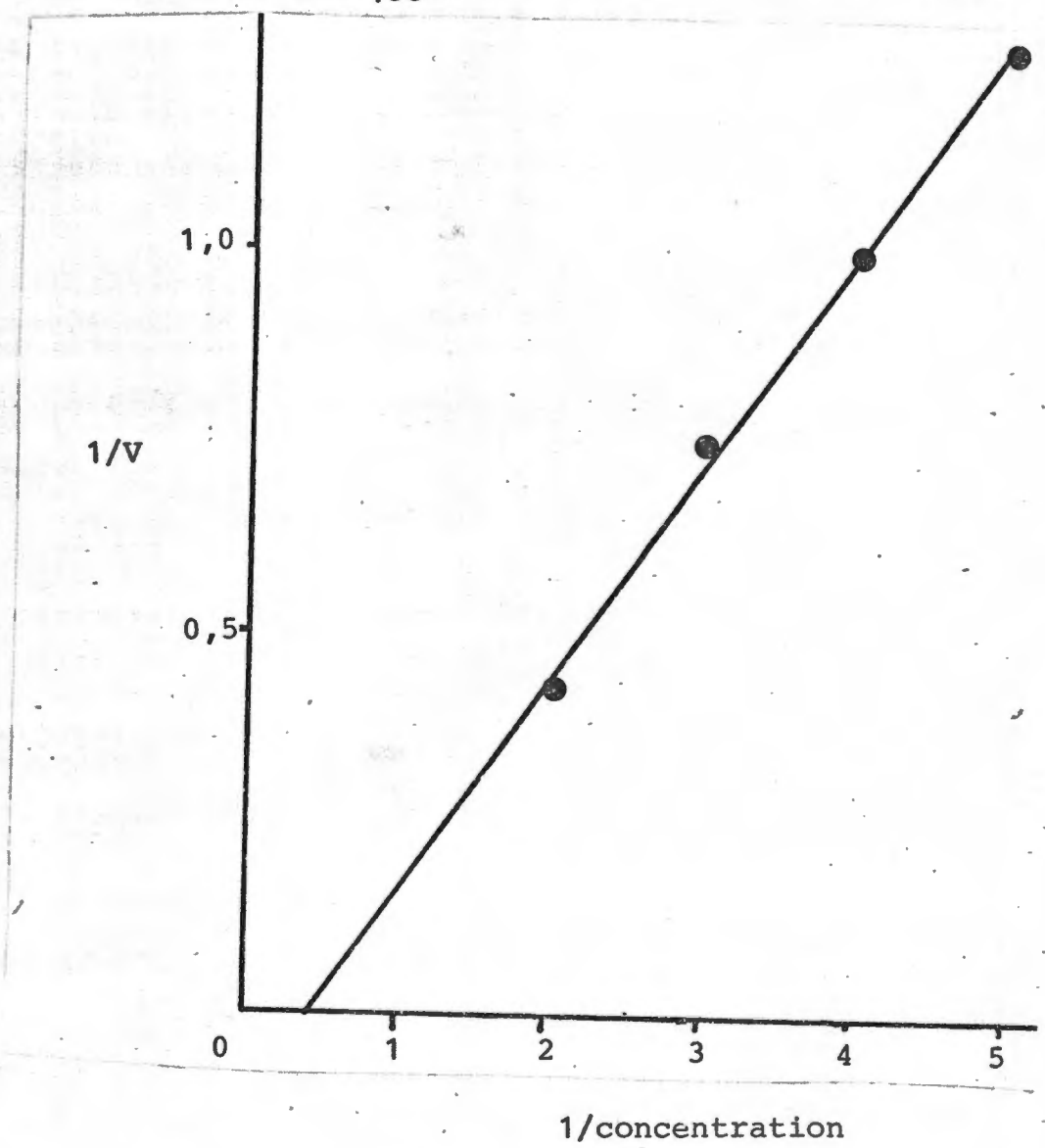


Figure 10-10: Double reciprocal relationship between enzyme concentration and velocity of fibrinolysis in the second phase of the colelysin-fibrin reaction.

10.4 Conclusions and summary

The composite cuvette method was developed for the study of enzyme kinetics at a sol/gel interface. This method was applied to the cholelysin/stabilised fibrin reaction, which was found to be biphasic. The degradation products of the two phases of the reaction were analysed by PAGE, and it was shown that cholelysin made a two-phase attack on the mole-

cule. The kinetics of the enzyme/substrate reaction were analysed by the composite cuvette method in terms of variation in enzyme concentration. A linear relationship was found between the reciprocal of enzyme concentration and the reciprocal of V (second phase).

CHAPTER ELEVEN

MOLECULAR WEIGHTS

11.1 Introduction

The molecular weights of cholelysin and of fibrin split products of digestion by cholelysin and by plasmin are reported in this chapter. To cut down unnecessary detail, workings are shown in Appendix 11; only the principal results are shown here.

11.2 The molecular weight of cholelysin was estimated by column chromatography (Petersen and Sober, 1962; Laurent and Killander, 1964). Experiments were done at widely varying intervals of time, on several preparations of cholelysin, and with and without sodium dodecyl sulphate (SDS). Experiments with SDS added are reported separately. Molecular weights were estimated from a graph supplied by the makers of 'Sephadex'.

11.2.1 Column chromatography without SDS

Table 11-1: Molecular weights without SDS, for cholelysin fractions.

Preparation	V_t of column	Sephadex	K_{av}	Molecular weight (estimated)
Ammonium sulphate precipitate	224	G50	0,3	23 000
Peak I	46,5	G75	0,5	11 000
Peak II	46,5	G75	0,45	13 000
Peak III	46,5	G75	0,58	8 000
Peak IV (i)	46,5	G75	0,62	7 500
(ii)	46,5	G75	0,005	50 000
Rechromatography of Peak I. Two experiments	224	G75	0,25	28 000
	224	G75	0,28	27 000

11.2.2 Column chromatography with SDS

SDS was added to the cholelysin preparation before the experiment to final concentration of 2 g/100 ml, and incubated together at room temperature for 30 minutes before the experiment was performed. All samples were batchwise Peak I. Chymotrypsin was used as a control. The results are shown in Table 11-2.

Table 11-2: Molecular weights of batchwise Peak I with SDS 2 g/100 ml. Chymotrypsin control.

Preparation	V_t of column	Sephadex	K_{av}	Molecular weight (estimated)
Batchwise Peak I	124	G75	0,4	14 300
Peak I	140,5	G75	0,39	14 500
Peak I	140,5	G75	0,63	7 000
Chymotrypsin	140,5	G75	0,40	13 000 (known)

11.2.3 Polyacrylamide gel electrophoresis of cholelysin with SDS or with SDS and mercaptoethanol, both 2 g/100 ml final concentration. This experiment was shown in Figure 9-1 (stained gels) and in Table 9-2 and Figure 9-2 (fibrinolytic activity of parallel experiments, with gel cut into sections and applied to a standard fibrin plate). Cholelysin stains poorly, but a line can be discerned in Figure 9-1, and this is confirmed in the parallel experiment reported in Table 9-2 and Figure 9-2. From these experiments, it was apparent that the molecular weight of the preparations of cholelysin studied was of the same order as chymotrypsin (~ 13 000).

11.2.4 Paper electrophoresis of batchwise Peak I was attempted, but definite stained bands could not be elucidated.

11.2.5 Discussion

These results cluster into three groups of molecular weights, with two values outside them. These are:

7 000
14 000
28 000

The discrepant results are

(1) 23 000, from a single experiment reported in Chapter Five, Figure 5-6. This experiment was performed on ammonium sulphate precipitated bile, with elution by tris/NaCl, and gave K_{av} = 0,3 on sephadex G50. These conditions were never repeated.

(2) 50 000, from a single experiment using Peak IV. This experiment gave two widely separated values for K_{av}, viz, 7 000 and 50 000. This was also a single experiment.

With or without these discrepant results, the possibility is raised that cholelysin in monomeric form has a molecular weight of ~ 7 000, with polymers of ~ 14 000 (~ 21 000) and ~ 28 000. The experiments with SDS, reported in section 11.2.2, are noteworthy, as they were done with particular care in view of the wide variations in molecular weight found in the experiments reported in 11.2.1. Even so, two values were found in the presence of adequate amounts of SDS.

The higher molecular weights (~ 28 000) found on rechromatography of Peak I could have been due to polymerisation of the molecule when in high purity and concentration.

The molecular weights of the four Peaks without SDS were only estimated once, and rechromatographed Peak I twice, before the decision was made to concentrate on Peak I for further experimentation. No attempt was made to correlate molecular weights with substrate activities. It is possible that certain molecular weights are associated with particular substrate activities; for example, high molecular weight (~ 50 000) Peak IV.

11.3 Molecular weights of fibrin split products

These experiments were shown in Chapters Nine and Ten. The PAGE technique used (linear gradient) gave high resolution, with a fixed end point for each band in relations to the gradient. This end point was independent of voltage and of prolongation of the procedure beyond the standard time. Thus, relative mobility need be determined only in relation to the length of the gel at the end of processing (Stead, 1981). Samples of split products were obtained from the composite cuvette at various times, and fibrinolysis was quenched by SBTI (see Method 8). Results are shown in Figures 11-1, 11-2, 11-3, and 11-4.

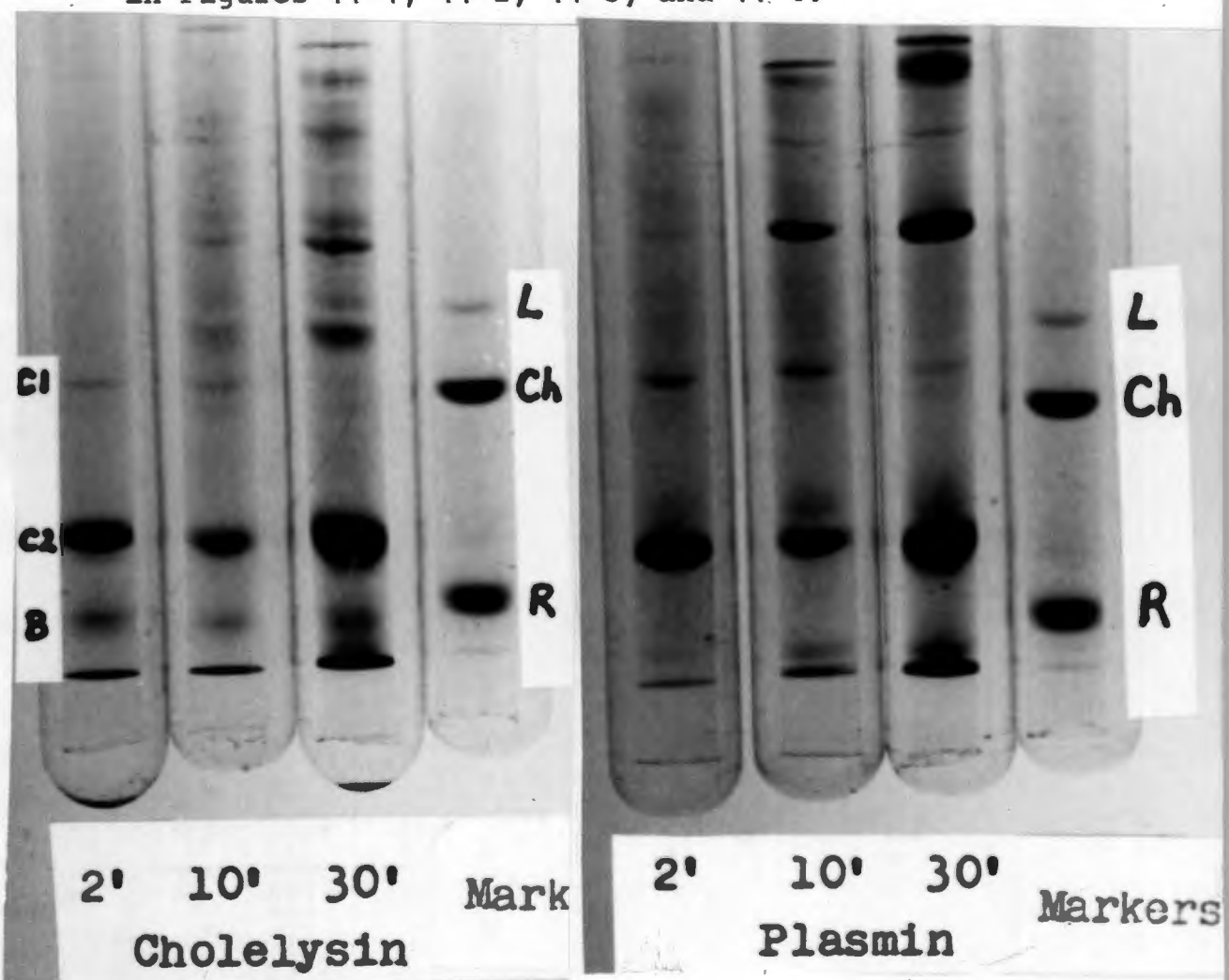


Figure 11-1: FSPC L = lactate dehydrogenase
Ch = chymotrypsinogen
Figure 11-2: FSPP R = ribonuclease

PAGE of cholelysin split products of fibrin digestion at the times indicated. Plasmin split products shown in Figure 11-2.

Figure 11-1 is a repeat of Figure 10-6.

Figure 11-3 is a repeat of Figure 9-5, and is one of a series from which relative mobilities and molecular weights were calculated, while Figure 11-4 shows the progress of FSPC over 24 hours.

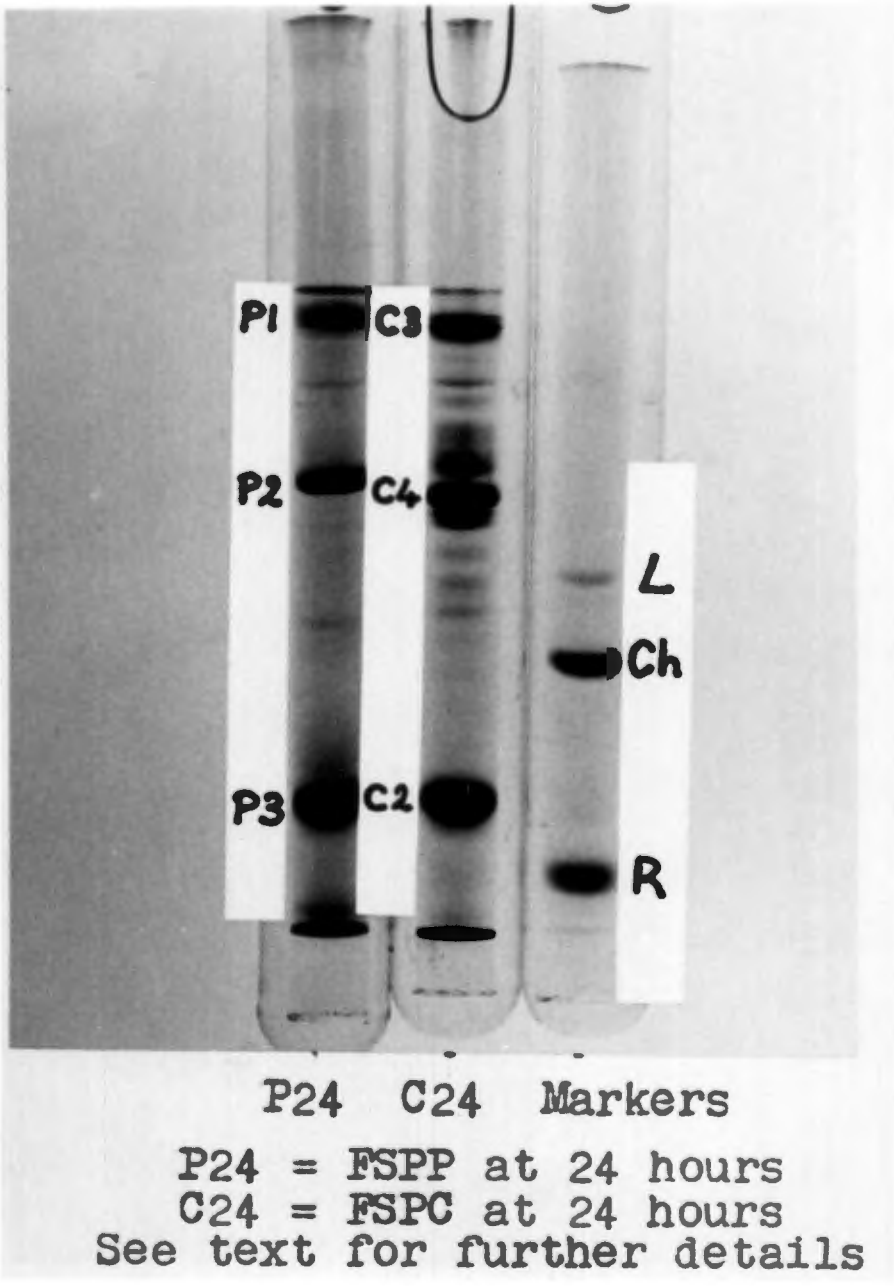


Figure 11-3: Comparison of FSPC and FSPP after 24 hours of incubation on the standard fibrin plate. Identified bands are discussed below. P1 = D dimer (γ") fragment
P2 = β" fragment
P3 = α" fragment

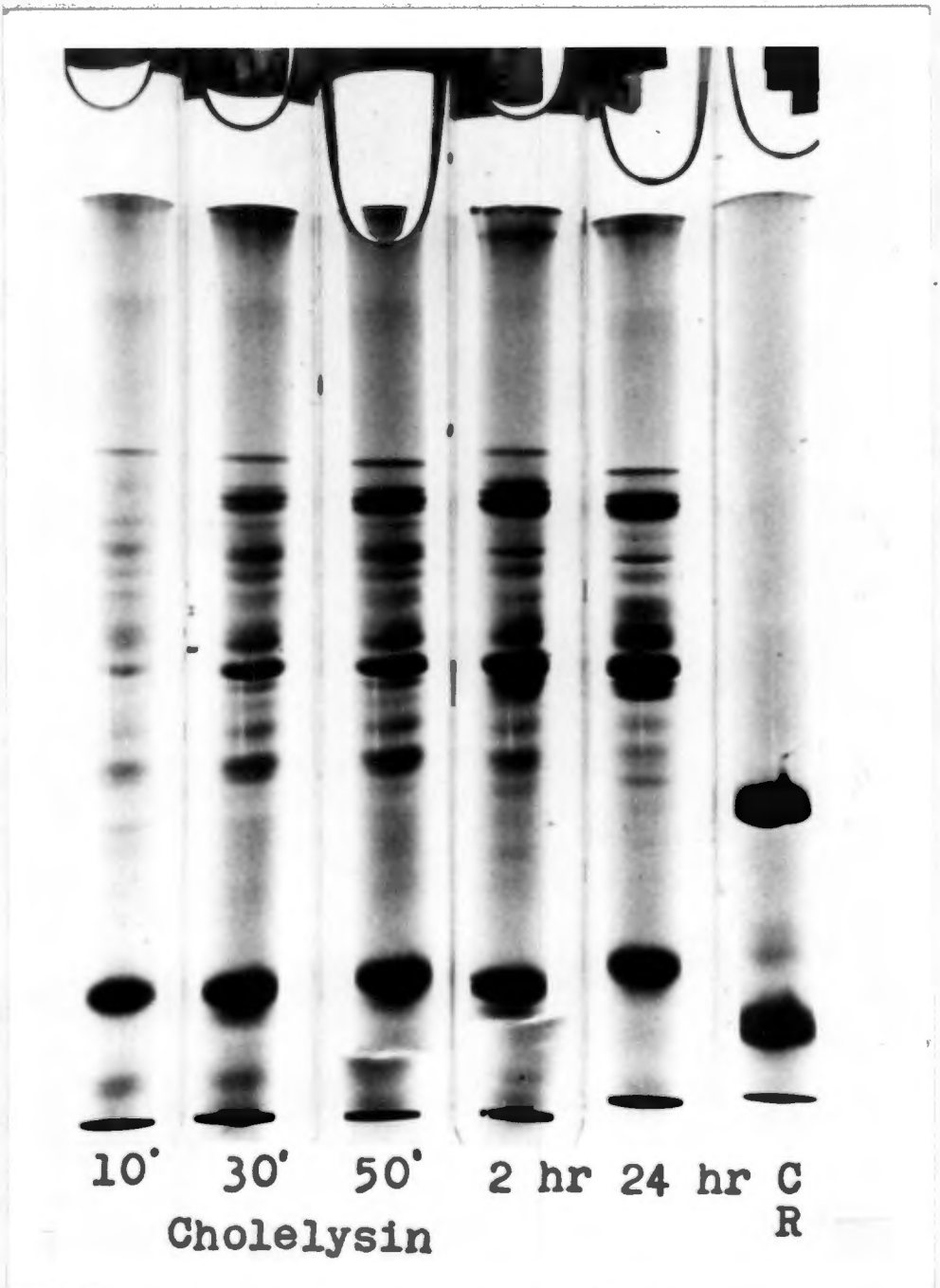


Figure 11-4: Progress of FSPC at the indicated periods of incubation with chymotrypsinogen and ribonuclease markers. Lactate dehydrogenase was unavailable when this experiment was performed. This photograph is included for interest.

11.3.1 The relative mobility of marker proteins was estimated as discussed in section 11.3 above, and plotted on a graph. This graph is shown in Figure 11-5. Relative mobility of protein bands marked in Figure 11-3 was then estimated, and the approximate molecular weights derived from the same graph. See also Table 11-3.

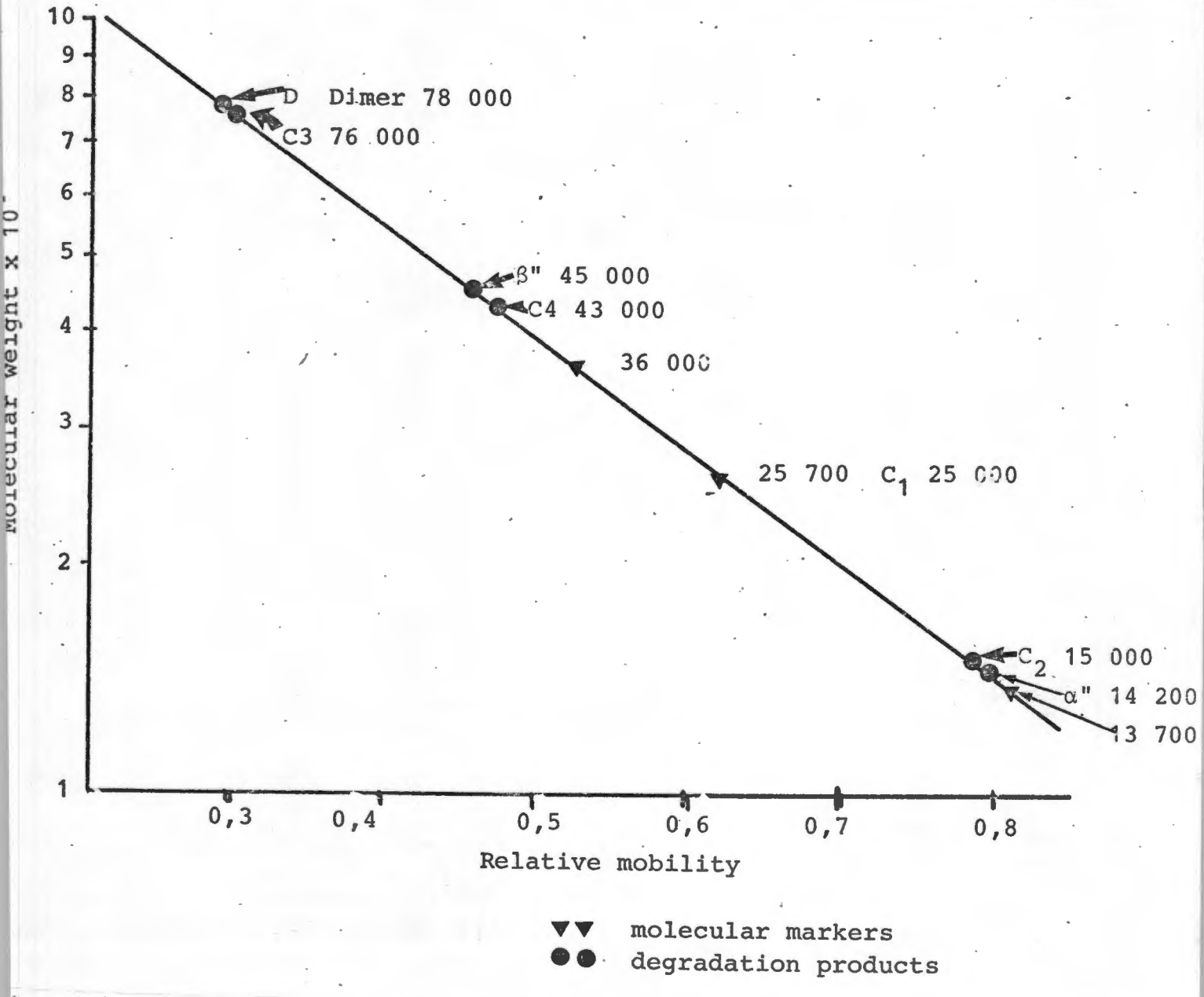


Figure 11-5: Major products of the degradation of stabilised fibrin by plasmin and cholelysin.
Molecular markers: 13 700 ribonuclease
25 700 chymotrypsinogen
36 000 lactate dehydrogenase
See Appendix 11 for detailed working.

The molecular weights of the identified bands of FSPP and PSFC, as derived from their relative mobilities, are shown in Table 11-3.

Table 11-3: Molecular weights of bands P1, P2, P3 of FSPP, and of C1, C2, C3, and C4 of FSPC as shown in Figures 11-1 and 11-3. The suggested identity of the FSPP bands is also shown.

FSPP			FSPC	
Band	Molecular weight	Suggested identity	Band	Molecular weight
P1	78 000	D-dimer (81 000)	C1	25 000
P2	45 000	β" (44 500)	C2	15 000
P3	14 200	α" (~15 000)	C3	76 000
			C4	43 000

11.3.2 Discussion

Pizzo, Taylor, Schwartz, Hill and McKee (op cit, 1973) found that three major fragments of stabilised fibrin remained after prolonged digestion with plasmin. These are listed in the column 'Suggested identity' of Table 11-3 above. Our findings, using the linear gradient gel technique of Stead (op cit, 1981) agreed reasonably with those of Pizzo, Taylor, Schwartz, Hill and McKee.

However, the products of cholelysin digestion of stabilised fibrin were entirely different.

(1) The first two products were C1 25 000 and C2 15 000.

(2) C1 had disappeared by 30 minutes.

(3) A large number of new split products had appeared by 10 minutes. Two of these showed a marked increase in density by 24 hours, and were labelled C3 and C4. C3 appears to be two products of closely similar molecular weight.

Thus, it is concluded that cholelysin makes a two phase attack on the stabilised fibrin molecule, and also shows a wider substrate specificity than plasmin.

11.4 Summary

The molecular weights of cholelysin and of the split products of the digestion of stabilised fibrin by cholelysin and plasmin were analysed.

(1) By column chromatography, cholelysin was found to have two molecular weights in the presence of SDS. One of these was probably a dimer of the other. A third molecular weight, probably a tetramer, was found in duplicate estimate of a rechromatographed sample of Peak I. Finally, two possibly discrepant estimates were found, which were not studied further because of changes of policy. The significance of these two discrepant estimates remains uncertain.

Cholelysin was found to have poor staining qualities, which hindered identification of the enzyme by PAGE or paper electrophoresis. However, in one experiment where a clear line was discernible in the gels containing cholelysin, the identity of the band was established by simultaneous assay of gels for fibrinolysis, with a chymotrypsin control.

(2) Using the linear gradient PAGE technique, in the presence of molecular markers, the 24 hour split products of plasmin digestion of stabilised fibrin (FSPP) were found to agree closely with those in published work. Cholelysin split products were analysed for molecular weight. All molecular weights found were different from those of FSPP. Two bands were present after two minutes of digestion by cholelysin. One of these bands had disappeared by 30 minutes; the other band remained and grew steadily larger over 24 hours. Numerous other bands were observed from 10 minutes onwards, confirming the two phase nature of the cholelysin/stabilised fibrin reaction as first observed by the composite cuvette technique.

CHAPTER TWELVE

SUMMARY

12.1 Summary

12.2 The nature of cholelysin

12.3 Possible future work

The study describes a new protease with some unusual properties, that has been found in the bile of 11 mammalian species studied, including man. It is a fibrinolysin, with an activity per milligram exceeding that of chymotrypsin; and it is also fibrinogenolytic. It has been given the tentative name of cholelysin to emphasise its fibrinolytic potential. However, it has a range of specifities against other substrates, including α-casein, and the synthetic esters of tyrosine (ATEE) and arginine (BAEE). It does not activate the zymogens plasminogen, chymotrypsinogen and trypsinogen.

CHAPTER FOUR

Ox-bile was used for almost all work reported in this study, because the bile of this species was available in large volumes from the Municipal Abattoir, Cape Town. From the point of view of this study, everything in bile was redundant except the protein carrying fibrinolytic potential, and much time and effort was expended to devise an effective system for the purification and concentration of this fibrinolysin (cholelysin).

CHAPTER FIVE

The final procedure contained the following steps:

desalting with sephadex A50 or AG 1-X-8
precipitation with ammonium sulphate
dialysis
column chromatography on sephadex G200
batchwise elution from Whatman DE 32
dialysis
flash freezing in liquid nitrogen
freeze drying

This produced a white powder, free of bilirubin, of which 10-50% by weight had fibrinolytic potential.

CHAPTER THREE

Quality control throughout was done by means of a standardised fibrin plate assay. Cholelysin was found to follow the semi-logarithmic dose response curve typical of most fibrinolysins tested by this method. Chymotrypsin had a closely similar dose-response curve, and this enabled cholelysin activity to be read in units/ℓ equivalent to the activity of chymotrypsin in mg/ℓ.

CHAPTER SIX

An earlier method of purification used NaCl gradient elution of cholelysin from Whatman DE 32. This produced a series (usually 4) of peaks of activity. When these were studied on various substrates, the interesting finding emerged that all four peaks were caseinolytic, though each peak showed apparently distinctive specificity for the two synthetic substrates. In particular, Peak IV hydrolysed neither ATEE nor BAEE, and Peak III was also inactive against BAEE though showing some ATEE esterase activity. Peaks I and II showed broad similarities with minor differences.

In a separate study, the ATEE esterase activity of Peak I was compared with its fibrinolytic activity. Two forms

of cholelysin were found with sharply differing esterase/lytic ratios; the second form showed a semi-logarithmic relationship between these two activities.

CHAPTER SEVEN

Extensive studies were made of the inhibition of cholelysin, using human serum and the serum of rat, rabbit and guinea-pig, platelet-rich and platelet-poor plasma; α_2-macroglobulin, soy bean trypsin inhibitor, and aprotonin.

Human serum was strongly inhibitory; the 50% inhibition point was reached at an average dilution of 1/70 in 10 sera studied. Heating the serum to 56°C for 30 minutes did not diminish the inhibitory potential. The serum of two patients with homozygous α1-antitrypsin deficiency was strongly inhibitory as well.

It was surprising that a broad spectrum inhibitor such as α1-antitrypsin played no part in the serum inhibition of cholelysin, and because of this paper electrophoresis of normal human serum was done to discover which area(s) were inhibitory. Chymotrypsin was run as a control. This experiment showed no inhibition amongst α-globulins, with strong inhibition in the inter-α-antitrypsin and β-globulin zones. The pattern of inhibition differed sharply from that demonstrated by chymotrypsin.

Rat, rabbit and guinea-pig sera were also inhibitory, but not as potently as human serum. Platelet-rich and platelet-poor human plasma were equally inhibitory. Simultaneous assay of serum inhibition of cholelysin ATEE-esterase and fibrinolytic activities was done; this did not show a dissociation of activities, or a semi-logarithmic relationship, as had been found when these activities were studied without serum inhibition. The point of 50% inhibition of ATEE-esterase activity of cholelysin by human serum was found at

a dilution of 1/90 in a single experiment.

α_2-macroglobulin, SBTI, and aprotonin, were studied for their inhibitory capacity. All were inhibitory, but to a variable degree among Peaks I, II, III, and IV of cholelysin. α_2-macroglobulin showed least inhibition of Peak I and most of Peak IV; SBTI produced erratic results when pre-incubated with cholelysin for 20 minutes, but consistent results when incubated for 24 hours at room temperature, when it showed a near-100% inhibition of Peaks I, II and III at a concentration of 6 mg/100 ml. Aprotonin resistance also differed among the peaks, Peak II being most resistant.

CHAPTER EIGHT

Biological studies were undertaken to discover if cholelysin had significant anticoagulant potential. In preliminary experiments, cholelysin had no effect on the partial thromboplastin time with kaolin, the prothrombin time, the thrombin time, and the fibrinogen assay. However, incubation of cholelysin and purified fibrinogen showed a marked fibrinogenolytic effect. Platelet aggregation with adrenaline, ADP and collagen was studied in the presence of cholelysin. A moderate inhibition of aggregation by ADP 10 µmol/ℓ was demonstrated, but none to aggregation by ADP 1 000 µmol/ℓ. Aggregation by collagen and adrenaline was unaffected.

A minimum lethal dose in the Wistar rat could not be determined, while the biological half-life of injected cholelysin was shown to be of the order of 5 minutes.

A limited study of the anticoagulant effect of cholelysin was done in a rat model originally described for ancrod ('arvin'). The quantities of purified Peak I required exceeded the modest manufacturing capacity of the programme at that time, and this experiment was possible only with

the kind co-operation of the South African Inventions Development Corporation and the Council for Scientific and Industrial Research. A statistically significant number of rats was studied, but with dose schedules of only about 10% of the average duration of those achieved with ancrod. The result approached, but did not reach, statistical significance.

CHAPTER NINE — Fibrinolysis by cholelysin was studied by a polyacrylamide gel system (PAGE) embodying a linear gradient. Fibrin split products at 24 hours were compared for cholelysin and plasmin, and were found to be totally different. The inhibitory effects of SDS and mercaptoethanol on cholelysin were studied during the PAGE work.

CHAPTER TEN — For further study of the dynamics of the cholelysin/fibrin reaction, a new test was devised, termed the composite cuvette method for the study of enzyme kinetics. In this method, the light path of the cuvette was half occupied by fibrin clot, the other half by cholelysin or plasmin solution. The reaction was followed in a temperature-controlled spectrophotometer, at a wavelength (335 nm) previously shown to distinguish fibrin from a reference standard of fibrin split products in a scanning spectrophotometer. A biphasic reaction was found; the biphasic nature of this reaction was proved by subsampling from the composite cuvette and subjecting the sample to PAGE, when only two products were found after 2 minutes of fibrin digestion, and multiple products at 10 minutes and later. Plasmin digestion of fibrin, on the other hand, showed a constant number of products from the commencement.

CHAPTER ELEVEN

Molecular weights of fibrin split by cholelysin and plasmin were studied through the standard method of protein markers, and estimating relative mobility of known and unknown bands. This showed that the major bands of plasmin digestion after 24 hours compared very closely with published work, while cholelysin split products were all different.

Finally, the molecular weight of cholelysin was estimated in three ways (column chromatography without SDS, (2) column chromatography with SDS (3) PAGE. At least three molecular weights of cholelysin were found by method (1), probably a dimer and tetramer of the basic molecular weight of ~ 7 000. Two discrepant results were only estimated once. By method (2), molecular weight of 7 000 and 13 000 were found, despite the presence of SDS. By method (3) a molecular weight of about 13 000 was found; the protein band stained poorly on PAGE, but the result was confirmed by simultaneous assay of a gel for fibrinolytic activity with a chymotrypsin control.

12.2 The nature of cholelysin

Cholelysin is a protein which acts as an anion at pH 7,4. It has a basic molecular weight of 7 000, with a probable dimer at ~ 13 000 and a tetramer at ~ 28 000. It is unrelated to trypsin, chymotrypsin or plasmin. It is present in variable amount in bile, and probably does not exceed 10% of total bile protein. The fibrinolytic potential of ox bile varies quite markedly from batch to batch, without reference to total protein or bilirubin concentrations.

The origin of cholelysin is uncertain; it is unlikely to be of dietary origin, as it was found in the bile of carnivores (cat, dog) herbivores (ox, sheep) and omnivores (man, pig). It is a previously undescribed protease, and

its significance in the biliary tree is uncertain. It may be there to act as a fibrinolysin, along the lines of the systems of fibrinolysis described in all other small tube systems in the body (though all previously described systems involve an activator of fibrinolysis by plasmin, and not a primary fibrinolysin). Alternatively, it is excreted in bile for physiological reasons having nothing to do with fibrinolysis.

12.3 Future work

This study has opened up many areas for future work. Those that appear to be of importance are:

(1) Why is there a potent inhibitor? Does this imply the intermittent presence of cholelysin in the blood stream? If so, is cholelysin present in the plasma of animals or man with obstructive jaundice? This could be tested by use of a specific antibody in an animal model, or in man with obstructive jaundice.

(2) Could cholelysin play a major role in thrombolysis? The ready availability of ox bile, and the development of relatively large scale techniques for the isolation of cholelysin, make it possible to study this field more intensively in the future. Both forms of thrombolysis currently available are unsatisfactory; urokinase is non-antigenic but very expensive, and steptokinase is a potent antigen as well as being very expensive. Cholelysin could be more effective than either, but the inhibitor would have to be overcome. This could be done *in vitro*, and by chronic infusion of cholelysin *in vivo* until a lasting effect appeared. The rat model of Chapter Eight, repeated under these conditions, might give a statistically significant result.

(3) Biochemically there is much of interest to follow up. The molecular weights need further intensive study;

in particular, the careful column chromatography with SDS producing two different molecular weights needs repetition. The relationship between the two peaks obtained on batchwise elution needs exploring; is the second peak a heterogenous collection of molecular weights? How do the substrate activities of the two (batchwise) peaks compare? The semi-logarithmic relationship between ATEE-esterase and fibrinolytic activities for batchwise Peak I needs further exploration. What is the nature of the serum inhibition of cholelysin? Is it a know inhibitor, if so which? What type of inhibition is it? Why does cholelysin stain poorly with standard PAGE techniques? Can this be remedied by the use of other stains? A new technique incorporating fibrin into PAGE, (Wilson, Becker, Hoal and Dowdle, 1980) might bypass the need for alternative staining techniques.

The dynamics of the cholelysin-fibrin reaction need further studying. What linkage is attacked by cholelysin, giving the reaction a biphasic nature? Can the reaction products be related to known degradation products? Differential staining of FSPC for eg carbohydrate might give further clues. Why does variation of substrate concentration in the composite cuvette give anomalous results? How do other fibrinolysins behave under similar condition (eg trypsin, chymotrypsin, plasmin).

It is apparent that there is no shortage of possible lines of research on this interesting protease.

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METHODS

FIBRINOLYSIS BY BILE

1 ACETONE precipitation of whole bile.
Acetone and raw ox bile pre-cooled to 0°C. Acetone added to bile 40% V/V. Precipitate formed immediately. Centrifuged at 0°C and 12 000 rpm (20 000 X G) for 10 minutes. Precipitate redissolved in tris buffer pH 8,5 M 0,006.

2 ATEE (N-acetyl-L-tyrosine ethyl ester monohydrate) esterase activity (Miles-Seravac Manual, 1972 p 14).
Principle: ATEE is hydrolysed at the ester linkage causing a decrease of extinction at 237 nm. Unit: That amount of enzyme causing a decrease of extinction at 237 nm of 0,0075 per minutes at 25°C.
The assay is carried out as follows:

Reagents: M 0,067 potassium phosphate buffer pH 7,0
M 0,001 HCI

Substrate: M 0,00934 ATEE in buffer
(25,2 mg ATEE is dissolved in buffer at 70°C. The solution is cooled rapidly and the volume adjusted to 100 ml with buffer)

Enzyme: A solution containing
10 - 20 NF units/ml M 0,001 HCI

Procedure: Into 10 mm quartz cells place the following at 25°C

	Test	Control
Substrate	3,0 ml	3,0 ml
M 0,001 HCI	-	0,2 ml
Enzyme at zero time	0,2 ml	-

Record the decrease of extinction at 237 nm for five minutes

Calculate the activity as follows:

$$\text{NF U/mg} = \frac{\Delta \text{ E 237 nm/min x 5}}{\text{0,0075 x 0,2 x mg enzyme/ml enzyme solution used}}$$

Determine the suitability of the substrate for this assay by carrying out a reference assay using a NF chymotrypsin standard.

Note: In terms of µmol units using N-benzoyl-L-tyrosine ethylester (BTEE) as substrate, crystalline α-chymotrypsin has an activity approximately 45 U/mg.

ATEE: revised method.
The standard test made provision for only 0,2 ml of test material, which was insufficient for testing cholelysin, and cholelysin + serum inhibition. Accordingly, the standard method was revised to allow for a larger volume of test material, as follows:

	ATEE mg/100 ml	Diluent (ml)	ATEE used (mg)	Volume for test (ml)	Unit of activity
Standard	25,2	3	0,757	0,2	0,0075
Revised	37,6	2	0,752	1,2	0,01

The unit of activity was increased to 0,01, as conditions were no longer standard, and this was a more convenient figure.

3 BAEE (N-benzoyl-L-arginine ethyl ester hydrochloride) esterase activity (Miles-Seravac manual, 1972 p 48).
Principle: BAEE is hydrolysed at the ester linkage, causing an increase of extinction at 253 nm. Unit: that amount of enzyme causing an increase of extinction at 253 nm of 0,003 per minute at 25°C.

The assay is carried out as follows:

Reagents: M 0,067 potassium phosphate buffer pH 7,6
M 0,001 HCI

Substrate: M 0,00025 BAEE in buffer (8,6 mg/100 ml)

Enzyme: A solution containing approximately 40 NF units/ml in M 0,001 HCI

Procedure: Into 10 mm quartz cells place the following at 25°C:

	Test	Control
Substrate	3,0 ml	3,0 ml
M 0,001 HCI	-	0,2 ml
Enzyme at zero time	0.2 ml	-

Record the increase of extinction at 253 nm for five minutes.

Calculate the activity as follows:

$$\text{NF units/mg} = \frac{\Delta \text{ E } 253 \text{ nm/min} \times 5}{0{,}003 \times \text{mg enzyme/ml enzyme solution}}$$

Determine the suitability of the substrate for this assay by carrying out a reference assay using a NF trypsin standard.

The potential activity of trypsinogen is determined by activating with trypsin at pH 8,4 and at 5°C for approximately 24 hours and then measuring the trypsin activity as described.

For acetyl trypsin the enzyme should be dissolved in M 0,067 potassium phosphate buffer pH 7,6.

4 BIURET

A) Reagents:

1) Biuret reagent:

Weigh off:

Potassium Sodium Tartrate	3,410 gm
Potassium Iodide (KI)	0,830 gm
Copper Sulphate ($CuSO4\ 5H_2O$)	2,345 gm
Sodium Hydroxide (NaoH)	4,000 gm

Dissolve and make up to 500 ml with distilled water.

(This reagent is commercially available as "Merckotest Total Protein kit" - Tauber and Corssen, Cape Town)

2) Standard:

Human albumin, 80 mg/ℓ in distilled water.

B) Method:

Using 10 x 75 mm glass test tubes, proceed as follows:

	Bank	Std	Test
Biuret reagent	1,05 ml	1,05 ml	1,05 ml
Distilled water	0,02 ml		
Versatol		0,02 ml	
Assay Serum			0,02 ml

Mix well and leave at room temperature for 30 minutes or at 37°C for 15 minutes Zero with blank at 545 nm and read (Two blanks should be prepared if a double beam instrument is used).

Calculation

$$\frac{\text{Reading of Test}}{\text{Reading of Standard}} \times \frac{\text{concentration of standard}}{1} = \text{gm/100 ml Total protein}$$

5 CASEINOLYSIS (Johnson, Kline and Alkjaersig, 1969)

Principle: casein is hydrolysed with the release of tyrosine. When plasmin is used as test enzyme, the unit is described

(1) as that quantity of plasmin that releases 0,1 microequivalent of tyrosine/minute from a specified casein preparation, or

(2) as that quantity of plasmin that, after 20 minutes of incubation with casein, causes an increase of extinction of 0,300 at 275 nm.

In the second definition, the reaction is stopped by the addition of perchloric acid after 20 minutes.

Reagents: Tris 7,265 g)
NaCl 5,26 g) pH 7,5 at 1 000 ml
Perchloric acid 0,5 M 42,8 ml of 70% + water to 1 000 ml.
α Casein 1,4 g in 100 ml of Tris NaCl buffer
Standard plasmin/plasminogen 10 u/ml.

Method: Dilute plasmin or test plasma - 1/10 with tris NaCl buffer. Place 1,0, 0,8, 0,6, 0,4, 0,2 in a series of test tubes. Add buffer to 2,5 ml total.
Add 2,5 ml α casein.
Mix by inversion.
Incubate 37,5°C.
At 2 minutes and 32 minutes blow 2 ml aliquots into 3,0 ml of 0,5 M perchloric acid.
Mix by shaking.
Stand at room temperature for 30 minutes.
Centrifuge at 2 000 XG for 15 minutes.
Remove supernatant through Pasteur pipette with cottonwool round tip. Read at 275 nm using 2 minute sample as the blank.

6 CHLOROFORM/METHANOL PARTITION (Bligh and Dyer, 1959)

Principle: In this method lipids are partitioned between these two solvents.

Materials: Chloroform, methanol.

Method: 100 ml of bile homogenised in a Waring blender with 100 ml chloroform and 200 ml methanol for 2 minutes. A further 100 ml chloroform then added, and, after another 30 seconds of blending, 100 ml of distilled water. Blend final mixture for another 30 seconds, and pour off into a glass cylinder. Fibrinolytic activity concentrates at the chloroform/methanol interface, along with black tarry material. Results are given in Appendix 5.

7 COAGULATION

Partial thromboplastin time with kaolin (Proctor and Rapaport, 1961). One stage prothrombin time (Poller and Thomson, 1969).

Thrombin time: The clotting time of control plasma with thrombin was adjusted by appropriate dilution of thrombin with barbitone buffer pH 7,4 until the control time was ~ 15 s. The clotting time of test plasma was then measured, using the same concentration of thrombin. A prolongation of > 2 s was regarded as significant.

Fibrinogen assay (Vermylen, De Vreker and Verstraete, 1963).

Fibrin split products (Merskey Johnson Kleiner and Wohl, 1967).

Platelet aggregation studies:

collagen (Packham, Warrior, Glynn, Senyi and Mustard, 1967)

ADP and adrenaline (Macmillan, 1966).

8 COMPOSITE CUVETTE (see Chapter Ten)

Principle:

For this test, a gel-phase substrate and soluble degradation products are required. These two are scanned against each other in a scanning spectrophotometer, and the wavelength of maximum difference selected for subsequent measurement. The gel-phase substrate is caused half to fill the cuvette in the light path; the enzyme used is introduced into the cuvette, and digestion of substrate commences. The reaction is followed at the selected wavelength, observing either a decrease of extinction (substrate scanned, degradation products as reference standard) or an increase (degradation products scanned, substrate as reference standard). Kinetics can be directly followed, and degradation products obtained from the outset of the reaction.

Reagents: Clotting fibrinogen solution as prepared for the standard fibrin plate (see Method 10).
Standard quartz cuvette.
Cholelysin Peak I in tris buffer pH 8,5 M 0,006
Split products of fibrinogen by cholelysin digestion (FSPC) obtained from standard fibrin plate after 21 hours of digestion of fibrin.

Method: (1) Establishment of point of maximum wavelength difference between fibrin and FSPC. Clotting fibrinogen solution poured into a cuvette and incubated at 37°C for 2 hours. After that time, the fibrin clot and FSPC each scanned against air, and then against each other. The point of maximum difference in absorption selected for further study. This was found to be at 335 nm with fibrin scanned, and FSPC as reference standard.

(2) Preparation of composite cuvette. Clotting fibrinogen solution poured into a quartz cuvette in sufficient quantity ot half fill it. The cuvette was then capped and laid on its side, with a side towards the light path upwards, and incubated in this position at 37°C for 2 hours. After this period of incubation the fibrin clot formed was firmly adherent to the cuvette, which was then removed from the incubator and placed in a thermostatically controlled spectrophotometer at 37°C.

(3) Kinetics of fibrinolysis by cholelysin were studied by pouring standard cholelysin solution, Peak I, tris buffer pH 8,5, into the vacant half of the cuvette and following the reaction on a recorder for decrease of extinction at 335 nm. Aliquots could be subsampled from the liquid (cholelysin) half of the composite cuvette at any time, and subsequently analysed by polyacrylamide gel electrophoresis (PAGE) (see 19 PAGE).

9 EUGLOBULIN LYSIS TIME (Chakrabarti, Bielawice, Evans and Fearnley, 1968)

Principle: plasminogen, plasminogen activator and most of the fibrinogen of plasma are precipitated by dilution with distilled water in the presence of CO_2. The precipitate is reconstituted, clotted with thrombin, and incubated at 37°C until fibrinolysis occurs.

Materials: Sodium citrate 3,8 g/100 ml
CO_2
Bovine thrombin

Method: Blood is collected without venous stasis by clean venepuncture and immediately mixed with

3,8% sodium citrate solution in a test tube standing in ice and water, the proportion of blood to anticoagulant being 4:1. The plasma is separated in a refrigerated centrifuge at 3 000 rpm for 10 minutes. One ml plasma is added to 10,0 ml distilled water in a conical flask of 50 ml capacity standing in a beaker of ice and water. Carbon dioxide is blown over the surface of the mixture for three minutes by means of a Pasteur pipette connected by rubber tubing and valve to the delivery cylinder. During this procedure, the flask is gently rotated to ensure full exposure of the mixture to the gas.

The mixture is then transferred to a centrifuge to be centrifuged at 4°C at 2 000 rpm for six minutes. The supernatant is discarded and the inner surface of the tube dried with no 1 Whatman filter paper. The euglobulin precipitate is resuspended in 2,0 ml of saline phosphate buffer. The suspension is equally divided into two test tubes 5 in x ½ in and is clotted with 5 units (0,1 ml) thrombin in saline. The whole procedure is done in an ice water bath. After five minutes the tubes are transferred to a water bath at 37°C and the time taken by the clot to dissolve completely is read as the lysis time.

10 FIBRIN PLATE

(1) Astrup (Astrup and Müllertz, 1952)
(2) This study
(3) Heated (Thomson, 1970)

(1) <u>Astrup</u>

Materials: Bovine fibrinogen prepared by ammonium

sulphate precipitation from oxalated bovine plasma. This preparation contained 80-90% clottable protein in a 0,6 g/100 ml solution.
Thrombin - commercial 100 units/ml in NaCl 0,9% solution .
Petri dishes - glass sterilised
Buffer: sodium diethyl barbiturate M 0,067 pH 7,8
Fibrinogen solution: 1 or 2 mg/ml (usually 2 mg/ml).

Method: In each Petri dish 9,0 ml of diluted fibrinogen solution (pH: 7,8 μ = 0,15, fibrinogen concentration: 0,2 or 0,1%) is measured from a buret. The Petri dishes are placed on a horizontal glass plate and clotted by means of 0,20 ml thrombin solution. With a 0,1 ml pipet graduated in 0,001 ml, exactly 0,030 ml of enzyme solution is placed as a small drop on the surface of the fibrin plate. Usually three single determinations are carried out on each plate. The area of digested fibrin is a quantitative measure of the enzyme activity. This is measured as follows: After incubation for 18-20 hr at 37^{o}C the product of two perpendicular diameters is recorded (in square millimeters) and the mean value of the three determinations are calculated. When especially accurate estimations are required, zones which are not quite circular have to be disregarded. The areas are converted into concentrations by interpolation on a reference curve.

Note: An attempt was made to prepare bovine fibrinogen by this technique, using fresh oxalated bovine plasma from the Cape Town Municipal Abattoir. For reasons that could not be established, these plates proved highly unstable, often showing spontaneous lysis within 24 hours of incubation at 37°C. Their use was therefore abandoned, and the method described below was adopted instead.

(2) This study

Materials: Commercial human fibrinogen (Kabi) 90% clottable Calcium thrombin solution: $CaCl_2.2H_2O$ 1,25 M in NaCl 0,9% solution, containing bovine thrombin (Parke-Davis) 1 unit/ml.
Tris buffer M 0,2 pH 7,8
Petri dishes: plastic presterilised, disposable 10 cm diameter
Plasmin (human): 0,1 unit/ml in glycerol 50% in tris buffer pH 8,5 M 0,006.

Method: (for making 10 plates)

1 Make a fibrinogen Kabi solution 0,2 g/100 ml by weighing out 0,52 g Kabi fibrinogen, and dissolve in 100 ml tris buffer.
2 Pipette 10 ml into each petri dish
3 Add 0,2 ml thrombin solution to each petri dish
4 Mix immediately by swirling 20 times evenly and gently
5 Stand open at room temperature for 2 hours
6 Incubate at 37°C for 2 hours

7 Store at 4°C until 30 minutes before use

8 Stable for at least 4 days, usually 5 days

Notes:

(i) Method 7 (Composite cuvette) refers to a 'clotting fibrinogen solution'. This is the solution obtained after step 3 above, which can be handled further for 2 minutes before clotting begins.

(ii) The relatively high electrolyte concentration of these plates meant some loss of sensitivity. However, they were very stable and reliable, and these qualities were preferred to the highest degree of sensitivity.

Test for fibrinolytic activity

1 Plate out 0,02 ml of test solution or plasmin standard on a standard fibrin plate.

2 Incubate for 21 hours at 37°C in a water-saturated container.

3 Read lysis diameter by ruler in two diameters at right angles to each other; report in mm average diameter of zone of lysis. For very accurate estimations, discard all results not circular.

4 Plasmin standard gave 12 ± mm zone of lysis under these conditons.

(3) Heated fibrin plate

Materials identical with (2), except that autoclaved glass Petri dishes (10 cm in diameter with perfectly flat bottom) were used.

Method: Standard fibrin plate heated to 80°C for for 45 minutes before use. This resulted in only slight loss of sensitivity.

11 FOLIN-LOWRY method of protein assay (Lowry, Rosebrough, Farr and Randall, 1951)

Materials: 'Reagent C' - 50 ml of Na_2CO_3 2 g/100 ml in NaOH 0,1 N + 1 ml of $CuSO_4$ 0,5 g/100 ml in potassium tartrate 1 g/100 ml.
'Reagent E' - Folin-Ciocalteu reagent diluted with distilled water to 1 N.

Method: To 1 ml of test material add 5 ml of Reagent C. Stand at room temperature for 10 minutes. Add 0,5 ml of Reagent E. Shake vigorously with each addition.

Between ½ and 2½ hours after addition of Reagent E, read extinction at 750 nm. Crystalline ovalbumin preparation used as standard.

12 FLASH FREEZING

Materials: Dialysed cholelysin solution
Liquid nitrogen

Method: Specimens of cholelysin in solution were plunged into liquid nitrogen in volumes of 10-20 ml. Freezing was almost instantaneous. Thereafter, these specimens were freeze dried.

13 FREEZE DRYING

Materials: Flash frozen preparations of cholelysin. Freeze drying was carried out in a Virtis laboratory freeze dryer. Thereafter, specimens were sealed in their plastic containers, and kept at $4^{o}C$.

14 INHIBITION

(1) Direct mixing of test material with cholelysin (Chapter Seven)

(2) Eluting of test material off filter paper following paper electrophoresis (Chapter Seven, Section 7.4.5).

(1) Direct mixing of test material with cholelysin.

Control: the same cholelysin fraction used in that specific experiment, diluted 1/1 with tris buffer pH 8,5 M 0,006.

Test: Control + inhibitory substance in equal volumes, incubated together at room temperature for 20 minutes before testing for fibrinolysis. Both Control and test applied in 0,02 ml quantities to a standard fibrin plate and incubated in the usual way. Results read as mm diameter of zone of lysis, and transformed into units of fibrinolytic activity, using the table of chymotrypsin equivalents in Chapter Three (Table 3-9). Calculation of inhibition % was done as follows:

$$\frac{\text{Control (units)} - \text{test (units)}}{\text{Control (units)}} \times 100 \qquad \text{Formula (A)}$$

Special instances:

(i) α2-macroglobulin possessed inherent fibrinolytic activity, which had to be allowed for. Formula (A) above was altered to allow for this:

$$\frac{\text{Control (units)} - (\text{test units} - \text{macroglobulin lytic activity in units})}{\text{Control (units)}} \times 100$$

Formula (B)

(ii) Soy bean trypsin inhibitor (SBTI).
Incubation of cholelysin + STBI was carried out
(a) for 20 minutes
(b) for 24 hours
Both mixtures were tested for fibrinolytic potential on the standard fibrin plate.

(iii) Aprotonin ('traylol')
Ampoules containing 20 000 units/ml (Bayer) were available. Equal volumes of aprotonin solution and cholelysin were mixed together, giving a final concentration of aprotonin of 10 000 units/ml.

(2) Eluting of test material off filter paper following paper electrophoresis.

Because the experimental details are highly relevant to the results, this experiment (with results) is described in detail in Chapter Seven, section 7.4.5. Lower volumes of cholelysin solution were used than with standard fibrin plate assays, to ensure that the enzyme percolated through the filter paper, and did not reach the fibrin plate by spilling over the edges of the paper. Calculations of inhibition were performed by using Formula (A), above.

The method for assessing inhibition was as follows:

(a) Test - Strips of paper containing fractions of serum after electrophoresis were laid on a fibrin plate, and 0,005 ml of chymotrypsin or cholelysin solution applied directly to the strip.

(b) Control - Strips of identical filter paper, unused, were laid on a fibrin plate, and 0,005 ml of cholelysin solution applied directly to the strip.

The paper strip was lifted off the fibrin plate before reading the result.

15 MOLECULAR WEIGHTS

(1) by K_{av} (Laurent and Killander, 1964)

(2) by PAGE (Weber and Osborn, 1969; Stead, 1981)

(1) (a) Estimation of molecular weights from molecular exclusion data.

The theory of Laurent and Killander was applied to certain gel exclusion data to provide an estimate of relative molecular weights. The Laurent-Killander treatment is based on a parameter (K_{av}) which represents the eluted volume of a solute (Ve) relative to the void volume of the column (Vo) and total column volume (Vt). This is in effect equivalent to the 'Rf' encountered in other forms of chromatography, and is calculated as follows:

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

Laurent and Killander describe a relationship between K_{av} and the Stokes' radius of spherical molecules:

$$\sqrt{- \ln K_{av}} = \alpha (\beta + rs)$$

where rs = Stokes' radius of spherical molecule

α = Constant made up from concentration of macromolecule rods constitution the supporting network

β = Constant made up from the radius of the macromolecule rods

Since for most proteins β is small compared to rs, a plot of $\sqrt{- \ln K_{av}}$ against rs will be linear. The slope of the plot should be dependent on gel concentration. Experimental confirmation of the linearity of the plot and the dependence of slope on gel concentration is found in the unpublished data of Sims, Bussey and Folkes (57) using agarose gel.

Since for a perfectly spherical molecule,

$MW = 4/3\pi r^3 \rho$, where r = molecular radius
ρ = density of molecule,

then $r \propto \sqrt[3]{MW}$,
and since r is closely related to the Stokes' radius rs,

$rs \propto \sqrt[3]{MW}$, and $\sqrt{-\ln K_{av}} \propto \sqrt[3]{MW}$

Thus the linear relationship also holds between $\sqrt{-\ln K_{av}}$ and $\sqrt[3]{MW}$. This provides a useful means of relating the elution properties of a solute to its approximate molecular weight.

Gel exclusion columns are normally calibrated by running standards of known molecular weight. However, in this study use was made of a graph provided by the manufacturers of sephadex, from which values of K_{av} could be directly converted into molecular weights for sephadex G50, G75, and G100.

(b) Estimation of void volume (Vo) and total volume (Vt) of gel column.

A dyed dextran preparation known as Blue Dextran 2 000 (Pharmacia, Uppsala, Sweden) was used for determination of void volume. The preparation consists of random polymers covering a wide range of molecular weights, a certain proportion of which are fully excluded by the column. These are used to estimate the elution volume of a totally-excluded solute. Five milligrams of Blue Dextran, dissolved in phosphate buffer for several hours, was applied to the column in 0,1 ml volume. The concentration of dextran in the effluent was estima-

ted spectrophotometrically at 260 mμ.

The total column volume was calculated from the dimensions of the column.

(2) The separation of proteins by PAGE in the presence of SDS is dependent on the molecular weights of their polypeptide chains (Weber and Osborn, 1969). Using the buffer system of O'Farrell (1975), a gradient of polyacrylamide from 2 tot 41% was established, and proteins run as described in Method 18. Proteins reached a position in the gel proportional to their molecular weight, when estimated by their mobility relative to the total length of the gel. The total length of the gel was measured, and the length travelled from the top of the gel by each protein band under study, was calculated as the simple ratio:

$$\text{relative mobility} = \frac{\text{distance travelled by band}}{\text{total length of gel}}$$

Using proteins of known molecular weight as markers in separate tubes in the same experiment, a graph was constructed from which the molecular weights of unknown proteins could be directly estimated (Stead, 1981).

16 SODIUM CHLORIDE GRADIENT and batchwise elution carried out by the methods of Petersen and Sober (1962).

17 NEUTRAL SUGAR (Dubois, Gilles, Hamilton, Rebers and Smith, 1956)

Materials: redistilled phenol
concentrated H_2SO_4

Method: To 1 ml of column effluent was added 0,5 ml of 5% (w/v) redistilled phenol and 2,5 ml of concentrated H_2SO_4. Contents of the tubes were thoroughly mixed and allowed to stand for 10 minutes. They were shaken and placed in a water bath at 25 - 30° for 10 - 20 minutes. Thereupon the extinction was read immediately in the Spectronic 20 spectrocolorimeter at 490 mμ.

18 PAGE (polyacrylamide gel electrophoresis) (O'Farrell, 1975)

Materials: Running tris buffer: Tris HCl M 1,5, pH 8,8 and 0,4% SDS; electrode buffer: Tris M ,025, glycine M 0,192, 0,1 SDS; 65% acrylamide stock: 64,2% acrylamide and 0,8% bisacrylamide in distilled water; 2,7% acrylamide: dilution of 65% stock in distilled water; sample application buffer: 0,1% SDS, Tris M ,002, 0.1% mercaptoethanol; reduction buffer: 4% SDS, EDTA M 0,1, Tris HCl M 0,04, pH 8,8, 2% mercaptoethanol.

For analysis of fibrin split products, final concentrations of reducing substances were: SDS 2 g/100 ml, mercaptoethanol 2 g/100 ml and urea M 6.

Samples were prepared as follows:

<u>cholelysin</u> - the most concentrated available preparation was applied to the gel to a total load of 0,010 - 0,020 mg of protein.

<u>fibrin split products</u> -

(1) The lysed fibrin solution was aspirated from the standard fibrin plate after 24 hours of incubation at 37°C with either

plasmin or cholelysin, and applied to the gel as above.

(2) Aliquots were aspirated from the liquid (fibrinolysin) side of the composite cuvette (see Method 7 above) at the stated times - usually 2, 10, 30 and 180 minutes and applied as above to PAGE. Either plasmin or cholelysin split products were studied in this way.

Fibrinolysis was quenched by the addition of an equal aliquot of soy bean trypsin inhibitor 0,2 g/100 ml.
Cholelysin solution usually 60+ u/ℓ
Plasmin solution usually 0,2 u/ml
Molecular markers used were ribonuclease, chymotrypsinogen and lactate dehydrogenase.

The solutions were mixed in the following manner to make 1 volume of running gel: 0,25 volume of running gel buffer; 0,75 volume of acrylamide stock (65% or 2,7%); 0,0033 volume of 10% ammonium persulphate. The mixtures were degassed, 0,0005 volume of TEMED was added, and 8,4 ml of the 2% mixture was added to the mixing chamber of the gradient former. This volume was half the filled volume of the seven electrophoresis tubes in each run, multiplied by a factor 1,176 to allow for the fact that gradient formation (theoretically from 2% to 48,75%) was stopped after removal of 0,85 volume. This ensured efficient mixing throughout formation of the gradient, and gave a final concentration range of 2,1 to 41%. An 8-channel peristaltic pump was used for gradient formation; seven channels removed the mixture

to the electrophoresis tubes, while the 8th channel pumped the heavy (48,75%) solution into the mixing chamber at half the total rate at which it was removed to the tubes.

The gradient was pumped through a capillary tube to the bottom of the electrophoresis tube, and the initial low concentration was displaced upwards by the increasing density of the mixture. The upper gel surface was covered by a layer of distilled water and the mixture allowed to polymerise overnight. Samples (prepared as described below) were applied in sample application buffer: glycerol (1:1) or reduction buffer: glycerol (1:1), and electrophoresis was run at a constant current of 2 mA (9V) per tube for 5 hours.

Gels were stained in 0,1% Coomassie Blue in 50% TCA for 20 minutes, and destained in 7% acetic acid.

Relative mobilities were determined (Stead, 1969, 1981) and molecular weights read from the graph provided by the molecular markers.

19 PAPER ELECTROPHORESIS

Principle: separation of plasma proteins on basis of charge. Used when relatively large-scale separation of plasma proteins is required.

Materials: Whatman No 1 filter paper 5 cm wide
Barbitone buffer M 0,083 pH 8,6
Serum
Amidoschwarz stain

Glacial acetic acid
Methanol
Bromphenol crystals

Method: Serum applied to separate strips
(1) 0,010 ml (staining)
(2) 0,200 ml (test)
Electrophoresis carried out at 4°C , with barbitone buffer, at 140 volts, 4-6 mA, run for 19 hours. Staining with amidoschwarz 1% in glacial acetic acid 10% and methanol 90%. Full experimental details given in the main text, Chapter Seven section 7.4.5.

20 PRECIPITATION with drugs capable of causing cholestatic jaundice in man (Clarke, Martiz and Denborough, 1972).

Principle: Chlorpromazine and other tranquillizers cause precipitation of the protein and glycoprotein components of bile in vitro; this results from interaction between the carboxyl groups of the protein and glycoprotein moieties, and the positively charged amine groups on the drug molecules.

Materials: The authors listed chlorpromazine, promazine, trifluperazine, amitryptilene and iprindole. The first four of these drugs were obtained from the manufacturers in pure form. They were mixed with 'Fraction G200' cholelysin to final concentrations that had been found by the authors to cause maximal precipitation of proteins and glycoproteins, as the following Table shows:

Drug	Molecular weight	Final concentrations (mM) Proteins	Glycoproteins
Chlorpromazine	318,9	10	30
Promazine	320,9	5	30
Trifluperazine	480,4	3	10
Amitryptiline	313,9	10	ineffective

Following incubation of mixture for 30 minutes at room temperature, the specimens were centrifuged at 12 000 X G for 20 minutes. Supernatants were pipetted off, and the precipitates redissolved in tris buffer M 0,006 pH 8,5, to the original volume.

Aliquots of cholelysin preparation diluted with buffer, and of redissolved precipitate, were tested for fibrinolysis. Results are repored in Appendix 5.

21 PRECIPITATION WITH POLYETHYLENE GLYCOL

Materials: polyethylene glycol in tris buffer pH 8,5
cholelysin 'fraction G200'.

Method: Aliquots of polyethylene glycol and cholelysin solution were mixed in varying final concentrations of polyethylene glycol and incubated for 30 minutes at room temperature. The mixtures were then centrifuged at 12 000 X G for 20 minutes, and supernatants pipetted off. Precipitates were redissolved in tris buffer pH 8,5 M 0,006 to the original volume, and both supernatants and redissolved precipitates tested for fibrinolysis. Results are shown in Appendix 5.

22 RAT MODEL (Chan, 1969, 1970)

Principle: Formation of clot in relation to a polyethylene tube chronically implanted in the carotid artery of the rat.

Treatment with cholelysin used in an attempt to diminish the rate of clot formation.

Materials: Wistar rats 200-250 g
Ether
Polyethylene tubing external diameter 0,965 mm (PE 50, Clay-Adams)
Heparin
Cholelysin Peak I

Basic method: Rats were anaesthetised with ether. The neck was shaved and the left carotid artery isolated through a midline incision down the front of the neck. A 10-15 mm segment of the artery was temporarily occluded between two "bulldog" clamps and a small transverse cut made into the wall of the occluded segment. The opened segment was then flushed clean of blood with heparin-saline (100 u/ml). Under a dissecting microscope, a 3-4 mm length of polyethylene tubing with an outer diameter of 0,965 mm and an internal diameter of 0,58 mm (PE 50, Clay Adams) was inserted into the lumen of proximal portion of the artery. The inserted tube was filled with heparin-saline and advanced into the lumen of the distal portion of the artery across the cut in the vessel wall and then secured on both sides of the arterial opening by cotton ties. When the occluding arterial clamps were released, normal blood flow was immediately established and nor-

mal pulsations of the artery before and beyond the polyethylene tube was observed. The time taken from the occlusion of the artery to release of the clamps after insertion of the tube, varied from 6-10 minutes. The wound was then closed and the animal had an uneventful recovery from anaesthetic, and remained healthy in the postoperative period.

Study of the progress of thrombus formation was by sacrifice of the animals at various intervals after the operation. The operated rats were put under anaesthetic, anticoagulated with 100 u of heparin intravenously, the wound reopened and the artery inspected carefully in situ. Patency of the blood vessel was assessed by the presence or absence of pulsatile flow and state of filling of the distal portion. Any thrombus visible through the vessel wall was noted. The artery was then removed in toto in its course in the neck and immediately opened longitudinally. The type of thrombus present was recorded.

Cholelysin anticoagulant test:

The jugular vein was cannulated with PE 50 Clay-Adams polyethylene tube filled with heparin saline 100 unit/ml. Infusion of cholelysin solution 364-482 units/ℓ was commenced, at a rate of 1 ml/hour with a slow infusion pump. Control animals were infused with sodium chloride 0,45 g/100 ml in glucose 5 g/100 ml, at the rate of 1 ml/hour. After the infusion had commenced, the basic method as outlined above was proceeded with. At the

end of the arterial implantation, the anaesthetic was continued for a further ~ 2 hours while the infusion of cholelysin or glucose/saline continued to total 3 ml. After that period of time, the anaesthetic and the infusion were discontinued, and the animal allowed to recover. It was killed either immediately ('2 hour' value) or at the stated periods, when the neck was opened and the state of the carotid artery recorded.

MATERIALS

acetic acid+
acetone+
ATEE - Miles Seravac Cape Town
acrylamide - Merck
ADP - Sigma Corp St Louis USA
A.G.1.X8 200-400 mesh chloride form - Biorad Laboratories
albumen (human) - Western Province Blood Transfusion Service Cape Town
α-casein - Worthington Biochemical Corporation New York USA
α-chymotrypsin - Miles Seravac Cape Town
α_2-macroglobulin - Natal Blood Transfusion Service Durban Natal
amitryptilene powder -Frosst MSD Halfway House Transvaal
amidoschwarz -Merck
ammonium sulphate+
ammonium persulphate+
aprotonin - Bayer Germany
BAEE - Miles Seravac Cape Town
bile -Human: Dept Surgery Groote Schuur Hospital Cape Town
ox, pig, goat, sheep: Municipal Abattoir Cape Town
other: Research Laboratories, Medical School, University of Cape Town
bilirubin - Tauber & Corssen Cape Town
bisacrylamide - Merck
blue dextran 2000 - Pharmacia Uppsala Sweden
bromphenol crystals - Merck
calcium chloride+
chymotrypsinogen - Miles Seravac Cape Town
chlorpromazine powder - Maybaker Port Elizabeth
collagen - Sigma Corp St Louis USA
Coomassie brilliant blue R250 - Merck
copper sulphate+
dialysis tubing - Arthur H. Thomas
EDTA - Merck
epinephrine - Dispensing chemist stock
ether+
fibrinogen (human) - Kabi Stockholm
Folin-Ciocalteu phenol reagent - Merck
glycine - Merck
glycerol+
heparin - DADE Laboratories
hydrochloric acid+

NOTE: + after a chemical = Analar grade

lactate dehydrogenase- Miles Seravac
liquid nitrogen - Afrox
mercaptoethanol - Merck
ovalbumin - Miles Seravac Cape Town
petri dishes - polyethylene and glass - Laboratory & Scientific Cape Town
perchloric acid+
phenol+
plasmin, plasminogen } American National Red Cross Washington DC USA
platelin + activator - General Diagnostics New Jersey USA
polyethylene glycol - British Drug Houses
polyethylene tubing PE 50 - Clay Adams USA
potassium sodium tartrate+
potassium tartrate+
potassium iodide+
potassium phosphate+
potassium oxalate+
promazine powder - Wyeth Laboratories Isando Transvaal
ribonuclease - Miles Seravac Cape Town
sephadex G50 G75 G100 G200 A50 - Pharmacia Uppsala Sweden
sodium carbonate+
sodium chloride+
sodium citrate+
sodium diethyl barbiturate+
sodium dodecyl sulphate - Serva Feinbiochemica Heidelberg West Germany
sodium hydroxide+
sodium glycocholate - Difco Laboratory
sodium phosphate+
sodium taurocholate
soybean trypsin inhibitor - Miles Seravac Cape Town
sucrose RNAse free- Miles Seravac Cape Town
sulfuric acid+
TEMED (N N N' N' tetramethylenediamine) - Merck
thrombin Hylands Laboratory
tricalcium phosphate+
trifluperazine powder - Smith Klin French Laboratories Isando Transvaal
tris : tris (hydroxy methyl)aminomethane - Tauber & Corssen
trypsin, trypsinogen } Miles Seravac Cape Town
versatol - General Diagnostics New Jersey
urea - Merck
Whatman DE 32 - Hickman & Kleber
Whatman filter paper- Hickman & Kleber
Wistar rats-UCT Medical School Animal House

Appendix 3

Table 3-3A PLASMIN ASSAY

Stability of fibrin plate after various periods of storage at 4°C. Standard plasmin used throughout, 0,1 U/ml. All results in mm diameter of zone of lysis.

Year	Day 0		Day 1		Day 2		Day 3		Day 4	
	14	mean	13	mean	12	mean	14	mean	12	mean
1970	15		13		14		15		12	
	14	14,3	13	13,0	12	12,7	14	14,3	12	12,0
	13		13		12		13		12	
1972	12		14		14		13		13	
	15	13,3	14	13,7	15	13,7	12	12,7	12	12,3
	14		14		12		13		14	
1974	15		15		12		12		12	
	13		13		13		14		13	
	14	13,5	13	13,8	-	12,3	13	13,0	14	13,3

Appendix 3

Table 3-5A

Dose-response curve of cholelysin. Five preparations of Peak I studied in doubling dilutions. Results in mm diameter of zone of lysis.

Preparation No 1

Dilution	Repetitive assays					Mean	x	SEM
1/1	36	39	36	37	38	37,2	1,3	0,58
1/2	32	34	33	34	32	33	1,0	1,0
1/4	27	30	30	29	28	28,8	1,3	0,58
1/8	25	22	24	24	23	23,6	1,1	0,51
1/16	20	21	19	18	18	19,2	1,3	0,58
Preparation No 2								
1/1	36	36	33			35	1,7	1,0
1/2	32	31	31			31,3		
1/4	26	27	26			26,3		
1/8	21	21	21			21		
1/16	18	16	16			16,7	1,2	0,7
Preparation No 3								
1/1	30	28	27	29		28,5	1,3	0,6
1/2	21	22	21	24		22	1,4	0,7
1/4	17	16	16	16		16,3		
1/8	10	9	8	-		9		
Preparation No 4								
1/1	27	25	25	24		25,25	1,2	0,6
1/2	20	18	17	21		19	1,8	0,9
1/4	15	14	14	13		14	0,8	0,4
1/8	10	9	9			9,3		
Preparation No 5								
1/1	22	19	21	20		20,5	1,3	0,6
1/2	15	15	15			15		
1/4	9	10	9	12		10	1,4	0,7
1/8	6	6	6	5		5,75		

Appendix 5

Precipitation with various agents

5.2.3 Acetone. The addition of acetone at 0°C in various concentrations to cholelysin led to precipitation of fibrinolytic activity. When equal quantities were mixed, the resultant precipitate contained all fibrinolytic activity when redissolved, but lost all activity within 30 minutes. The method was not pursued further.

5.2.4 Chloroform/methanol partition. This method (see Methods) led to the partition of a tarry black liquid at the interface between the two solvents. Fibrinolytic activity was present in this black liquid, and could be recovered by redissolving in tris buffer. It could be subjected to exclusion chromatography, but was found to lose activity thereafter (in two experiments). The successful development of ammonium sulphate precipitation caused this method to be abandoned.

5.2.5 Polyethylene glycol precipitation. (PEG)
Experimental design: 10ml of cholelysin solution, in tris buffer M 0,006 pH 8,5. precipitated by polyethylene glycol solutions 10ml of varying strength to final concentrations as shown. The mixture was stood for 30 minutes at room temperature, and then centrifuged at 4°C, 12 000 X G, for 20 minutes. Precipitates were redissolved in tris buffer to 0,5ml, and both supernatant and precipitate were tested for fibrinolytic activity in the usual way.
Results:

PEG%	Control	Supernatants			Precipitates		
		1	2	units/l	1	2	units/l
2	27,28, 29,28 Mean 28 = 62 u/l	26	26,5	49	16,5	16,5	15,0
5		26	26	48,5	16,5	16,5	15,0
10		25,5	27	49	16	16,5	14,5
15		25	24,5	41	17	17	16,0
20		25,5	24,5	42,9	18,5	17,5	18,2
25		25	25,5	43,5	19	19,5	20,9
30		24	24	37,9	20,5	21	25,1
35		24	23,5	36,2	22	22	29,7

Comment: this poor separation was repeated several times, and this technique was then abandoned.

Appendix 5

5.2.6 Phenothiazines

In view of the report by Clarke Maritz and Denborough (1972) that chlorpromazine and other tranquillizers cause a precipitation of the protein and glycoprotein components of bile in vitro, 4 of these drugs in clinical use were studied for their effect on cholelysin in vitro. These were: chlorpromazine
promazine
trifluperazine
amitryptilene

In all cases, the local agents kindly procured the pure compound in powder form, and this was dissolved in tris buffer to the concentrations found to be optimal for precipitation by the authors. These optimal concentrations were:

	mM	
	protein	glycoprotein
chlorpromazine	10	30
promazine	5	30
trifluperazine	3	10
amitryptilene	10	0

Tests using these drugs in the concentrations given failed to precipitate cholel ysin to more than a very slight degree ($\leq$ 10%) and the experiments were then abandoned.

Appendix 6

Table 6-1A

Attempt to activate trypsinogen, chymotrypsinogen and plasminogen with cholelysin, trypsin, chymotrypsin and plasmin, respectively. Results in mm diameter of zone of lysis.

Cholelysin on substrate	Repetitive assays					Mean
None	11	12	14	12	12	12,2
Trypsingeon	10	11	12	12	13	11,6
Chymo-trypsinogen	11	12	10	12	10	11,0
Plasminogen	15	12	13	12		13
Trypsin on substrate						
None	14	15	16	16	13	14,8
Trypsinogen	26	21	24	24	23	23,6
Chymotrypsin on substrate						
None	14	16	15	13	14	14,4
Chymo-trypsinogen	21	23	23	22	22	22,2
Plasmin on substrate						
None	12	13	12	12	12	12,2
Plasminogen	21	21	21			21,0

Appendix 7

7.4.2

Table 7-1A

Inhibitory effect of human serum on lytic action of cholelysin. Control measured in 1/1 dilution in tris buffer M 0,006. Test serum introduced in place of tris buffer, and lytic effect measured. Inhibition calculated from the formula: All tests in duplicate.

$$\% \text{ Inhibition} = \frac{\text{Control (units)} - \text{test (units)}}{\text{Control (units)}} \times 100$$

Serum No	Dilution	Lytic activity(mn)				Mean	Units	Inhibition %
Control	1/1	22,	22,	21,	22	21,75	29,1	
1	1/20	7		8		7,5	5,0	83
	1/40	15		16		15,5	13,4	54
	1/80	17		19		18	18,2	37
	1/100	19		19		19	20,5	30
	1/160	21		22		21,5	27,6	5
	1/320	22		22		22	29,7	0
2	1/20	6		(2)		4	3,2	89
	1/40	12		11		11,5	8,2	72
	1/80	17		17		17	16,0	45
	1/100	21		21		21	26,3	10
	1/160 } 1/320 }	not done						
3.	1/20	0		0		0	0	100
	1/40	10		13		11,5	8,2	72
	1/80	-		15		15	12,6	57
	1/100	20		17		18,5	19,3	34
	1/160	20		20		20	23,2	20
	1/320	22		22		22	29,7	0
4.	1/20	7		8		7,5	5,0	83
	1/40	14		11		12,5	9,1	69
	1/80	13		11		12	8,7	70
	1/160	21		23		22	29,7	0
	1/320	22		23		22,5	31,7	0

Appendix 7

Serum No	Dilution	Lytic activity(mm)		Mean	Units	Inhibition%
5	1/20	0	0	0	0	100
	1/40	13	16	14,5	11,8	59
	1/80	19	20	19,5	21,8	25
	1/100	19	19	19	20,5	30
	1/160	-	19	19	20,5	30
	1/320	20	21	20,5	24,7	15
6	1/20	0	0	0	0	100
	1/40	5	5	5	3,7	87
	1/80	16	17	16,5	15,1	48
	1/100	17	18	17,5	17,1	41
	1/160	18	20	19	20,5	30
	1/320	22	21	21,5	27,6	5
7	1/20	0	0	0	0	100
	1/40	10	9	9,5	6,4	78
	1/80	17	15	16	14,2	51
	1/100	19	16	17,5	17,1	41
	1/160	19	20	19,5	21,8	25
	1/320	20	21	20,5	24,7	15
New control						
	1/1	21,22,22,19,21,21,20,21,21,21		Mean 20,9	Units 25,7	
8	1/20	0	0	0	0	100
	1/40	8	8	8	5,3	79
	1/80	18	17	17,5	17,1	33
	1/100	18	19	18,5	19,3	25
	1/160	18	21	19,5	21,8	15
	1/320	19	21	20	23,2	9
9	1/20	0	0	0	0	100
	1/40	4	7	5,5	3,9	85
	1/80	10	12	11,0	7,7	70
	1/100	15	16	15,5	13,4	48
	1/160	18	18	18	18,2	29
	1/320	20	19	19,5	21,8	15
New control		21,21,21,22		Mean 21,3	Units 27,2	
10	1/20	6	0	3	2,8	90
	1/40	12	11	11,5	8,2	70
	1/80	18	18	18	18,2	33
	1/100	20	21	20,5	24,7	9
	1/160	21	21	21	26,3	3

Appendix 7

Table 7-2A

Inhibitory effect of human serum heated to 56°C for 30 minutes on fibrinolytic effect of cholelysin. Experimental design and calculations as in Table 7-1A. Sera are from Table 7-1A, and are numbered from that Table.

Serum No	Dilution	Lytic activity(mm)		Mean	Units	Inhibition%
Control	1/1	22,22,21,22		21,75	29,1	
3	1/20	0	0	0	0	100
	1/40	12	11	11,5	8,2	72
	1/80	18	20	19	20,5	30
	1/100	20	21	20,5	24,7	15
	1/160	21	22	21,5	27,6	5
Control	1/1	21,22,22,19,21,21,20,21,21,21		Mean 20,9	Units 25,7	
9	1/20	7	8	7,5	5,0	80
	1/40	13	9	11	7,7	70
	1/80	17	17	17	16,0	38
	1/100	17	17	17	16,0	38
	1/160	17	19	18	18,2	29
	1/320	19	19	19	20,5	20
Control	1/1	21,21,22,21		21,3	27,2	
10	1/20	0	0	0	0	100
	1/40	8	7	7,5	5,0	82
	1/80	10	10	10	7,7	72
	1/100	12	14	13	9,8	64
	1/160	20	21	20,5	24,7	9

Appendix 7

7.4.6

Inhibitory effects of platelet rich plasma (PRP), platelet poor plasma (PPP) and of serum derived from the same plasma, on the fibrinolytic action of cholelysin.

Experimental design: blood was taken into sodium citrate solution 3,1g/100ml in the proportion of 9 parts of blood to 1 part of sodium citrate solution. This was centrifuged at 900 X G for 3 minutes. The platelet rich supernatant was harvested, and the blood remaining was centrifuged at 12 000 X G for 20 minutes to give PPP. An aliquot of fresh blood from the same donor was placed separately into a glass test tube and incubated in a waterbath at 37°C for 2 hours, after which it was centrifuged and the serum removed. The PRP had a platelet count of 500 X 10^9/l.

Inhibition was then tested as set out in sections 7.3, 7.4.1 and 7.4.2 in the main text. Results are shown below, in mm diam zone of lysis and units.

Dilution of sample	Serum		mean	units	Inhibition (serum)	Inhibition (PRP)	Inhibition (PPP)
Control	14	15 14	14,3	11,5			
1/10	0	0	0	0	100	100	100
1/20	10	10	10	6,8	41	41	44
1/40	13	13	13	9,8	15	15	0
1/100	13	13,5	13,3	10,5	9	0	0
	PRP						
1/10	0	0	0	0	100		
1/20	10	10	10	6,8	41		
1/40	13	13	13	9,8	15		
1/100	14	14	14	11,1	0		
	PPP						
1/10	0	0	0	0	100		
1/20	10	9	9,5	6,4	44		
1/40	14	14	14	11,1	0		
1/100	15	14	14,5	11,8	0		

Table 7-6A

Inhibitory effect of human serum on ATEE-esterase activity of cholelysin. The revised ATEE-esterase assay was used (Section 6.4 p). In this table, serum volume (µl) + cholelysin volume (µl) = vol.incubation mixture. In the test, vol.incubation mixture made up to 1,2ml with tris buffer. Cholelysin volume was 0,2ml. Thus units of activity/ml was derived from: $\frac{1,0}{0,2} \times \frac{\text{Initial rate}}{0,01} = \text{I.R.} \times 500$

% Inhibition calculated as in Table 7-1A.

INCUBATION MIXTURE					ATEE ESTERASE ACTIVITY			
Serum dilution	Serum volume (µl)	Cholelysin volume (µl)	Ratio cholelysin/serum	Incubation time at 22° (minutes)	Volume incubation mixture	Initial rate	Units activity per ml	% Inhibition
		200			200	0,0832	41,60	
		200	(CHOLELYSIN CONTROLS)		200	0,0816	40,80	Mean
		100			100	0,0441 (X 2)	44,10	42,2
1	200				200	0		
20	200				200	0		
50	200		(SERUM CONTROLS)		200	0		
100	200				200	0		
500	200				200	0		
500	200	200	500	5'	400	0,0810	40,50	
500	200	200	500	9'	400	0,0840	42,00	Mean
500	200	200	500	2'	400	0,0802	40,10	40,9 3,1
100	50	200	400	2'	250	0,0771	38,55	
100	50	200	400	6'	250	0,0728	36,40	37,5 11,1
100	100	200	200	2'	300	0,0675	33,75	
100	100	200	200	6'	300	0,0628	31,40	32,6 22,7
100	200	200	100	5'	400	0,0451	22,55	
100	200	200	100	10'	400	0,0444	22,20	22,4 46,9
20	50	200	80	2'	250	0,0351	17,75	
20	50	200	80	5'	250	0,0368	18,38	18,1 57,1
50	200	200	50	3'	400	0,0168	8,42	
50	200	200	50	10'	400	0,0181	9,05	8,7 79,4
20	100	200	40	2'	300	0,0058	2,90	
20	100	200	40	2'	300	0,0048	2,40	2,7 93,6
20	200	200	20	2'	400	0,0003	0,15	
20	200	200	20	5'	400	0,0002	0,10	0,1 100,0

Comment: Various periods of incubation from 2' to 10' made no difference to I.R.

APPENDIX 7

Table 7-7A

Inhibitory effect of serum of rat, rabbit, guinea pig and mouse on ox cholelysin, Peak I. Experimental design identical to that set out in sections 7.3, 7.4.1 and 7.4.2 of main text above.

	Dilution	Lytic activity (mm diameter)			Mean	Units (u/l)	Inhibition%
Control		33	33,5	32	32,8	112	
RAT	1/1	0	0	0		0	100
	1/2	0	0	0		0	100
	1/4	15	15	15	15	12,6	89
	1/10	27	26	28	27	54,8	51
	1/100	36	30	35	33,7	122	0
RABBIT							
	1/1	0	0	0		0	100
	1/2	0	0	0		0	100
	1/4	21	17	20,5	19,5	21,8	81
	1/10	28	28,5	27,5	28	62	45
	1/100	29	31	34	31,3	93	17
	1/1000	33	32	32	32,7	108	0
Control		32	28	27	29	68,7	
GUINEA PIG							
	1/1	0	0	0		0	100
	1/2	0	0	0		0	100
	1/4	0	0	0		0	100
	1/10	22,5	21	23	22,2	30	56
	1/100	30	31	27	29,3	71,3	0
MOUSE							
	1/1	0	0	0		0	100
	1/2	0	0	0		0	100
	1/4	5	3	3	3,7	3,1	95
	1/10	24	26	26	25,3	44	36
	1/100	30	28	29	29	68,7	0

Appendix 7

Table 7-8A

Inhibitory effect of α_2-macroglobulin solution on cholelysin preparations. Experimental detail as set out in sections 7.3, 7.4.1 and 7.4.2 in main text. Because macroglobulin showed inherent fibrinolysis, this had to be allowed for, and the formula for calculating inhibition % was modified as follows:

$$\text{Inhibition\%} = \frac{\text{Control(units)} - (\text{test (units)} - \text{macroglobulin lysis (units)})}{\text{control (units)}} \times 100$$

As the α_2-macroglobulin solution showed an inherent fibrinolytic potential of 6,8 units/l, the equation read:

$$\text{Inhibition\%} = \frac{\text{Control(units)} - (\text{test(units)} - 6{,}8)}{\text{control(units)}} \times 100$$

All test results reported in mm diameter of zone of lysis

Control α_2-macroglobulin: 9, 10, 11, 9 Mean 10 Units 6,8

Preparation								Inhibition%
G200 - Control			28	28	Mean 28		62	
Test	19	19	18	20 18	18,8		20,3	78
Peak I Control			30	31	30,5		84,4	
Test	26	27	25	26 26	26		48,5	51
Peak II Control			18	21	19,5		22,2	
Test	17	15	16	16 15	15,8		13,7	69
Peak III Control			17	18	17,5		17,1	
Test	16	15	15	14 15	15		12,6	66
Peak IV Control			10	11	10,5		7,3	
Test	11	10	11	12	11		7,7	88

Appendix 7

Table 7-9A

Inhibitory effect of soy bean trypsin inhibitor (SBTI) on cholelysin. Incubation for 60 minutes at 20°C, otherwise conditions as stated in sections 7.3. and 7.4.1 and 7.4.2 in main text. SBTI in g/100ml.

(a) SBTI 0,0005%

Cholelysin fraction	Control 1	Control 2	Control mean	Control units	Test 1	Test 2	Test mean	Test units	Inhibition
G200	29	30	29,5	74,5	24	23	23,5	35,7	52
Peak I	29	29	29	70,1	23	22	22,5	31,7	55
Peak II	19	18	18,5	19,3	13	13	13	9,8	49
Peak III	22	22	22	29,7	10	10	10	6,8	77

(b) SBTI 0,001%

G 200				74,5	17	19	18	18,2	76
Peak I	(Same controls)			70,1	22	23	22,5	31,7	55
Peak II				19,3	12	12	12	8,6	55
Peak III				29,7	9	9	9	6,0	80

(c) SBTI 0,002%

G200	30	29,5	29,8	78	18	17	18	16	18	17,4	16,8	78
Peak I	30	30	30	79,2	19	20	22	21	21	20,6	25	68
Peak II	18	18	18	18,2	10	10	11	11	10	10,4	7,2	60
Peak III	18	16	17	16	5	2	4	5		4,0	3,0	81

(d) SBTI 0,004%

G200	29	30	29,5	74,5	10	10	9	10	10	9,8	6,7	91
Peak I	28	29	28,5	66	17	14	16	14	14	14,8	12,2	82
Peak II	17	18	17,5	17,1	5	6	5	5	5	5,2	3,8	78
Peak III	16	16	16	14,2	0	0	0	0	0	0	0	100

(e) SBTI 0,006%

G200	29	29	29	70,1	10	9	10	12	8	9,8	6,7	90
Peak I	28	28	28	62	0	2	3	2	3	2	2,5	96
Peak II	25	24	24,5	39,7	0,	3	2	3	0	1,6	2,4	94
Peak III	16	16	16	14,2	0	0	0	0	0	0	0	100

(f) SBTI 0,008%

G200	29	29	29	70,1	11	11	8	9	7	9,2	6,2	91
Peak I	28	28,5	28,3	64,5	10	10	9	9	9	9,4	6,4	90
Peak II	17	17	17	16	5	5	4	5	5	4,8	3,6	78
Peak III	16	16	16	14,2	0	0	0	0	0	0	0	100

Appendix 7

Table 7-10A

Inhibitory effect of soy bean trypsin inhibitor (SBTI) on cholelysin. Incubation with cholelysin fractions for 24 hours, otherwise conditions as stated in sections 7.3, 7.4.1 and 7.4.2 of main text. SBTI in g/100ml.

Cholelysin fraction	CONTROL 1	2	mean	units	TEST					mean	units	Inhibition%
(a) SBTI 0,002%												
G200	29,5	30	29,8	78	15	13	16	16	15	15	12,5	84
Peak I	30	30	30	79,2	14	17	15	16	14	15,2	13,8	83
Peak II	18	18	18	18,2	6	8	5	6		6,3	4,3	76
Peak III	18	16	17	16	4	0	0	0		1	0	100
(b) SBTI 0,004%												
G200	29	30	29,5	74,5	13	12	12			12,3	9,0	88
Peak I	28	29	28,5	66	9	8	10			9	6,0	91
Peak II	17	18	17,5	17,1	3	4	3			3,3	3,0	82
Peak III	16	16	16	14,2	0	0	0			0	0	100
(c) SBTI 0,006%												
G200	29	29	29	70,1	10	6	7			7,7	5,1	93
Peak I	28	28	28	62	2	3	2			2,7	2,8	96
Peak II	17	18	17,5	17,1	2	2	2			2,0	2,5	85
Peak III	16	16	16	14,2	0	0	0			0	0	100
(d) SBTI 0,008%												
G200	29	29	29	70,1	4	4	4			4	3,3	95
Peak I	28	28,5	28,3	64,3	0	2	4			2	2,5	96
Peak II	17	17	17	16	0	0	0			0	0	100
Peak III	16	16	16	14,2	0	0	0			0	0	100

Appendix 7

Table 7-11A

Inhibitory effect of aprotonin (Bayer) on cholelysin. Experiments were done with prior incubation at (a) 20°C for 60 minutes
(b) 37°C for 60 minutes
In both cases, 0,2 ml of commercial 'Trasylol' (Bayer) containing 2000 units of aprotonin was added to 0,2ml of cholelysin solution. The mixture was plated out in the usual way after incubation. Aprotonin itself failed to lyse a fibrin plate. Results in mm diameter of zone of lysis, converted into units/l. Inhibition calculated as described in section 7.4.2 in main text.

(a) Room temperature (20°C).

Cholelysin fraction	1	2	mean	units	Test					mean	units	Inhibition%
G200	27	27	27	54,8	11	11	11	11	11	11	7,7	86
Peak I	37	33	35	146,4	0	0	0	0	0	0	0	100
Peak II	19	21	20	23,2	10	11	10	10	11	10,4	7.2	69
Peak III	16	16	16	14,2	0	0	0	0	0	0	0	100
Peak IV	10	10	10	6,8	0	0	0	0	0	0	0	100
(b) at 37°C												
G200	26	26	26	48,5	14	15	14	15		14,5	11,8	76
Peak I	26	28	27	54,8	8	9	8	8		8,5	5,3	90
Peak II	18	18	18	18,2	12	13	13	12,5		12,7	9,4	48
Peak III	20	20	20	23,2	0	0	0	0		0	0	100
Peak IV	5	5	5	3,7	0	0	0	0		0	0	100

Table 11-1A

Molecular weight of cholelysin - various preparations. Column chromatography.
Section A: no added SDS. Section B: added SDS.

Preparation	Sephadex	Column Height (cm)	Column Diam (cm)	V_t (ml)	V_o (ml)	Volume applied (ml)	Flow rate ml/hr	Fractions (ml)	Front at Fraction No	V_e (ml)	K_{av}	Molecular weight X 10^3
Section A												
G200	G50	71,3	2,0	224	95	2	28	1,5	26	39	0,3	23
Peak I	G75	26,3	1,5	46,5	13,5	2	36	1,2	25	30	0,5	11
Peak II	G75	26,3	1,5	46,5	13,5	2	34,5	1,15	25	28,75	0,46	13
Peak III	G75	26,3	1,5	46,5	13,5	2	34	1,03	26	26,78	0,58	8
Peak IV (i)	G75	26,3	1,5	46,5	13,5	2	36	1,1	25	27,5	0,62	7,5
(ii)								1,1	13	14,3	0,04	50
Section B												
Batchwise												
Peak I	G75	70,0	1,5	124	44	2	42	2,0	38	76	0,4	14,3
-do-	G75	79,5	1,5	140	58	2	45	2,0	45	90	0,39	14,5
-do-	G75	79,5	1,5	140	58	2	40	2,0	55	110	0,63	7,0
Chymotrypsin	G75	79,5	1,5	140	45	2	42	2,0	45	90	0,39	13,0

Comment: Data for rechromatographed Peak I not available (see Figure 11-1 in main work).
Molecular weight of chymotrypsin is known.

Appendix 11

Figure 11-5A

The relative mobility of the various bands studied was estimated from measurement of several gels. In each case, the position of the band studied was estimated as a simple ratio of

$$\frac{\text{movement of band (mm)}}{\text{total length of gel (mm)}}$$

There was variability in this result until the technique was established. Results on which Figure 11-5 is based were as follows:

			n	s	SEM
Markers:	Lactate dehydrogenase	0,528	4	0,21	0,011
	Chymotrypsinogen	0,621	4	0,17	0,009
	Ribonuclease	0,820	4	0,024	0,012
	Plasmin split products-				
	P1 (D-dimer)	0,280	4	0,017	0,009
	P2 (")	0,458	4	0,014	0,007
	P3 (")	0,797	4	0,024	0,012
	Cholelysin split products				
	C3	0,302	5	0,017	0,008
	C4	0,475	5	0,020	0,009
	C1	0,624	5	0,020	0,009
	C2	0,785	5	0,033	0,015